UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

HUMAN BIOMONITORING:

BIOMARKERS, INDIVIDUAL SUSCEPTIBILITY, AND NUTRIGENETICS

Carina Alexandra Fernandes Ladeira

DOUTORAMENTO EM BIOLOGIA

(GENÉTICA)

2014

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

HUMAN BIOMONITORING:

BIOMARKERS, INDIVIDUAL SUSCEPTIBILITY, AND NUTRIGENETICS

Carina Alexandra Fernandes Ladeira

DOUTORAMENTO EM BIOLOGIA

(GENÉTICA)

Tese orientada por:
Professor Doutor Manuel Carmo Gomes (FCUL)
Professor Doutor Rui Miguel Brito (ESTeSL),
especialmente elaborada para a obtenção do grau de doutor em Biologia especialização Genética

2014

"The main aim of my life has been to free man from the bonds which bind him to the surface, allowing him to escape from his natural limits, breathe in an unbreathable atmosphere and withstand increasingly great pressures. Just not place man in this new atmosphere but to adapt him teaching to explore it, to subsist, to survive and to study what lies around him."

Jacques Ives Cousteau, The Living Sea 1964.

DISSERTAÇÃO APRESENTADA À UNIVERSIDADE DE LISBOA PARA OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA (ESPECIALIDADE GENÉTICA)

CARINA ALEXANDRA FERNANDES LADEIRA

OUTUBRO 2014

O PRESENTE TRABALHO FOI FINANCIADO PELO PROGRAMA DE APOIO À FORMAÇÃO AVANÇADA DE DOCENTES DO ENSINO SUPERIOR POLITÉCNICO (PROTEC) EM PARCERIA COM O INSTITUTO POLITÉCNICO DE LISBOA E PELA AUTORIDADE PARA AS CONDIÇÕES DE TRABALHO COM A REFERÊNCIA ACT 036APJ/09.

AGRADECIMENTOS

A gratidão pode ser explicada como a recognição abrangente pelas situações e dádivas que a vida proporciona. Este é o espaço que disponho para fazê-lo e será claramente insuficiente para poder expressar a minha gratidão a todas as pessoas que de alguma forma me acompanharam nestes últimos quatro anos.

Existe uma pessoa à qual tenho de agradecer em primeiro lugar, pois sem o seu convite, o meu percurso académico teria seguido uma direcção diferente. À Professora Maria Fernanda Quintino, por ter acreditado em mim quando eu própria não o fazia. Pela sua amizade, sabedoria, sentido visionário e todas as qualidades humanas que ela tem a um nível excepcional. A ela agradeço a oportunidade de profissionalmente estar onde estou, apesar de ela discordar comigo.

Um agradecimento muito especial às pessoas que estão directamente envolvidas neste projecto comigo, os meus orientadores. Aos Professores Miguel Brito e Manuel Carmo Gomes por sempre me terem acompanhado, apoiado e ajudado ao longo de toda esta jornada de forma incansável, mesmo em alturas mais complicadas. Estou muito grata e feliz por ter tido a oportunidade de ter trabalhado com eles. A minha sorte foi imensa.

Um agradecimento especial à Professora Susana Viegas, que me inspirou a investigar e a perseguir os meus objectivos na área de investigação, integrando-me nos seus projectos e fazendo-me ver a minha contribuição como muito importante. O tempo que passamos juntas é sempre um excelente investimento, pois surgem sempre mais ideias de linhas de investigação.

Ao Professor Mário Pádua que me ensinou e ajudou a optimizar os métodos analíticos para quantificar vitaminas. Passamos um óptimo tempo juntos, ao longo de 4 anos.

À Professora Elisabete Carolino pelos seus ensinamentos, paciência e amizade ao longo de todas as reuniões de tratamento estatístico.

Aos meus colegas de laboratório Ana Oliveira, Paula Ferreira, Joana Malta Vacas e Bruno Carmona, por me terem transmitido os seus conhecimentos e me terem ajudado.

À Professora Luísa Veiga que realizou a quantificação da vitamina D por ELISA.

A todos os meus colegas de trabalho e amigos que me ajudaram e mostraram-se disponíveis sempre que eu precisei: Amadeu Ferro, Paula Casimiro, Gilberto Matias, Mário Matos, Nélia Nunes, Luísa Pedro, Paula Mendonça, Dora Pinto, Luísa Fialho.

Tenho a agradecer aos voluntários que participaram no estudo e que o tornaram possível com as suas amostras. Ainda agradecer a amizade e a assistência técnica no processo de recolha do sangue aos meus colegas Renato Abreu, Ana Almeida, Maria do Céu Leitão e Fernando Bellém. Ainda às enfermeiras Sandra Dantas e Maria Cristina Lacerda.

Agradecer à minha família, à minha mãe, Mª Alice e pai, Joaquim, pois fazendo o percurso que fizeram foi possível que eu hoje possa estar a trilhar este. À minha irmã Susana, por estar sempre disposta a aplacar os meus voos de Ícaro. E à família que escolhemos, que são os amigos. Sem eles esta seria uma missão não impossível mas infinitamente menos prazeirosa.

Tenho um agradecimento ainda a fazer à Escola Superior de Tecnologia da Saúde de Lisboa – Instituto Politécnico de Lisboa (ESTeSL/IPL) e à Caixa Geral de Depósitos (CGD) pela atribuição de uma Bolsa de Mérito durante o meu último ano de doutoramento.

A todos o meu Muito Obrigada por tudo!

NOTAS PRÉVIAS

I

A presente dissertação inclui o aproveitamento de trabalhos publicados, nos termos do n.º 3, do Artigo 36º, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – 2ª série, n.º 65 de 30 de Março de 2012, os quais consistem na secção II. State of the Art, Chapter 7 – Nutritional Research e na secção IV. Results, e cujas referências completas se encontram abaixo e as publicações originais na secção VIII. Annexes.

Para efeitos do disposto n.º 2 do Art. 8º do Decreto-Lei 388/70, a autora da dissertação declara que interveio na concepção do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados e submetidos para publicação.

II. STATE OF THE ART, CHAPTER 7 – NUTRITIONAL RESEARCH

Ladeira, C., Gomes, M.C. & Brito, M. (2014). Human Nutrition, DNA damage and Cancer: a review. N. Tomlekova, I. Kozgar and R. Wani (eds). *Mutagenesis: exploring novel genes and pathways.* Wageningen Academic Publishers. ISBN: 978-90-8686-244-3.

IV. RESULTS, CHAPTER 1 – FORMALDEHYDE

Ladeira, C., Viegas, S., Carolino, E., Gomes, M.C. & Brito, M. (2011). Genotoxicity biomarkers in occupational exposure to formaldehyde – the case of histopathology laboratories. *Mutation Research.* 721 p.pp. 15–20.

Ladeira, C., Viegas, S., Carolino, E., Gomes, M., Prista, J., Gomes, M.C. & Brito, M. (2012). Exposição Ocupacional a Formaldeído: Avaliação da Exposição e Efeitos Genotóxicos. *Revista Saúde & Tecnologia*. 7. p.pp. 18-27.

Ladeira, C., Viegas, S., Carolino, E., Gomes, M.C. & Brito, M. (2012). Genotoxicity biomarkers: application in histopathology laboratories. *Biomarker*. Chapter 7. Croatia: Intech. p.pp. 133-154. ISBN 979-953-307-612-5.

Ladeira, C. Viegas, S., Carolino, E., Gomes, MC. & Brito, M. (2013). The influence of genetic polymorphisms in XRCC3 and ADH5 genes on the frequency of genotoxicity biomarkers in workers exposed to formaldehyde. *Environmental Molecular Mutagenesis.* 54. p.pp. 213-221.

IV. RESULTS, CHAPTER 2 – CYTOSTATICS DRUGS

Ladeira, C., Viegas, S., Pádua, M., Gomes, M.C., Carolino, E., Gomes, MC. & Brito, M. (2014). Assessment of genotoxic effects in nurses handling cytostatic drugs. *Journal of Toxicology and Environmental Health, Part A: Current Issues*. 77. p.pp. 879-887.

II

De modo a melhor compatibilizar os restantes capítulos da dissertação com aqueles que foram objecto de publicação em capítulos de livro e jornais científicos e, existindo um encadeamento subsequente ao longo da dissertação, optou-se pela escrita em língua inglesa. A apresentação da dissertação numa língua estrangeira está prevista no n.º 5, do Artigo 45º, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, no âmbito do qual se requereu e obteve a respectiva autorização. Como previsto no n.º 5 do Artigo supra-citado inclui-se, na secção seguinte, um resumo alargado da dissertação, em português.

RESUMO

A utilização dos métodos da biologia molecular na investigação epidemiológica – designada de epidemiologia molecular – tem enorme potencial para o estabelecimento de associações entre patologias oncológicas e exposição ambiental relacionada com estilos de vida, ocupação profissional ou poluição ambiental.

A biomonitorização humana consiste, por um lado, na pesquisa e identificação de condições ambientais perigosas e, por outro, na estimação do risco de desenvolvimento de cancro por exposição a estas condições. Dado que a carcinogénese é um processo prolongado, os biomarcadores a que se tem recorrido para reconhecer eventos biológicos anormais têm sido desenvolvidos no âmbito de estudos epidemiológicos moleculares. Estes biomarcadores são quantificáveis e permitem a identificação da progressão de condições biológicas normais para anormais ao nível molecular. De uma forma geral, subdividem-se em biomarcadores de exposição, de efeito e de susceptibilidade genética. Os biomarcadores de genotoxicidade são um caso particular de biomarcadores de efeito e utilizam-se na avaliação de efeitos genómicos provocados por exposição, ambiental ou ocupacional, sendo em geral considerados preditores de desenvolvimento cancerígeno.

Duas técnicas importantes são utilizadas neste estudo – o ensaio dos micronúcleos por bloqueio da citocinese (CBMN) e o *comet assay*. O CBMN é uma das técnicas mais sensíveis para detecção de dano no DNA, sendo amplamente utilizada na investigação de efeitos genotóxicos de uma ampla variedade de agentes químicos, físicos e biológicos. O *comet assay,* permite a quantificação de dano no DNA de células individuais. Grande parte das lesões detectadas por esta técnica podem não ser posteriormente corrigidas pelos mecanismos normais de reparação do DNA, não se detectando, portanto, alterações genéticas que sejam necessariamente permanentes. A combinação destes dois ensaios genotóxicos é recomendada para a monitorização de populações com exposição crónica a agentes genotóxicos, sendo considerados os testes de genotoxicidade que, a curto prazo, são os mais promissores na avaliação de risco em humanos.

Factores como a idade, género e estilos de vida, tais como o consumo de tabaco e álcool, são variáveis que devem ser alvo de avaliação em estudos de biomonitorização pela sua capacidade para gerar confundimento no estabelecimento de associações. Também com relevância crescente, como factor capaz de condicionar a resposta a agentes genotóxicos, encontram-se os hábitos alimentares. Através da aplicação de técnicas de biologia molecular, pode-se investigar a interacção funcional entre o genoma e macro e micronutrientes, quer a nível molecular, quer celular e sistémico. A nutrigenética

estuda a resposta a padrões alimentares específicos e a forma como os genes e polimorfismos genéticos podem influenciar a bioavaliabilidade dos micronutrientes e dos cofactores enzimáticos envolvidos na reparação e metabolismo do DNA.

Os laboratórios são importantes locais de exposição ocupacional, uma vez que se manipula uma panóplia de agentes químicos que conferem um risco permanente de exposição por parte dos trabalhadores. Neste estudo, foram investigadas as consequências genómicas de exposição de trabalhadores a dois tipos particulares de químicos usados em meio laboratorial – o formaldeído e os citostáticos.

O formaldeído está classificado pela *International Agency for Research on Cancer* (IARC) como carcinogénico para humanos (grupo 1), baseado na evidência existente de associação com a incidência de cancro nasofaríngeo e, mais recentemente de leucemia mielóide. Relativamente aos citostáticos, são um grupo de fármacos cada vez mais utilizados, quer no tratamento de neoplasias quer no de doenças não malignas. São um grupo heterogéneo que compreende diversos agentes não relacionados mas que têm em comum a capacidade de inibir o crescimento celular, afectando, directa ou indirectamente, o genoma. A IARC classifica como carcinogénico para humanos (grupo 1) a ciclofosfamida e o paclitaxel, não considerando carcinogénico para humanos o 5-fluoracil (grupo 3).

É objectivo desta investigação contribuir para o desenvolvimento de um programa de biomonitorização da genotoxicidade destas substâncias que contemple biomarcadores de susceptibilidade e estilos de vida, nomeadamente dieta e nutrição.

O planeamento experimental aqui utilizado pode ser descrito como um estudo caso-controlo. Em cada um de dois contextos ocupacionais estudados – um para formaldeído, outro para citostáticos – foram constituídas duas amostras. Uma de trabalhadores expostos (os casos) e outra de não expostos (os controlos), tendo sido quantificados vários biomarcadores moleculares de genotoxicidade e avaliado o risco danos genómicos nos expostos por comparação com os controlos.

O estudo do formaldeído compreendeu 56 trabalhadores de seis laboratórios de Anatomia Patológica da região de Lisboa e Vale do Tejo e 85 trabalhadores da Escola Superior de Tecnologia da Saúde (ESTeSL) sem exposição a este agente químico (controlos). No contexto ocupacional de exposição a citostáticos foram reunidos 46 trabalhadores expostos a citostásticos das unidades de Farmácia, Hospital de dia e Pediatria de dois hospitais da região de Lisboa e Vale do Tejo e o grupo de controlo foi igualmente formado por 46 indivíduos da ESTeSL. Todos os participantes preencheram um termo de consentimento informado acerca da participação no estudo e na recolha das amostras, assegurando princípios de confidencialidade. Foi preenchido um questionário para caracterização de dados demográficos e de possíveis variáveis de confundimento, tais como exposições na sua ocupação laboral e/ou de tempos livres que pudessem enviesar os resultados, contacto com terapias

antineoplásicas, entre outros. Foi também preenchido um questionário que permitisse caracterizar os hábitos alimentares dos participantes.

A avaliação da genotoxicidade foi realizada com recurso ao ensaio CBMN e ao *comet assay*. Ambas as técnicas foram realizadas em linfócitos recolhidos e isolados de sangue periférico recolhido por venipunctura. Para avaliar os biomarcadores de susceptibilidade individual, nomeadamente os polimorfismos dos genes de reparação de DNA (*XRCC3* e *OGG1*), enzimas metabólicas (*ADH5*) e do receptor da vitamina D (*VDR*), foi realizado PCR em Tempo Real após extracção de DNA pelas técnicas de fenol-clorofórmio e por mancha de sangue. A quantificação de vitaminas A e E no soro humano foi realizada por HPLC e da vitamina D feita por ensaio imunológico (ELISA).

Nos dois contextos ocupacionais encontraram-se diferenças estatisticamente significativas entre trabalhadores expostos e não expostos ($p < 0.05$), indicando que a exposição é um factor de risco para o aumento dos biomarcadores de genotoxicidade avaliados pelo CBMN. O *comet assay*, aplicado no estudo da exposição ocupacional a citostáticos, não apresentou diferenças estatisticamente significativas entre expostos e não expostos.

No que respeita aos biomarcadores de susceptibilidade individual, o estudo da exposição a formaldeído encontrou associações estatisticamente significativas entre os genótipos *XRCC3* e protusões nucleares, nomeadamente entre *XRCC3* Met/Met (OR = 3.975, IC=1.053-14.998, p=0.042) e *XRCC3* Thr/Met (OR = 5.632, IC=1.673-18.961, p=0.005) comparativamente com o genótipo *XRCC3* Thr/Thr. Para os genótipos do *VDR BsmI* foi encontrada uma associação significativa no grupo dos expostos (p=0.041, teste Mann-Whitney), em que os portadores do genótipo CT+T apresentaram maior média de micronúcleos em linfócitos comparativamente com o genótipo CC. Não foram obtidos resultados significativos para os polimorfismos do *ADH5*. Não foi encontrada também qualquer associação significativa entre os genótipos *OGG1* e os biomarcadores quantificados pelo CBMN e pelo *comet assay* no estudo da exposição a citostáticos.

Os resultados referentes ao estudo nutricional indicam, de forma geral, que a vitamina A actua como factor de risco e a vitamina E como protector. Especificamente no estudo do formaldeído, a vitamina A obteve uma correlação positiva com as pontes nucleoplásmicas ($r=0.557$, $p < 0.01$) e a vitamina E diminui a média de protusões nucleares ($r=-0.297$, $p<0.05$). No estudo dos citostáticos, a vitamina A está positivamente correlacionada com o aumento de dano oxidativo no DNA, enquanto a vitamina E revelou uma correlação negativa ($p < 0.05$) com os micronúcleos. O folato e a vitamina B12 funcionam como factores protectores, ao contrário do ferro.

Os dados obtidos permitem concluir que é exequível e necessário implementar um programa de biomonitorização humana em contextos de exposição ocupacional. Este deve integrar biomarcadores

de exposição, efeito, genotoxicidade e susceptibilidade. Hábitos de vida, com particular ênfase na nutrição, devem ser incluídos, uma vez que têm influência sobre os biomarcadores estudados.

Palavras-Chave: Biomonitorização, genotoxicidade, exposição ocupacional, nutrigenética.

ABSTRACT

Cytokinesis blocked micronucleus (CBMN) assay and comet assay are the most promising short-term genotoxicity assays for human risk assessment and their combination is recommended to monitor populations chronically exposed to genotoxic agents. Nutrition is recognized to be an important lifestyle factor that influences cancer risk, and should be taken into account at an individual level.

Laboratories are occupational settings where chemical agents are handled and workers are exposed. Formaldehyde and cytostatic drugs, in particular, are chemical agents handled in laboratories that are considered carcinogenic for humans and special protective measures should thus be adopted against them.

The aim of this investigation is to contribute to the development of a biomonitoring programme that includes genotoxicity assessment related with genetic susceptibility biomarkers, and lifestyle factors, namely nutrition. The experimental planning used was a case-control blinded study. Four separated samples were formed comprising two samples of subjects exposed (n = 56 for formaldehyde; n = 46 for cytostatics), and two samples of non-exposed controls (n = 85 and n = 46, respectively). Participants filled-in a personal and a food frequency questionnaires. CBMN and comet assays were used to assess genotoxicity. Individual susceptibility was investigated by Real Time PCR. Measurements of serum vitamins A and E were performed by HPLC, and vitamin D by ELISA. The risk of genotoxicity in those exposed was then compared with the risk in the controls, allowing for the quantitative measurement of association between exposure and genotoxicity.

In both occupational settings, the genotoxicity biomarkers were significantly higher in the exposed than in the non-exposed controls ($p<0.05$). In the formaldehyde occupational context, significant associations were found between *XRCC3* genotypes and nuclear buds; but that was not the case either for *ADH5* or *VDR*. Comet assay did not identify significant differences between those exposed to cytostatics and controls, and the same lack of association applies to the *OGG1* genotypes. Micronutrients association to the genotoxicity biomarkers was controversial; we have found positive correlations for vitamin A and negative ones for vitamin E.

Our findings emphasize the need for the implementation of a regular biomonitoring programme of personnel occupationally exposed to drugs like formaldehyde and cytostatics.

Keywords: Biomonitoring, genotoxicity, occupational exposure, nutrigenetics.

LIST OF ABBREVIATIONS

ACGIH – American Conference of Governmental Industrial Hygienists

ADH – Alcohol Dehydrogenase

ALARA – As Low As Reasonable Achievable

ALDH – Aldehyde Dehydrogenase

AP – Apurinic/apyrimidinic

ATSDR - Agency for Toxic Substances and Disease Registry

BER – Base Excision Repair

CARET - Beta-Carotene and Retinol Efficacy Trial

CBMN – Cytokinesis Block MicroNucleus

CI – Confidence Interval

CIIT – Chemical Industry Institute of Toxicology

CP - Cyclophosphamide

DAD – Diode Array Detector

DNA – Deoxyribonucleic Acid

DRI – Dietary Reference Intake

ECD - Electrochemical Detection

ESCODD - European Standards Committee on Oxidative DNA Damage

ESTeSL – Escola Superior de Tecnologia da Saúde de Lisboa

EPA – Environmental Protection Agency

FA – Formaldehyde

FDH – Formaldehyde Dehydrogenase

FFQ – Food Frequency Questionnaire

FISH – Fluorescent *in situ* Hibridization

FPG - Formamidopyrimidine DNA glycosylase

FaPyG - 2,6-Diamino-4-hydroxy-5-formadopyrimidine

FaPyA - 4,6-Diamino-5-formamidopyrimidine

GS – Gas Chromatography

GS-MS - Gas Chromatography-Mass Spectrometry

GSH – Gluthathione

GST – Gluthathione S- Transferase

GSTM1 - Gluthathione S- Transferase Mu I

GSTT1 - Gluthathione S- Transferase Theta I

HA – Heterocyclic Amines

HOPE - Heart Outcomes Prevention Evaluation

HOPE-TOO - Heart Outcomes Prevention Evaluation Ongoing Outcomes

HPLC - High Performance Liquid Chromatography

HR – Homologous Recombination

HUGO – Human Genome Organization

HUMN – Human MicroNucleus

IARC – International Agency for Research on Cancer

ILO - International Labour Organization

ISO - International Standardization Organisation

IUPAC – International Union of Pure and Applied Chemistry

LOD – Limit of Detection

LOQ – Limit of Quantification

MGG – May-Grünwald Giemsa

MN – Micronuclei

MNT – Micronucleus Test

MRL - Minimal Risk Level

MS - Mass Spectrometry

NAT – N-acetyltransferase

NBUD – Nuclear Buds

NCI - National Cancer Institute

NER – Nucleotide Excision Repair

NHEJ - Non Homologous End Joining

NIOSH – National Institute for Occupational Safety and Health

NNK – Nicotine derived Nitrosamino Ketone

NOAEL – No-Observable Adverse Effect Limit

NPB – Nucleoplasmic Bridges

OECD - Organization for Economic Cooperation and Development

OEL - Occupational Exposure Limits

OGG1 - 8-Oxoguanine DNA Glycosylase 1

OR – Odds Ratio

OSHA – Occupational Safety and Health Administration

PAH - Polycyclic Aromatic Hydrocarbons

PCR – Polimerase Chain Reaction

PEL - Permissible Exposure Limit

PID – Photo Ionization Detection

PTX - Paclitaxel

RAGE - Receptor for Advanced Glycation End-products

RAR - Retinoic Acid Receptors

RDA - Recommended Dietary Allowances

RFLP – Restriction Fragment Length Polymorphism

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

RXR - Retinoid X Receptors

SCE – Sister Chromatid Exchanges

SCGE – Single Cell Gel Electrophoresis

SCOEL - Scientific Committee on Occupational Exposure Limits

SNP – Single Nucleotide Polymorphisms

SPSS – Statistical Package for the Social Sciences

STEL – Short Term Exposure Limit

TA – Tocopheryl Acetate

TLV - Threshold Limit Value

TWA – Time Weighted Average

VDR – Vitamin D Receptor

WHO - World Health Organization

XRCC3 – X-Ray Cross-Complementation 3

5-FU - 5- Fluorouracil

8-OHdG - 8-hydroxydeoxyguanosine

8-OHG - 8-hydroxyguanine

8-oxoG – 2-oxo-7,8-dihydro-2’-deoxyguanosine

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

1. GENERAL INTRODUCTION

Molecular epidemiology intends to provide reliable and specific information regarding the etiology and mechanism of disease processes, which can be associated with environmental exposures related to lifestyle, occupation, or ambient pollution, in order to achieve prevention at the community level; molecular epidemiology employs laboratory methods to document the molecular basis and preclinical effects of environmental carcinogenesis (Portier & Bell, 1998; Vainio, 1998; Bartsch, 2000; Dusinska & Collins, 2008).

The possibility to use a biomarker to substitute classical endpoints, such as disease incidence or mortality, is the most promising feature and one that is most likely to affect public health. Resorting to events that are on the direct pathways from the initiation to the occurrence of disease as surrogates for disease incidence is a very appealing approach, being investigated in different fields (Bonassi & Au, 2002). The ultimate goal of molecular epidemiology is the prevention of disease, and particularly cancer.

Human biomonitoring consists in the search for specific biomarkers in biological samples, with the goal of preventing the harmful accumulation of dangerous substances (Sexton *et al.,* 2004; Bertazzi & Mutti, 2008; Manno *et al.,* 2010). Biological monitoring has applications in exposure assessment and in occupational health; by measuring human exposure to chemical substances and providing unequivocal evidence that both exposure and uptake of chemical or physical substances have been taken place (Sexton *et al.*, 2004; Angerer *et al.,* 2007). In order to predict disease risk and/or to monitor the effectiveness of control procedures aimed at avoiding exposure to genotoxic chemicals in occupational and environmental settings, biomarkers of effect, specifically of genotoxicity, have been used (Manno *et al.*, 2010). The most frequently used endpoints in human biomonitoring studies have been the cytogenetic biomarkers (Barrett *et al.*, 1997; Battershill *et al.*, 2008).

Major goals of many of the research programmes on biomonitoring are the development and validation of biomarkers that reflect specific exposures and predict the risk of disease in individuals and in population groups (Watson & Mutti, 2004). From an individual point of view, it has long been speculated that genetically determined susceptibility may predispose some workers to occupational disease whereas others in the same environment seem to be unaffected. Recognition of the role of genetic factors in disease (both occupationally and non-occupationally related disease) presents new opportunities for detection, prevention, and treatment (U.S. Congress, 1990; Vähäkangas, 2008).

For environmentally induced diseases, biomarkers of susceptibility play a key role in understanding the relationships between exposure to toxic environmental chemicals and the development of chronic diseases as well as in identifying individuals at increased risk. They may also inform about inter-individual variation in response to a variety of factors (Dusinska & Collins, 2008; Vähäkangas, 2008).

Dietary habits are recognized to be an important modifiable environmental factor influencing cancer risk and tumour behaviour, being estimated that about 30-40% of all cancers are related to dietary habits (Strickland & Groopman, 1995; Davis & Milner, 2007; Sutandyo, 2010). Nutrition science has evolved into a multidisciplinary field that applies molecular biology and integrates individual health with epidemiologic investigation at the population level (Go *et al.*, 2003).

In laboratory context, there are many chemical agents that are handled occupationally by a wide range of workers. The International Agency for Research on Cancer (IARC) classifies two important chemical agents handled in occupational settings as being human carcinogens (group 1): formaldehyde (IARC, 2006) and cytostatics drugs (IARC, 1981; 1987).

Formaldehyde is a colourless gas that has been considered carcinogenic to humans, making it a subject of major environmental concern, namely in occupational settings where employees in industrial and medical areas, in particular anatomists and medical students may be highly exposed to formaldehyde gas (Gulec *et al.,* 2006). Epidemiological studies linked formaldehyde exposure to higher risk of nasopharyngeal, lung, brain, pancreas, prostate, colon, and lymphohematopoietic cancers (Walrath & Fraumeni, 1983; Hall *et al.*, 1991; Hauptmann *et al.*, 2004; Pinkerton *et al.*, 2004; Orsière *et al.*, 2006; Freeman *et al.*, 2009; Hauptmann *et al*., 2009; Zhang *et al.*, 2009a, 2010; NTP, 2011) in the industry, for embalmers, pathologists and anatomist workers.

Cytostatics drugs allow the inhibition of tumour growth by disrupting cell division and killing actively growing cells, being for that reason widely used in the treatment of cancer and in some non-neoplasic diseases too. Nevertheless, have been proved to be also mutagens, carcinogens and teratogens (Fucic *et al.*, 1998; Burgaz *et al.*, 1999; Sessink & Bos, 1999; Bouraoui *et al.*, 2011; Gulten *et al.,* 2011; Buschini *et al.,* 2013).

Epidemiological studies (NIOSH, 2004) have related cytostatic workplace exposure health effects such as skin rashes, hair loss, irritation, hypersensitivity, headaches, spontaneous abortions, malformations, infertility, and possibly leukemia, as well as other cancers (Kolmodin-Hedman *et al.,* 1983; Stücker *et al.,* 1990; Froneberg, 2006; Harrison, 2006;

Fransman *et al.*, 2007; Hedmer *et al.*, 2008; Kopjar *et al.*, 2009; Chu *et al.*, 2011; Stover & Achutan, 2011).

2. RESEARCH QUESTION

The main purpose of the research herein presented was to address the following question:
Is it possible to implement a human biomonitoring programme using genotoxicity, susceptibility, and nutrigenetic biomarkers to assess occupational health?

3. OBJECTIVES

3.1. GENERAL OBJECTIVE

The main objective of this investigation was the development of biomonitoring methodologies for genotoxicity assessment of chemical agents in occupational settings with exposure to formaldehyde and to cytostatic drugs. For that purpose, genotoxicity biomarkers and oxidative damage in DNA were studied, as well as how they relate to genetic susceptibility biomarkers and lifestyle factors, such as tobacco habits, alcohol consumption and diet.

3.2. SPECIFIC OBJECTIVES

To achieve the general objective and address the investigation question, more specific objectives have been established:

1. Determine genotoxicity by cytokinesis-blocked micronucleus assay, measuring micronuclei, nucleoplasmic bridges, and nuclear buds in peripheral blood lymphocytes and the micronucleus test in exfoliated cells from buccal mucosa;

2. Determine DNA damage and DNA oxidative damage (8-hydroxydeoxyguanine) by comet assay in peripheral blood lymphocytes;

3. Investigate the association between genomic damage measured by the previous biomarkers, and genetic polymorphisms in DNA repair genes (*XRCC3, OGG1*), genes of metabolic enzymes (*ADH5*) and vitamin D receptor (*VDR*) by Real Time PCR;

4. Verify if antioxidants such as vitamins A, D and E, measured in human serum by HPLC and ELISA, reflect differences in the effect biomarkers measured;

5. Substantiate the association between the vitamins quantified (Vitamin D) and genetic polymorphisms;

6. Investigate the influence of dietary intake of calories, vitamins A, D, E, B12, folate, iron, and selenium assessed by food frequency questionnaire, upon the genotoxicity biomarkers measured.

The conduction of these objectives is intended to contribute for the building of a battery of biomarkers to be considered in human biomonitoring programmes.

4. THESIS ORGANIZATION

The thesis is organized in VIII sections: I. Introduction, II. State of the art, III. Methodology, IV. Results, V. Discussion, VI. Conclusions and Perspectives, VII. References, and VIII. Annexes.

The Introduction provides a general contextualization of the study focusing in the adequacy and relevance of this, and of the questions that will be addressed later.

The state of the art section is divided in nine chapters, each providing background to understand and contextualize corresponding topics in this study, namely: Molecular epidemiology, Human biomonitoring, Biomarkers, Genomic instability, Genotoxicity assessment methods, Individual susceptibility, Nutritional research, Formaldehyde, and Cytostatics.

The section on Methods presents the study design, describes how samples were collected and processed, which variables were measured, as well as the statistical methods used.

Since there are two distinct occupational contexts under study, the Results section is divided into chapter 1 for the formaldehyde occupational setting, and chapter 2 for the cytostatics setting.

The same structure was used in the Discussion section, as it was divided by chemical agent. Finally, Conclusions and Future perspectives are presented in section VI. Questionnaires, informed consent, and publications are in the Annexes (section VIII), right after the References (section VII).

II. STATE OF THE ART

CHAPTER 1 - MOLECULAR EPIDEMIOLOGY

1. CLASSICAL EPIDEMIOLOGY

Traditional epidemiology has always been the hallmark approach to demonstrate associations between exposure to hazardous substances and development of disease. When disease is cancer, the endpoints for such investigations are usually mortality and disease incidence. However, such traditional approach is beset by limitations. For example, the results are meant to translate into the implementation of measures of disease prevention to an entire community, with little or no regard for inter-individual variations in response to the exposure. Since such variations play a significant role in determining who is more likely to be affected, they should be taken into account if we are to improve our predictions regarding environmental disease. Furthermore, with increasingly stringent regulations on environmental exposure and with the automation of hazardous processes, namely in the workplace, the exposure concentrations and availability of exposed individuals may become too small to conduct meaningful traditional epidemiological investigations (Bonassi & Au, 2002).

Classical epidemiology studies have made seminal contributions to identifying the etiology of the most common types of cancer and have had substantive public health impact. The IARC evaluated the cancer-causing potential of more than 900 likely candidate items, placing them into the following groups: Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), Group 3 (unclassifiable as to carcinogenicity in humans) and Group 4 (probably not carcinogenic to humans) (IARC, 1989).

Although there has been growing recognition for the need to incorporate complex interactions between environmental exposures together with genetic factors, in order to fully understand cancer causation, the molecular tools to explore these associations were yet to be developed. There is now growing recognition that environmental challenges not only interact with genes but may also modulate genetic effects and influence phenotypes. It is also increasingly recognized that environmental exposures may not only damage DNA but additionally may alter gene expression through epigenetic mechanisms that could be reversible (Spitz & Bondy, 2010).

2. MOLECULAR EPIDEMIOLOGY

The term “molecular epidemiology” made its appearance in the literature in the early 1980s. It was originally conceived as an extension of traditional (classical) epidemiology to incorporate biomarkers (biochemical and molecular) with conventional questionnaire data, in order to further our understanding of mechanisms of carcinogenesis and of events throughout the continuum between exposure and cancer development (Hussain & Harris, 1998; Vineis & Perera, 2007; Spitz & Bondy, 2010).

Molecular biology is a potentially useful tool in epidemiological studies as it can be used to strengthen the identification of cancers associated with environmental exposures related to lifestyle, occupation, or ambient pollution. In molecular epidemiology, laboratory methods are employed to document the molecular basis and preclinical effects of environmental carcinogenesis (Portier & Bell, 1998; Vainio, 1998; Bartsch, 2000; Dusinska & Collins, 2008).

A major objective of molecular epidemiological investigations is to provide reliable and specific information regarding the etiology and mechanism of disease processes in order to achieve disease prevention. The possibility to use a biomarker to substitute classical endpoints, such as disease incidence or mortality, is the most promising feature and one that is most likely to affect public health. The use of events that are on the direct pathways from the initiation to the occurrence of disease as surrogates for disease incidence is a very appealing approach, being investigated in different fields (Bonassi & Au, 2002).

The ultimate goal of molecular epidemiology is the prevention of cancer. Various lines of evidence indicate that the great majority of cancers are, in principle, preventable because the factors that determine cancer incidence are largely exogenous. This evidence comes mainly from epidemiologic studies and includes: (i) time trends in cancer incidence and mortality; (ii) geographic variations and the effects of migration; (iii) the identification of specific causative factors such as cigarette smoking, occupational and environmental chemicals, radiation, dietary factors and viruses; and (iv) the observation that the majority of human cancers do not show simple patterns of inheritance. Genetic factors are clearly important in terms of influencing individual susceptibility to carcinogens; and in certain rare forms of human cancer, hereditary factors play a decisive role. However, external factors represent also the greatest opportunity for primary prevention. This is an optimistic message because it means that the development of cancer is not an inherent consequence of the aging process *de per se*, and the human species is not inevitably destined to suffer a high incidence of cancer. This awareness has lent greater urgency to the search for more powerful tools for primary prevention, for

early warning systems to identify causal environmental agents and flag risks well before the malignant process is entrenched (Perera & Weinstein, 2000).

Molecular epidemiology has the advantage of being directly relevant to human risk, unlike animal or other experimental models that require extrapolation to humans. In contrast to traditional epidemiology that relies on cancer incidence or mortality as the endpoint, molecular epidemiology has the potential to give early warnings by flagging the preclinical effects of exposure and increased susceptibility, thus signalling opportunities to avert cancer through timely intervention. Moreover, biomarker data on the distribution of procarcinogenic changes and of susceptibility factors in the population can improve the estimation of cancer risk from a given exposure. However, molecular epidemiology is also subject to many of the limitations of epidemiology, such as the vulnerability to confounding factors that give rise to misleading results (Perera, 1996).

Molecular epidemiology has become a major field of research, leading to considerable progress in the validation and application of biomarkers. One of its greatest contribution has been the insight provided into inter-individual variation in human cancer risk and into the complex interactions between environmental factors and host susceptibility factors, both inherited and acquired, throughout the multistage process of carcinogenesis (Perera, 1996; Bartsch, 2000; Perera & Weinstein, 2000; Weis *et al.*, 2005). Figure 1 compiles the multiplexicity of human cancer risk assessment using molecular epidemiology tools (interligation between internal exposure assessment and susceptibility), and bioethical issues associated and intervention strategies. Increasingly, molecular epidemiology studies are incorporating panels of biomarkers relevant to exposure, preclinical effects and susceptibility, using samples of blood cells, exfoliated cells, tissues and body fluids. These biomarkers are now being widely used in cross-sectional, retrospective, prospective and nested case-control epidemiologic studies, with the aim of improving our understanding of the causes of specific human cancers (Perera & Weinstein, 2000; Au, 2007).

Many of the biomarkers used in molecular epidemiologic studies require further validation (Perera & Weinstein, 2000) as it will be discussed in the Biomarkers chapter.

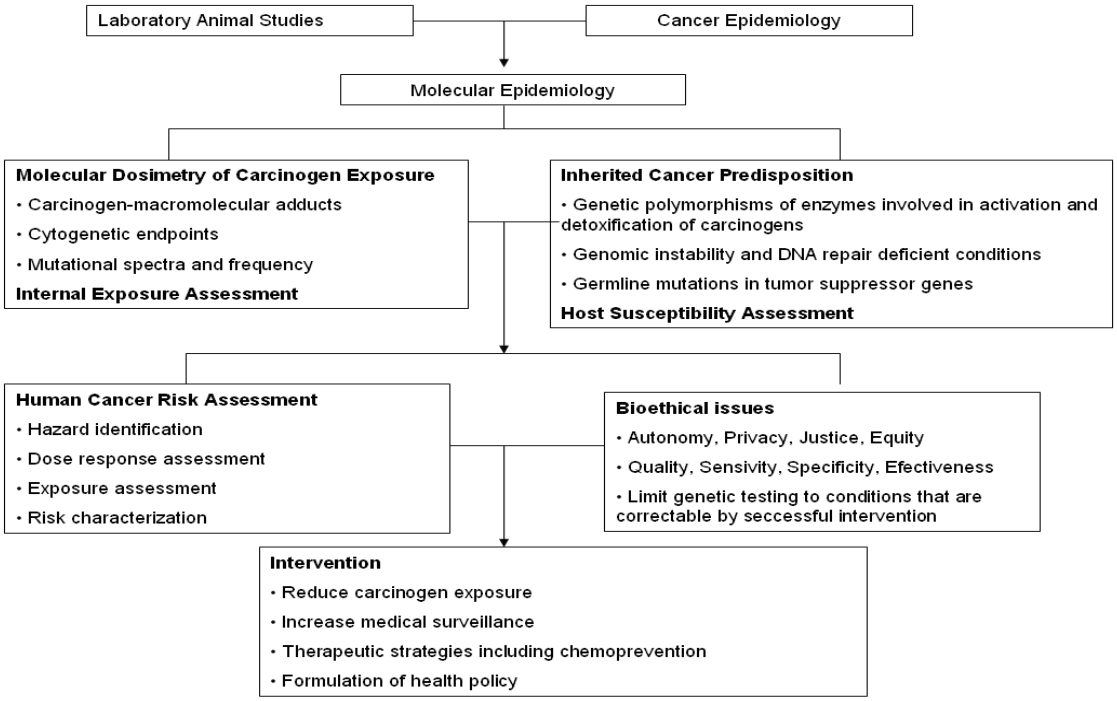


Figure 1 - Paradigm of human cancer risk assessment and bioethical issues associated with molecular epidemiology and human cancer. Adapted (Hussain & Harris, 1998).

2.1. CAPABILITIES OF MOLECULAR EPIDEMIOLOGY

Molecular epidemiology has many capabilities, namely the delineation of a continuum of events between exposure and disease, the identification of exposures and dose reconstruction, the identification of events earlier in the natural history of disease, the reduction of variable misclassification, the indication of mechanisms, and the enhanced individual and group risk assessment (Spitz & Bondy, 2010).

Using different combinations of biomarkers, molecular epidemiology has reinforced prior evidence that risk from carcinogenic exposures can vary significantly with ethnicity, age or stage of development, gender, pre-existing health impairment and nutritional factors. Biologically based inter-individual variation in only a few susceptibility factors can lead to a significant increase in population risk over what would be expected based on the assumption of uniform susceptibility, possibly by an order of magnitude or more (Perera, 2000; Perera & Weinstein, 2000; Weis *et al.*, 2005).

The availability of risk estimates based on the frequency of a biomarker in healthy individuals would be a formidable tool for any cancer prevention initiative and is the reason that justifies

the interest of molecular epidemiologists in prospective human cohort studies. The validation of candidate biomarkers for long-term risk prediction is a priority with special attention given to those biomarkers – as in the case of micronuclei that are possibly affected by the presence of the disease, i.e. the so called "reverse causality"; in this case, cohort studies are preferable for biomarker validation (Bonassi & Au, 2002) as all individuals are known to be disease-free at the time of biomarker evaluation, with disease eventually arising thereafter.

There has been dramatic progress in the application of biomarkers to studies of cancer causation in humans. Progress has been made in the development and validation of biomarkers that are directly relevant to the carcinogenic process and that can be used in large-scale epidemiologic studies. Study designs have become increasingly complex, with greater attention to the need to incorporate appropriate controls and account for potential confounders. A number of longitudinal or nested case-control studies have been undertaken to establish the predictive value of biomarkers. However, as knowledge of mechanisms in carcinogenesis has evolved, the available armamentarium of biomarkers is no longer sufficient. The majority of the available biomarkers used in molecular epidemiology studies relate to agents that cause DNA damage and are mutagenic (Perera & Weinstein, 2000).

A recent trend that brings together cancer researchers interested in cancer epidemiology, chemoprevention and therapy is the increasing recognition that biomarkers developed in the field of molecular epidemiology may also be useful as early or intermediate endpoints in studies of cancer prevention by identifying "at risk" populations and then assessing the efficacy of various types of intervention (Perera & Weinstein, 2000).

The field of molecular epidemiology is especially relevant to the very promising and rapidly expanding field of cancer chemoprevention, i.e. the use of specific, synthetic or naturally occurring compounds to inhibit the carcinogenic process before the development of malignant tumours (Perera & Weinstein, 2000). The molecular epidemiology approach, measuring molecular or cellular biomarkers as indicators of disease risk or of exposure to causative or preventive factors, has applications in studies of environmental and occupational exposure, disease etiology, nutrition, lifestyle and others.

It is a valuable adjunct to conventional epidemiology, and has the advantage that it requires far fewer subjects and much less time (being therefore more cost-effective) than the traditional approach. In addition, the biomarkers, if carefully chosen, can give useful information about molecular mechanisms involved in disease etiology, like for example if they reflect an early stage in disease progression (Collins & Dusinska, 2009).

In conclusion, the potential benefits of biomarkers and molecular epidemiology in cancer prevention justify a major commitment to the further development and use of this approach and to addressing the ethical concerns involved in its application to cancer prevention (Perera & Weinstein, 2000).

3. CANCER EPIDEMIOLOGY

Most cancers results from man-made and natural environmental exposures (such as tobacco smoke; chemical pollutants in air, water, food, drugs; radon; and infectious agents) acting in concert with both genetic and acquired characteristics of an individual (Perera, 1996, 2000). It has been estimated that without these factors, cancer incidence would be dramatically reduced, by as much as 80%-90%. Cancer risk from these environmental carcinogens is strongly influenced by many factors, including genetics, age, ethnicity, gender, immune function, pre-existing disease, and nutrition (Perera, 1996; Wild, 2009). The majority of cancer epidemiology studies were limited to assessing possible causative associations between two types of events: exposure to potential causative "environmental" agents (i.e. cigarette smoke, specific chemicals in the workplace, dietary factors, etc.) and disease outcome (i.e. clinically apparent cancer incidence or cancer mortality). In the past, the modulation of environmental factors by host susceptibility was rarely evaluated, but in recent years the interaction between environmental factors and host susceptibility has become a very active area of research (Perera & Weinstein, 2000).

3.1. MECHANISMS IN CARCINOGENESIS

Cancer is a multistage process that results from an accumulation of multiple genetic changes. The concept that genetic susceptibility to development of cancer is related to genomic instability was initially supported by rare disorders such as ataxia telangiectasia and *xeroderma pigmentosum*, which are associated with *in vivo* and *in vitro* chromosomal instability and defective DNA repair capacity. It is now established that maintaining the integrity of the genome is essential for normal cell function and any disruption in the process can lead to either cell death or cancer development (El-Zein *et al.*, 2011).

Carcinogenesis would thus be a sequence of more or less stochastic transitions including gene mutations and cell proliferation. In addition, carcinogenesis would be a multifactorial process, i.e., different external exposures would be able to affect it and few of them, if any, would be necessary (Vineis & Porta, 1996). As a consequence, confounding, (i.e., the interplay of

multiple concurrent exposures in the interpretation of cause-effect relationships) is necessarily required to be considered in any study of carcinogenesis (Vineis & Porta, 1996). The carcinogenic process was pictured as an orderly progression of the cell through three distinct stages: initiation by exposure to genotoxic agents; tumour progression by agents that stimulated the initiated cell to proliferate and expand clonally to form a benign tumor; and progression, in which the accumulation of additional genetic damage in the expanding population of initiated cells caused the tumor to become malignant. This simplified model has been modified by the discovery that cancer results from a succession of genetic and epigenetic events whose order may vary. Carcinogens are now understood to be remarkably versatile, able to derail gene function by inducing mutations or by disrupting gene expression or both. So-called "nongenotoxic" agents, such as chlorinated organic compounds, hormones, and asbestos, are known to indirectly damage the genes via a number of different mechanisms, including alterations in gene expression and oxidant formation (Perera, 1996; Waters *et al*., 1999).

The current paradigm holds that cancer results from the accumulation of changes in the structure or expression of certain key genes by mechanisms as varied as point mutation induced by carcinogen-DNA binding, gene amplification, translocation, chromosomal loss, somatic recombination, gene conversion, or variation in DNA methylation patterns. At the center of the paradigm are the oncogenes and tumor suppressor genes that code for proteins serving as "relays" in the regulatory circuitry of the cell. Damage to these target genes can result in altered protein products or abnormal amounts of normal proteins, leading to deregulation of cell growth and differentiation (Perera, 1996).

4. RISK ASSESSMENT

Risk assessment has been the traditional tool to derive acceptable tolerable levels of exposure to environmental chemicals. It entails the evaluation of available scientific information on the biological, epidemiological and toxicological properties of an agent leading to an informed judgement about the potential for adverse effects in humans under defined exposure conditions (Greim *et al.,* 1995; Di Marco *et al.,* 1998). Effectively linking molecular epidemiology to risk assessment and health policy formulation will, in most cases, require additional research to confirm and to further elucidate many of the reported interactions between specific environmental exposures and susceptibility factors (e.g. gene-environment, gene-gene-environment, and gene-nutrition-environment interactions). But the data, taken

together, have bearing on fundamental principles of risk assessment (WHO, 1995; Perera, 2000).

Risk assessment aims to quantify the probability that a specific agent or chemical will give rise to an adverse effect. The principal factors that affect this include the chemical itself, its use and exposure levels; and susceptibility of exposed individuals. Even very toxic substances may not pose any significant risk to human health if exposure levels are negligible or the number of exposed and susceptible subjects is very low. On the other hand, even substances with low toxicity may cause serious concern when exposure is at levels sufficient to give rise to biologically effective doses and involves a high number of susceptible individuals (Mutti, 1999; Watson & Mutti, 2004).

The carcinogenic process involves the accumulation of genetic changes that can be facilitated by many susceptibility factors that render individuals vulnerable (Perera, 1996). However, conventional risk assessment methods may underestimate the individual's risk for exposures to environmental carcinogens because of the default assumption that all individuals within a certain population possess equal susceptibility to a specific carcinogen dose (Perera, 1996; Ketelslegers *et al.*, 2008). An individual's risk for developing cancer depends on both inherited and environmental factors; exposure to specific carcinogens is clearly related to increased cancer risk. Individual susceptibility varies greatly, however, and may be the factor that determines who will develop cancer (Vainio, 1998).

Occupational risk assessment may be defined as the qualitative and quantitative characterization of an occupational risk, i.e. the probability that an adverse health effect may result from human exposure to a toxic agent which is present in the occupational setting. It has three fundamental tools: environmental monitoring, health surveillance and biological monitoring. Risk assessment is meant to quantify the likelihood that a quantitatively defined occupational exposure of an individual (or group of individuals) to a chemical might result in adverse health effects. The level of probability essentially depends on three elements: the intrinsic potency/characteristics of the risk factor itself (hazard identification/assessment), the level/type/duration of exposure (dose-response/exposure assessment) and the degree of individual susceptibility, as represented by the following simple equation (Di Marco *et al.*, 1998; Perera, 2000):

Risk = Hazard x Exposure x Susceptibility

Each of the three components must be different from zero. It also indicates that the same level of risk may be achieved by various associations of different levels of each of the three components. Highly intrinsically toxic chemicals, including carcinogens, at low levels of exposure and/or susceptibility may provide a similar level of risk as would less toxic ones at higher levels of exposure and/or susceptibility (Perera, 2000).

Exposure assessment is much more complex in epidemiologic studies because human exposure does not occur in controlled conditions. Focusing on improving exposure assessment in human studies is worthwhile because, although human studies are usually more difficult to conduct, they provide valuable additional information to the risk assessment process. Typically, human studies substantially decrease the uncertainties of human risk assessment (Pirkle & Sampson, 1995).

In occupational situations, the number of individuals is usually relatively small, and therefore the main determinant is the exposure level. However, for environmental exposures a large number of individuals might receive a biologically effective dose resulting from relatively low exposure levels, and individual susceptibility may represent the main risk determinant (Watson & Mutti, 2004).

Major gains in cancer prevention should stem from theoretically important strategies, namely regulations, public education programs, health surveillance, behaviour modification, and chemoprevention programs and other interventions that adequately protect these groups from environmental carcinogens (Perera, 1996, 2000).

Despite eventual weaknesses and methodological liabilities, molecular biomarkers studies are likely to provide valuable tools for risk assessment and the prevention of environmental cancer (Husgafvel-Pursiainen, 2002).

Regarding risk assessment, other than the concept of susceptibility it is also worth mentioning the possible existence of very vulnerable sub-populations who are embedded in the main population. Certain groups (those with genetic, ethnic, or gender-related traits conferring susceptibility, the young; the elderly; and persons with pre-existing disease or immunologic or nutritional deficits) are likely to be at greater risk than other members in the population who are similarly exposed (Perera, 1996; Ketelslegers *et al.*, 2008).

Risk estimates may become seriously inflated when such subgroups comprise an important proportion of the main population (Hines *et al.,* 2010a).

Molecular epidemiology and biomonitoring studies have provided mechanistic data on carcinogens that have been used in risk assessment and in some cases regulation of these same carcinogens. The Monographs on the Evaluation of Carcinogenic Risks to Humans have

been published by the IARC since 1971 as a guide to regulatory and public health agencies in their decision making. Since 1997 (Monograph 54), mechanistic evidence, including biomarker data in humans or animals, has been used to "upgrade" or "downgrade" the classification of carcinogens (Vineis & Perera, 2007). Future research will improve the current assays used by molecular epidemiology, making them simpler, cheaper, and more reliable, but these are only tools needed for human biomonitoring (Albertini *et al.*, 1996).

CHAPTER 2 - HUMAN BIOMONITORING

1. BIOMONITORING

Human biomonitoring has its roots in the analysis of biological samples, aimed at looking for markers of pharmaceutical compounds and occupational chemicals, in an effort to prevent the harmful accumulation of dangerous substances (Sexton *et al.*, 2004). Biological monitoring is defined as the repeated, controlled measurement of chemical or biochemical markers in fluids, tissues or other accessible samples from subjects exposed (or exposed in the past or to be exposed) to chemical, physical or biological risk factors in workplace and/or the general environment (Bertazzi & Mutti, 2008; Manno *et al.,* 2010). Major goals of many of the research programmes on biomonitoring are to develop and validate biomarkers that reflect specific exposures and to predict the risk of disease in individuals and in population groups (Watson & Mutti, 2004).

Biomonitoring has many advantages over traditional methods. For example, biological samples reveal the integrated effects of repeated exposure. Also, this approach documents all routes of exposure – inhalation, absorption through the skin and ingestion, including hand-to-mouth transfer in children. Such specimens also reflect modifying influences in physiology, bioavailability and bioaccumulation, which can magnify the concentrations of some environmental chemicals enough to raise them above detection thresholds. Perhaps most importantly, these tests can help establish correlations between exposure and subsequent illness in individuals – which is often the key observation to prove whether or not a link exists between both (Sexton *et al.*, 2004; Angerer *et al.*, 2007). The advantages of human biomonitoring for the individuals being studied include: identification of exposure, identification of environmental mutagens/carcinogens, and determination of the possible range of susceptibility of humans to specific mutagens and carcinogens (Valverde & Rojas, 2009). In summary, nowadays human biomonitoring of dose and biochemical effect has tremendous utility providing an efficient and cost effective means of measuring human exposure to chemical substances providing unequivocal evidence that both exposure and uptake have been taken place (Sexton *et al*., 2004; Angerer *et al.*, 2007). Human biomonitoring considers all routes of uptake and all sources which are relevant making it an ideal instrument for risk assessment and risk management. It can identify new chemical exposures, trends and changes in exposure, establish distribution of exposure among the general population, identify vulnerable groups and populations with higher exposures and

identify environmental risks at specific contaminated sites with relatively low expenditure (Angerer *et al.*, 2007).

1.1. BIOLOGICAL MONITORING IN OCCUPATIONAL CONTEXT

Biological monitoring has applications in exposure assessment and in occupational health. The term "biological monitoring" has come into use as a natural adaptation of the term environmental monitoring, i.e. the periodic measurement of the level or concentration of a chemical, physical or biological risk factor in the workplace environment, which is traditionally used as an indirect measure of human exposure. Measurements of the concentration of substances or their metabolites in urine, for example, can provide useful information to assess inadvertent ingestion, but only in conjunction with measurements of exposure by other relevant routes such as inhalation and/or dermal. When compared with environmental monitoring, biological monitoring provides additional information which can be effective in improving occupational risk assessment at the individual and/or group level (Manno *et al.*, 2010).

Biological monitoring of workers has three main goals: the first is individual or collective exposure assessment, the second is health protection, and the ultimate objective is occupational health risk assessment. It consists of standardized protocols aiming to the periodic detection of early, preferably reversible, biological signs which are indicative, if compared with adequate reference values, of an actual or potential condition of exposure, effect or susceptibility, possibly resulting in health damage or disease. These signs are referred to as biomarkers (Manno *et al.*, 2010).

Another important application of biological monitoring, besides exposure assessment, is the use of biomarkers, at either individual or group level, for the correct interpretation of doubtful clinical tests. These are usually performed as part of occupational health surveillance program when exposure assessment data are unavailable or are deemed unreliable. Biomarkers are usually more specific and sensitive than most clinical tests and may be more effective, therefore, for assessing a causal relationship between health impairment and chemical exposure when a change is first detected in exposed workers (Valverde & Rojas, 2009a; 2009b; Manno *et al.*, 2010).

Experience in biological monitoring gained in the occupational setting has often been applied to assess (the effects of) human exposure to chemicals in the general environment. The use of biological fluids/tissues for the assessment of human exposure, effect or susceptibility to chemicals in the workplace represents, together with the underlying data (e.g. personal

exposure and biological monitoring measurements), a critical component of the occupational risk assessment process, a rapidly advancing science (Manno *et al.*, 2010). In environmental epidemiological studies, biological measures of exposure should be preferred, if available, to environmental exposure data, as they are closer to the target organ dose and provide greater precision in risk estimates and in dose-response relationships (Manno *et al.*, 2010).

Based on the recognition that certain disease can be caused by exposure to environmental contaminants, the movement for prevention of environmental disease has gained broad-based public support for decades and, the public and the regulatory agencies are demanding more reliable information on health risk from environmental contaminants (Au *et al*., 1998).

Au *et al.* (1998) advise on putting more emphasis upon monitoring populations which are known to be exposed to hazardous environmental contaminant and on providing reliable health risk evaluation. The information can also be used to support regulations on protection of the environment. Two issues are crucial in the application of predictive biomarkers to public health policies. The first is dealing with the meaning of altered levels of predictive biomarkers at individual level. A conservative and traditional approach is that of considering risk predictions valid only at group level. This interpretation allows cutting down the effect of inter-individual variability and reduces the variability due to technical parameters. On the other hand, variability is a fundamental source of information. In addition, differences among individual should not be viewed as a nuisance but should be seen as useful hints in the hypothesis generation and as an enhanced possibility to apply preventive measures in subsets of high risk subjects. The second is crucial aspect is the validation issue. A biomarker must be validated before it can be used for health risk assessment, especially as far as regulatory aspects are involved. Despite the characterization of valid biomarkers is a leading priority in environmental research, defining validity is troublesome. Validity is a general concept that refers to a range of characteristics of the biomarker, and an impressive amount of literature has been published on the concept of biomarker validity and the various aspects of the validation process (Bonassi & Au, 2002).

The International Labour Organization (ILO) has recommended that occupational health goals for industrial nations focus on the hazards of new technology among which pharma and biopharma products are leaders. Their unchecked growth cannot continue without parallel commitment to the health and safety of workers encountering these “high tech” hazards. Improving the present state therefore requires: (i) recognizing healthcare as a “high-hazard” employment sector; (ii) fortifying voluntary safety guidelines to the level of enforceable regulation; (iii) “potent” inspections; (iv) treating hazardous pharmaceuticals like the chemical

toxicants they are; and (v) protecting health care workers at least as well as workers in other high-hazard sectors (McDiarmid, 2006).

2. HUMAN GENOME-ENVIRONMENT

The relative contribution of genetics *versus* the environment to human illness has been debated for decades. The importance of environmental exposures has been supported by geographic differences in incidence of disease, by variation in incidence trends over time, and by studies of disease patterns in immigrant populations (Olden & Guthrie, 2001).

Genetic polymorphisms (changes in DNA sequence) often affect the function of a gene but some may change the level of expression of a gene or change the activity of the gene product, for example, an enzyme. Genetic polymorphisms that are functionally significant are quite important when the gene controls the response of an organism to environmental hazards (Barrett *et al.*, 1997). Given that a large number of genes are involved in responses to environmental hazards and that a large number of polymorphisms exist in these genes, genetic differences are important susceptibility factors in environmental responses (Barrett *et al*., 1997).

There is an important difference between individuals with genetic alterations that lead to disease susceptibility and individuals with genetic susceptibility to environmental factors. Individuals who inherit a mutation in a disease susceptibility gene have a high risk of developing that disease regardless of environmental exposures, although environmental factors may increase the incidence or rate of disease development (Barrett *et al.*, 1997).

Conceptually, the relationship between genes and the environment can be described as a loaded gun and its trigger. A loaded gun by itself causes no harm; it is only when the trigger is pulled that the potential for harm is released or initiated. Likewise, one can inherit a predisposition for a devastating disease, yet never develop the disease unless exposed to the environmental trigger(s) (Olden & Guthrie, 2001). Individuals who have a mutation or polymorphism in genes involved in response to environmental hazards will only have an increased risk of disease development when they are exposed to specific environmental hazards (Barrett *et al.*, 1997). Particularly in the case of low-dose toxicants, the interactions of susceptibility genes with specific environmental factors are probably the dominant cause of any resultant human illness. Therefore, the identification of susceptibility alleles can provide a unique opportunity to tear apart the effects of genes and the environment on the risk of disease (Olden & Guthrie, 2001). However, the probability that an environmental exposure will cause illness is dependent on the capacity of the genetically-controlled metabolic

machinery and repair mechanisms of the cell to modulate adverse influences of xenobiotics (Olden & Guthrie, 2001).

Therefore, risk to these individuals is influenced strongly by gene-environment interaction. Also, because multiple genes are involved in response to the same environmental hazard, two individuals with the same genetic susceptibility and environmental exposure may have different risks because of the interplay between genes involved in response to xenobiotics. For example, two individuals may both have a polymorphism in a gene that increases the rate of carcinogen activation but different polymorphisms in a gene that inactivates the same carcinogen (Barrett *et al.*, 1997). Twin-cohort studies, the *"gold standard"* for distinguishing between the contributions of genetics *versus* the environment, suggest that the environment plays a prominent role in disease development (Olden & Guthrie, 2001).

Understanding risks to human health in light of the human genome-environment interaction is one of the most compelling challenges in environmental public health. With approximately 99.9% of human genomes being identical, the remaining 0.1% (or about 3 million base pairs) appears to dictate differences in susceptibility to environmental challenges among human populations (Toscano & Oehlke, 2005). It is now apparent that most diseases are not carried in our genes as if these were deterministic factors of disease, but rather our genomes carry variations that result in differences in susceptibility to disease. With the sequencing of the human genome, renewed interest in understanding the role of the environment as a cause of human disease has re-emerged. Genes are expressed in response to the environment (Toscano & Oehlke, 2005) and there are two kinds of susceptibility genes; those that predispose to disease without exposure to environmental factors and those that increase risk only by interaction with environmental agents (Olden & Guthrie, 2001). Information about environmental risk factors should point to genes that might modify the risk, and identification of susceptibility genes should help identify previously unrecognized environmental risk factors (Olden & Guthrie, 2001).

3. VARIABILITY

Variability is an intrinsic feature of both biological and exposure measurements. Several biological and sampling/analytical sources of variability may influence biomarker levels and, therefore, taking variability factors into consideration will make the interpretation of biological monitoring data easier. In fact, it is important, when interpreting new biological monitoring data, not to "remove" biological variance but rather to uncover and explain it. In

other words, variability in biological monitoring may become a resource more than a limitation. Many of the variables that affect biological monitoring results are actually helpful to achieve a better indication of systemic exposure (Manno *et al.*, 2010). Study design is critically important: exposed groups should be matched with respect to gender, age, smoking habit, alcohol consumption, nutrition and lifestyle with control (referent) groups. Inclusion and exclusion criteria have to be clearly defined and confounding factors (such as age, gender and smoking), which influence the background level of DNA damage and may bias the study, should be taken into consideration. Environmental and occupational monitoring relies on data from exposure measurement and personal monitoring, and information on dose-response relationships is valuable, if available (Dusinska & Collins, 2008).

4. LIMITATIONS

Biological monitoring advantages are matched by some important limitations. One of them is that one cannot tell from biological monitoring data what source the exposure originated from, e.g. whether the exposure was generated by occupational or non-occupational sources. In order to keep track of what source is investigated, the researcher can use questionnaires to get individual information, collect pre-exposure samples to establish baseline or background levels and/or involve “non-exposed” controls (Manno *et al.*, 2010).

Biomarkers may not be sufficiently specific for assessing exposure to a particulate chemical (e.g. hippuric acid is not very useful as an urinary biomarker of toluene exposure due to high background values from diet usually found in workers). It may not be easy to relate some exposure biomarkers to external exposure levels, and it may be even more difficult to establish a relationship between exposure biomarkers and a biological endpoint such as an adverse response or effect (Manno *et al.*, 2010).

Biomonitoring strategies are not useful at all if the toxic effects are local and/or acute, such as in the case of irritating agents. The collaboration with other disciplines has been extremely fruitful in developing early effect biomarkers (e.g. nephrotoxicity, neurotoxicity, etc.) or biomarkers that may be associated more closely with the development of pathology (e.g. neurobehavioural, reproductive, etc.). In routine use, however, there is a need for standardized, robust methodologies for comparison of test methods between different laboratories. Uniform protocols for establishing detection limits are necessary. Standardized reporting procedures and measurement units as well as an expanded database on “normal” or reference values are all important. Availability of biological reference materials, the benchmarks of accuracy, is also needed. In summary, important infrastructure that is already

available in other areas of routine testing is sometimes needed for a more efficient and effective biological monitoring in occupational health (Manno *et al.*, 2010).

5. GENETIC MONITORING

During the past decades the understanding of genetics has advanced remarkably as new methods for identifying, manipulating, and analyzing DNA have developed. Less well understood, however, is the interaction between the environment and heredity, and the role each plays in sickness and health (U.S. Congress, 1990; Barrett *et al.,* 1997).

Is has long been recognized that there are substantial health risks posed by various workplace environments, risks often associated with exposure to harmful agents such as chemicals and radiations (U.S. Congress, 1990). Indeed, as early as 1938, geneticist J.B.S. Haldane discussed *"sorting out workers according to their susceptibility to occupational hazards"*.

An occupational illness is defined by the USA Department of Labor's Bureau of Labor Statistics as "*any abnormal condition or disorder, other than one resulting from an occupational injury, caused by exposure to environmental factors associated with employment".* This includes acute and chronic illnesses or disease that can be caused by inhalation, absorption, ingestion, or direct contact (U.S. Congress, 1990).

Genetic monitoring involves periodically examining employees to evaluate modifications of their genetic material, e.g., chromosomal damage or evidence of increased occurrence of molecular mutations – that might have evolved in the course of employment. The putative cause is workplace exposure to hazardous substances and the premise is that such changes could indicate increased risk of future illness (U.S. Congress, 1990).

All genetic is not definitely a result of the workplace because ambient exposures, personal habits and lifestyle decisions (e.g., tobacco habits, alcohol consumption, etc.), and age can also induce changes in genetic material; genetic monitoring could detect changes that arise from exposures outside of the workplace. In general, current techniques are not exposure-specific but serve merely as an indicator of recent exposure (U.S. Congress, 1990).

Genetic monitoring could be performed on groups of employees to identify the risk for the exposed group as a whole, to target work areas for increased safety and health precautions, and to indicate a need to lower exposure levels for a group exposed to a previously unknown hazard (U.S. Congress, 1990). Genetic monitoring ascertains whether and individual's genetic material has altered over time. Workplace genetic monitoring is designed to detect the effects of a toxic substance or its byproducts, and to evaluate the genetic damage caused by such a

substance. The objective of these techniques, ultimately, is to predict risk of disease due to genetic damage. When hazards are identified via genetic monitoring, prevention programs can be considered that will reduce exposures. This is of particular concern for certain occupational groups exposed to hazardous substances over many years at much higher concentrations than the general population (U.S. Congress, 1990).

It is well-documented that exposure to some chemical substances and to radiation at high doses causes cancer and genetic mutations (changes in genetic information). Not all mutations, however, cause disease. The damage will be resolved in one of three ways: cell death, successful DNA repair, or viable mutation. It is difficult to establish the causal relationships between the mutation and cancer because of the long latency of human cancer. Nonetheless, the rationale behind the use of genetic damage assays as indicators of exposure is that events observed initially and at high frequencies trigger a process that may ultimately produce abnormal growth (neoplastic changes) in a smaller subset of cells (Figure 2). Such relationships between genes, mutations, and disease are becoming clearer with the development of molecular techniques (U.S. Congress, 1990).

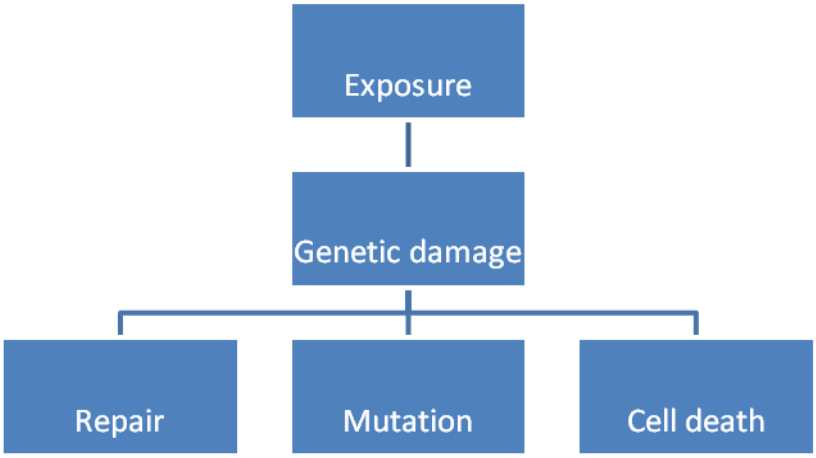


Figure 2 – Biological consequences of exposure to mutagenic agents.

Genetic testing includes a number of technologies to detect genetic traits, changes in chromosomes, or changes in DNA. As used in the workplace, it encompasses two activities: monitoring and screening. Thus, genetic testing of employee populations involves both examining persons for evidence of induced change in their genetic material (monitoring) and methods to identify individuals with particular inherited traits or disorders (screening) (U.S. Congress, 1990). Periodic genetic monitoring of workers could be used to detect induced

genetic change that could indicate an increased risk of certain diseases, in particular cancer. Genetic testing to identify workplace susceptibility and predisposition to disease in essentially healthy people is occurring and the results have been used to making employment decisions. In the UK, the Human Genetic Advisory Commission in 2002 confirmed that this is a real issue by stating that: *"(…) people should not be required to tale a genetic test for employment purposes (…) employers should offer a genetic test if it is known that a specific working environment or practice, while meeting health and safety requirement, might pose specific risks to individuals with particular genetic constitutions"* (Bertazzi & Mutti, 2008).

Genetic monitoring can be viewed as an extension of several types of biological monitoring in the workplace to detect changes or assess exposures that could be associated with increased exposure to occupational or non-occupational risk. Genetic screening, on the other hand, can be used to detect both traits that indicate a predisposition to occupational disease, as well as traits not associated with workplace illness (U.S. Congress, 1990).

Certain environmental agents are known to mutate previously normal somatic cells that could, in some cases, cause disease (U.S. Congress, 1990). The recognition of genetic factors in disease presents new opportunities for detection, prevention, and treatment; being the most validation efforts undertaken in genetic monitoring have been designed to quantify the correlation of mutagenesis with carcinogenesis.

The diseases most associated with genotoxic substances are various forms of cancer. Several types of mutational changes (i.e., point mutations, chromosomal rearrangements) have been associated with the early stage of tumour development, as well as with the following steps of tumour promotion and progression.

Cancers resemble other common diseases in so far as some forms are associated with chromosomal anomalies, others with single mutant genes, or environmental agents. The vast majority, however, are best explained by a genetic-environmental interaction. Clearly, some individuals are predisposed to certain types of cancer given the right environmental exposure.

There are at least 50 human genetic diseases that have been identified as having the potential to enhance an individual's susceptibility to toxic or carcinogenic effects of environmental agents. Occupational exposures have been implicated in lung, bladder, testicular, and laryngeal cancers, as well leukemias. As the connections between cancer and genetics become clearer, so may the relevance of occupational exposure to genetic disease (U.S. Congress, 1990).

Gene-environment interactions will continue to play an increasingly important role in understanding the risk of cancer from environmental and occupational exposures. Studies

involving genetically sensitive populations help to clarify epidemiological data by strengthening the finding and providing insight into mechanisms which can lead to observed patterns of dose *versus* response. Application of these findings to risk characterization will be difficult; for instance, if we know a specific genotype increases the risk of developing cancer in a particular occupational setting, should the worker be dissuaded from taking the employment? Use of this information in environmental settings causes similar problems but provides an advantage in terms of more precise estimates of risk to the general population (Portier & Bell, 1998).

6. SOCIAL, ETHICAL, AND LEGAL IMPLICATIONS

Biomonitoring is one of the best, and probably the most rapidly growing tool available today for the prevention of health effects resulting from occupational exposure to chemicals. Therefore, there is a growing attention towards scientific, ethical issues and social implications that must include individual risk estimation, the communication of epidemiological results, and the translation of epidemiologic data into clinical or occupational health practice (Manno *et al.*, 2010)**.**

The use of human biological samples implies special considerations of information, consent, confidentiality and follow-up as stated in the Declaration of Helsinki (www.wma.net). The collection of samples and personal information about health status used for research and/or surveillance must be preceded by a notification of the project to the ethical committee, including a protocol describing, e.g., the risk of the persons participating, the information (oral or written) given to persons participating and the way of obtaining informed consent (Watson & Mutti, 2004; Knudsen & Hansen, 2007).

According to the International Code of Ethics, biomarkers must be chosen for their validity and relevance for protection of the health of the worker concerned, with due regard to their sensitivity, their specificity and their predictive value and should not be used as screening tests or for insurance purposes (Olden & Guthrie, 2001; Manno *et al*., 2010).

Some of the most relevant ethical issues faced by those involved in biological monitoring, particularly for research purposes, are the following: planning the study, informed consent, confidentiality, communication and susceptibility (Manno *et al.*, 2010). The information about exposure and susceptibility gained by biological monitoring is personal and may predict health impairments. Such information may therefore be discriminative and thus sensitive in relation to future opportunities in occupational health insurance. It is therefore of utmost importance

to keep all information confidential with precise guidelines on who is allowed to use the information (Knudsen & Hansen, 2007).

In straightforward routine biomonitoring programs, communication of individual results (including their interpretation) to each worker and of collective results/interpretation to the employer and to the workers' representatives would be sufficient in most cases. Finally, it is crucial a correct interpretation of individual or collective biomarker data requires a comparison of the results with appropriate reference values obtained in non-exposed but otherwise comparable subjects (Manno *et al.*, 2010).

The study of susceptibility in human populations poses a number of ethical challenges. A special attention should be given to the ethical aspects related with the use of susceptibility biomarkers, namely the benefit to the worker in terms of preventive action and the cost in terms of their possible removal from the job. In principle, biological monitoring should not result in discrimination or reduction of job opportunities for the workers involved.

The recognition of individuals who are subjected to a potentially increased risk of cancer from exposure, particularly occupational exposure, poses the ethical dilemma common to much of the present development of biomarker applications: how to prevent susceptible individuals from being exposed to these chemicals (Barrett *et al.*, 1997).

About genetic screening of workers, many critics have noted the importance of controlling workplace exposures instead of removing susceptible workers ("hypersusceptible") from the workplace.

Ethical considerations should always be borne in mind before biomonitoring programs are to be planned and implemented, particularly when new or partially validated biomarkers are involved. Since the primary purpose of biological monitoring is the protection of the worker's health, it must be avoided that biological monitoring data, whether from exposure or effect or susceptibility biomarkers, could result in an adverse impact on the worker's status of employment and/or quality of life (Manno *et al.*, 2010).

CHAPTER 3 – BIOMARKERS

1. BIOMARKERS – AN OVERVIEW

Biomarkers have been defined by the U.S. National Academy of Sciences Committee on Biological Markers as an alteration in cellular or biochemical components, processes, structure or functions that is measurable in a biological system or sample (National Research Council, 1987), but is not a measure of the disease, disorder or condition itself (Fergurson, 2008). A biomarker can be any substance, structure or process that can be monitored in tissues or fluids and that predicts or influences health, or assesses the incidence or biological behaviour of a disease. Ideally, biomarkers should be accessible (non-invasive), non-destructive and easy and cheap to measure. Identification of biomarkers that are on causal pathway, have a high probability of reflecting health or the progression to clinical disease, and have the ability to account for all or most of the variation in a physiological state or the preponderance of cases of the specified clinical outcome, have largely remained elusive, as one is never quite sure if they fulfill such requirements (Schulte & Mazzuckelli, 1991; Davis & Milner, 2007).

Biological markers can contribute to quantitative risk assessment by helping to: determine the forms of dose-time-response relationships; assess the biologically effective dose; make interspecies comparison of effective dose, relative potency, and effects; resolve the quantitative relationships between human interindividual variability; and identify subpopulation that are at enhanced risk (Schulte & Mazzuckelli, 1991). Nowadays, most research on biomarkers is concerned with markers which will increase our ability to identify long-term risks due to toxicant exposure, in particular the risk of developing cancer; and identify early markers of toxicity in the field of environmental or ecotoxicology. For the past 25 years, biomarkers have been used to identify biological changes due to toxic chemicals and, as part of an integrated approach, in the assessment of environmental health. In the future, many more biological markers predictive of long-term effects, such as chromosomal changes and DNA adducts, will be available, allowing risk assessment judgments to be made (Waterfield & Timbrell, 1999).

The challenge in biomarker research is to facilitate the identification of environmental and genetic factors which modulate cancer risk, a challenge which must be seen in the context of the fact that most environmental carcinogens appear to be associated with relative risks which are so low as to be detectible with difficulty by classical epidemiological methods

(Kyrtopoulos, 2006). A goal in the use of biomarkers must be to identify adverse effects of chemical contaminants at the lowest levels of biological organization, so avoiding toxicological problems at a higher stage (Waterfield & Timbrell 1999).

The traditional, generally accepted classification of biomarkers divides them into three main categories - biomarkers of exposure, effect, and susceptibility; depending on their toxicological significance (Schulte & Mazzuckelli, 1991; Timbrell, 1998; Manno *et al.*, 2010). A biomarker of exposure is defined as *"an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism"* (National Research Council, 1987; Manno *et al.,* 2010). A biomarker of effect is a measurable biochemical, structural, functional, behavioural or any other kind of alteration in an organism that, according to its magnitude, can be associated with an established or potential health impairment or disease. A sub-class of biomarkers of effect is represented by biomarkers of early disease (or early biomarkers of disease), i.e. tests which are more closely indicative of a subclinical effect or even an early, reversible clinical response (Manno *et al.,* 2010). A biomarker of susceptibility may be defined as an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a chemical (Manno *et al.*, 2010). A further discussion of biomarkers of exposure and of effect will be provided below. Although the different types of biomarkers are considered for classification purposes, as separate and alternative, in fact it is not always possible to attribute them to a single category. The allocation of a biomarker to one type or the other sometimes depends on its toxicological significance and the specific context in which the test is being used (Manno *et al*., 2010).

With respect to prevention, the use of biomarkers to quantify interindividual variability in response to exposure has significant implications for carcinogenic risk assessment and associated regulatory actions. The assumption underlying current risk assessment models, that all humans respond homogeneously to a specific carcinogen or mixture of carcinogens, is belied by the large interindividual variation observed within human populations exposed to similar levels of diverse carcinogens.

1.1. BIOMARKERS OF EXPOSURE

The fundamental role of biomarkers of exposure in occupational health practice is to assess exposure by all routes and to complement information obtained by workplace environmental monitoring. For many reasons, such as being more informative, particularly at the individual level; biomarkers of exposure are often used, when available, as a better substitute for

environmental monitoring (Manno *et al.*, 2010), often indicating exposures to environmental pollutants which are important to public health (Angerer *et al.*, 2007).

Exposure biomarkers can reflect bioavailability and be influenced by numerous parameters such as route of exposure, physiological characteristics of the receptor and chemical characteristics of the xenobiotic. Exposure biomarkers have the advantage of providing an integrated measure of chemical uptake, a consideration that is important in the case of agents that exhibit large route-dependent differences in absorption (DeCaprio, 1997; DeCaprio, 1999). Another valuable application of exposure biomarkers is in evaluating the potential of intervention strategies. In either case, biomarkers can be used as endpoints, permitting a proof of principle to be established in advance of long-term interventions where pre-cancerous lesions or cancer itself might be the outcome (U.S. Congress, 1990).

Biomarkers of exposure can be divided into markers of internal dose and effective dose. The former gives an indication of the occurrence and extent of exposure of the organism and thus likely concentration of a parent compound or metabolite at the target site. The simplest indicator of internal dose is the blood concentration of a chemical agent measured following exposure. The latter is an indication of the true extent of the exposure of what is believed to be the target molecule, structure or cell. Both markers of internal and effective dose are therefore preferable to measuring external levels of the compound in question, for example in the workplace, as they take into account the biological variations in absorption, metabolism and distribution of the compound in an individual (Timbrell, 1998; Waterfield & Timbrell 1999).

1.2. BIOMARKERS OF EFFECT

The International Programme on Chemical Safety has defined a biomarker of effect as *"a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease"*. This is a very broad definition. Biomarkers of effect can be elicited as a result of interaction of the organism with a host of different environmental factors (including chemical, physical, and biologic agents); this definition encompasses biomarkers of effect at the level of the whole organism, at the level of organ function, at the level of tissue and individual cells, and at the subcellular level (Barrett *et al.,* 1997).

Biomarkers of effect, which measure processed genetic damage, are sometimes used to define exposures; because of that, classification in more mechanistic terms, such as reversible (transient) genotoxic responses (exposure/dose) and irreversible (permanent) genotoxic

responses (effect) may be used (Albertini *et al.*, 1996). DNA adducts are better representations of penetration of the agent to the target molecules of genotoxic concern than are protein adducts, however, the fact that DNA molecules are repaired, which must be considered when using DNA adducts as *in vivo* dosimeters, are examples of reversible genotoxic response (Albertini *et al.*, 1996). Effect or irreversible genotoxic endpoints require host processing of DNA lesions into informational changes in the cell (e.g. mutations) and therefore may be relatively insensitive when used as dosimeters (Albertini *et al.*, 1996).

Historically and in practical terms these biomarkers are those which have been used most widely and routinely. They can be grouped into different categories. Hence those markers which are the result of pathological damage could be considered separately from markers which indicate a metabolic lesion (Waterfield & Timbrell, 1999).

Other potential uses of biomarkers of effect are in monitoring of disease progression and prognosis, and as adjuncts to other biomarkers in providing refinements of epidemiology and risk assessments. At the very last, biomarkers, offer the opportunity to provide scientific confirmation of proposed exposure-disease pathways *in vivo* in human populations. Biomarkers of effect may be particularly useful for demonstrating the biologic influence of preceding susceptibility factors, for instance, genetic polymorphisms of xenobiotic-metabolizing enzymes (Barrett *et al.*, 1997).

This type of biomarker indicates early biochemical or functional alterations including a wide array of biological responses, ranging from physiological adaptation to disease. They represent a heterogeneous group of indicators and have different applications depending on the toxicological significance. Some of them have been used for decades as indirect biological signs of exposure rather than markers of effect. This is because they are well and promptly correlated with the degree of exposure, sometimes, but not always, even at levels of exposure without any toxicological significance (Manno *et al*., 2010).

An important group of effect biomarkers which have been developed in animals, even *in vitro*, and are now increasingly applied to occupationally exposed populations, are genotoxicity biomarkers in workers exposed to mutagens or genotoxic carcinogens. These tests, including chromosomal aberrations, micronuclei and the more recent comet assay, may be effective in distinguishing exposed from non-exposed subjects at high exposure. Mainly used as group indicators they are sensitive but not specific and in some cases difficult to interpret correctly, although new techniques, such as the alkaline comet assay, appear to be promising in distinguishing between different mechanisms of DNA damage (covalent binding *versus* oxidative stress) (Manno *et al.*, 2010).

There is also growing interest in the use and identification of "non-invasive" biomarkers. These allow more routine sampling in human studies and may overcome ethical issues, for example in screening children. Thus biomarkers identified in urine, breath or saliva are potentially more useful than those measured in blood (Waterfield & Timbrell, 1999). Some of the simplest biomarkers can be very important tools in biomonitoring as they may indicate more subtle or complex changes taking place in response to external stressors.

Chromosomal abnormalities can also be identified in peripheral lymphocytes and may act as surrogate biomarkers of changes in other tissues. Micronuclei and translocations and sister chromatid exchanges, which can be induced by a wide range of exposures, reflecting cumulative response to a variety of environmental factors are also important biomarkers in this field (Timbrell, 1998; Waterfield & Timbrell, 1999; Wild, 2009). Indeed, there are aspects of exposure assessment that are best accomplished by irreversible genotoxic endpoints (Albertini *et al.*, 1996). In summary, effect biomarkers used as early predictors of clinical disease can improve occupational health risk assessment and contribute to implement new effective disease prevention policies in occupational and environmental settings, but they must be first validated (Manno *et al.*, 2010).

1.2.1. BIOMARKERS OF GENOTOXICITY

Over the past decades, biomarker-based approaches have been applied in the assessment of exposure to genotoxic agents and increases of these biomarkers are considered early events associated with disease-related changes (Bonassi *et al.,* 2011). For surrogate biomarkers to have disease predictability, it must be demonstrated that genotoxic events actually measured really mimic disease-causing genotoxic events (Albertini *et al.*, 1996).

Biomarkers of genotoxicity are used to measure specific occupational and environmental exposures or to predict the risk of disease or to monitor the effectiveness of exposure control procedures to genotoxic chemicals (Manno *et al.*, 2010). Cytogenetic biomarkers are the most frequently used endpoint in human biomonitoring studies and are used extensively to assess the impact of environmental, occupational and medical factors on genomic stability (Barrett *et al.*, 1997; Battershill *et al.*, 2008). Lymphocytes, in particular, are used as a surrogate for the actual target tissues of genotoxic carcinogens (Barrett *et al.*, 1997; Hagmar *et al.,* 1998).

Genotoxicity biomonitoring endpoints such as micronuclei, chromosomal aberrations and 8-hydroxydeoxyguanosine (8-OHG) and DNA repair measured by comet assay are the most commonly used biomarkers in studies evaluating environmental or occupational risks associated with exposure to potential genotoxins. A review by Knudsen and Hansen (2007) on

the application of biomarkers of intermediate endpoints in environmental and occupational health concluded that micronuclei in lymphocytes provided a promising approach to assess health risks, but concluded that the use of chromosomal aberrations is likely to be limited by the laborious and sensitive procedure of the test and the lack of trained cytogeneticists. Nevertheless, methodologies like comet assay in peripheral blood lymphocytes, urine and tissues are increasingly being used as markers of oxidative DNA damage (Battershill *et al*., 2008; Ersson, 2011).

1.3. BIOMARKERS OF SUSCEPTIBILITY

A biomarker of susceptibility is defined as an indicator or a measure of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance (Barrett *et al.*, 1997; Waterfield & Timbrell 1999). Thus any variation in the response of an individual to identical exposures may represent some difference in susceptibility due either to the genetic make-up of the individual or to variables and environmental influences such as diet or the uptake and absorption of the xenobiotics (Waterfield & Timbrell, 1999).

Biomarkers of susceptibility are concerned with factors in kinetics and dynamics of uptake and metabolism of exogenous chemicals. Thus the concept encompasses enzymes of activation and detoxification, repair enzymes, and changes in target molecules for toxic chemicals (Barrett *et al.*, 1997).

Toxicological research in experimental animals and humans over many years has revealed that individuals can often differ markedly in their qualitative and quantitative responses to chemical exposure. Such interindividual differences can be genetically mediated or can be result of some environmental stressor, disease process or other epigenetic factor. While these interindividual differences can complicate safety evaluation and risk assessment activities, they can also be usefully employed as biomarkers of individual susceptibility to xenobiotics (DeCaprio, 1997).

Hyper-susceptibility can be defined as a lack of capacity, beyond the limits of human variability, to tolerate or respond effectively to exogenous toxicants or pathogens. The concept of individual variability is intrinsic to the interpretation of chemical biomonitoring data as well as to that of any biological or clinical test. Mechanisms of susceptibility to chemical agents are of two kinds: toxicokinetic and toxicodynamic. Biomarkers of susceptibility may be of either type. A group of potential susceptibility biomarkers with a toxicokinetic mechanism for use in humans exposed to chemicals is represented by the *in vivo*

measurement of the specific drug metabolizing enzymes or enzyme activities involved in the chemicals' activation or detoxification reactions (Manno *et al.*, 2010).

Interindividual variation occurs as a result of different genetically inherited background modified by dietary and environmental exposure and revealed by genotypic and phenotypic variation. Susceptibility markers are useful because they can partially explain interindividual variation inherent in the general population and thus provide a biological rationale for investigation of inherent vulnerability prior to exposure to environmental hazards (Barrett *et al.*, 1997).

Biomarkers of susceptibility do not represent stages along the dose-response mechanistic sequence, but instead represent conditions that alter the rate of transition between the stages or molecular events. The kinetics of transition is often governed by specific enzymes or other gene products. Consequently, determination of relative enzyme activities or the presence or absence of other gene products is often employed as susceptibility biomarkers. Enzymes involved in xenobiotic metabolism can be particularly important in the overall mechanism of action of xenobiotics, and genetic polymorphism in metabolic enzymatic activity is a common basis for interindividual differences in toxicity (DeCaprio, 1997; DeCaprio, 1999).

An example of xenobiotic metabolizing enzymes as susceptibility biomarkers is the cytochrome P450 enzyme system. This system is responsible for oxidative (i.e. Phase I) metabolism of a multitude of xenobiotics and endogenous molecules, primarily in the liver but also in others bioactivated metabolites. Another important detoxification enzyme system with significant use in susceptibility studies is glutathione-S-transferase (GST), which catalyses the conjugation (i.e. Phase II metabolism) of cellular thiol glutathione (GSH) with oxidizied xenobiotics (DeCaprio, 1999; Manno *et al.*, 2010).

In addition to enzymes involved in biotransformation, other potential susceptibility biomarkers have been explored or proposed in human and animal studies. These include DNA repair enzymes activities, nuclear and cytoplasmic receptor protein levels, oncogenes and corresponding gene products, tumour suppressor genes and humoral and cellular immune system components.

Much hope has been vested in the development of genetic biomarkers, but for environmental and occupational field the research so far has not led to any routinely usable biomarkers. Many known genetic traits, such as polymorphisms of drug metabolism, are individually only weak associated with disease and many probably remain unknown, due to the requirement for environmental factors. Especially if the risk of disease is associated more with exposure

than with the genotype, limiting exposure is the only feasible approach to prevention and benefits all (Vähäkangas, 2008).

These methods cannot easily identify all individuals at risk in a hazardous environment due to lack of understanding of the interaction of compensatory genetic and cellular mechanisms and complex environmental influences; to determine the role of genetic variations to explain interethnic differences associated with susceptibility to chemical exposures and to predict population vulnerability and; to improve the detection of environmental hazards by increasing the sensitivity of epidemiological studies which in turn will result in (i) reduction of risk through avoidance or limitation of chemical exposure, (ii) changes in dietary and social habits to improve health or reduce risk (iii) improved drug treatment to maximize response and minimize toxicity. Better knowledge of xenobiotic metabolism and pharmacokinetics of elimination of toxins will speed progress of this work (Barrett *et al.*, 1997). Possible consequences of differential inter-individual and inter-ethnic susceptibilities may be related to (i) individual expression of clinical signs of chemical toxicity, (ii) biological monitoring data in exposed workers, and (iii) interpretation of results of epidemiological or molecular epidemiological studies (Manno *et al.*, 2010).

2. BIOLOGICAL PLAUSIBILITY

One of the criteria for establishing association between an exposure and disease is biological plausibility. In this context, biomarkers may contribute by illuminating some of the carcinogenic steps linked to a particular risk factor. This is possibly an undervalued area where biomarkers can make significant contributions to cancer epidemiology. If a particular chemical exposure from ambient air is associated with increased risk, the additional information that exposed individuals have higher levels of DNA damage would add support to the exposure-disease association (U.S. Congress, 1990). If genetic polymorphisms in carcinogen metabolizing or DNA repair enzymes are associated with both an increased cancer risk and higher levels of a biomarker on the presumed causal pathway, e.g. DNA adducts, this would provide support for the original association (Wild, 2009).

3. VALIDITY, RELIABILITY, AND PREDICTABILITY OF BIOMARKERS

Validity has been defined as the (relative) lack of systematic measurement error when comparing the actual observation with a standard (reference) method, which represents the "truth" (U.S. Congress, 1990; Vineis, & Garte, 2008; Wild, 2009). Validity has two components, one is sensitivity and the other is specificity. Sensitivity is the ability to avoid false negative results, and it is fundamental for preventive purposes, whereas specificity, the capacity to avoid false positive results, is usually more important for diagnostic purposes (Manno *et al.,* 2010).

In order to ensure a rational occupational risk assessment, it is important to use validated biomarkers. This means that before biomarkers can be routinely used for workers' protection they must be tested in suitable studies. It must be demonstrated that a biomarker of exposure indicates the actual exposure, a biomarker of effect truly predicts the actual risk of disease and a biomarker of susceptibility reliably suggests a modification on the risk (Manno *et al.,* 2010). It must be stressed that exposure or effect biomarkers are really useful risk assessment tools when the metabolic fate of the compound (toxicokinetics) or the mechanisms of a resultant disease (toxicodynamics) are completely understood (Manno *et al.,* 2010).

The predictive value of an effect biomarker is the probability that a biomarker, which has identified a subject as having an impairment or disease, is actually correct in having done so (Manno *et al*., 2010). The predictive value mostly depends on the prevalence of the disease, on type of chemical being measured and on the quality of the method. Generally speaking, with prevalence in the reference population below 5% the negative predictive value of any biomarkers is high, whereas the positive predictive value is poor. The opposite occurs when prevalence is high. Only highly specific and sensitive biomarkers should be used when decisions have to be made on the worker's job fitness or their removal from work or other important personal risk management issues, in order to avoid misjudgement, particularly with low prevalence diseases (Manno *et al.*, 2010).

The presence of long-term risks associated with human exposure to mutagenic and carcinogenic agents has been revealed by classic epidemiologic studies. A number of progressive changes occurred in workplaces, in the environment and in life-styles during the past few decades, this has resulted in different exposures patterns and exposures to new substances, and therefore new and more sensitive tools should be utilized to investigate cancer risk from these substances. In addition, there are aspects of metabolism and susceptibility previously unknown or poorly understood, such as metabolic polymorphisms,

which can dramatically modify individual responses to toxic and carcinogenic agents. These features have made the identification of etiological factors more difficult, especially for cancers with a long induction period, and the use of traditional epidemiologic outcomes, such as cancer incidence or mortality, in many cases no longer seem sufficient for the evaluation of cancer risk in human populations (Fenech *et al.*, 1999b). Biomarkers that have been validated for their predictive value may be used for the timely identification of increased cancer risk, and can be used in the prevention or control of disease. The assumption underpinning the use of a biomarker as a surrogate of disease is that the observed relationship between exposure and the marker will translate into a similar relationship between exposure and disease (Fenech *et al.*, 1999b).

The advantages of using biomarkers as tools for exposure assessment are well established. Biomarkers are particularly useful when their toxicological significance is sufficiently understood, including the following: toxicokinetic fate of the chemical or its metabolites (for exposure biomarkers), or the mechanism of disease/adverse effect (for effect biomarkers), or the modulating factors linking the chemical to the disease/adverse effect (for susceptibility biomarkers) (Manno *et al.,* 2010).

CHAPTER 4 – GENOMIC INSTABILITY

1. MUTAGENETICITY AND GENOTOXICITY

Current evidence suggests that the mutagenic events involved in carcinogenesis are themselves produced by one or two broad modes of action. The first may be mediated by the covalent binding of a chemical or its metabolites to DNA or chromatin, or by their interference with DNA-related processes, such as spindle function or transcription, thereby directly affecting the integrity of the genome (i.e., the structure or content of DNA). The second mechanistic process involves chemical alterations in homeostasis that may be mediated via tissue necrosis, apoptosis, or cellular turnover leading indirectly to the expression of mutations in DNA (Waters *et al.*, 1999).

A genotoxic agent is a chemical or another agent that damages cellular DNA resulting in mutation and/or, consequently, cancer. Genotoxic substances are known to be potentially mutagenic or carcinogenic when inhaled, ingested or penetrate the skin. A mutagen is an agent that is responsible for inducing a change to the genetic material of an organism. That agent might be physical, chemical or biological. As many mutations may ultimately result in cancer (or be part of the multistep process of carcinogenesis), mutagens are typically also carcinogens (Friedberg *et al.*, 2006).

While genotoxicity is often confused with mutagenicity, all mutagens are genotoxic; however, not all genotoxic substances are mutagenic. The alteration can have direct or indirect effects on the DNA. The permanent, heritable changes can affect either somatic or germ cells, being the latter passed on to future generations. Mechanisms of cell defense, such as DNA repair or apoptosis can prevent expression of the genotoxic mutations and, consequently enabling the damage to be fixed and leading to mutagenesis (Friedberg *et al.,* 2006).

Although some carcinogens are primarily associated with genotoxic mechanisms, while others are considered nongenotoxic, recent knowledge indicates that many chemical carcinogens operate via a combination of both mechanisms with the prevailing mechanism dependent upon the target cell type (Waters *et al.*, 1999). With evidence that gene mutations, gene amplifications, chromosomal rearrangements, and aneuploidy are associated with numerous types of tumours, it remains essential to identify chemicals and other agents that are capable

of inducing the types of genetic alterations that could damage the genes involved in carcinogenesis (Waters *et al.*, 1999).

Markers of general DNA damage include chromosomal aberrations, micronuclei, and sister chromatid exchanges, which can be induced by a wide range of exposures, reflecting cumulative exposure to a variety of environmental factors (U.S. Congress, 1990).

The increasing demand for the information about health risk derived from exposure to complex mixtures calls for the identification of biomarkers to evaluate genotoxic effects associated with occupational and environmental exposure to chemicals.

Lymphocytes are the most commonly used cells in human biomonitoring studies for the assessment of genetic damage, since they are easy to sample and can be used as surrogate cells of damaged target tissues. In fact, they circulate throughout the body, have a reasonably long life span, and can therefore be damaged in any specific target tissue by a toxic substance (Cavallo *et al.*, 2009). In addition to lymphocytes, exfoliated cells from epithelial tissues are often exposed to chemical agents and have been used to evaluate genotoxic effects from xenobiotic exposure. The exfoliated buccal cells can be rapidly and noninvasively collected in large number, more easily than lymphocytes (Cavallo *et al.*, 2009).

2. OXIDATIVE DAMAGE

Free radicals are unstable molecule species with an unpaired electron and are produced in living cells by normal metabolism and by exogenous sources such as carcinogenic compounds and ionizing radiations (Dizdaroglu *et al.*, 2002; Bender, 2006).

It is generally accepted that oxidative stress is an inevitable feature of life, induced by reactive forms of oxygen released during normal respiration, by the oxidative burst of the macrophages in response to infection, and by a variety of exogenous agents (Poulsen *et al.,* 1998; Volkovová *et al*., 2006; Valavanidis *et al*., 2009). Normal cellular metabolism is well established as the source of endogenous reactive oxygen species (ROS), and is these (normally non-pathogenic) cellular processes that account for the background levels of oxidative DNA damage detected in normal tissues (Poulsen *et al.,* 1998; Cooke *et al.,* 2003).

ROS is a collective term which comprises oxygen species like oxygen (O_2), superoxide anion ($O^{\cdot}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$) and other species that are easily converted into radicals such as hypochlorite (HOCl) and peroxynitrite ($ONOO^-$) (Poulsen *et al.*, 1998; Boiteux & Radicella, 1999).

Multiple exogenous and endogenous sources generate ROS in mammalian cells. The exogenous sources include radiation, air pollution, tobacco smoke, and a wide range of chemicals, whereas endogenous sources of ROS include mitochondrial respiration, inflammatory responses involving the immune system, apoptosis, oxidation of reduced flavin coenzymes, biotransformation and other metabolic processes (Collins, 1999; Marnett, 2000; Gedik *et al.*, 2002; Cooke *et al.,* 2003; Bender, 2006; Loft *et al.*, 2008; Sedelnikova *et al.*, 2010). It has been proposed that ROS and reactive nitrogen species (RNS) play a key role in human cancer development, especially as evidence is growing that antioxidants may prevent or delay the onset of some types of cancer (Wiseman & Halliwell, 1996; Mohrenweiser, 2004).

Elevated ROS levels can create oxidative stress in a cell and chronic exposure to this stress can result in permanent changes in the genome. It is generally accepted that the accumulation of oxidative DNA lesions may promote mutagenesis, human pathogenesis and loss of homeostasis. High levels of oxidative stress contribute significantly to the age-related development of some cancers through DNA damage (Fergurson, 2008). Lipids, proteins and DNA are the major target of free radicals and active oxygen species. Above all, the polyunsaturated fatty acids and their esters are quite susceptible to radical attack and oxidation (Marnett, 2000; Niki, 2000).

Mutagenesis by ROS could contribute to the initiation of cancer, in addition to being important in the promotion and progression phases. ROS can have the following effects: (i) cause structural alterations in DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification. ROS can produce gross chromosomal alterations in addition to point mutations, and thus could be involved in the inactivation or loss of the second wild-type allele of a mutated proto-oncogene or tumour-suppressor gene that can occur during tumour promotion and progression, allowing expression of the mutated phenotype; (ii) affect cytoplasmic and nuclear signal transduction pathways; (iii) modulate the activity of the proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis (Wiseman & Halliwell, 1996). However, it is important to refer that the oxidation of DNA is not only a consequence of the production of ROS as decreases in antioxidant defence and inhibition of repair of oxidative damage should also be taken into account. Xenobiotics can produce ROS, decrease antioxidant defences or inhibit the repair of oxidative damage (Azqueta *et al.,* 2009).

Oxidative damage probably constitutes the most varied class of DNA damage with at least 20 different lesions identified. These include single or double strand breaks, single base

modifications, abasic sites, and DNA-protein-cross-links (Boiteux & Radicella, 1999; Lloyd & Phillips, 1999).

The targets for ROS that may result in initiation of cancer, coronary heart disease and autoimmune disease are nucleic acids, polyunsaturated fatty acids (in cell membranes and plasma lipoproteins) and proteins. Both purine and pyrimidines bases in DNA are susceptible to chemical modifications by reactive oxygen species, resulting in the formation of derivatives (e.g. 8-dGuo) that, if not detected and excised by the DNA-repair mechanisms, will result in the incorporation of incorrect bases during DNA replication (Bender, 2006). In germline cells, this may result in a heritable mutation and, in somatic cells, it may initiate cancer. Radical damage can also cause strand breaks in DNA that accelerate the normal age-related shortening of telomeres (the repetitive sequences at the end of chromosomes that stabilize them). Polyunsaturated fatty acids are highly susceptible to oxidation, leading to the formation of lipid peroxides. These break down to form highly reactive dialdehydes, which cause chemical modification of nucleic acid bases and proteins. Amino acid side chains in proteins are susceptible to direct oxidation by radicals (Bender, 2006).

Oxidative damage may play an important role in the pathogenesis of several neurodegenerative diseases, and growing evidence points to the involvement of free radicals in mediating neuronal death in these illnesses (Mecocci *et al.,* 2002; Valavanidis *et al.,* 2009). Biological systems develop enzymatic systems – superoxide dismutase, catalase, peroxidases, glutathione peroxidase – in combination with other antioxidants (vitamin E, glutathione, ascorbate) to protect against oxidative damage. In general, biological systems are in a state of approximate equilibrium between pro-oxidant forces and the antioxidant capacity of biological systems (Floyd, 1990).

2.1. 8-HYDROXYDEOXYGUANOSINE (8-OHdG)

Among free radicals, the highly reactive hydroxyl radical (·OH) causes damage to DNA and other biological molecules. This type of DNA damage is also called “oxidative damage to DNA” and is implicated in mutagenesis, carcinogenesis, and aging (Fairbairn *et al.,* 1995; Jaruga *et al.,* 2000; Dizdaroglu *et al.,* 2002; Cooke *et al.,* 2003; Thompson, 2004; Collins, 2004; Azqueta *et al.*, 2009; Collins, 2009).

“Oxidative stress” refers to a state where the balance is upset, either by an excessive production of free radicals, or by deficient antioxidant defences. In such circumstances – notably in a variety of diseases states – it would be expected to find an elevated level of oxidative damage to biomolecules (Collins, 2009).

The interaction of ·OH with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanosine). Initially, the reaction of the ·OH addition leads to the generation of radical adducts then, by one electron abstraction, the 8-hydroxy-2'-deoxyguanosine (8-OHdG) is formed (Valavanidis *et al.,* 2009). The 8-OHdG undergoes keto-enol tautomerism, which favours the oxidized product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG). In the scientific literature 8-OHdG and 8-oxodG are used for the same compound (Valavanidis *et al.,* 2009).

Therefore, the most commonly base lesion, and the one most often measured as an index of oxidative DNA damage, is 8-hydroxyguanine (8-OHG). The popularity of 8-OHG as an indicator of DNA oxidation is probably attributable to the ease with which it can be measured (Collins, 2004). It can pair A rather C, and so if it is present during replication, C > A transversions may result. It is sometimes measured as the nucleoside, 8-hydroxydeoxyguanosine (8-OHdG) (Wiseman & Halliwell, 1996; Persinger *et al.,* 2001; Volkovová *et al.*, 2006; Sedelnikova *et al.,* 2010; Ersson, 2011).

Oxidative DNA base damage (measured as 8-OHdG) has been detected in mitochondrial DNA at steady-state levels several-fold higher than in nuclear DNA (Wiseman & Halliwell, 1996; Collins, 1999; Sedelnikova *et al.*, 2010). 8-OHG and 8-OHdG are the products most frequently measured in isolated DNA as an indicator of oxidative DNA damage (Wiseman & Halliwell, 1996), and as a possible indicator of cancer risk (Collins, 2009) since it has a pro-mutagenic potential (Ersson, 2011). The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to examine critically the different approaches to measuring base oxidation in DNA, in particular 8-OHdG (ESCODD, 2003). 8-OHdG has thus been established as an important biomarker of oxidative stress, of cancer risk to humans by mechanisms of oxygen-free radicals, of aging processes including degenerative diseases, and in general as a biological marker of lifestyle and the effect of diet (Valavanidis *et al.*, 2009).

Measurement of 8-OHdG by High Performance Liquid Chromatography (HPLC) coupled to electrochemical detection (ECD) is a highly sensitive method. One alternative is Gas Chromatography-Mass Spectrometry (GS-MS) with selected ion monitoring, which can measure a wide spectrum of modified DNA bases (methylated, oxidized, deaminated, etc.). Both methods are sufficiently sensitive to measure steady-state levels of oxidative base damage in human cells and tissues (Wiseman & Halliwell, 1996; Collins, 1999; Gedik *et al.*, 2002; Azqueta *et al.*, 2009). Also HPLC linked to tandem mass spectrometry (HPLC-MS/MS) is another method that can be used (Azqueta *et al.*, 2009). The enzyme comet assay is often

regarded as being less specific when measuring oxidative DNA lesions than HPLC based techniques because the enzymes used recognise a range of different damages. The comet assay using formamidopyrimidine DNA glycosylase (FPG) was included in the ESCODD trial set up to optimize methods for measuring background levels of oxidative damage, particularly 8-OHG in humans. The trial concluded that the FPG-based methods seemed less prone to spurious oxidation than other methods including HPLC-ECD, GC-MS or HPLC-MS/MS (Collins, 1999; Smith *et al.*, 2006; Dusinska & Collins, 2008; Ersson, 2011).

The ESCODD study provided a realistic estimate of the actual background level of damage in lymphocytes; it is likely to lie somewhere between 4.2 and 0.3 8-OHdG per 10^6 guanines, these are medians of the means from different laboratories, for HPLC and the comet assay, respectively (ESCODD, 2003).

CHAPTER 5 – GENOTOXICITY ASSESSMENT METHODS

1. CYTOKINESIS-BLOCK MICRONUCLEUS CYTOME ASSAY (CBMN)

The cytokinesis-block micronucleus cytome (CBMN) assay is a comprehensive system for measuring DNA damage; cytostasis and cytotoxicity-DNA damage events are scored specifically in once-divided binucleated cells. The endpoints possible to be measured are micronuclei (MN), a biomarker of chromosome breakage and/or whole chromosome loss, nucleoplasmic bridges (NPB), a biomarker of DNA misrepair and/or telomere end-fusions, and nuclear buds (NBUD), a biomarker of elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios (Fenech, 2006, 2007).

The CBMN assay has become one of the most commonly used methods for assessing chromosome breakage and loss in human lymphocytes both *in vivo* and *ex vivo* (Fenech *et al.*, 1999a; 1999b).

In the CBMN assay, once-divided cells are recognized by their binucleated appearance after blocking cytokinesis with cytochalasin-B, an inhibitor of microfilament ring assembly required for the completion of this step (Fenech, 2007). The restriction of scoring just micronuclei in binucleated cells prevents confounding effects caused by suboptimal or altered cell division kinetics. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells (Fenech & Crott, 2002; Fenech, 2007) and has been extensively used to evaluate the presence and the extent of chromosome damage in human populations exposed to genotoxic agents in various occupational settings, in the environment, or as a consequence of lifestyles (Bonassi *et al.*, 2011). The biological meaning of micronuclei presence in mononucleated cells indicate DNA damage that was present in the cells before they were put into culture with cytochalasin-B while binucleated cells may contain pre-existing micronuclei as well as micronuclei expressed during culture as a result of chromosome breaks accumulated during G0 phase *in vivo* (Kirsch-Volders & Fenech, 2001; Fenech *et al.*, 2003).

CBMN assay is visualized as a "cytome" concept, that implies that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, binucleated, multinucleated) and its chromosomal damage or instability status (presence of micronuclei, nucleoplasmic bridges, nuclear buds and number of centromere probe signals among nuclei or micronuclei of binucleated cells if such molecular tools are used in combination with the assay) (Fenech, 2007).

The use of the CBMN assay in *in vitro* genetic toxicology testing is well established and in fact it has become an accepted standard method to assess the genotoxic hazard of chemicals which led to the development of a special guideline by the Organization for Economic Cooperation and Development (OECD), the OECD 487 guideline (Kirsch-Volders *et al.*, 2014).

The CBMN assay is an effective tool for the study of cellular and nuclear dysfunction caused by *in vitro* or *in vivo* aging, micronutrient deficiency or excess, genotoxin exposure and genetic defects in genome maintenance. It is also fruitful in the emerging fields of nutrigenomics and toxicogenomics and their combinations, as it becomes increasingly clear that nutrient status also impacts on sensitivity to exogenous genotoxins (Fenech, 2005, 2007).

Many results obtained by this assay indicate the potential predictive value of the CBMN assay with respect to cancer risk and validate its use as a test for detecting nutritional, environmental and genetic factors that are potentially carcinogenic. Also it is used by pharmaceutical industry, human biomonitoring of genotoxic exposures and its increasing application in preventive medicine and nutrition and the increased investment in the automation of the CBMN assay are indicative of the increasing importance of this test (Fenech, 2007).

The CBMN assay is also widely used in human biomonitoring of *in vivo* exposure to genotoxins and has become a standard biodosimetry method endorsed by the International Atomic Energy Agency and the World Health Organization (WHO) for measuring exposure to ionizing radiation (Vral *et al.*, 2011). The assay measures micronuclei and other nuclear anomalies in *ex vivo* mitogen stimulated lymphocytes from *in vivo* systemic exposed persons, integrating in this way *in vivo* systemic exposure of lymphocytes and *in vivo/ex vivo* response to the genotoxic stress. Its predictivity for the detection of genetic risks is supported by the fact that it allows measurement at the single cell level of both structural and numerical chromosome aberrations (Kirsch-Volders *et al.*, 2014).

CBMN assay is a robust assay for genetic damage with applications in ecotoxicology, nutrition, radiation sensitivity testing both for cancer risk assessment and optimization of radiotherapy, biomonitoring of human populations and importantly testing of new pharmaceuticals and

other chemicals. There are expectations regarding the future development of an automated system that can reliably score the various end points which are possible with the CBMN assay (Fenech, 2007).

There are some limitations and misconceptions regarding CBMN assay that have been reported in literature. The use of the CBMN assay for detecting *in vivo* exposure to genotoxic chemicals is somewhat controversial because of the extremely wide diversity of chemicals, the multitude of direct or indirect mechanisms of their interaction with the genome, the wide spectrum of DNA lesions they may induce and the variety of cellular death/survival responses they may trigger (Vral *et al.*, 2011; Kirsch-Volders *et al.*, 2014).

Other limitation is the needing for a thorough calibration of scorers and standardization of scoring procedures aimed at reliably compare micronuclei frequencies among different laboratories and studies. One way to exclude scorer variability might be the use of an automatic image-analysis system, although a good correlation between visual and automatic scoring has been reported by experienced scorers. Automatic scoring is a useful contribution to the standardization of the assay and should become a quality standard for future biomonitoring studies with the CBMN assay (Speit *et al.,* 2012).

Other limitations of the CBMN assay include: (i) the exclusion of micronuclei being scored in non-divided cells, (ii) the micronuclei produced *in vivo* do not substantially contribute to micronuclei frequency measured in binucleated lymphocytes in the *ex vivo* CBMN assay, (iii) the sensitivity of the CBMN assay for detection of micronuclei in binucleated cells is diminished because cytochalasin-B is added late during the culture period so that the binucleated cells scored do not always represent cells that have completed one cell cycle only; (iv) the delay in adding cytochalasin-B means that damaged cells can be eliminated by apoptosis and/or DNA damage induced *in vivo* can be repaired prior to the production of a micronuclei in the presence of cytochalasin-B this may render the CBMN assay to be insensitive; (v) a comparison with the *in vitro* CBMN assay used for genotoxicity testing leads to the conclusion that it is unlikely that DNA damage induced *in vivo* is the cause of increased micronuclei frequencies in binucleated cells after occupational or environmental exposure to genotoxic chemicals (Kirsch-Volders *et al.*, 2014).

A recommendations for the future, is the establishment of an international network including several cytogenetic reference laboratories establishing and optimising International Standardization Organisation (ISO) standards for the conventional and automated CBMN assay. By creating such a network of trained laboratories using similar equipment for micronuclei automation and the same classifiers, standardised fixation protocols, etc.,

comparable results can be obtained and the throughput of automated micronuclei scoring can be increased to allow a rapid response to large-scale radiation accidents (Vral *et al.*, 2011).

1.1. MICRONUCLEI (MN)

Micronuclei originate from chromosome fragments or whole chromosomes that lag behind anaphase during nuclear division and are not included in the main nuclei (Fenech, 1997, 2000; Fenech & Crott, 2002).

Micronuclei are small, extranuclear bodies that arise in dividing cells from acentric chromosome/chromatid fragments or whole chromosome/chromatid that lag behind in anaphase, and are not included in the daughter nuclei in telophase (Mateuca *et al.*, 2006).

At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term "micronucleus" (Fenech, 2000). Micronuclei harbouring chromosomal fragments may result from direct double strand DNA breakage, conversion of single strand breaks into double strand breaks after cell replication, or inhibition of DNA synthesis (Mateuca *et al.*, 2006).

Micronuclei can be formed via different pathways, namely from acentric chromosome or chromatid fragments. A small proportion of acentric chromosome fragments may simply arise from unrepaired double-stranded DNA breaks. Other mechanisms that could lead to micronuclei formation from acentric fragments include simultaneous excision repair of damaged (e.g. 8-OHdG) or inappropriate bases incorporated in DNA (e.g. uracil) that is in proximity and on opposite complementary DNA strands (Fenech *et al.*, 2011).

Other mechanism that may lead to micronuclei from chromosome loss events is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher order repeats of satellite DNA in centromeric DNA (Fenech *et al.,* 2011).

Given the central role of kinetochore proteins in the engagement of chromosomes with the spindle, it is probable that mutations leading to defects in kinetochore and microtubule interaction dynamics could also be a cause of micronuclei formation due to chromosome loss at anaphase. Other variables that are likely to increase micronuclei from chromosome loss are defects in mitotic spindle assembly, mitosis check point defects and abnormal centrosome amplification (Fenech *et al.*, 2011).

The fate of micronuclei after their formation in the micronucleated cell is poorly understood. Their post-mitotic fate includes: (i) elimination of the micronucleated cell as a consequence of apoptosis; (ii) expulsion from the cell (when the DNA within the micronuclei is not expected to be functional or capable of replication owing to the absence of the necessary cytoplasmic components); reincorporation into the main nucleus (when reincorporated chromosome may be indistinguishable from those of the main nucleus and might resume normal biological activity); (iii) retention within the cell's cytoplasm as an extra-nuclear entity (when micronuclei may complete one or more rounds of DNA/chromosome replication) (Mateuca *et al.,* 2006; Shimizu, 2011).

The key advantage of the CBMN assay lies in its ability to detect both clastogenic and aneugenic events, leading to structural and numerical chromosomal aberrations, respectively (Mateuca *et al.,* 2006). Clastogens induce micronuclei by breaking the double helix of DNA, thereby forming acentric fragments that are incapable of adhering to the spindle fibers and integrate in the daughter nuclei and are thus left out during mitosis. The same occurs to whole chromosomes with damaged kinetochores; they cannot attach to the microtubules that pull the chromatids toward the daughter cells during mitosis and thus they remain outside the new nuclei. This damage could be generated by chemicals reacting with proteins forming the kinetochores (Serrano-García & Montero-Montoya, 2001; Utani *et al.,* 2010).

Aneugens are chemicals that prevent the formation of the spindle apparatus during mitosis. These agents generate not only whole chromatids that are left out of the nuclei, thus forming micronuclei, but also the formation of multinucleated cells, in which each nucleus would contain a different number of chromosomes. These agents are also likely to induce an increase in mitotic figures that are clearly seen in the same slides (Serrano-García & Montero-Montoya, 2001; Utani *et al.*, 2010).

With CBMN assay it is possible to distinguish between micronuclei originating from whole chromosomes and those originating from acentric fragments as well as to determine whether malsegregation of chromosomes is occurring between nuclei in a binucleated cell that may not contain micronuclei by using pancentromeric DNA probes (Fenech *et al.*, 1999b; Fenech, 2000, 2006). The use of chromosome-specific centromeric DNA probes allows both the determination of specific chromosome loss events resulting in micronuclei, as well as unequal segregation of specific chromosomes among daughter nuclei even in the absence of micronuclei formation (Fenech *et al.*, 2011). Pancentromeric probes should be used only to distinguish between micronuclei originating from chromosome breaks (centromere negative) and chromosome loss (centromere positive). Chromosome-specific centromere probes should

be used only to measure malsegregation (owing to non-disjunction or chromosome loss) involving unique chromosomes (Fenech *et al.*, 1999b; Fenech, 2000; Norppa & Falck, 2003; Fenech, 2006; Fenech *et al.,* 2011). Evaluation of the mechanistic origin of individual micronuclei by centromere and kinetochore identification contributes to the high sensitivity and specificity of the method (Mateuca *et al.,* 2006).

It is essential to refer that there are important factors influencing the baseline micronuclei frequency in human lymphocytes. Age and gender are the most important demographic variables affecting the micronuclei index, with frequencies in females being greater than those in males by a factor of 1.2 to 1.6 depending on the age group (Bolognesi *et al.,* 1999). Micronuclei frequency was significantly and positively correlated with age in males and females, and is affected by dietary factors such as folate deficiency, and plasma levels of vitamin B12 and homocysteine. It was also proposed that the micronuclei index can be influenced by the propensity of individual's cells to undergo apoptosis and genetic factors, such as genetic polymorphisms (Fenech, 1998; Fenech *et al.,* 1999b; Mateuca *et al.,* 2006).

In general, the formation of micronuclei is attributed to a variety of insults to the genetic material, which could be classified as exogenous and endogenous factors. Exogenous factors include radiation, chemical agents, microorganism invasion, etc. Endogenous factors include genetic defects, pathological changes, deficiency of essential nutritional ingredients (e.g. folic acid) and injuries induced by deleterious metabolic products (such as ROS) (Huang *et al.,* 2011).

The hypothesis of a predictive association between the frequency of micronuclei in CBMN assay in lymphocytes and cancer development is supported by a number of findings: (i) an association between micronuclei frequency and cancer risk was inferred from mechanistic similarities with chromosomal aberrations, which were shown to be predictive for cancer; (ii) *in vitro*, a high concordance is observed between chromosomal aberrations and micronuclei; (iii) an increase in micronuclei frequency is observed in lymphocytes of cancer patients and in patients with syndromes that make them cancer prone such as the Bloom syndrome and ataxia telangiectasia; (iv) micronuclei frequency is significantly associated with the blood concentration of vitamins such as folate, whose deficiencies are associated with increased risk for some cancers; (v) a direct link between micronuclei frequencies and early stages of carcinogenesis, namely a significant association between increasing of micronuclei frequencies and low-grade and high-grade diagnostic categories of cervical carcinogenesis in women (Mateuca *et al.,* 2006).

Formation of nuclear anomalies such as micronuclei, chromosomal rearrangements, and anaphase bridges (leading to breakage-fusion-bridge cycles and generation of more micronuclei) are events commonly seen in the early stages of carcinogenesis. Elevated levels of micronuclei are indicative of defects in DNA repair and chromosome segregation which could result in generation of daughter cells with altered gene dosage, or deregulation of gene expression that could lead to the evolution of the chromosome instability phenotype often seen in cancer. These considerations give mechanistic support to a possible causal association between micronuclei frequency and the risk of cancer. Study from Bonassi *et al.* (2007) observed an association between micronuclei frequency and cancer risk in non-haematological malignancies suggested that genome damage events in lymphocytes may be correlated with cancer initiating events in other tissues via a common genetic, dietary, or environmental factor.

Figure 3 shows the aspect of micronuclei in peripheral binucleated lymphocytes when observed at 1000 magnification with immersion oil in optical microscope.

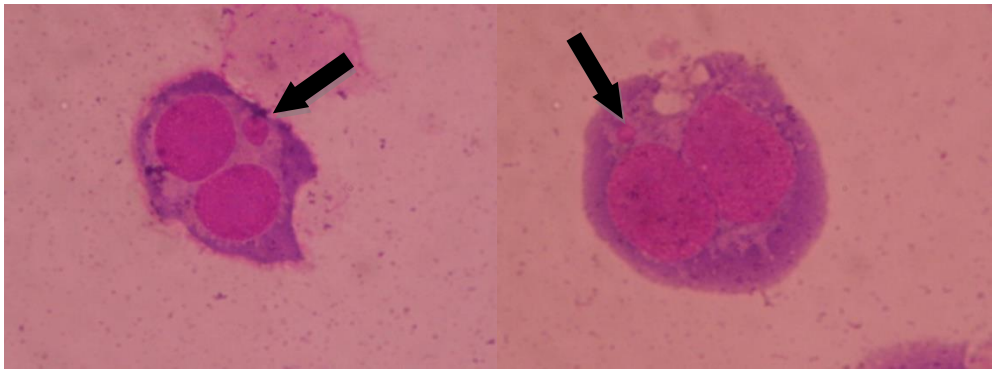


Figure 3 – Micronuclei in peripheral binucleated lymphocytes (1000X). May-Grünwald Giemsa staining technique.

1.2. NUCLEOPLASMIC BRIDGES (NPB)

Nucleoplasmic bridges occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. In the absence of breakage of the anaphase bridge, the nuclear membrane eventually surrounds the daughter nuclei and the anaphase bridge and in this manner, a nucleoplasmic bridge is formed (Fenech *et al.,* 2011). There are various mechanisms that could lead to nucleoplasmic bridges formation following DNA misrepair of strand breaks in DNA. Typically, a dicentric chromosome and an acentric chromosome fragment are formed that result in the formation of a nucleoplasmic bridge and a

micronucleus, respectively (Fenech, 2000; Fenech & Crott, 2002; Fenech, 2006, 2007). Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosomes and concatenated ring chromosomes which could also result in the formation of nucleoplasmic bridges. An alternative mechanism for dicentric chromosome and nucleoplasmic bridges formation is telomere end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion (Thomas *et al.*, 2003; Fenech, 2007). The study of Rudolph *et al.,* (2001), in models of rodent and human intestinal cancer *in vivo*, correlates with telomere length, indicating that nucleoplasmic bridges formation may also be used as a surrogate measure of critically short telomeres (Fenech, 2007).

The two mechanisms of nucleoplasmic bridges formation can be distinguished in binucleated cytokinesis-blocked cells using telomere probes. Nucleoplasmic bridges arising from telomere end fusions are expected to be telomere positive if they retain telomere dysfunction due to loss of telomere-binding proteins without telomere attrition. In contrast, nucleoplasmic bridges caused by misrepair of DNA breaks has a low probability of occurring within the telomeric sequences and is therefore likely to be telomere negative. Furthermore, nucleoplasmic bridges arising from misrepair of DNA breaks are also likely to be associated with micronuclei originating from the acentric fragment generated during misrepair (Fenech *et al.*, 2011). Nucleoplasmic bridges can break and form micronuclei (Fenech, 2006; Lindberg *et al.*, 2007). About 40% of micronuclei, two or more arise from a single nucleoplasmic bridge. When two or more micronuclei are observed after a nucleoplasmic bridge resolution, normally micronuclei in each daughter cell remain (Hoffelder *et al.*, 2004).

Umegaki and Fenech (2000) validated the use of nucleoplasmic bridges as a biomarker of DNA damage in human WIL2-NS cells treated with hydrogen peroxide, superoxide or after co-incubation with activated human neutrophils. Therefore, the importance of scoring nucleoplasmic bridges should not be underestimated because it provides direct evidence of genome damage resulting for misrepaired DNA breaks or telomere end fusions, which is otherwise not possible to deduce by scoring micronuclei only (Umegaki & Fenech, 2000; Fenech & Crott, 2002; Fenech, 2006). Nucleoplasmic bridges formation has been shown to be increased by a wide range of exposures including endogenous oxidants, ionising radiation, polycyclic aromatic hydrocarbons, the cigarette smoke carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, vanadium pentoxide, as well as deficiencies in folate and selenium (Fenech *et al.*, 2011).

Figure 4 shows the aspect of nucleoplasmic bridges in peripheral binucleated lymphocytes when observed at 1000 magnification with immersion oil in optical microscope.

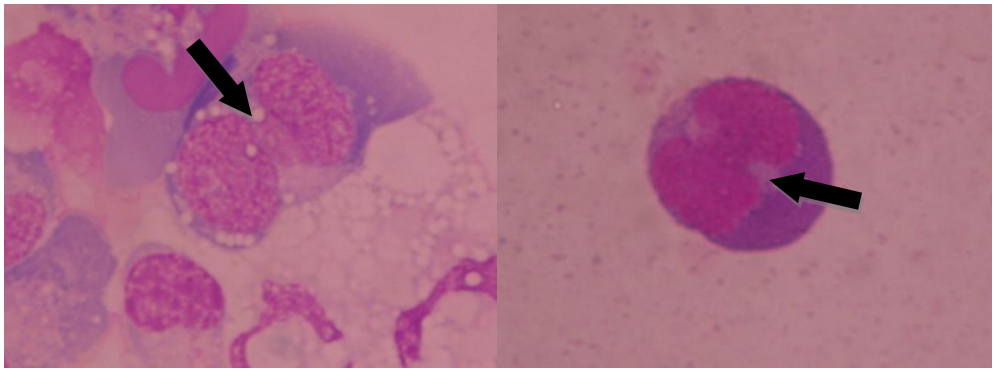


Figure 4 – Nucleoplasmic bridges in peripheral binucleated lymphocytes (1000X). May-Grünwald Giemsa staining technique.

1.3. NUCLEAR BUDS (NBUD)

Nuclear buds are biomarkers of elimination of amplified DNA and/or DNA repair complexes. The nuclear budding process has been observed in cultures grown under strong selective conditions that induce gene amplification as well as under moderate folic acid deficiency (Fenech & Crott, 2002; Fenech, 2007). Gene amplification plays a crucial role in the malignant transformation of human cells as it mediates the activation of oncogenes or the acquisition of drug resistance (Utani *et al.,* 2007). Studies conducted by Shimizu *et al.* (1998, 2000, 2005) showed that amplified DNA is selectively localized at specific sites of the periphery of the nucleus and eliminated via nuclear budding to form micronuclei during S phase of mitosis. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes), which localized at distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The process of nuclear budding occurs during S phase and the nuclear buds are characterized by having the same morphology as an micronuclei with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Shimizu *et al.*, 1998, 2000, 2005). Excess of DNA may in general be expelled from the nucleus by the formation of nuclear buds, and subsequent micronucleation (Lindberg *et al.,* 2007). The duration of the nuclear budding process and the extrusion of the resulting micronuclei from the cell remain largely unknown (Fenech & Crott, 2002; Fenech, 2006, 2007),

although Utani *et al.* (2007) provided evidence that at least some of the cytoplasmic micronuclei may be eliminated from the cell by extrusion.

Nuclear buds are also classified as tentative precursors of micronuclei, being morphologically similar to micronuclei, namely in shape, structure, and size; with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Serrano-García & Montero-Montoya, 2001; Lindberg *et al.,* 2007; Fenech *et al.,* 2011). Nuclear buds may also be explained by the conventional model of micronuclei formation, assuming they derive from anaphase laggards that independently form a nuclear envelope in telophase before fully integrating into the nucleus or from remnants of broken anaphase bridges (Lindberg *et al.,* 2007). The DNA in these buds is replicated and can subsequently be released as micronuclei in the cytoplasm. Nuclear buds have also been shown to be formed when a nucleoplasmic bridge between two nuclei breaks and the remnants shrink back towards the nuclei (Fenech *et al.*, 2011).

Nuclear buds originate from interstitial or terminal acentric fragments. Such nuclear buds may possibly represent nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division or from excess DNA that is being extruded from the nucleus. Whether nuclear buds are also a mechanism to eliminate excess chromosomes in a hypothesized process known as aneuploidy, rescue remains unclear as there is only limited evidence for this possibility. Finally, it is also plausible that nuclear buds might occur transiently after breakage of nucleoplasmic bridges (Fenech *et al.,* 2011). Figure 5 shows the aspect of micronuclei in peripheral binucleated lymphocytes when observed at 1000 magnification with immersion oil in optical microscope.

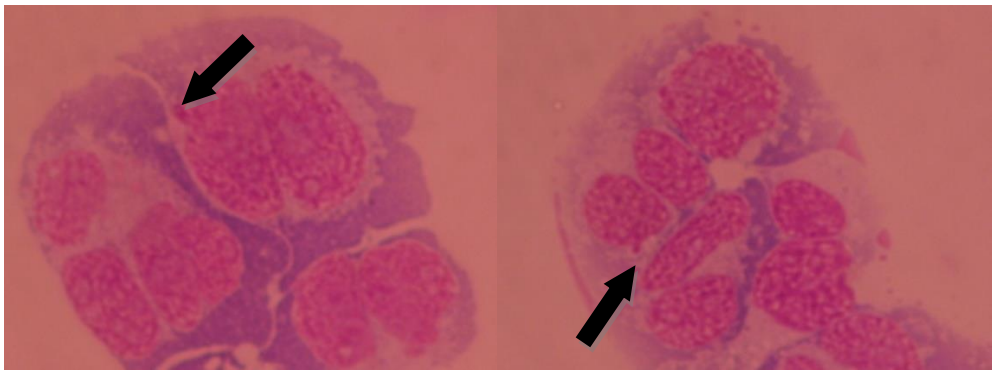


Figure 5 – Nuclear buds in peripheral binucleated lymphocytes (1000X). May-Grünwald Giemsa staining technique.

In conclusion, the CBMN assay has evolved into an efficient "cytome" assay of DNA damage and misrepair, chromosomal instability, mitotic abnormalities, cell death and cytostasis, enabling direct and/or indirect measurement of various aspects of cellular and nuclear dysfunction such as: unrepaired chromosome breaks fragments and asymmetrical chromosome rearrangement (micronuclei or nucleoplasmic bridges accompanied by micronuclei originating from acentric chromosomal fragments); telomere end fusions (nucleoplasmic bridges with telomere signals in the middle of the bridge and possibly without accompanying micronuclei); malsegregation of chromosomes due to spindle or kinetochore defects or cell-cycle checkpoint malfunction (micronuclei containing whole chromosomes or asymmetrical distribution of chromosome-specific centromere signals in the nuclei of binucleted cells); nuclear elimination of amplified DNA and/or DNA repair complexes (nuclear buds); chromosomal instability phenotype and breakage-fusion-bridge cycles (simultaneous expression of micronuclei, nucleoplasmic bridges, and nuclear buds); DNA hypomethylation; altered mitotic activity and/or cytostasis and cell death by necrosis or apoptosis (ratios of necrotic and apoptotic cells) (Fenech, 2007).

2. MICRONUCLEI TEST IN EXFOLIATED BUCCAL CELLS

2.1. EXFOLIATED BUCCAL CELLS

Up to 90% of cancers arise in epithelial tissues, often these tissues are the actual targets of carcinogens, as can be deduced by relating the sites of cancers with the exposures. Epithelial tissues are in immediate contact with inhaled and ingested genotoxic agents, and kidney and bladder cells are also in contact with metabolites of the chemicals (Tolbert *et al.*, 1991; Fenech *et al.*, 1999b; Burgaz *et al.,* 2002; Proia, 2006; Holland *et al.,* 2008; Kashyap & Reddy, 2012). Therefore, buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products (Burgaz *et al.*, 2002; Holland *et al.,* 2008; Kashyap & Reddy, 2012).

Exfoliated buccal cells have been effective in showing the genotoxic effects of lifestyle factors such as tobacco smoking, alcohol, medical treatments, such as radiotherapy as well as occupational and environmental exposure, namely exposure to potentially mutagenic and/or carcinogenic chemicals, and by studies of chemoprevention of cancer (antioxidants) and evaluation of malignant transformation of preneoplastic lesions with oral squamous cell

carcinoma (Fenech *et al.*, 1999a; Majer *et al.*, 2001; Burgaz *et al.,* 2002; Proia, 2006; Fenech, 2007; Holland *et al.*, 2008; Thomas & Fenech, 2011; Cerqueira & Meireles 2012; Kashyap & Reddy, 2012). For these reasons, exfoliated cells hold strong potential as a tool for biomonitoring human populations exposed to genotoxic agents or undergoing preventive treatments, furthermore they can be easily collected from the mouth, nose, and bladder by non-invasive procedures.

The buccal mucosa is a stratified squamous epithelium consisting of four distinct layers. The *stratum corneum*, or keratinized cell layer, lines the oral cavity comprising cells that are constantly being shed as a result of wear and tear of the surface tissue. Below this layer, lies the *stratum granulosum*, or the granular cell layer, and the *stratum spinosum*, or the prickle cell layer, containing population of differentiated, apoptotic and necrotic cells. Beneath these layers are the *rete pegs* or *stratum germinativum*, containing actively dividing basal cells and basal stem cells, which produce progeny that differentiate and maintain the profile, structure and integrity of the buccal mucosa (Thomas & Fenech, 2011; Cerqueira & Meireles, 2012; Kashyap & Reddy, 2012).

The mucosa represents a permeability barrier for xenobiotics due mainly to the flattened surface cell layers and intercellular material. Cells of the epithelia are covered by mucus, whose main components are complexes made up of proteins and carbohydrates. Thus, soft tissues are well protected from chemicals and from abrasion by rough materials. Consequently, the detection of increased micronuclei frequencies in exfoliated epithelial cells requires that the genotoxic agent overwhelms the permeability barrier, reaches the basal layer on paracellular and/or transcellular routes, and induces DNA lesions that become micronuclei during cell division. These cells then have to migrate to the surface to be collected for the micronuclei test (Speit & Schmid, 2006).

2.2. MICRONUCLEUS TEST

The micronucleus test (MNT) is a cytogenetic method for measuring genetic damage, cell proliferation, cell differentiation and cell death in exfoliated buccal cells. This technique is particularly attractive as buccal cells can be collected in a minimally invasive manner. The MNT has been widely applied since 1980 in biomonitoring inhalation or local exposure to genotoxic agents. It has also been applied to investigate and evaluate the impact of nutritional status and lifestyle factors on DNA damage, cellular proliferation and cell death (Bolognesi *et al.,* 2013). The MNT has been used to measure biomarkers of DNA damage (micronuclei and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell

frequency) and/or cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells) (Thomas *et al.,* 2009; Bolognesi *et al.*, 2013).

The MNT is also frequently used for monitoring genetic damage in humans. The MNT with exfoliated epithelial cells, in particular in buccal cells, is used as a minimally invasive method for monitoring genotoxic effects at the site of first contact, being expected that the MNT is a site-specific biomarker of exposure to genotoxic agents and for cancer risk and a useful tool to establish human exposure limits for genotoxic substances (Majer *et al.,* 2001; Speit *et al.,* 2007; Holland *et al.,* 2008; Speit *et al.,* 2011).

Micronuclei are formed in damaged cells of the basal layer during cell division. These cells then have to migrate to the surface to be collected for the MNT.

The use of biomarkers to identify genetic damage in individuals at higher risk of developing oral squamous cell carcinoma and to evaluate the malignant transformation potential of precancerous lesions is considered to be an important tool for cancer prevention. MNT on exfoliated cells from oral epithelium has been widely used for these purposes (Cerqueira & Meireles 2012) in all human tissues from which exfoliated cells can be obtained (Majer *et al.,* 2001).

Compared with other genotoxicity assays which are currently used for human biomonitoring, the MNT in exfoliated buccal cells is potentially an excellent candidate to serve as such a biomarker having many advantages:

(i) it is a simple and fast test system. The cells can be obtained easily and do not have to be cultivated. Processing and staining of the cells are less time-consuming compared to other test systems and can be performed in laboratories with basic equipment; (ii) the endpoint is well defined and can easily be recognised; (iii) cells can be fixed and stored for long periods of time; (iv) unlike other cytogenetic measurements, micronuclei are found in the interphase of the cell cycle. Therefore, all samples usually contain a sufficiently high rate of countable cells; (v) the simplicity of sample collection with non-invasive methods makes the test applicable to large sample sizes (Majer *et al.,* 2001; Holland *et al.*, 2008; Cerqueira & Meireles, 2012).

The time of sampling is also an important variable to consider. As the buccal cells turn over every 7-21 days, it is theoretically possible to observe the genotoxic effects of an acute exposure approximately 7-21 days later (Shojaei, 1998; Thomas *et al.*, 2009; Cerqueira & Meireles, 2012). Unlike lymphocytes which must be stimulated to undergo mitosis, and thus introduce problems of interpretation, epithelial cells do not need to be stimulated; micronuclei in exfoliated cells reflect genotoxic events that occurred in the dividing basal cell

layer 1 – 3 weeks earlier. Furthermore, at many sites, the technique is completely non-invasive, and repeated sampling is acceptable (Tolbert *et al.*, 1991).

There is some concern regarding the staining techniques in the MNT since there are many false-positive results in micronuclei frequency as a result of using Romanowsky-type stains such as Giemsa, May-Grunwald Giemsa (MGG) and/or Leishmann's, and Papanicolaou (Ayyad *et al.,* 2006; Nersesyan *et al.*, 2006) which leads to inaccurate assessment of DNA damage (Nersesyan *et al.*, 2006). Romanowsky stains have been shown to increase the number of false positives as they positively stain keratin bodies that are often mistaken for micronuclei and are therefore not appropriate for this type of analysis (Majer *et al.*, 2001; Holland *et al.,* 2008; Thomas *et al.,* 2009). The staining technique recommended is Feulgen because it is a DNA-specific stain and because permanent slides can be obtained and be viewed under both transmitted and/or fluorescent light conditions (Nersesyan *et al.,* 2006).

Figure 6 shows the aspect of micronuclei in exfoliated buccal cell when observed at 1000 magnification with immersion oil in optical microscope.

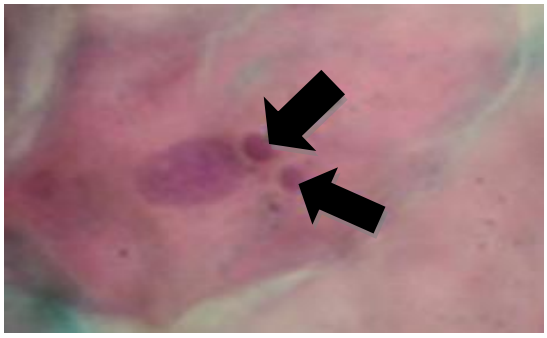


Figure 6 – Micronuclei in exfoliated buccal cell (1000X). Feulgen staining technique without counterstain.

3. COMET ASSAY

Rydberg and Johanson (1978) were the first to directly quantify levels of DNA damage in individual cells by embedding them in agarose on slides and then lysing under mild alkali conditions to allow for the partial unwinding of DNA (Valverde & Rojas, 2009a).

Östling and Johanson in 1984 developed a microgel electrophoresis technique for detecting DNA damage at the level of a single cell. In their technique, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salt, and the liberated DNA was electrophoresed under neutral conditions. Cells with an increased

frequency of DNA double-strand breaks displayed increased migration of DNA toward the anode. The migrating DNA was quantified by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. The neutral conditions used greatly limited the general utility of the assay though (Tice *et al.*, 2000; Valverde & Rojas, 2009a).

Subsequently, Singh *et al.* (1988) introduced a microgel technique involving electrophoresis under alkaline (pH>13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with incomplete excision repair sites, and alkali labile sites (Tice *et al.,* 2000; Hartmann *et al.,* 2003).

Because almost all genotoxic agents induce orders of magnitude more single strand breaks and/or alkali labile sites than double strand breaks, this version of the assay offered greatly increased sensitivity for identifying genotoxic agents (Valverde & Rojas, 2009a). Two years later, Olive and colleagues introduced another alkaline version of this assay in which DNA is electrophoresed at a pH of ≈12.3. Since the introduction of alkaline (pH > 13) comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include: (i) its demonstrated sensitivity for detecting low levels of DNA damage; (ii) the requirement for small numbers of cells per sample; (iii) flexibility; (iv) low costs; (v) ease of application; (vi) the ability to conduct studies using relatively short time period (a few days) needed to complete an experiment (Tice *et al.*, 2000).

The comet assay or single-cell gel electrophoresis (SCGE) is a simple, sensitive method for detecting DNA-strand breaks. Cells embedded in agarose on a microscope slide are lysed with detergent and 2.5 M NaCl and fresh Triton X-100 to remove membranes and soluble cell constituents, including most histones, leaving the DNA, still supercoiled and attached to a nuclear matrix, as a nucleoid. A break in one strand of a DNA loop is enough to release the supercoiling, and during electrophoresis the relaxed loops are able to extend towards the anode (Fairbairn *et al.*, 1995; Collins *et al.*, 1997; Moller *et al.,* 2000; Azqueta *et al.*, 2009; Collins & Dusinska, 2009). Electrophoresis causes DNA loops containing breaks to move towards the anode, forming “comets” when stained and visualised by fluorescence microscopy. The relative content of DNA in the tail indicates the frequency of breaks (Gedik *et al.*, 2002; Kumaravel & Jha, 2006; Collins & Dusinska, 2009).

DNA strand breaks can originate from the direct modification of DNA by chemical agents or their metabolites; from the processes of DNA excision repair, replication, and recombination; or from the process of apoptosis. Direct breakage of the DNA strands occurs when ROS

interact with DNA. In what refers to alkaline labile sites, those can be generated by depurination of an adducted base of the nucleotide and a subsequent conversion of the abasic site to a strand break detected by alkaline treatment (pH above 13.1) (Moller *et al.,* 2000).

This assay was adapted to measure oxidised purines and oxidised pyrimidines by the incubation of the nucleoids with bacterial DNA repair enzymes (Azqueta *et al.,* 2009), including formamidopyrimidine DNA glycosylase (FPG), which recognizes the oxidised purine 8-OHdG, Endonuclease III do detect oxidised pyrimidines, T4 endonuclease V to detect UV-induced pyrimidines dimmers, AlkA (3-methyladenine DNA glycosylase) for alkylated bases, or uracil DNA glycosylase, which removes misincorporated uracil from DNA (Collins & Dusinska, 2009).

Comet assay has become one of the standard methods for assessing DNA damage, with a wide range of applications, namely in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair (Collins, 2004, 2009); studying the mechanisms of action of genotoxic chemicals; investigating oxidative damage as a factor in disease; monitoring oxidative stress in animals or human subjects resulting from exercise, or diet, or exposure to environmental agents; studying the effects of dietary antioxidants; and monitoring environmental pollution by studying sentinel organisms (Dusinska & Collins, 2008; Azqueta *et al.*, 2009a). This assay is useful for evaluating xenobiotic impacts based on its use of small cell samples, and its ability to evaluate DNA damage in non-proliferation cells such as lymphocytes. In addition, the ability to obtain sufficient numbers of cells for analysis from different tissues, for instance lymphocytes and buccal cells provides a relatively non-invasive procedure for analysis (Valverde & Rojas, 2009a). Most of the studies assayed human blood cells because they circulate in the body, and the cellular, nuclear, and metabolic state of the blood cells can reflect the overall extent of body exposure (Valverde & Rojas, 2009). Although lymphocytes are, like all tissues, highly specialized, they can be seen as reflecting the overall state of the organism, insofar as they circulate through the whole body (Collins *et al.,* 2008). In addition, in biomonitoring studies, nasal epithelial cells and buccal cells have drawn the most attention because they are cells from tissues that come into direct contact with ingested or inhaled compounds (Moller *et al.*, 2000).

The congruence of results between the comet assay and other endpoints such micronuclei or sister chromatid exchanges (SCE), has been one of the principal reasons to increase the use of the comet assay as a biomarker for hazard assessment, particularly in monitoring the effects of occupational hazards (Valverde & Rojas, 2009a; 2009b). Biological monitoring has been an

important tool for the surveillance of medical health programs in European countries and for monitoring occupational hazards in the USA (Valverde & Rojas, 2009b).

3.1. FORMAMIDOPYRIMIDINE DNA GLYCOSYLASE (FPG)

Measuring DNA strand breaks gives limited information. Breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined. They may in fact be apurinic/apyrimidinic sites (i.e. AP sites or baseless sugars), which are alkali labile and therefore appear as breaks. Or they may be intermediates in cellular repair, because both nucleotide and base excision-repair processes cut out damage and replace it with sound nucleotides (Collins *et al.,* 2001; Collins, 2004). AP-sites are alkali-labile, so in principle they are expected to appear among the strand breaks detected in the standard alkaline comet assay. But it has not been convincingly demonstrated that all AP-sites are converted under these conditions (Azqueta *et al.*, 2009; Johansson *et al.*, 2010).

To make the assay more specific as well as more sensitive, an extra step was introduced of digesting the nucleoids with an enzyme that recognizes a particular kind of damage and creates a break. FPG detects the major purine oxidation product 8-OHG as well as other altered purines (Moller *et al.*, 2000; Collins *et al.*, 2001; Collins, 2004; Collins *et al.*, 2008). This enzyme was named for its ability to recognize imidazole-ring-opened purines, or formamidopyrimidines, namely 8-OHdG, 2,6-diamino-4-hydroxy-5-formadopyrimidine (FaPyG) and 4,6-diamino-5-formamidopyrimidine (FaPyA), which occur during the spontaneous breakdown of damaged purines; however, a major substrate in cellular DNA is 8-OHG (Smith *et al.*, 2006; Dusinska & Collins, 2008; Azqueta *et al.*, 2009; Collins, 2009).

A mammalian analogue of FPG, 8-Oxoguanine DNA glycosylase 1 (OGG1), has been applied in the comet assay, however studies performed comparing FPG and OGG1 reveals ineffectiveness of OGG1 (Azqueta *et al.*, 2009). For that reason, FPG continues to be the enzyme of choice for oxidised purines.

Figure 7 shows the aspect of comets observated at 400X magnification with DAPI fluorochrome in fluorescence microscope.

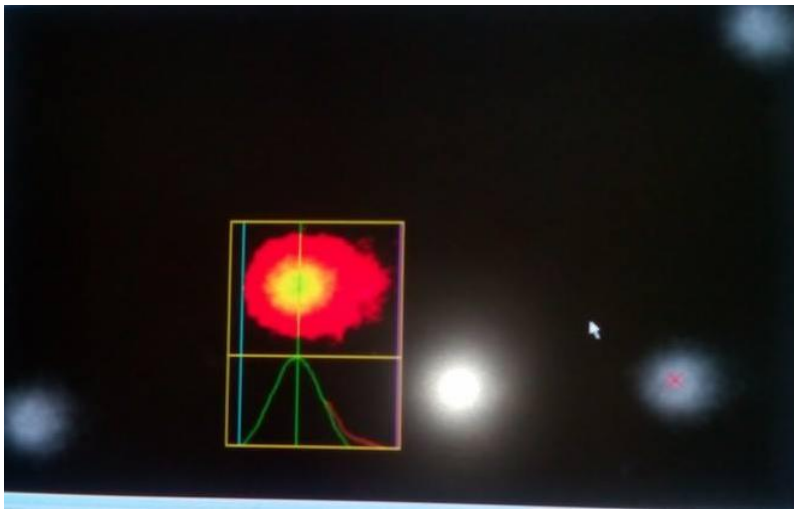


Figure 7 – Comets measured by Perceptive Instruments® software at 400X magnification. DAPI fluorescent staining technique.

CHAPTER 6 – INDIVIDUAL SUSCEPTIBILITY

1. GENETIC SUSCEPTIBILITY

Susceptibility is genetic when stemming from differences at the DNA level. Subareas of genetic susceptibility include: inheritable variation in carcinogen metabolizing enzymes (activation and detoxification); mutations of proto-oncogenes or tumour suppressor genes; hormonal and in immunologic factors and inherited differences in DNA adduct formation and DNA repair mechanisms (Ishibe & Kelsey, 1997; Vainio, 1998; Berwick & Vineis, 2000; Perera, 2000).

Given equal exposure to the same carcinogen, individuals will vary in their internal processing of the agent, depending on genetic background, acquired characteristics, and other past or ongoing exposures (Perera, 1996).

Individual variations in uptake of reactive chemicals, metabolism of environmental mutagens and repair of DNA damage, and others can affect the study outcome significantly (Au *et al.,* 1998). Those subjects who biotransform absorbed chemicals into effective doses even at the low exposure levels exhibit adverse effects comparing with the vast majority of the population who do not exhibit any adverse effects at the same exposure level (Mutti, 1999).

The development of molecular techniques and data from the human genome project, have contributed to a better understanding of the genetic basis of the variation in environmental disease outcome, namely based on the inheritance of different versions of polymorphic metabolizing genes, such as the cytochrome P450, the GST and the N-acetyl transferase genes. Inheritance of “unfavourable” versions of these genes is significantly associated with the development of a variety of environmental cancers, such as lung and bladder cancers. Data suggest that inheritance of the “unfavourable” genes caused individuals to have increased body burden of reactive metabolites from exposure to specific environmental mutagens. Therefore, these individuals have significantly increased risk for environmental cancers (Au *et al.*, 1998; Mutti, 1999).

There are certain cancers, such as colon, breast and prostate cancers that are due to a genetic predisposition. These very strong susceptibility genes may not be influenced by environmental factors, but there is evidence for environmental influences in some diseases even when there is strong genetic predisposition. Many genes in the genome of humans and other species influence the impact of environmental agents on the organism. Genetic controls on the

uptake, activation, detoxification, or repair of environmental insults are known. The exact number of genes involved in the organism's response to environmental hazards is unknown but could be very large (Barrett *et al.*, 1997).

1.1. SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

Single Nucleotide Polymorphisms (SNP) are of interest for a variety of reasons. First, a SNP, particularly when found in a functional gene region, may itself encode differences in protein form and expression, which in turn lead to disease and other, often subtler, phenotypic differences. Second, SNPs may mark or track the presence of other, perhaps less easily detected and processed genetic differences that cause phenotypes of interest. Third, they are useful in studying mutation rates and evolutionary history (Salisbury *et al.*, 2003).

Polymorphic variation in several types of genes may influence cancer susceptibility at the population level. In addition to the metabolism of xenobiotics, these polymorphisms can affect the metabolism of various dietary factors, the endogenous synthesis, metabolism and action of hormones, DNA repair, immune and inflammatory processes, oxidant stress, signal transduction and cell-cycle control (Perera, 2000).

An example of a main contribute to interindividual susceptibility is "Phase II" detoxifying enzymes, such as GST and N-acetyltransferase (NAT) that may add a more substantial attributable risk in a carcinogen-exposed population (Perera, 1996; Ishibe & Kelsey, 1997; Hussain & Harris, 1998). Different sources indicated that about 40% of the population has a deletion at this *locus* which has been linked to increased risk of bladder and lung cancers (Perera, 2000) or approximately 50% of Caucasians have a deletion in the GSTM1 gene (Perera, 1996; Au *et al.*, 1998) and in GSTT1 approximately 30% (Au *et al.,* 1998).

Certain combinations of metabolic polymorphisms are increasingly being linked to increased cancer risk. Acquired or inherited variations in the efficiency or fidelity of DNA repair can also influence individual susceptibility to cancer (Vineis, 1995; Perera, 2000). Common DNA variation, as opposed to rare mutations, could be responsible for a proportion of common human diseases [i.e., the common variant/common disease (CV/CD) hypothesis] (Crawford *et al.,* 2005). The risks associated with single SNPs in molecular epidemiology studies are generally expected to be less than the risks associated with reduced repair capacity phenotypes assuming that the repair capacity and mutagen sensitivity phenotypes are the sum of the impact of multiple variants in multiple genes along a pathway (Mohrenweiser, 2004).

2. GENETIC POLYMORPHISMS

A genetic polymorphism is defined as a gene variant that is prevalent at least at a frequency of 1% in a given population (Brooks, 2003; Thier *et al.*, 2003). The discovery of polymorphisms in genes for chemical metabolisms and for DNA repair has generated tremendous interest in understanding the phenomenon of genetic susceptibility in populations (Au *et al.,* 2004). There are several reasons why incorporating common genetic polymorphisms into epidemiologic studies will enhance our understanding of the relationship between environmental exposures and cancer: (i) by characterizing the effects of established carcinogens among people with particular genetic variants, one can gain mechanistic insights into the origins of cancer; (ii) by identifying and studying population subgroups that are genetically susceptible to a particular carcinogen, one can uncover the low levels of risk associated with certain common exposures; and (iii) by determining which susceptibility genes are associated with a given cancer, one can generates insights into the potential carcinogens acted upon by these gene products (Rothman *et al.*, 2001; Zijno *et al.*, 2006). Genetic polymorphisms may be particularly important for low exposures levels, which could influence the whole process of risk assessment, a process that is now starting to take individual variability in susceptibility into account (Garte, 2008).

3. DNA DAMAGE REPAIR SYSTEMS

Genes involved in DNA repair play an important role in carcinogenesis. Major research activities have been focused on polymorphisms in these genes as an important component of the susceptibility phenomenon because DNA repair activities are critically involved with protection of the genome and in cancer prevention (Au *et al.*, 2004). Their primary duty is to maintain the integrity of the genome by removing lesions created by chemicals and other environmental exposures. These lesions, left unrepaired, contribute to cell death, mutation, chromosome damage, and carcinogenesis (Ishibe & Kelsey, 1997; Berwick & Albertini, 2008).

At least four major, partly overlapping damage repair pathways operate in mammals – nucleotide excision repair (NER), base-excision repair (BER), homologous recombination (HR), and non homologous end joining (NHEJ) (Hoeijmakers, 2001).

3.1. X-RAY CROSS COMPLEMENTATION GROUP 3 (XRCC3)

DNA double-strand breaks are considered to be particularly important because their repair is intrinsically more difficult than that of other types of DNA damage posing a particular threat to genomic integrity (Hoeijmakers, 2001; Johnson & Jasin, 2001; Khanna & Jackson, 2001). There are two distinct and complementary mechanisms for DNA double strand breaks repair - HR and NHEJ pathways (Hoeijmakers, 2001; Khanna & Jackson, 2001). HR (e.g. *RAD51* gene) repairs double strand breaks with a template, such as a sister chromatid or homologous chromosome, found elsewhere in the genome, whereas NHEJ uses no repair template at all. NHEJ can be mutagenic; HR is more accurate and increases in S-G_2 cell cycle phases. HR also can result in deletions and rearrangements (Berwick & Albertini, 2008). Notably, a growing number of mammalian proteins are related to *RAD51*, and some of them, such as *RAD51B, XRCC2* and *XRCC3*, are involved in HR and may contribute to the maintenance of genome stability (Khanna & Jackson, 2001).

The X-ray repair cross-complementing gene 3 (*XRCC3*) participates in DNA double-strand break/recombination repair and is a member of an emerging family of Rad-51-related proteins that participate in the HR pathway to maintain chromosome stability, repair DNA damage, and correct chromosome segregation in mammalian cells (Bolognesi *et al.,* 2013; Catalán *et al.,* 2000b; Matullo *et al.,* 2001; Bonassi *et al.*, 2003; El-Zein *et al.*, 2006; Iarmarcovai *et al.,* 2006; Kirsch-Volders *et al*., 2006; Battershill *et al*., 2008; Mateuca *et al.*, 2008). This pathway is of great importance in preventing chromosomal fragmentation, translocations, and deletions, which can lead to carcinogenesis (Winsey *et al.,* 2000). The *RAD51* paralogue *XRCC3* promotes the HR repair of double strand breaks induced either directly or indirectly following replication of closely spaced single strand breaks (Mateuca *et al.,* 2008). *XRCC3* is also required for the assembly and stabilization of *RAD51* (Winsey *et al.*, 2000; Shen *et al.*, 2002). In addition to repairing double strand-breaks, *XRCC3* also plays a role in the repair of more global DNA damage arising from carcinogen treatment (Araujo *et al.,* 2002). It was reported that *XRCC3* mutation causes severe chromosome instability and increased sensitivity to DNA cross-linking drugs (Liu *et al.*, 1998; Brenneman *et al.,* 2000).

XRCC3 is on chromosome 14 (14q32.3) and its most studied polymorphism is a transition between cytosine and thymine in exon 7 (*XRCC3*-18067C> T) at codon 241 that results in the substitution of a threonine by a methionine (Shen *et al.*, 2002; Bonassi *et al.*, 2003; Wang *et al.,* 2003; Battershill *et al.*, 2008; El-Zein *et al.*, 2008). The *XRCC3* Thr241Met variation does not reside in the adenosine triphosphate-binding domain, the only functional domain identified in the resulting protein (Manuguerra *et al.*, 2006); however, conversion from a hydroxyl amino

acid to one with a sulfhydryl group represents a substantial change in protein functional characteristics (Winsey *et al.,* 2000). This polymorphism has been proposed as an allele of low penetrance associated with breast and lung cancer, acute myeloid leukemia, risk of upper aerodigestive tract cancer (Stich & Rosin, 1983; Ramirez & Saldanha, 2002; Wang *et al.*, 2003; Au *et al.*, 2004; El-Zein *et al*., 2008) and risk for melanoma skin cancer and bladder carcinoma (Wang *et al.*, 2003).

3.2. HUMAN 8-OXOGUANINE DNA GLYCOSYLASE 1 (OGG1)

The cellular defense system against 8-oxoguanine (8-OHdG) mutagenesis involves BER, NER, mismatch repair and prevention of incorporation. BER via DNA glycosylase (OGG1) represents the main mechanism of protecting the integrity of the human DNA with respect to 8-OHdG. Repair of oxidative damage is initiated by OGG1 (Hu & Ahrendt, 2005; Jiao *et al.*, 2007), and its activity is responsible for the excision of 8-OHdG and the structurally related lesion 2,6-diamino,4-hydroxy-5-formamidopyrimidine, a hydrolytic ring-opening product of guanine. BER is the main guardian against damage due to cellular metabolism, including that resulting from reactive oxygen species, methylation, deamination and hydroxylation (Hoeijmakers, 2001). It is the key repair system for removing small-sized base damage that includes oxidized or reduced bases and non-bulky DNA adducts. BER employs DNA lesion-specific glycosylase to recognize and hydrolytically cleaves and removes the altered base, giving rise to an abasic site (Cooke *et al.*, 2003; Au *et al.,* 2004; Sedelnikova *et al.*, 2010). OGG1 is considered to be the main enzyme responsible for the removal of 8-OHdG in humans, removing it when it is paired with cytosine. However, OGG1 does not release 8-OHdG when misrepaired with an adenine or a guanine (Boiteux & Radicella, 1999; Ersson, 2011). In general, the glycosylase mechanism of action acts by sliding along the DNA chain, frequently forming an “interrogation” structure where bases flip out from the DNA helix and are inspected by the enzyme extra-helically. When encountering a damaged base, it is transferred from the interrogation complex to the enzyme’s active site. Bifunctional glycosylases, such as OGG1 and FPG, possess both glycosylase and AP-lyase activity, cleaving first the glycosidic bond between the base and the sugar and then the DNA backbone (at the 3’-carbon of the abasic sugar). FPG also have an attached AP-endonuclease activity cleaving the other phosphodiester bond at the DNA backbone (at the 5’-carbon of the abasic sugar) (Wiseman & Halliwell, 1996; Ersson, 2011).

Some findings indicate that the inactivation of *OGG1* plays a role in the multistage process of carcinogenesis. The human *OGG1* gene is located on chromosome 3 (3p26), and encodes a bifunctional DNA glycolylase endowed with a AP lyase activity. This is a region frequently lost

in various types of cancer, especially in small-cell lung cancers where loss of heterozygosity in nearly 100% of the cases can be observed. Loss of one *OGG1* allele may lead to a moderate generation of 8-OHdG in DNA. However, loss of both alleles would abrogate *OGG1* activity imposing an increased risk of mutagenicity on the cell due to the imbalance of oxidative burden and accumulation of 8-OHdG in DNA (Pilger & Rüdiger, 2006).

The product of *OGG1* gene exhibits specificity and activity for the excision of 8-OHdG (Boiteux & Radicella, 1999; Cooke *et al.*, 2003; Au *et al.*, 2004), and has a major role in the prevention of ROS-induced carcinogenesis (Cooke *et al.*, 2003).

A common polymorphism is Ser326Cys, which affects over 50% of Chinese and Japanese and approximately 33-41% of Caucasian population (Hu & Ahrendt, 2005). The *OGG1* has a C→G polymorphism at position 1245 in exon 7 which causes the substitution of serine by cysteine at codon 326 (Kohno *et al.,* 1998) and it is associated with increased risk for cancer (Macpherson *et al.*, 2005).

4. ALCOHOL DEHYDROGENASE 5 (ADH5)

Genetic polymorphisms of metabolically relevant enzymes may lead to relevant shifts in the critical balance of activation and inactivation, and subsequently to altered individual disease susceptibility (Thier *et al.,* 2003). To prevent the lethal and mutagenic effects of formaldehyde, several repair mechanisms are involved. Detoxification of formaldehyde can be carried out by enzymes like formaldehyde dismutase, methylformate synthase, or glutathione-independent formaldehyde dehydrogenase (Gonzalez *et al.*, 2006; Neuss & Speit, 2008).

The glutathione-dependent formaldehyde dehydrogenase (FDH, also known as alcohol dehydrogenase 5, ADH5; EC 1.2.1.1) is the most important enzyme for the metabolic inactivation of formaldehyde (Just *et al.,* 2011; NTP, 2011).

According to the Human Genome Organization (HUGO) Gene Nomenclature Committee (www.genenames.org) this gene is called alcohol dehydrogenase 5 (*ADH5*). Previous names and aliases are: formaldehyde dehydrogenase (FDH); chi isozyme of ADH; ADH, class III and S-nitrosoglutathione reductase, and even alcohol dehydrogenase 3 (ADH3) is still frequently used in the scientific literature (Just *et al.,* 2011). *ADH5* is composed of nine exons and eight introns (Hur & Edenberg, 1992), is located on chromosome 4 (4q23) (Just *et al.*, 2011), and has been detected in all human tissues and at all stages of development. This enzyme is an important component of cellular metabolism for the elimination of formaldehyde serving as the prime guardian against formaldehyde (Hedberg, 2001) and offering enzymatic defence against both formaldehyde and nitrosative stress in human oral tissue and in epithelial cell

lines. Although formaldehyde is rapidly metabolized, it is an electrophile that reacts with a variety of endogenous molecules, including glutathione, proteins, nuclei acids, and folic acid (NTP, 2011). This is the only ADH identified thus far that is capable of oxidizing formaldehyde in a glutathione dependent reaction (Kaiser *et al*., 1991; Engeland *et al.*, 1993; Lee *et al.,* 2003).

ADH5 oxidizes S-hydroxymethylglutathione (which is formed spontaneously from formaldehyde and glutathione) to S-formylglutathione. Formation of S-hydroxymethylglutathione efficiently counteracts the presence of free formaldehyde; a reaction that is determined by the fact that free glutathione is present in cells in abundance. S-formylglutathione is then further metabolized by S-formylglutathione hydrolase to yield formic acid and reduced glutathione. The activities of ADH5 are two to three orders of magnitude lower than those of S-glutathione hydrolase and thus the ADH5-catalysed step is rate-limiting. An alternative pathway involves aldehydes dehydrogenase (ALDHs) (Just *et al.,* 2011; NTP, 2011).

Formaldehyde is rapidly metabolized by ADH5 and S-formyl-glutathione hydrolase to formic acid, which enters the one-carbon pool and can be either excreted in the urine or oxidized to carbon dioxide and exhaled. ADH5 has been detected in all human tissues at all stages of development, from embryo through adult. Although formaldehyde is rapidly metabolized, it is an electrophile that reacts with a variety of endogenous molecules, including glutathione, proteins, nucleic acids, and folic acid (Hedberg, 2001; Gonzalez *et al*., 2006; NTP, 2011).

Two *ADH5* polymorphisms are known: *ADH5* Val309Ile, a transition of a cytosine to a thiamine in codon 309 that consists in the substitution of a valine by an isoleucine; and *ADH5* Asp353Glu, a transversion of an adenine to a cytosine in codon 353, that results in the substitution of an aspargine by a glutamine. To our knowledge, no association has been found between *ADH5* polymorphisms and disease (Wang *et al.*, 2010).

5. VITAMIN D RECEPTOR (VDR)

$1,25(OH)_2D_3$ is the biologically active form of vitamin D, and it exerts its effects mainly through binding to nuclear vitamin D receptor (*VDR*) and further binding to specific DNA sequences, namely vitamin D response elements. Through this genomic pathway, $1,25(OH)_2D_3$ exerts transcriptional activation and repression of target genes by binding to the *VD*R (Cui & Rohan, 2006; Polidori & Stahl, 2009).

The *VDR* (OMIM 601769) is a crucial mediator for the cellular effects of vitamin D and additionally it interacts with other cell-signalling pathways that influence cancer development (Raimondi *et al.*, 2009; Orlow *et al.*, 2012).

The *VDR* is an intracellular hormone receptor that specifically binds the biologically active form of vitamin D, $1,25(OH)_2D_3$ and interacts with specific nucleotide sequences of target genes to produce a variety of biologic effects. The *VDR* gene is located on chromosome 12q12-q14 and several SNPs in this gene have been identified that may influence cancer risk (Kállay *et al.*, 2002; Uitterlinden *et al.*, 2004; Maruyama *et al.,* 2006; Raimondi *et al.*, 2009). Numerous studies *in vitro* and *in vivo* have shown proapoptotic and anticancer effects upon biding of $1,25(OH)_2D_3$ to the *VDR* for many types of cancer, namely in cells derived from tumours of the breast, prostate, pancreas, colon, bladder, cervix, thyroid, pituitary, skin (squamous cell carcinoma, basal cell carcinoma and melanoma), glioma, neuroblastoma, leukemia and lymphoma cells (Maruyama *et al.*, 2006; Raimondi *et al.,* 2009). Also *VDR* polymorphisms have been implicated in several immune and inflammatory disorders, including mycobacterial and human immunodeficiency virus susceptibility, diabetes, psoriasis, and Crohn's disease, although the precise mechanisms of action of these diverse disease-related effects remain speculative, such as asthma and atopic risk (Raby *et al.*, 2004).

The involvement of *VDR* in multiple pathways and points of convergence within these pathways indicates the potential importance of *VDR* in the etiology of cancer (Raimondi *et al.,* 2009). Binding of *VDR* by $1,25\ (OH)_2D_3$ leads to increased differentiation and apoptosis as well as reduced proliferation, invasiveness, angiogenesis and metastasis (Bao *et al.,* 2010).

VDR polymorphisms have been identified and analysed so far mostly in Caucasians and, to a lesser extent, in other ethnic groups (Uitterlinden *et al.*, 2004). The *VDR* gene has two sets of polymorphisms, one at the 3' end of the gene involving a series of polymorphic sites and the other at 5' end of the gene affecting the start codon. The 3' polymorphisms are defined by the enzymes *BsmI, ApaI*, and *TaqI,* and a poly-A microsatellite, all of which are in linkage disequilibrium (Medeiros *et al.*, 2002). Although neither the *TaqI* RFLP in exon 9 nor the linked *BsmI* and *ApaI* restriction fragment length polymorphism (RFLPs) in intron 8 are known to have functional consequences themselves, these sequence polymorphisms have been shown to be associated with varying levels of the circulating *VDR* ligand 1,25-D (Medeiros *et al.,* 2002).

BsmI polymorphism which is located at the 3' end of the gene apparently does not change the translated protein and has no known function on *VDR*. This G to A polymorphisms is located in

intron 8 and is linked in a haplotype with variable-length polyA sequence within the 3'-untranslated region that has an impact on *VDR* mRNA stability (Shahbazi *et al.*, 2013).

BsmI gene polymorphism is one of the most important subtype of *VDR* gene polymorphisms, and it is genotyped as *BB*, *Bb*, or *bb* by polymerase chain reactions based on polymorphism at the *BsmI* restriction site (Qin *et al.*, 2013).

Biomonitoring and molecular epidemiology could therefore play an important role in identifying susceptible individuals, particularly those suffering a combination of high risk factors, namely a high level of exposure to chemicals, inherited cancer predisposing genes and a deficiency of protective factors, such as those arising, for example, from diet. Individual susceptibility factors can influence all stages between exposure and the onset of disease (Watson & Mutti, 2004). The strength of this field is that it has allowed for the investigation of how genes and the environment may interact either positively or negatively in humans. By identifying susceptible subpopulations and their association with cancer prevalence, epidemiologists have gained a better understanding of this heterogeneous disease, extending what could not be determined via traditional methods (Ishibe & Kelsey, 1997).

CHAPTER 7 – NUTRITIONAL RESEARCH

1. NUTRITION RESEARCH

Dietary habits are recognized to be an important modifiable environmental factor influencing cancer risk and tumour behaviour. Although some studies have estimated that about 30-40% of all cancers are related to dietary habits, the actual percentage is highly dependent on the foods consumed and the specific type of cancer (Strickland & Groopman, 1995; Davis & Milner, 2007; Sutandyo, 2010).

Nutrition science has evolved into a multidisciplinary field that applies molecular biology and integrates individual health with the epidemiologic investigation of population health (Go *et al.,* 2003). Nutritional genomics studies the functional interaction of food and its components, macro and micronutrients, with the genome at the molecular, cellular, and systemic level (Ordovas & Corella, 2004). In nutritional genomics, two terms are used: nutrigenomics and nutrigenetics. Nutritional genomics, defined as the interaction between nutrition and an individual's genome or the response of an individual to different diets, will likely provide important clues about responders and non-responders (Davis & Milner, 2004).

Nutritional genomics provides the means to develop molecular biomarkers of early, pivotal changes between health maintenance and disease progression (Elliott & Ong, 2002), applying systems biology to build models that will integrate information about intake, gene polymorphisms, gene expression, phenotypes, diseases, effect biomarkers and susceptibility biomarkers (Ordovas & Corella, 2004).

A nutritional biomarker can be any biological specimen that indicates the nutritional status with respect to intake or metabolism of dietary constituents. It can be a biochemical, functional or clinical index of status of an essential nutrient or other dietary constituent. Nutritional biomarkers can have three categories depending on their use: (i) a means of validation of dietary instruments; (ii) surrogate indicators of dietary intake; or (iii) integrated measures of nutritional status for a nutrient (Potischman & Freudenheim, 2003).

1.1. NUTRIGENETICS

Nutrigenetics refers to the impact of genetic variability between individuals in their response to a specific dietary pattern, functional food or supplement for a specific health outcome (Bull & Fenech, 2008). It examines the effect of genetic variation on the interaction between diet

and disease. The specific fields of genome-health nutrigenomics and genome-health nutrigenetics are proposed on the premise that a more useful approach to the prevention of diseases caused by genome damage is to take into consideration. Inappropriate nutrient supply can cause sizeable levels of mutation or alter expression of genes required for genome maintenance. Genetic polymorphisms may alter the activity of genes that affect the bioavailability of micronutrients and/or the affinity for micronutrient cofactors in key enzymes involved in DNA metabolism or repair (Bull & Fenech, 2008).

Nutrigenetics attempts to identify and characterize gene variants associated or responsible for differential responses to nutrients. The goal of nutrigenetics is to generate recommendations regarding the risks and benefits of specific diets or dietary components to the individual as a personalized or individualized nutrition (Ordovas & Corella, 2004).

Genetic polymorphisms may be partially responsible for variations in individual response to bioactive food components (Davis & Milner, 2004). Several genetic polymorphisms have been identified like folate metabolism (Guerreiro *et al.*, 2007; Carmona *et al.,* 2008), iron homoeostasis, bone health, lipid metabolism, immune function and others (Elliott & Ong, 2002), that can have significant association with nutrients in health/disease outcomes. Some common polymorphisms in genes involved in nutrient metabolism, metabolic activation and/or detoxification could establish the magnitude whether there is a positive or negative response to a food component (Davis & Milner, 2004). An example is the case of folate metabolism, there are common polymorphisms in genes that control folate metabolism that have been linked to conditions such as neural tube defects, Down's syndrome, homocystineamia, and cancer (Elliott & Ong, 2002). If the mechanisms by which these polymorphisms disturb folate mechanism and alter disease risk can be elucidated, it should be possible to develop dietary or therapeutic strategies for "at risk" individuals to redress the balance. Polymorphisms have also been identified in genes involved in lipid metabolism that are important in determining an individual's plasma low density lipoprotein cholesterol concentration, a marker of cardiovascular disease risk. It is important to consider the logistics and costs of routine genetic screening for many genes, the provision of appropriate counselling, and public attitudes and ethical issues associated with such screening in relation to, say, life insurance and family planning (Elliott & Ong, 2002).

1.2. NUTRIGENOMICS

Nutrigenomics focuses on the effect of nutrients on the genome, proteome, metabolome, epigenome and transcriptome. Because it is a complex area of knowledge there are many different definitions regarding this concept (Ordovas & Corella, 2004). The term nutrigenomics emerged from the mapping of the human genome and provides researchers with the tools for using systems biology into exploitation of the relationship between nutrition and health *(Go et al.*, 2003).

An integrated framework that simultaneously examines genetics and associated polymorphisms with diet-related diseases (nutrigenetics), nutrient induced changes in DNA methylation and chromatin alterations (nutritional epigenomics), nutrient induced changes in gene expression (nutritional transcriptomics), and altered formation and/or bioactivation proteins (proteomics) will allow for greater understanding of the interrelationships between diet and cancer risk and tumour behaviour (Davis & Milner, 2004).

Since the response to a bioactive food component may be subtle, careful attention will need to be given to characterizing how the quantity and timing of exposure influence small molecular weight cellular constituents (metabolomics). Managing this enormous amount of information will necessitate new and expanded approaches to bioinformatics (Davis & Milner, 2004).

Nutrigenomics will promote and increase understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phases of diet-related disease and the extent to which individual sensitizing genotypes contribute to such diseases. Eventually, nutrigenomics will lead to evidence-based dietary intervention strategies for restoring health and fitness for preventing diet-related disease (Afman & Müller, 2006).

In short, nutrigenomics is the study of molecular relationships between nutritional stimuli and the response of the genes by application of high-throughput functional genomic technologies in nutrition research. Applied wisely, it will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phase of a diet-related disease, and to what extend individual sensitizing genotypes contribute to such disease (Ordovas & Corella, 2004). Such techniques can facilitate the definition of optimal nutrition at the level of populations, particular groups, and individuals. This in turn should promote the development of new food derived treatments and functionally enhanced foods to improve health (Elliott & Ong, 2002).

2. DIET AND DNA DAMAGE

Dietary patterns involve complex interactions of food and nutrients summarizing the total diet or key aspects of the diet for a population under study. In that sense it is important to focus in each nutrient, but also in the whole diet itself. For instance, several studies have highlighted the protective effect of the so call Mediterranean diet (high consumption of vegetables, legumes, fruits, nuts and minimally processed cereals, and mono-unsaturated lipids, moderately high consumption of fish, low consumption of dairy and meat products and regular but moderate intake of alcohol) in cancer prevention (Couto *et al.*, 2011, 2013). Of major importance are also epidemiological studies on the role of environmental exposure to carcinogens in diet and specific cancers whose incidence is known to vary considerably among countries (Strickland & Groopman, 1995). The link between diet and cancer is revealed by the large variation in incidence and by the observed changes in incidence in those communities who migrated to a different geographic area and culture (Anand *et al.*, 2008). The substantial increases in the risk of cancers are observed in populations migrating from low- to high-risk areas, suggesting that international differences in cancer incidence can be attributed primarily to environmental or lifestyle factors rather than genetic factors (Strickland & Groopman, 1995; Anand *et al*., 2008).

Diet can influence cancer development in several ways, namely direct action of carcinogens in food that can damage DNA, diet components (macro or micronutrients) that can block or induce enzymes involved in activation or deactivation of carcinogenic substances (Willett & Giovannucci, 2006). Moreover, inadequate intake of some molecules involved in DNA synthesis, repair or methylation can influence mutation rate or changes in gene expression. Other mechanism that diet can influence DNA mutation, and consequently cancer risk, are energy balance and growth rates, since nutrition will influence hormone levels and growth factors that will influence the rate of cell division, cell cycling, and consequently influence time for DNA repair and/or replication of DNA lesions (Willett & Giovannucci, 2006). High levels of insulin like growth factor are associated with some cancers, namely colon cancer (Pollak, 2000).

Several studies support the idea that diet can influence the risk of cancer; however information concerning the precise dietary factor that determines human cancer is an ongoing debate (Ames, 2001; Key *et al.,* 2004; Anand *et al*., 2008; Couto *et al.*, 2011). A lot of epidemiological studies, involving food frequency questionnaires, have been developed providing important information concerning diet and cancer, however, diet is a complex

composite of various nutrients (macro and micronutrients) and non-nutritive food constituents that makes the search for specific factors almost limitless.

The definition of nutrient is variable and continues to evolve. A nutrient is classically defined as a constituent of food necessary for normal physiological function and essential nutrients are those required for optimal health. The postgenomic era classifies nutrient as a "fully characterized (physical, chemical, physiological) constituent of a diet, natural or designed, that serves as a significant energy yielding substrate or a precursor for the synthesis of macromolecules or of other components needed for normal cell differentiation, growth, renewal, repair, defence and/or maintenance or a required signalling molecule, cofactor or determinant of normal molecular structure/function and/or a promoter of cell and organ integrity" (Go *et al*., 2003).

3. ENERGY BALANCE

Calorie restriction (undernutrition without malnutrition) prevents a variety of cancers in experimental animal model. The influence of calorie restriction on carcinogenesis is effective in several species, for a variety of tumour types, and for both spontaneous tumours and chemically induced cancers (Hart *et al.*, 1999; Hursting *et al.*, 2003). In rodents, experiments with caloric restriction showed suppression in the carcinogenic action of diethylnitrosamine (Lagopoulos & Stalder, 1987) and also inhibition of radiation induced cancers (Gross & Dreyfuss, 1990).

Calorie restriction reduces metabolic rate and oxidative stress, improves insulin sensitivity, and alters neuroendocrine and sympathetic nervous system function in animals (Heilbronn & Ravussin, 2003).

A number of molecular processes also change with changes in energy consumption. Regardeless of the source and nature of DNA damage, DNA repair is better preserved and/or enhanced when caloric consumption decreases (Hart *et al*., 1999).

The possible mechanisms associating calorie restriction to cancer prevention evolve regulation of cellular proliferation and apoptosis (decrease in DNA replication), reduction in metabolic rate, in oxidative damage and in inflammation mediators (reduction in ROS and consequent reduction in DNA damage) (Masoro, 2005). Inversely, the association between obesity (a positive energy balance) and cancer can be partially explained by alterations in hormone levels and metabolism that could influence cell proliferation, differentiation and apoptosis (Willett & Giovannucci, 2006).

Moreover, it is well established that excess calorie intake, resulting in fat deposits, is a risk factor for cancer. Digestion, absorption, metabolism and excretion of excess nutrients require oxidative metabolism and produce more active oxygen species which cause DNA damage (Sugimura, 2000; Hwang & Bowen, 2007; Sutandyo, 2010). Since oxidative DNA damage is suggested to have a role in carcinogenesis, this may be one mechanism by which dietary change can reduce cancer risk (Djuric & Kritschevsky, 1993).

Calorie restriction is hypothesized to lessen oxidative damage by reducing energy flux and metabolism, or the "rate of living", thereby influencing the aging process. Calorie restriction is linked to oxidative stress, reducing it in various species, including mammals. Therefore, reducing metabolic rate by using calorie restriction may reduce oxygen consumption, which could decrease ROS formation and potentially increase life span (Heilbronn & Ravussin, 2003).

4. MICRONUTRIENTS

Micronutrients are a set of approximately 40 substances, including vitamins, essential minerals and other compounds required in small amounts for normal metabolism, that are essential for human health (Ames, 1998; Lal & Ames, 2011). Micronutrients are capable of acting via a number of mechanisms to block DNA damage, mutation, and carcinogenesis by oxygen radicals, PAHs, and other chemical carcinogens (Perera, 2000; Collins & Ferguson, 2004). Mutations have been related to the deficit, rather than the excess, of micronutrients (Ferguson & Philpott, 2008).

Epidemiological studies performed by the American Institute for Cancer Research/World Cancer Research Fund have also shown that individuals who consume large amounts of fruits and vegetables rich in micronutrients with antioxidant properties, (such as vitamin C, vitamin E, carotenoids and flavonoids) show a lower incidence of lung, stomach, oesophagus, breast, colon, liver, pancreas, endometrium, oral cavity and pharynx cancer (Prado *et al.*, 2010; Sutandyo, 2010), presumably because many micronutrients are required as cofactors in DNA maintenance reactions, including DNA synthesis, DNA repair, DNA methylation and apoptosis (Ferguson, 2002).

Micronutrient deficiency or excess can have modifying effects on genomic integrity that may involve nutrient-nutrient or nutrient-gene interactions and may depend on an individual's genetic constitution (Fenech *et al.,* 2005; Thomas *et al.*, 2011). Therefore, determining the intake levels of micronutrients required to maintain genome stability is an essential step in the definition of optimal diets for the prevention of cancer and other diseases caused by genome damage (Fenech *et al*., 2005).

Complex gene-environment and nutrient-nutrient interactions are also risk determinants for most disease states. Thus, the individual's genes, environmental exposures and physiological state must all be considered when determining disease risk.

In a biological system, an antioxidant can be defined as any substance which, when present at low concentration in relation to oxidizable substrates, would significantly inhibit or delay oxidative processes, while often being oxidized itself (Wanasundara & Shahidi, 2005; Kumar, 2011). The oxidazable substrate may be any molecule that is found in foods or biological materials, including carbohydrates, DNA, lipids and proteins (Wanasundara & Shahidi, 2005).

Antioxidants delay autoxidation by inhibiting formation of free radicals or by interrupting the propagation of free radical by one (or more) of several mechanisms: (i) scavenging species that initiate peroxidation, (ii) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (iii) quenching O_2^- preventing formation of peroxides, (iv) breaking the autoxidative chain reaction, and/or (v) reducing localized O_2 concentration (Brewer, 2011).

Antioxidants defend against both enzymatic and non-enzymatic reactions protecting the body against oxidative damage. Cellular DNA may be protected against oxidation by antioxidants, and oxidised DNA lesions are removed by several repair systems such as base excision repair and nucleotide excision repair that have overlapping specificity and may interact or function as back-up systems (Guarnieri *et al.*, 2008). Antioxidants may be molecules that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized (Lü *et al.,* 2010).

Non enzymatic antioxidants are frequently added to the food to prevent lipid oxidation. Several lipid antioxidants can exert pro-oxidant effect towards other molecule under certain circumstances thus antioxidants for food and therapeutic use must be characterized carefully (Kumar, 2011).

Antioxidants can be classified as primary or natural and secondary or synthetic. Natural antioxidants comprise enzymes, such as superoxide dismutase, catalase and glutathione peroxidase; they also include low molecular weight antioxidants, such as lipid and water soluble antioxidants (Hamid, 2010; Lü *et al.*, 2010; Kumar, 2011).

The antioxidant enzymes are complemented by small-molecule antioxidants, some of which are derived exclusively from diet and are vitamins. These small-molecule antioxidants are extra and intra-cellular, and include ascorbic acid (vitamin C), glutathione, and tocopherols

(vitamin E mostly). The mechanisms by which these antioxidants act at the molecular and cellular level include roles in gene expression and regulation, apoptosis, and signal transduction, being thus involved in fundamental metabolic and homeostatic processes (Frei, 1999; Hamid, 2010).

Hamid *et al.* (2010) classifies the natural antioxidants in three categories: mineral antioxidants, vitamins and phytochemicals. Mineral antioxidants are co-factor of antioxidant enzymes. Their absence will definitely affect metabolism of many macromolecules such as carbohydrates, and examples include selenium, copper, iron, zinc and manganese. Antioxidant vitamins are needed for most body metabolic functions; they include vitamin B, C and E (Cooke *et al.*, 2003; Hamid, 2010).

Chemoprevention is the process of using natural or synthetic compounds to block, reverse, or prevent the development of cancers through the action on multiple cellular mechanisms. Generally, these cellular mechanisms can be grouped in two: (i) Anti-mutagenesis, that includes the inhibition of the uptake, formation/activation of carcinogens, their detoxification, the blockage of carcinogen-DNA binding, and the enhancement of fidelity of DNA repair; (ii) Anti-proliferation/anti-progression, that includes modification of signal transduction pathways, inhibition of oncogene activity, and promotion of the cellular modulation of hormone/growth factor activity (Bartsch & Gerhäuser, 2009).

Potential chemopreventive agents are to be found both among nutrients and non-nutrients in diet (Tanaka *et al.*, 2001). Dietary components with potential cancer chemopreventive activity include vitamins, fibre, and minerals. If chemopreventive agents, as supplements, are to be suitable for the large-scale prevention of cancer in the general population, they should have high acceptance, low cost, oral consumability, high efficacy, no or low toxicity, and a known mechanism of action (Bartsch & Gerhäuser, 2009). Promising chemopreventive agents currently investigated in preclinical and clinical studies include naturally occurring anti-inflammatory agents, antiestrogens, micronutrients, phytochemicals, and some synthetic analogues (Banakar, 2004; Bartsch & Gerhäuser, 2009).

4.1. VITAMINS

The role of vitamins in cancer chemoprevention has been increasingly under scrutiny. Antioxidants (vitamins A, D, E) are known to be reducing agents and these molecules are capable of slowing or preventing the oxidation of other molecules (Awodele *et al.*, 2010).

Vitamins A, D and E belong to the family of fat-soluble vitamins. Their intakes have been associated with reduced risk of several chronic diseases, particularly some cancers and heart

diseases. In contrast to water-soluble vitamins, fat-soluble vitamins are stored in the liver and fatty tissues and are only slowly excreted from the body. Thus, they may have deleterious or toxic consequences if consumed at very high levels (Jenab *et al.*, 2009). They are at the end of oxidative chain reactions, removing free radicals and preventing the oxidation of unsaturated fats; and are clearly documented anti-genotoxic and antimutagenic potential antioxidants (Awodele *et al.*, 2010).

4.1.1. VITAMIN A

Retinol (vitamin A) and its metabolites (retinoids) are important micronutrients that regulate many biological processes such as cellular growth and differentiation. The classical mechanism of action by retinoids is through activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Fritz *et al.*, 2011; Pasquali *et al.,* 2013). In experimental models, retinoids suppress the transforming effects of carcinogens, inhibit growth of premalignant cells, enhance differentiation of malignant cells and induce apoptosis (Stich *et al.,* 1984a; Kristal, 2004).

The term vitamin A (all-*trans* retinol) is often used as a general term for all compounds that exhibit the biological activity of retinol, while the term retinoid refers to both naturally occurring and synthetic compounds bearing a structural resemblance to all-*trans* retinol. Biologically important oxidation products of retinol are retinal and retinoic acids that occur in several isomeric forms such as 11-cis retinal, 9-cis, or all-trans-retinoic acid, but the important biologically active form of vitamin A is retinoic acid (Polidori & Stahl, 2009).

The oxidant activity of retinol and its derivatives is moderate; however, the compound plays a major role in cellular signalling, for example, as a ligand of a family of nuclear receptors involved in the regulation of gene expression. *In vitro* cell culture studies, studies on animal's models, and different types of human studies; all support the idea that carotenoids and vitamin A play a role in the prevention of cancer (Polidori & Stahl, 2009), namely because of the antigenotoxic and anticarcinogenic effects of both (De Flora *et al.*, 1999). Genotoxic effects include a variety of endpoints which can be evaluated both *in vitro* and in animal models, such as DNA damage, point mutation of differential specificity, numerical and structural chromosomal alterations and impairment of DNA repair mechanisms (De Flora *et al.*, 1999).

Vitamin A and its derivatives are essential to processes such as vision and cell differentiation, particularly during embryological development, as well as in carcinogenesis, glycoprotein synthesis, epithelial cell integrity, immune cell maintenance and human growth hormone

production (Kristal, 2004; Jenab *et al.,* 2009; Polidori & Stahl, 2009; Fritz *et al.,* 2011). Deficiency of this vitamin is associated with night blindness, loss of vision, xeropthalmia, growth retardation, foetal reabsorption, and immunodeficiency (Kristal, 2004; Polidori & Stahl, 2009; Fritz *et al.,* 2011). Almost all epithelial tissues contain receptors for retinoic acid, and a deficiency of vitamin A has consistently been implicated as an important causal factor of cancers in human beings (Zhang *et al.,* 2012). Experiments in animals have also shown that vitamin A deficiency predisposes to the development of squamous intraepithelial lesions (Zhang *et al.,* 2012). The body obtains vitamin A from two sources: preformed vitamin A (retinol and retinal in the form of retinyl esters), and provitamin A carotenoids (beta-carotene, alpha-carotene, beta-cryptoxanthin) (Jenab *et al.,* 2009; Fritz *et al.,* 2011). Retinol itself is rarely found in foods. Preformed vitamin A is found in cod liver oil, butter, eggs, animal products and fortified grains. Provitamin A carotenoids are found in highly pigmented vegetables such as carrots, squash, tams, and green leafy vegetables. Once in the body, retinol is ultimately converted into retinoic acid and its isoforms, collectively known as retinoids (Fritz *et al.,* 2011).

In recent years many studies have described a protective role of vitamin A in several diseases related to lung development (e.g. asthma, chronic obstructive pulmonary disease, and parenchymal lung diseases). These studies exploit the ability of vitamin A as a scavenger of toxic metabolites widely known as free radicals (Pasquali *et al.*, 2013).

Vitamin A has been recommended in a wide range of doses for treatment of some conditions, mainly in the field of dermatologic disturbances and oncology (infants, children and young adults during leukaemia treatment). Retinoids were claimed to exert important antioxidant functions in biological systems, and this belief stimulated the use of retinoids as antioxidants and nutritional supplements in the prevention and treatment of diverse diseases (Pasquali *et al.*, 2013). Preneoplastic and neoplastic diseases successfully treated with retinoids include oral leukoplasia, cervical dysplasia and *xeroderma pigmentosum* (premalignant), and acute promyelocitic leukemia. Modest but encouraging results have been found in the treatment of other cancer types including: head and neck cancer, oesophageal, cutaneous T-cell lymphoma, neuroblastoma and mesothelioma (Kristal, 2004; Fritz *et al.,* 2011;). However, some studies observed that vitamin A may induce toxic effects to different cell types. Retinol and its derivatives may exert pro-oxidant effects which may cause oxidative damage, cell cycle disruption, and transformation and/or cell death (Pasquali *et al.,* 2013).

Cell culture as well as other *in vitro* assays confirmed that retinoids also presented cytotoxic and/or pro-oxidant effects, causing oxidative damage to biomolecules. The explanation could

be the increased lipid peroxidation by retinol, as well as the protein carbonylation, and decreased protein thiol content. Moreover, the activities of antioxidant enzymes, such as catalase and superoxide dismutase were also modulated by retinol. It is known that retinol auto-oxidation *in vitro* increases $O_2^{-\cdot}$ (Pasquali *et al.,* 2013).

The study of Pasquali *et al.* (2013) demonstrate that retinol causes an increase ROS/RNS production in human lung cancer A549 cells, which leads to NF-kB activation and decreased the receptor for advanced glycation end-products (RAGE) expression, this has recently been considered a key event in lung cancer development and progression; retinol, on the other hand, was previously considered an antioxidant, anticancer agent, but it has been observed to induce deleterious and pro-neoplastic effects. Also the review made by De Flora *et al.* (1999), concluded that the impact of supplementation with vitamin A could vary significantly, ranging from showing benefit to producing small but significant increases in lung cancer incidence amongst high risk individuals such as tobacco smokers and asbestos-exposed workers. This highlights the importance of just proceeds to supplementation under nutritional or medical specialist advice.

Beta-Carotene and Retinol Efficacy Trial (CARET) results showed that participants receiving the combination of β-carotene and vitamin A had no chemopreventive benefit and had excess lung cancer incidence and mortality (Omenn *et al.*, 1996). Also the Cheng & Neuhouser (2012) study which investigated the association between vitamin A intake and serum hydroxyvitamin D, showed a limited statistical evidence of the beneficial association of vitamin D with reduced lung cancer mortality, nevertheless this association may be diminished among those who are supplement users with excess circulating vitamin A or vitamin A/β-carotene (Cheng & Neuhouser, 2012). The study of Fritz *et al.* (2011) suggested that there is no evidence for an association between treatment and prevention of lung cancer and vitamin A and related retinoids. This study also enlightened important factors regarding daily supplementation of vitamin A, such as the increase of several parameters of oxidative stress in rat lungs (Fritz *et al.,* 2011). Furthermore, data from clinical trials also indicate an increase in incidence of lung cancer and colorectal cancer in smokers and asbestos-exposed men that receive oral supplementation with vitamin A and/or beta-carotene, the same being true for cardiovascular disease incidence (Pasquali *et al.,* 2013). Results obtained from Klerk *et al.* (1998) investigation suggested that retinol supplementation in subjects exposed to crocidolite (blue asbestos) may reduce the incidence of mesothelioma, however there was a small increase in risk of mesothelioma for those on β-carotene (Klerk *et al.*, 1998), similar to what was found in the CARET study (Omenn *et al.,* 1996). The study by Miyazaki *et al.* (2012), demonstrated a clear

positive association between dietary vitamin A intake and the incidence of gastric cancer in the general Japanese population. However this data should be critically interpreted because such association was enhanced by the positivity for *Helicobacter pylori*, a well-established powerful risk factor for gastric cancer. Although this study also suggested that dietary vitamin A was significantly associated with the incident of gastric cancer, such association was not observed for dietary retinol or carotenoids alone, suggesting that the combination of both may act as a risk factor for gastric cancer (Miyazaki *et al.*, 2012).

Miyazaki *et al.* (2012) justified their results by the autoxidation of retinoids, generating free radicals, which play a role in DNA damage, coupled with a higher dietary vitamin A intake promoting mucosal damage in the stomach. It is well known that infection with *H. pylori* also induces DNA damage in gastric mucosal cells, through oxidative stress, acting together in synergy. A study by Park *et al.* (2012) showed that dietary supplementation with vitamin A inhibits colon cancer metastasis to the liver, the major storage site for vitamin A and the target organ for colon cancer metastasis, in a mouse model. Taken together, these data suggest that dietary vitamin A supplementation may prove useful for reducing the number of metastatic tumours that develop, and thus the overall amount of cancerous tissue per liver in patients prone to colorectal cancer metastasis (Park *et al.*, 2012).

Deregulation of retinoid metabolism has been found in several cancers, including the prostate cancer and prostate cancer tissue is known to have a lower concentration of retinoic acid than a normal prostate. Studies of multivitamin supplements, mostly using retinyl palmitate as a source of vitamin A, have failed to find an association with prostate cancer risk (Patterson *et al.*, 1999). Finally, prospective studies based on serum retinol have revealed increased, decreased and no prostate cancer risk associated with higher retinol concentrations. Since serum retinol is homeostatically controlled, it is difficult to interpret these associations as a reflection of dietary retinol intake (Kristal, 2004). Neither dietary nor supplemented vitamin A intake is related to prostate cancer risk, and there is no evidence that they are useful as chemopreventive agents. Currently available synthetic retinoids will also not be useful as prostate cancer chemopreventive agents due to their high toxicity (Kristal, 2004).

The results from the meta-analysis performed by Zhang *et al.* (2012) indicated that vitamin A intake is inversely associated with risk of cervical cancer; however there was no significant association between blood retinol level and cervical cancer risk.

Administration of topic vitamin A revealed regression and even remission of leukoplakia, a white lesion of the mucosa that does not represent a histological condition but due to the possibility of malignant transformation, these lesions must be assessed and managed closely

(Epstein & Gorsky, 1999). It was observed that in hipervitaminosis A, the levels of plasma and tissue retinol do not correlate with the increased intake, suggesting that retinol is converted to several other metabolites when increasing doses are ingested (Pasquali *et al.,* 2013). These data reinforce the importance of keeping retinol status within the normal physiologic range and the importance of carefully observing the outcome of vitamin supplementations in epidemiologic and experimental studies.

4.1.2. VITAMIN D

Biological and epidemiological data suggest that vitamin D levels may influence cancer development. Vitamin D is not a true micronutrient for most mammals, since it is primarily synthesized in skin cells in the presence of adequate sunlight providing UVB. Vitamin D deficiency, and insufficiency, has become a well-recognized problem worldwide (Polidori & Stahl, 2009). Besides its "classical" role in mediating calcium and phosphate homeostasis, 1,25-dihydroxyvitamin D3 has "nonclassical" roles that include antiproliferative, antiangiogenic, and prodifferentiating effects in a wide range of tumour cells (Uitterlinden *et al.,* 2004; Polidori & Stahl, 2009; Bao *et al.*, 2010), it can also activate apoptotic pathways and inhibit cell migration, supporting claims of its potential role in cancer prevention and cure (Deeb *et al.*, 2007; Raimondi *et al.*, 2009). These effects are mediated through perturbation of several important cellular signalling pathways.

There are many terms and synonyms used in the description of various compounds referred to broadly as *vitamin D*. In general, the letter D without a numeral modifier is used when a distinction between D_2 and D_3 forms is not necessary. Therefore, the common term Vitamin D may be inclusive of all forms of vitamin D, including ingestible forms or serum levels. Vitamin D2 is ergosterol, Vitamin D3 is calcitriol, 25(OH)D or 25-Hydroxyvitamin D are synonymous of 25-(OH) vitamin D (calcidiol) and 1,25$(OH)_2$D3 is 1,25(OH)2D3 or 1,25$(OH)_2D_3$ (Hines *et al.*, 2010).

1,25$(OH)_2D_3$ is the biologically active form of vitamin D, and it exerts its effects mainly through binding to nuclear *VDR* and further binding to specific DNA sequences, namely vitamin D response elements. Through this genomic pathway, 1,25$(OH)_2D_3$ exerts transcriptional activation and repression of targeted genes by binding to the *VDR* (Cui & Rohan, 2006; Polidori & Stahl, 2009). Normal respiratory epithelial cells have high levels of *VDR*, however in lung cancer tissues; these components of the vitamin D pathway are suppressed, leading to a decrease in 1,25$(OH)_2D_3$, deterring vitamin D's anti-proliferative function. These contrasts

between normal lung and malignant cells suggest that vitamin D may be important for maintenance of normal and anti-proliferative functions in the lung (Cheng *et al.,* 2012).

The study of Cheng *et al.* (2012) concluded that serum 25(OH)D concentrations were inversely associated with lung cancer mortality in non-smokers. The mechanism by which vitamin D reduces lung cancer risk and progression may involve modulating the immune function of lung epithelial cells and inhibiting tumour proliferation, angiogenesis, and metastasis. The study of Bao *et al.* (2010) higher 25(OH)D score was associated with a significantly lower risk of pancreatic cancer.

There are biological reasons to suspect that the active form of vitamin D, may be related to ovarian cancer incidence and mortality. The study by Cook *et al.* (2010) demonstrated absence of a consistent or strong evidence to support the claim made in numerous review articles that vitamin D exposures reduce the risk for ovarian cancer occurrence or mortality (Cook *et al.*, 2010). However, Grant (2010) claims the existence of good evidence that solar UVB and vitamin D reduces the risk of ovarian cancer (Grant, 2010).

4.1.3. VITAMIN E

Vitamin E was first described by Evans and Bishop as an essential nutrient for reproduction in rats (Polidori & Stahl, 2009) and is a general term including α-, β-, δ- and γ-forms of the tocopherol and tocotrienol chemical classes (Jenab *et al.*, 2009). Vitamin E has the ability to chemically act as a lipid based (lipoprotein and membranes) free radical chain breaking molecule and to exert its action by protecting the organism against the attack of those radicals. Vitamin E has been shown to influence cellular signalling, enzymatic activity and gene expression.

The claim that vitamin E has, like vitamin A and vitamin D derivatives, cell regulatory properties unrelated to its radical chain breaking potential, is supported by a number of experimental results (Zingg & Azzi, 2004). The most potent form of natural vitamin E, α-tocopherol, is taken up, transported and retained by the body much more efficiently than the other natural and synthetic derivatives (Zingg & Azzi, 2004; Singh *et al.,* 2005). Since they all have equal radical chain breaking properties, it is to date still unexplained why nature specifically selected the α form of tocopherol, and it is an open question whether vitamin E deficiency syndromes could be completely prevented by supplying β-, γ- and δ-tocopherols or tocotrienols. On the one hand α-tocopherol has some specific characteristics, for instance the fully methylated chromanol-head group may be required for optimal interactions with enzymes and/or "α-tocopherol receptors" (Zingg & Azzi, 2004).

On the other hand, the β-, γ- and δ-tocopherols and the tocotrienols may have biological effects that interfere with normal cellular processes, so that they need to be specifically recognized, metabolized by the liver and later eliminated. A unique feature of δ-tocopherol is the location of the reactive –OH group between two methyl groups; after reacting with lipid peroxide the unpaired electron can delocalize over the fully substituted chromanol ring which is known to increase its stability and chemical reactivity (Zingg & Azzi, 2004).

In the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study, vitamin E and β-carotene failed to prevent upper aerodigestive tract cancers (Wright *et al.*, 2007). Vitamin E was recognized as possible blocking and suppressing agent for oesophageal cancer on account of its antioxidative function of scavenging electrophiles and inhibiting oxidative DNA damage (Yang *et al.,* 2012).

Yang *et al.* (2012) demonstrated that vitamin E and selenium supplementation was time selective in the chemopreventive of N-Nitrosomethylbenzymanine-induced oesophageal carcinogenesis. An early-stage supplementation significantly prevented cancer development, whereas late-stage supplementation did not show a clear benefit. Data present from the animal model provide further experimental support to the hypothesis that the efficacy of cancer chemoprevention by nutrients may time selective during the multistage of carcinogenesis. Tomasetti *et al.* (2004) observed that vitamin E analogue efficiently kills malignant mesothelioma cells and sensitises them to immunologic inducer of apoptosis tumour necrosis factor-related apoptosis-inducing ligand, showing therefore anticancer activity (Tomasetti *et al.,* 2004).

Lotan *et al.* (2012) found no preventative effect of selenium or vitamin E, alone or in combination on bladder cancer in men. The SELECT research group had previously reported no reduction in cancer risk following the long-term supplementation with vitamin E or selenium, reporting a non-significant trend for increased prostate cancer risk with vitamin E supplementation (Lippman *et al.*, 2009). Gaziano *et al.* (2009) showed in a long-term trail of male physicians that neither vitamin E nor vitamin C supplementation reduced the risk of prostate cancer or even total cancer, namely colorectal, or other common cancers. Klein *et al.* (2011), reported that men who used vitamin E supplements were at 17% increased risk for cancer as compared to men taking placebo, with the increased risk for developing cancer being seen as soon as 3 years after enrolling in the trial. Beilby *et al.* (2010) also reported null associations between prostate cancer and serum folate, lycopene, β-carotene, retinol and vitamin E. Rodriguez *et al.* (2004) showed lack of support for a strong role of vitamin E in

prostate cancer prevention, although a modest protective effect among smokers could not be ruled out.

The Heart Outcomes Prevention Evaluation (HOPE) trial and the HOPE-Ongoing Outcomes (HOPE-TOO) studies, which administered a daily dose of natural source Vitamin E (400IU) and a matching placebo, concluded that in patients with vascular disease or *diabetes mellitus*, long-term vitamin E supplementation not only does not prevent cancer or major cardiovascular events but also may increase the risk for heart failure, therefore the investigators recommended that vitamin E supplements should not be used in patients with these diseases (Lonn *et al.*, 2005). Miller *et al.* (2005) also studied the effect of a high-dosage of vitamin E supplementation and concluded that a high-dosage (≥ 400 IU/d) vitamin E supplements may increase all-cause mortality and should therefore be avoided.

4.2. OTHER MICRONUTRIENTS BY-SUBSTANCES AND CANCER

The vitamin B9, commonly folate, is known to be involved in a wide variety of reactions within the cell, including DNA synthesis, repair and methylation (Wasson *et al.*, 2008). *In vitro* studies have shown that folic-acid deficiency causes a dose-dependent increase in uracil incorporation into human lymphocyte DNA, with subsequent single-strand breaks in DNA formed during base-excision repair. Low folate results in excessive uracil misincorporation into DNA, a lesion which is both mutagenic and leads to DNA strand breaks (Fenech *et al.,* 1999b; Ames & Wakimoto, 2002; Cooke *et al.,* 2002; Ames, 2004). Folate deficiency also causes expression of chromosomal fragile sites, chromosome breaks, micronuclei formation and mitochondrial DNA deletions (Duthie & Hawdon, 1998a; Fenech, 2001; Ferguson & Fenech, 2012). Poor folic acid status in human lymphocytes *in vitro* is associated with increased DNA strand breakage, misincorporated uracil, and reduced DNA repair efficiency (Duthie & McMillan, 1997; Duthie & Hawdon, 1998).

An animal study also suggested that folate supplementation at early stage (prior to the existence of preneoplastic lesions) could inhibit colorectal cancer formations; however, supplementation at a later stage could promote carcinogenesis (Fenech & Ferguson, 2001; Kim, 2004; Cole *et al.,* 2007). A low folate concentration has been implicated as a potential promoter of carcinogenesis, for example, in colorectal cancer, lung, breast, pancreatic, gastric, oesophageal, cervical, brain, and prostate malignancies (Ames, 1998, 2001; Ames & Wakimoto, 2002; Duthie *et al.,* 2002; Ferguson & Philpott, 2008; Beilby *et al.*, 2010; Sutandyo, 2010).

Vitamin B12 is required for the synthesis of S-adenosylmethionine, a common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation. Low concentrations of S-adenosylmethionine lead to DNA hypomethylation, associated with abnormal gene expression and chromosomal segregation in specific regions such as the centromere, chromosomes 1, 9 and 16, and fragile sites (Fenech *et al.,* 1999c; Milić *et al.*, 2010). Deficiencies of vitamin B12, which is common in the population, also causes uracil misincorporation into human DNA and chromosome breaks by the same mechanism that folate deficiency, since a deficiency of vitamin B12 can mimic chemicals in damaging DNA by causing single and double strand breaks (Ames, 2004; Courtemanche *et al.,* 2004; Minnet *et al.,* 2011). Therefore, vitamin B12 deficiency, like folic acid deficiency, causes uracil to accumulate in DNA, chromosome breaks, excessive uracil in DNA, micronuclei formation and DNA hypomethylation; an increased level in homocysteine status, an important risk factor for cardiovascular disease. These same defects may also play an important role in developmental neurological abnormalities (Fenech, 2001; Milić *et al.*, 2010).

Minerals can be considered to be essential micronutrients, albeit typically with a somewhat narrow dose range of efficacy as compared with toxicity, include iron, selenium and zinc.

Iron is the most abundant trace element in the human body, being essential because iron cofactors activate enzymes involved in most of the major metabolic processes in the cell (Prá *et al.*, 2012), being required in the synthesis of organic and inorganic cofactors, such as heme and iron-sulfur clusters. Iron is required narrow range for maintaining metabolic homeostasis and genome stability, and participates in oxygen transport and mitochondrial respiration as well as in antioxidant and nuclei acid metabolism; whereby iron deficiency leads to oxidative stress. Iron is extremely reactive, interacting with hydrogen peroxide, and generating hydroxyl radicals that can lead to DNA strand breaks and reduction in telomere length. Iron can damage biomolecules mainly through Fenton and Haber-Weiss chemistry, leading to the production of hydroxyl radicals and other ROS. Iron is capable of inducing a wide array of DNA lesions, from base modifications to strand breaks and adducts. Iron also seems to alter the methylation pattern of several genes (Prá *et al.,* 2012).

Iron deficiency causes decreased heme levels in the mitochondria, which results in dysfunctional mitochondria and neurodegeneration. Iron deficiency in mitochondria appears at higher iron intakes than anemia. Many reports show that inadequate iron intake causes cognitive dysfunction in rats and humans by altering metabolic processes such as mitochondrial electron transport and neurotransmitter synthesis and degradation (Ames, 2004; Prá *et al.*, 2011).

The epidemiological data on iron and cancer are mainly limited to studies of iron excess. Iron overload has been linked to genome instability (Prá *et al.*, 2011, 2012). The increased risk of hepatic carcinoma in individuals with cirrhosis caused by haemochromatosis indicates a link between iron overload and cancer. Several studies have reported associations between increased levels of iron and colorectal cancer. But iron deficiency, as well as iron excess, leads to oxidative DNA damage (Ames, 2001; Ames & Wakimoto, 2002), namely being associated with diminished immune function and neuromuscular abnormalities (Ames, 2006).

It is a matter of concern that current Recommended Dietary Allowances (RDA) for iron does not consider the concept of genomic stability (Ferguson & Fenech, 2012).

Selenium is an important component of antioxidant enzymes, namely selenoproteins such as selenocysteine (Se-Cys) and selenomethionine (Se-Met), and functioning as a co-factor for the reduction of antioxidant enzymes, including glutathione peroxidises, thioredoxin reductases, and selenoprotein P, which contain molecular selenium in the form of selenocysteines within their active centre (Hwang & Bowen, 2007; Ferguson *et al.,* 2012). They are involved in the defence of ROS, which otherwise may cause DNA damage alterations of protein function (Ames, 2001; Hwang & Bowen, 2007). It appears that over one third of all known selenoproteins are antioxidant in nature, while certain Se metabolites induce ROS. The antioxidant nature of selenoproteins is essential in minimising the levels of hydroperoxides (Ferguson *et al.*, 2012).

Plant foods like rice and wheat are the major dietary sources of selenium in most countries. Selenium supplementation has moved from the realm of correcting nutritional deficiencies to one of pharmacological intervention, especially in the clinical domain of cancer chemoprevention and in the control of heart failure (Hamid, 2010). Dietary selenium significantly inhibits the induction of skin, liver, colon, and mammary tumours in experimental animals by a number of different carcinogens, as well as the induction of mammary tumours by viruses (Ames, 1983).

Populations with low selenium status have been found to have an increased risk of several cancers, including prostate, breast, lung and colorectal. It is possible that these levels are particularly important in different parts of the cancer progression, because of variable effects on genomic stability. For example, serum selenium levels were studied in relation to markers of neoplastic progression among persons with Barrett's oesophagus (Ferguson *et al.,* 2012).

As with iron, selenium shows a "U" shaped curve for functionality, whereby too little is as damaging as too much. At optimal levels, selenium may protect against the formation of DNA

adducts, DNA or chromosome breakage, chromosome gain or loss, mitochondrial DNA, and telomere length and function (Ferguson & Fenech, 2012).

Current dietary recommendations do not consider the concept of genome stability which is of concern because damage to the genome has been linked to the origin and progression of many diseases and is the most fundamental pathology (Prá *et al.,* 2012).

In 2005, Fenech suggested that the concept of recommended diet should be based on the prevention of genomic instability, not merely in relation to spontaneous chromosomal anomalies, but also in terms of genomic damage induced by chemical agents or radiation. Therefore, based on this proposal, the traditional concepts of mutagenicity, which were focused only on the interaction between gene and toxin, were enlarged to include diet as an influencing factor (Minozzo *et al.,* 2010).

CHAPTER 8 – FORMALDEHYDE

1. GENERAL CONSIDERATIONS

Formaldehyde (CAS number: 50-00-0), also known as formalin, formol, and methyl aldehydes, is the most simple yet most reactive of all aldehydes, with the chemical formula CH_2O. It is a colourless gas at room temperature, flammable, and has a strong pungent smell (Liteplo *et al.,* 2002; Pala *et al.*, 2008; Zhang *et al.*, 2009b). Aleksander Butlerov synthesized the chemical in 1859, but it was August Wilhelm von Hofmann who identified it in 1867, as the product formed from passing methanol and air over a heated platinum spiral. This method is still the basis for the industrial production of formaldehyde today, in which methanol is oxidized using a metal catalyst. By the early 20th century, the explosion of knowledge in chemistry and physics, coupled with demands for more innovative synthetic products, set the scene for the birth of new material plastics (Zhang *et al.,* 2009a).

Formaldehyde is an economically important chemical with an annual production of approximately 46 billion pounds worldwide. According to the Report on Carcinogens (NTP, 2011), formaldehyde ranks 25th in overall U.S. chemical production with more than 11 billion pounds produced each year. Formaldehyde production has increased steadily in China in recent years, with 7.5 million tons (16.5 billion pounds) of formaldehyde produced in 2007 (Zhang *et al.*, 2009a, 2010; NTP, 2011).

Commercially, formaldehyde is manufactured as an aqueous solution called formalin, usually containing 37% by weight of dissolved formaldehyde. It is commonly used as a tissue preservative or as a bactericide in embalming fluid and medical laboratories (IARC, 2006; NTP, 2011).

Formaldehyde has different formulations, such as casein formaldehyde, phenolic resins, urea formaldehyde, melamine formaldehyde and all have played an important role in the production of domestic and industrial goods that have become vital to everyday life. Casein formaldehyde (buttons, buckles, and knitting needles); phenolic resins (electrical and automobile insulations and other heavy industrial products, appliances toasters and radios); urea formaldehyde (picnic-ware, lampshades, varnishes, laminates and adhesives); and melanine formaldehyde (plastics more resistant to heat, water and detergents, cups, saucers and other domestic items) are formaldehyde-based materials that are broadly used (Zhang *et al.*, 2009a; Kim *et al.*, 2011; NTP, 2011). It is also widely used in molding compounds, glass wool and rock wool insulation, decorative laminates and textile treatments. Formaldehyde is

now extensively used by industries across the globe. There are regulatory decisions regarding formaldehyde, such as occupational exposure limits (OELs) and drinking water standards, which have an economic impact that runs into millions of dollars (Zang *et al.*, 2009).

2. HUMAN EXPOSURE TO FORMALDEHYDE

Given its economic importance and widespread use, many people are exposed to formaldehyde environmentally and/or occupationally. Occupational exposure involves not only individuals employed in the direct manufacture of formaldehyde and products containing it, but also those in industries utilizing these products, such as construction (Liteplo *et al.,* 2002; Zhang *et al.*, 2009).

2.1. OCCUPATIONAL EXPOSURE AND SAFETY STANDARDS

The U.S. Occupational Safety and Health Administration (OSHA) has estimated that approximately 2.1 million workers in the U.S. and many more in developing countries are occupationally exposed to formaldehyde. The exposed workers, commonly found in resin production, textiles or other industrial settings, inhale formaldehyde as a gas or absorb the liquid through their skin. Other exposed workers include health-care professionals, medical lab-specialists, morticians and embalmers, all of whom routinely handle bodies or biological specimens preserved with formaldehyde (Zang *et al.,* 2009a). In occupational environments, formaldehyde occurs mainly as a gas; however formaldehyde particulates can be inhaled when paraformaldehyde or powdered resins are used or when formaldehyde adsorbs to other particles, such as wood dust (IARC, 2006). Occupational exposure to formaldehyde is highly variable and can occur in numerous industries, including the manufacture of formaldehyde and formaldehyde-based resins, wood composite and furniture production, plastics production, embalming, foundry operations, fibreglass production, construction, agriculture, firefighting, and histology, pathology, and biology laboratories, among others (Kim *et al.*, 2011; NTP, 2011). In the past, the highest continuous exposure levels were measured during the varnishing of furniture and wooden floors, during the finishing of textiles, in the garment industry, during the treatment of furs, and in certain jobs in manufactured board mills and foundries (NTP, 2011).

The formaldehyde occupational exposure limits of many countries are available on the International Labour Organization website and through the Registry of Toxic Effects of Chemical Substances database (RTECS#: LP8925000) maintained by National Institute for Occupational Safety and Health (NIOSH) (Zang *et al.*, 2009a).

The U.S. OSHA has established the following standards that have remained the same since 1992: the permissible exposure limit (PEL) is 0.75 ppm (parts per million) in air as an 8-h time-weighted average (8h TWA) and the short-term (15 min) exposure limit (STEL) is 2 ppm. The American Conference of Governmental Industrial Hygienists (ACGIH) threshold limit value (TLV) recommended is 0.3 ppm as an 8 h TWA. The Scientific Committee on Occupational Exposure Limits (SCOEL) has recommended a health-based OEL of 0.2 ppm (Bolt *et al.*, 2010).

2.1.1. PATHOLOGY ANATOMY OCCUPATIONAL SETTINGS

Formaldehyde has been considered carcinogenic to humans, making it a subject of major environmental concern, namely in the occupational setting where employees in industrial and medical areas, in particular anatomists and medical students may be highly exposed to formaldehyde gas (Gulec *et al.*, 2006). Pathology Anatomy laboratories conduct diagnosis based on a wide range of biological specimens removed from a subject by biopsy, surgery or necropsy. Formaldehyde is used as preservative, avoiding autolysis and putrefaction phenomena and mimetizing an *in vivo* state. Pathology Anatomy laboratories are mainly in hospitals, and are important formaldehyde occupational exposure settings, namely to pathologists, anatomy pathology technicians, and auxiliary staff. The professionals have contact with formaldehyde specifically during macroscopy exams, also known as grossing, working out formaldehyde solution dilutions, and discard and exchange of formalin in biological samples or in the processing equipment.

2.2. ENVIRONMENTAL EXPOSURE AND AMBIENT LEVELS

Formaldehyde is ubiquitous in the environment and has been detected in indoor and outdoor air, soil, food, treated and bottled drinking water, surface water, and groundwater (Liteplo *et al.,* 2002; Kim *et al.,* 2011; NTP, 2011). Although environmental exposure to formaldehyde typically occurs at much lower levels than occupational exposure, a greater number of people are exposed to these lower levels in their daily lives. Environmental sources of formaldehyde include: (i) off gassing from new mobile homes; (ii) automobile engines, especially those burning biofuels; (iii) smoke from cigarettes and the burning of forests and manufactured wood products; and (iv) various consumer products such as furniture, carpeting, fibreglass, permanent press fabrics, paper products and some household cleaners. Of these, the most significant source of global formaldehyde exposure is indoor air pollution from modern home furnishings and incomplete fuel combustion in older homes, where air concentrations could exceed occupational levels (Liteplo *et al.*, 2002; Gulec *et al.*, 2006; Zang *et al.*, 2009a; NTP,

2011). In what concerns indoor sources of formaldehyde exposure, the most important are many construction materials (e.g., medium-density fiber board, particleboard, and plywood), which contain phenol-formaldehyde or urea-formaldehyde resin glues, and glass wool insulation (with similar types of binders) which are known to emit large quantities of formaldehyde (Liteplo *et al.*, 2002; Kim *et al.*, 2011; Nielsen *et al.*, 2013). Formaldehyde is also formed in the early stages of residual plant decomposition in the soil and in the troposphere during oxidation of hydrocarbons that react with hydroxyl radicals and ozone. It ultimately becomes part of smog pollution.

It has been considered carcinogenic to humans, making it a subject of major environmental concern, namely for employees in occupational industrial and medical settings anatomists and medical students in particular may be highly exposed to formaldehyde gas (Liteplo *et al.*, 2002; Gulec *et al.*, 2006; Zhang *et al.*, 2009a; NTP, 2011).

The general population could also be exposed to formaldehyde by handling consumer products that contain formaldehyde as an antimicrobial agent (such as laundry detergents, wallpaper adhesive, or sanitizers) or from its use as a mildewcide for clothing and linens or in vacation homes. Although formaldehyde *de per se* now is rarely used in cosmetics, the use of formaldehyde releasers is common. An analysis of data from the U.S. Food and Drug Administration's Voluntary Cosmetic Registration Program Database indicated that nearly 20% of cosmetic products contained formaldehyde-releasing preservatives (Kim *et al.*, 2011; NTP, 2011).

Formaldehyde in food exists mostly in a bound form, and it is considered to be unstable in aqueous solution. Formaldehyde present in food can occur naturally or through inadvertent contamination; it can also be added as a preservative, disinfectant, or bacteriostatic agent and can result from cooking or smoking of foods. Generally, higher levels were reported in fish, seafood, and smoked ham, shiitake mushrooms, some Italian cheeses, and dried food (Liteplo *et al.*, 2002; Norliana *et al.*, 2009; Zhang *et al*., 2010; Kim *et al.*, 2011; NTP, 2011). There have also been instances of formaldehyde found in fruits, vermicelli noodles, and even beer (Zhang *et al.*, 2010).

3. CLASSIFICATION OF FORMALDEHYDE

The programme of the IARC "Monographs on the Evaluation of Carcinogenic Risk to Humans" represents a major example of hazard identification procedure (http://monographs.iarc.fr). For some genotoxic carcinogens the existence of a "practical" threshold is supported by studies on mechanisms and/or toxicokinetics. Such is the case of formaldehyde, for which a

No-Observable Adverse Effect Limit (NOAEL) may be established from which a health-based exposure limit is derived (Bertazzi & Mutti, 2008).

The U.S. Environmental Protection Agency (EPA) classified formaldehyde as a B1 compound, probable human carcinogen under the conditions of unusually high or prolonged exposure, on basis of limited evidence in humans but with sufficient evidence in animals (US EPA, 2000).

Formaldehyde was long considered as a probable human carcinogen (Group 2A chemical) based on experimental animal studies and limited evidence of human carcinogenicity. Toxic responses induced by formaldehyde in the respiratory tract at high airbone concentrations, which include ulceration, hyperplasia, and squamous metaplasia, are *"considered to contribute to the subsequent development of cancer"* (IARC, 1995). However, the IARC reclassified formaldehyde as a human carcinogen (Group 1) in June 2004 based on *"sufficient epidemiological evidence that formaldehyde causes nasopharyngeal cancer in humans"* (IARC, 2006). The sufficient evidence comes from a statistically significant excess of deaths from nasopharyngeal cancer in the largest and most informative cohort study of industrial workers by the U.S. National Cancer Institute (NCI), with a strong exposure-response correlation between the cancer mortality rate and peak cumulative exposures (Hauptmann *et al.*, 2004, 2009). Collins & Lineker (2004) found an overall meta-relative risk for nasopharyngeal cancer also. After a thorough discussion of the epidemiologic, experimental and other relevant data, the IARC panel concluded that formaldehyde is a carcinogen in humans. However, it should be noted that some studies have argued that the IARC conclusion was premature and that the largest and most influential NCI study should be re-evaluated (Marsh & Youk, 2004; Marsh *et al.*, 2010). Indeed, results in the literature are somewhat inconclusive. For example, Bosetti *et al.* (2008) concluded that workers and professionals exposed to formaldehyde showed no appreciable excess risk for oral and pharyngeal, sinonasal or lung cancers. However, for brain cancer and lymphohematopoietic neoplasms, there were modestly elevated risks in professionals, but not in industry workers. Walrath & Fraumeni, (1983) showed a slightly elevated mortality by cancer in embalmers, but no excess of mortality from cancers of the respiratory tract including the nasal passages.

In their review, IARC also concluded that there was *"strong but not sufficient evidence for a causal association between leukemia and occupational exposure to formaldehyde".* Much of the evidence for an association between formaldehyde exposure and leukemia comes from epidemiology studies, of which there are primarily three types: case-control studies in the general population, proportionate mortality studies of professionals, (e.g. funeral industry workers and pathologists) and cohort studies of industrial workers (Pinkerton *et al.*, 2004;

Zhang *et al.*, 2010). Zhang *et al.* (2009) state that formaldehyde may induce leukemia by damaging hematopoietic stem/progenitor cells circulating in the peripheral blood or by damaging the primitive pluripotent stem cells present within the nasal turbinates and/or olfactory mucosa. Since the previous evaluation (IARC, 2006), the NCI cohort of industrial workers in the USA has been updated with an additional ten years of mortality data. As in the previous analysis of leukemia (Hall *et al.*, 1991), the association was stronger for myeloid leukemia and situations of peak exposure (Pinkerton *et al.*, 2004; Freeman *et al.*, 2009; NTP, 2011).

Other studies have argued that it is biologically implausible for formaldehyde to cause leukemia, being the primary arguments: (i) it is unlikely to reach the bone marrow and cause toxicity due to its highly reactive nature; (ii) there is no evidence that it can damage the stem and progenitor cells, the target cells for leukemogenesis; (iii) there is no credible experimental animal model for formaldehyde-induced leukemia; (iv) absence of an increase of blood formaldehyde levels after inhalative exposure as formaldehyde converting enzymes such as formaldehyde dehydrogenase and aldehydes dehydrogenase are present in many human tissues and also in human erytrocytes (Collins & Lineker, 2004; Heck & Casanova, 2004; Franks, 2005; Speit & Schmid, 2006; Neuss *et al.*, 2010; NTP, 2011; Rhomberg *et al.*, 2011) and cannot be directly related to occupation (Hayes *et al.,* 1990). Indeed, IARC itself concluded *"based on the data available at this time, it was not possible to identify a mechanism for the induction of myeloid leukemia in humans"* and stated that *"this is an area needing more research"* (IARC, 2006). The last evaluation performed by IARC, concluded that there is "*sufficient evidence in humans for the carcinogenicity of formaldehyde. Formaldehyde causes cancer of the nasopharynx and leukemia. Also, a positive association has been observed between exposure to formaldehyde and sinonasal cancer"* (Baan *et al.,* 2009; IARC, 2012).

4. FORMALDEHYDE METABOLISM

Almost every tissue in the body has the ability to breakdown formaldehyde, once absorbed it is usually converted to formate (a nontoxic chemical), which is excreted through the urine and can be exhaled via conversion to carbon dioxide (Kim *et al.,* 2011). Formaldehyde is rapidly metabolized, namely in the site of first contact such as respiratory tract, where the formation of adducts with mucus glycoproteins can take place. By reaching blood and tissues the delivery of significant quantities of reactive formaldehyde to distant sites can happen (Liteplo *et al.*, 2002; Heck & Casanova, 2004; NTP, 2011).

Formaldehyde is carcinogenic at the site of contact as a consequence of epithelial cell regenerative proliferation resulting from cytotoxicity and mutation (Li *et al.,* 2007). It has been estimated that as much as 22 – 42% of inhaled formaldehyde may be removed by mucus flow (Schlosser, 1999). Thus, the fundamental issue is the dose (Heck & Casanova, 2004). A minimum exposure concentration is required for formaldehyde to induce a toxic response; actually, formaldehyde is normally present in all cells and pathways for its metabolism already exists in all tissues and cells examined (Heck & Casanova, 2004).

Formaldehyde is accepted as being toxic above certain doses and the chances of harmful effects are increased at room temperatures because of its volatility. When ingested, formaldehyde is rapidly metabolized and removed from the liver (Gulec *et al*., 2006).

The metabolic pathways of formaldehyde have been extensively studied (IARC, 1995), but the involvement of these pathways in the detoxification of formaldehyde has generally not been closely correlated with cytotoxicity. Cytosolic alcohol dehydrogenase, mitochondrial aldehyde dehydrogenase, and the GSH-dependent formaldehyde dehydrogenase are important pathways of formaldehyde metabolism in the hepatocyte system. Like the liver, the nasal mucosa is a very active metabolic system for inhaled compounds. Formaldehyde is metabolized by similar pathways in the nasal respiratory mucosa, which supports the relevance of the hepatocyte model *in vivo*, but owing to the many anatomical and biochemical differences between the two systems, the hepatocyte model can provide only qualitative information about formaldehyde metabolism in the tissue of interest (Teng *et al.*, 2001; Heck & Casanova, 2004; Franks, 2005). Formaldehyde is also a naturally occurring compound that is present in human plasma at concentrations ranging from 13 to 97 µM (Heck & Casanova, 2004). Endogenous formaldehyde and its oxidation product, formic acid, are intermediates in the "one-carbon-pool", central to many biological processes, including the biosynthesis of purines, thymidine - essential components of nuclei acids - the biosynthesis of certain amino acids, and the demethylation of a variety of important biological compounds that are central to cell function and survival (Zhang *et al.*, 2010; NTP, 2011).

Formaldehyde is an extremely reactive chemical, and reacts with monoamines or amines to form methylene bridges and produces covalently cross-linked complexes with proteins and DNA. It is also known that formaldehyde is produced by the metabolism of L-methionine, histamine, methanol, and methylamine (Gonzalez *et al.,* 2006; Gulec *et al.,* 2006; NTP, 2011). Graaf *et al.* (2009) showed that following acute formaldehyde exposure, repair and/or tolerance of DNA-protein crosslinks proceeds via formation of nucleotide excision repair-dependent single-strand break intermediates and without a detectable accumulation of

double-strand breaks, demonstrating a differential pathway response to chronic versus acute formaldehyde exposures. Therefore, the relative contribution of each pathway differs depending on the dose and duration of exposure (Graaf *et al.,* 2009).

5. HEALTH EFFECTS

Formaldehyde irritates the nose and pharynx in humans and laboratory animals under a variety of circumstances (IARC, 2006) and also its inhalation is associated with respiratory symptoms, and eye, nose and throat irritation, dry skin, tearing eyes, conjunctivitis, sneezing, and coughing (Zhang *et al.*, 2009; Kim *et al*., 2011).

Nasal biopsies of workers chronically exposed to formaldehyde showed chronic inflammation, loss of cilia, mild dysplasia, hyperplasia and squamous metaplasia, although the latter finding has been inconsistent and may have been confounded by others exposures, such as to wood dust (IARC, 2006; 2012). The cytotoxicity of formaldehyde has been confirmed in numerous *in vitro* systems (Sul *et al*., 2007; IARC, 2006; IARC, 2012). Irritation of the nasal and upper respiratory tract is also noted in animal studies. Dose-dependent pathological findings include inflammation, hyperplasia, degenerative changes, necrosis and squamous metaplasia (IARC 2006; 2012). There is also some controversy regarding occupational asthma due to formaldehyde exposure. Asthma induced by inhaled formaldehyde may be classified as irritant induced asthma, as short exposures to high level formaldehyde are identified to cause a sudden onset of asthmatic symptoms called “Reactive airways dysfunction syndrome”. Because of its airways-irritating properties, it may also aggravate preexisting asthma (Kastner *et al.*, 2011; Kim *et al.*, 2011).

Formaldehyde is a known cause of allergic contact dermatitis. Skin sensitization to formaldehyde has been associated with many situations of dermal exposure, including with formaldehyde, formaldehyde-containing resins, formaldehyde-treated fabrics, formaldehyde containing household products, facial tissues, and others. Its exposure has been widely reported to cause dermal allergic reactions in occupationally exposed nurses, doctors, and dentists, as well as cosmetic workers, textile workers, and construction workers (Groot *et al.*, 2009; Kim *et al.*, 2011).

Formaldehyde exposure can cause a wide range of toxic effects such as formation of DNA-/protein cross-links, cytotoxicity, immune activation and sensory irritation (Gulec *et al.*, 2006). Formaldehyde toxicity is thought to be mediated by the activation of free radical producing

enzymes, and also by the inhibition of free radical scavenging systems, thereby enhancing the production of the reactive oxygen species (Gulec *et al.*, 2006).

6. GENOTOXIC EFFECTS OF FORMALDEHYDE

Formaldehyde is a direct-acting genotoxic compound and has been associated with positive results for almost all genetic endpoints evaluated in bacteria, yeast, fungi, plants, insects, nematodes, and cultured mammalian cells. It has caused base-pair gene mutations in *Salmonella typhimurium* and DNA adducts, DNA-protein crosslinks, DNA-DNA crosslinks, DNA single-strand breaks, unscheduled DNA synthesis, inhibition of DNA repair, gene mutations, cell transformation, and cytogenetics effects - chromosomal aberrations, sister chromatid exchanges, and micronuclei - in cultured mammalian cells (Suruda *et al.,* 1993; Conaway *et al.*, 1996; Heck & Casanova, 1999; Saito *et al.,* 2005; Orsière *et al.*, 2006; Speit *et al.*, 2007; Zhang *et al.*, 2009a; Jiang *et al.*, 2010; Zhang *et al.*, 2010; Kim *et al.*, 2011; NTP, 2011). The mechanism by which formaldehyde causes cancer is not completely understood and most likely involves several modes of action. Formaldehyde exposure is associated with key events related to carcinogenicity, such as DNA reactivity, gene mutation, chromosomal breakage, aneuploidy, epigenetic effects, glutathione depletion, oxidative stress, and cytotoxicity-induced cellular proliferation (Saito *et al.*, 2005; NTP, 2011). In summary, formaldehyde is genotoxic and induces both DNA damage and chromosome changes, frequently expressed as DNA-protein crosslinks. In recent years a number of reports indicate that formaldehyde can induce damage in circulating lymphocytes (Orsière *et al.*, 2006; Zhang *et al.,* 2009a).

CHAPTER 9 – CYTOSTATICS DRUGS

1. ANTINEOPLASTIC DRUGS

Chemical agents have a successful history of use in the treatment of illnesses and injuries, namely in the treatment of cancer, where they have been used for decades and were responsible for many advances in therapy during the past century. Many drugs with diverse modes of action have been synthesized and adapted for clinical use. Antineoplastic or cytostatics drugs are an heterogeneous group of chemicals widely used in the treatment of cancer and in some non-neoplasic diseases too, having in common an ability to inhibit tumour growth by disrupting cell division and killing actively growing cells. These drugs have nevertheless been proved to be also mutagens, carcinogens and teratogens (Fucic *et al.*, 1998; Burgaz *et al.*, 1999; Sessink & Bos, 1999; Bouraoui *et al.*, 2011; Gulten *et al.*, 2011; Buschini *et al.*, 2013).

Antineoplastic drugs include alkylating agents (e.g., cyclophosphamide, melphalan, chlorambucil), antimetabolites (e.g., thioguanine, 5-fluorouracil, methotrexate), antibiotics (e.g., doxorubicin), mitotic spindle inhibitors (e.g. vincristine), hormones (e.g., diethylstilbestrol), free radical generators (e.g., bleomycin) and topoisomerase inhibitors (e.g., irinotecan, etoposide) (Villarini *et al.*, 2012). In general, chemicals that interact directly with DNA by binding covalently or by intercalating, or indirectly by interfering with DNA synthesis, were among the first chemotherapeutics developed. Compounds that inhibit mitotic spindle formation and those that affect endocrine function are also used in cancer chemotherapy (Jackson *et al.*, 1996). Also, these drugs can induce reactive oxygen species that can lead to DNA damage and, consequently, mutations (Rombaldi *et al.*, 2008).

These drugs are often used in combination to achieve synergistic effects on tumour cells resulting from their differing modes of action. However, most if not all of these chemical agents are generally nonselective and, along with tumour cells, normal cells may undergo cytotoxic/genotoxic damage (Connor, 2006; Kopjar *et al.*, 2006; Villarini *et al.*, 2012). The *in vivo* exposure to antineoplastic drugs has been shown to induce different types of lesions in DNA, depending on the particular stage of cell cycle at the time of treatment.

The majority of lesions occur during the DNA synthesis (S) phase, often due to misreplication. Both neoplasic and non-neoplasic cells attempt to repair them but, if unrepaired, DNA lesions

may give rise to chromatid-type aberrations during S-phase which interfere with the transcription and replication of DNA, resulting in cytotoxic and mutagenic effects. Growing evidence suggests that secondary neoplasms may arise as a complication of successful chemotherapy (Kopjar *et al.,* 2006).

Virtually all drugs have side effects associated with their use. Both patients and workers who handle them are at risk of suffering such effects. In addition, it is known that exposures to even very small concentrations of certain drugs may be hazardous for workers who handle them or work near them (NIOSH, 2004; Villarini *et al.*, 2011, 2012). Accordingly, several antineoplastic drugs have been classified by the IARC, on the basis of epidemiological reports, animal carcinogenicity data, and the outcomes of *in vitro* genotoxicity studies, as belonging to the group of human carcinogens (Group 1), probable human carcinogens (Group 2A), or possible human carcinogens (Group 2B). In addition, investigational agents have also to be considered as potentially hazardous until their safety can be established.

According to European Guidelines (Corrigendum to Directive 2004/37/EG), any use of carcinogenic, mutagenic or teratogenic substances, including the application in health care settings, are assigned to the highest risk level (Sessink & Bos, 1999; Kiffmeyer & Hadtstein, 2007; Bouraoui *et al.*, 2011; Gulten *et al.*, 2011; Buschini *et al.*, 2013).

1.1. CYCLOPHOSPHAMIDE

Cyclophosphamide (CAS no. 50-18-0) is one of the most frequently used alkylating antineoplastic agents for different types of tumours (Sessink *et al.,* 1995). It is administered as monotherapy or in combination with other drugs to treat neoplasic and non-neoplasic diseases (Hedmer *et al.*, 2008). It is a potent alkylating agent that induces a variety of DNA base modifications. These DNA lesions are repaired by a set of enzymes that specifically recognise alkylated bases, often producing sites of base loss (Kopjar *et al.,* 2006).

Cyclophosphamide is carcinogenic in rats after oral or intravenous administration, producing benign and malignant tumours at various sites, including the bladder. It is carcinogenic in mice following its subcutaneous injection, also producing benign and malignant tumours at the site of injection and at distant sites. There was some evidence of its oncogenicity in mice and rats following intraperitoneal injection. The combined oral administration of cyclophosphamide intraperitoneally and 2-naphthylamine to mice resulted in the induction of carcinomas of the bladder at doses which, when given individually, did not produce bladder cancer (IARC, 1981).

The teratogenic effects of cyclophosphamide are well established in many animal species. The drug can also be embryolethal at nontoxic doses to the mother (IARC, 1981).

Cyclophosphamide exhibited mutagenic activity in several different assays (bacteria, yeast and mammalian cells *in vitro*, and *Drosophila* and mice *in vivo*). The agent also induced chromosomal aberrations in mammalian cells of several species *in vitro* and *in vivo*. Moreover, it induced morphological transformation of mammalian cells *in vitro* (IARC, 1981). Cyclophosphamide has been widely used since the early 1950s in the treatment of malignant lymphoma, multiple myeloma, and cancers of the breast, ovary and lung. It has also been used in the treatment of certain chronic diseases, such as rheumatoid arthritis and chronic glomerulonephritis and other non-malignant diseases (IARC, 1981). Although two cases of limb reduction defects have been reported among the offspring of women treated with cyclophosphamide during pregnancy, no epidemiological data were available for assessing the embryotoxic risk to man. Increases in chromosomal aberrations and sister chromatid exchanges were seen in peripheral blood lymphocytes of patients treated with cyclophosphamide though (IARC, 1981).

Furthermore there are many case reports of cancer, particularly bladder cancer and acute nonlymphocytic leukemia, following cyclophosphamide therapy (IARC, 1981). All added up, there appears to be sufficient evidence for the carcinogenicity of cyclophosphamide in mice and rats as well as in humans (IARC, 1981). Due to its reactivity with DNA and mutagenicity in various short-term tests, cyclophosphamide is classified as a genotoxic carcinogen (Sessink *et al.,* 1995).

1.2. 5 – FLUOROURACIL

Heidelberger and co-workers began do develop pyrimidine analogs that could be used to inhibit uracil utilization by tumour cells. The investigators theorized that uracil analogs would be preferentially utilized by tumour cells, thus inhibiting tumour growth. They further postulated that a fluorine-substituted analogue might block the formation of thymine nucleotides since fluoroacetic acid is poisonous to rats, yet acetic acid is harmless (Heidelberger *et al.,* 1957; Heidelberger, 1965). It was found that fluorine could be substituted at the 5 or 6 position of the uracil ring and that substitution at position 5 was more straightforward and stable. This led to the synthesis of 5-fluorouracil, an effective anticancer drug (Jackson *et al.,* 1996). 5-Fluorouracil (CAS no. 51-21-8) has been used as the main antineoplastic agent in the treatment of gastrointestinal tumours; it is used frequently in combination with other agents for the treatment of a variety of solid tumours (IARC, 1981). Data on chromosomal aberrations produced by 5-fluorouracil, though limited, suggest that the drug has clastogenic potential, but no evaluation can be made so far on the carcinogenic

risk of 5-fluorouracil to humans (IARC, 1981, 1987). Additionally, 5-fluorouracil is one of the most frequently antineoplastic agents used and can be easily absorbed through the skin. Given these two factors, this drug can be used as an indicator of surfaces contamination and exposure and have been extensively discussed in other studies (Larson *et al.,* 2003; Castiglia *et al.,* 2008; Schierl *et al*., 2009; Hedmer & Wohlfart, 2012; Kopp *et al.*, 2013).

5-Fluorouracil is an antimetabolite that interferes with the production of nucleic acids, increasing the number of short DNA fragments by reducing the availability of essential nucleotide precursors and thereby also the activity of DNA polymerase (Kopjar *et al.,* 2006).

5-Fluorouracil was tested by intravenous administration in mice and rats and by oral administration in rats. No evidence of carcinogenicity was found, but the studies suffered from limitations regarding duration or dose (IARC, 1981; 1987).

5-Fluorouracil can induce embryotoxic and teratogenic effects in several animal species and may be embryolethal in monkeys at nontoxic doses to the mother. The available experimental data on the mutagenicity of 5-fluorouracil are inconclusive; the agent did however induce transformation in a mouse cell line (IARC, 1981; 1987).

5-Fluorouracil has been associated in a few case reports with a variety of subsequent neoplasms but In almost all cases the drug was given together with other agents known or suspected of being carcinogens, thus raising doubts on the effect of 5-fluorouracil alone. No epidemiological study was available to the IARC Working Group. There was no evidence for the carcinogenicity of 5-fluorouracil in the limited studies available in experimental animals and, because the data from case reports in humans were insufficient to arrive at a conclusion, no evaluation could be made of the carcinogenic risk of 5-fluorouracil to humans (IARC, 1981; 1987).

1.3. PACLITAXEL

Paclitaxel (generic name Taxol) was discovered as part of the new cancer drugs screening and discovery program of the USA NCI in the 1960s. In this program many plant extracts were screened for anticancer activity, which included a crude extract from the bark of *Taxus brevifolia* (Pacific or Western yew). This crude extract showed antitumour activity against several cancer lines and the chemical structure of the active ingredient of the extract was identified as paclitaxel, being one of the most important compounds to emerge from a natural source (Panchagnula, 1998; Bayat *et al.,* 2011; Al-Sharif, 2012). Paclitaxel (CAS no. 33069-62-4) is a well-established *in vitro* and *in vivo* antineoplastic agent approved in numerous countries worldwide for the first- and second-line treatment of advanced breast cancer (Panchagnula,

1998; Simpson & Plosker, 2004). As adjuvant therapy for early breast cancer, paclitaxel is approved in the USA, Japan and other countries and is generally administered sequentially to anthracycline-containing regimens; it has also antineoplastic activity in a wide spectrum of cancer types (Simpson & Plosker, 2004; Bajic *et al.*, 2010), such as ovarian cancer, and some activity in head and neck cancer, gastric cancer, haematological cancer (Al-Sharif, 2012), and non-small-cell lung (Digue *et al.,* 1999).

Paclitaxel blocks the cell cycle during mitosis in the transition from prometaphase to metaphase since it is an antimicrotubule agent, binding specifically to the β-subunit of the protein tubulin promoting the assembly of microtubules (Digue *et al.,* 1999; Cunha *et al.,* 2001; Simpson & Plosker, 2004). These microtubules are stable, although non-functional, preventing normal mitotic spindle formation and function (Cunha *et al.,* 2001; Simpson & Plosker, 2004; Al-Sharif, 2012).

This disruption of normal spindle function, which is the primary mechanism of the antitumour activity of paclitaxel, leads to chromosome breakage and inhibition of cell replication and migration. Apoptosis induction also contributes to the antitumour action of paclitaxel, although the mechanism by which it interrupts signal transduction pathways to promote this process is poorly understood (Simpson & Plosker, 2004).

Paclitaxel is clastogenic, genotoxic, embryotoxic and fetotoxic (CCO Formulary, 2013). Can induce chromosome damage and aneuploidy, thereby enhancing the probability of damaged cells to survive (Bajic *et al*., 2010), also induced an increase in the frequency of micronuclei in the mouse bone-marrow micronucleus assay, indicating a possible carcinogenic potential of paclitaxel in humans(Tinwell & Ashby, 1994; Al-Sharif, 2012).

2. OCCUPATIONAL EXPOSURE TO CYTOSTATICS DRUGS

Although the potential therapeutic benefits of hazardous drugs outweigh the risks of side effects for ill patients, exposed health care workers risk these same side effects with no therapeutic benefit. The NIOSH has compiled several case studies that suggest both acute and long-term health effects associated to antineoplastic drug exposures, and various studies have associated workplace exposure with health effects such as skin rashes, hair loss, irritation, hypersensitivity, and headaches after reported skin contact (Kolmodin-Hedman *et al.*, 1983; Stücker *et al.*, 1990; NIOSH, 1994; Hedmer *et al.,* 2008; Chu *et al.*, 2011). Negative reproductive health outcomes are also associated with antineoplastic exposure (Kolmodin-Hedman *et al.*, 1983; Fransman *et al.,* 2007; Stover & Achutan, 2011). Spontaneous abortions have been reported approximately twice more often among exposed pregnancies than

unexposed ones (Stücker *et al.,* 1990); the same goes for congenital malformations, infertility, and possibly leukemia, as well as other cancers (Stücker *et al*., 1990; NIOSH, 1994; Froneberg, 2006; Harrison, 2006; Kopjar *et al.*, 2009).

Workers may be exposed to a drug at different stages of its life cycle – from manufacture to transport and distribution, during its use in health care or home care settings, or at its final waste disposal. These workers include shipping and receiving personnel, pharmacists and pharmacy technicians, nursing personnel, environmental services personnel, workers in veterinary practices where hazardous drugs are used (NIOSH, 1994; Sessink & Bos, 1999;). workers employed in the synthesis and production of these products, and staffs involved in cleaning, transport, and disposal of hazardous drugs or contaminated material, they all may face health risks (Sessink & Bos, 1999; Connor, 2006; Kiffmeyer *et al.*, 2012). The main focus of concern has dwelled upon the pharmacy and nursing personnel who mix and administer drugs and who are likely to experience the highest exposure intensity, and little attention has been paid to para-professional personnel, such as nursing assistants who have been assumed to be at a lesser risk (Kusnetz & Condon, 2003).

Health care workers who prepare or administer hazardous drugs or who work in areas where these drugs are used may be exposed to these agents in the air, on work surfaces, contaminated clothing, medical equipment, patient excreta, and other surfaces (NIOSH, 2004; Kopjar *et al.*, 2009; Mahboob *et al.,* 2012). Exposures may occur through inhalation resulting from aerosolization of powder or liquid during reconstitution and spillage taking place while preparing or administering to patients, through skin contact, skin absorption, ingestion, or injection. Inhalation and skin contact/absorption are the most likely routes of exposure, but unintentional ingestion from hand to mouth contact and unintentional injection through a needle stick or sharps injury are also possible (NIOSH, 2004; Kopjar *et al.*, 2009; Mader *et al.*, 2009; El-Ebiary *et al.*, 2013). Hand contact with contaminated equipment used in preparing and administering these drugs, or contaminated food or cigarettes, all lead to oral ingestion. Furthermore, patients may excrete these drugs and their metabolic by-products in body wastes, exposing personnel who handle such items (Kopjar *et al.,* 2009; El-Ebiary *et al*., 2011).

Contamination of the work surfaces and also permeation of gloves to some antineoplastic drugs were reported already in several studies (Laffon *et al.*, 2005; Kopjar *et al.*, 2009; Gulten *et al.*, 2011). Moreover, vaporization of spilled antineoplastic drugs may represent an additional route of exposure to healthcare workers through inhalation. However, contact with contaminated surfaces seems to have the most important role in exposure due to dermal absorption (Sessink & Bos, 1999; Kromhout *et al.*, 2000; Fransman *et al.*, 2004, 2005).

Therefore, the monitoring of surfaces contamination is a common way to assess occupational exposure, being the wipe sampling the most common method used (Hedmer *et al.,* 2004; Connor, 2006; Hedmer *et al.*, 2008) allowing for the demonstration of widespread workplace contamination, even when strict protocols and standard operating procedures have been applied (Schierl *et al.*, 2009).

Exposure in a hospital setting is normally due to the use of several antineoplastic drugs simultaneously. Nevertheless, the effects of such mixtures at the cell level and on human health in general are unpredictable and unique due to differences in practice of hospital oncology departments, in the number of patients, protection devices available, and the experience and safety procedures of medical staff (Kopjar *et al.,* 2009).

Since no occupational exposure limits have been established for airborne concentrations of antineoplastic drugs and for their concentration in the urine, there is no exposure level can be considered safe, and thus zero contamination should be the target (Sessink *et al.*, 1992; Santos-Burgoa, 2006; Turci *et al.*, 2010; Davis *et al.*, 2011). Exposure to these compounds should be avoided, and safety guidelines and protective measures like wearing masks, gloves, gowns, caps, protective eyewear and the preparation of drugs in biological safety cabinets are normally available in the workplaces in order to prevent exposure (Sessink *et al.*, 1992; Sorsa *et al*., 2006; Gulten *et al.*, 2011).

The growing use of complex mixtures of known and new antineoplastic drugs in cancer treatment, emphasize concerns about the occupational exposure and the genotoxic risks of workers handling such mixtures. The presence of drugs in different amounts and with different mechanisms of action suggests the need to study the relationship between the presence of genotoxic components in the mixture and the ensuing effects, taking into account the mechanism of action of each component *de per si* (Cavallo *et al.,* 2007).

NIOSH and U.S. OSHA have developed guidelines to protect nursing staff against antineoplastic drug exposure. These include (i) the use of two protective gloves and gowns when preparing, administering, or handling waste or excreta containing antineoplastic agents, (ii) training all employees who are involved in their use upon start of employment, and (iii) continuing education on how to handle hazardous drugs (Stover & Achutan, 2011).

3. GENOTOXIC EFFECTS OF ANTINEOPLASTIC DRUGS

Many anticancer agents have the potential to cause genetic alterations, which may lead to the development of cancer if they interact with proto-oncogenes or tumour suppressor genes, which are involved in controlling cell growth or differentiation (Moretti *et al.,* 2011).

There are six broad categories of anticancer chemicals, grouped by their mode of action: (i) covalent DNA-binding agents, (ii) noncovalent DNA-binding agents, (iii) topoisomerase II inhibitors, (iv) antimetabolites, (v) mitotic spindle inhibitors, and (vi) endocrine disrupters (Jackson *et al.*, 1996). Exposure to any of these genotoxic agents may initiate a sequence of events that leads to adverse health effects. The biological effects may vary depending on the drug(s), its dose, and individual genetic sensitivity/susceptibility (Kopjar *et al.,* 2009), but it is difficult to assess how much drug is absorbed in the course of handling agents at the workplace.

Because a safe threshold of occupational exposure cannot be defined for the majority of anticancer drugs, the search for appropriate monitoring parameters is an ongoing challenge. Although various methods of monitoring biological effects have been established, none points to a direct correlation between exposure level and the development of cancer, therefore they should be considered as an internal dosimeter in the detection of genotoxic and, presumably, carcinogenic risks (Bouraoui *et al.*, 2011).

Cytogenetic assays can be used for this goal; the conceptual basis for application is that DNA damage is the initial event towards pathogenesis and disease (Padjas *et al.*, 2005; Bouraoui *et al.*, 2011). Thus, cytogenetic surveillance can be viewed as an indicator enabling the early detection of exposure to genotoxic agents (Rekhadevi *et al.*, 2007; Kopjar *et al.*, 2009; Bouraoui *et al.*, 2011).

The monitoring of genotoxic risks should be done combining environmental and biological monitoring with procedures of biological effect monitoring (primary DNA damage and chromosome damage). In this integrated chemical/biotoxicological approach, the use of genotoxicity biomarkers measuring changes in cellular or molecular endpoints (e.g., DNA and/or chromosome damage) will allow us to combine environmental and biological monitoring with biological effect monitoring. In this context, comet assay represents a highly sensitive technique for detecting low levels of DNA damage in individual cells and is used in this thesis to accurately monitor interaction of antineoplastic drugs with DNA (biomarker of biologically effective dose). Among biotoxicological tests, the frequency of micronuclei in peripheral lymphocytes is recognized to be a predictor of cancer risks in humans, and because of its ability to detect both clastogenic (e.g., chromosome breakage) and aneugenic (e.g., spindle disruption) effects, it could have a role in occupational health surveillance programs for workers exposed to antineoplastic drugs to monitor long-term exposure effects (a so-called biomarker of early/preclinical biological effects) (Villarini *et al.,* 2012).

Several reports have addressed the relationship of cancer occurrence with the exposure of health care workers to antineoplastic drugs. A significant increased risk of leukemia has been reported among oncology nurses identified in the Danish cancer registry for the period 1943-1987 (Skov *et al.*, 1992). The same authors found an increased, but not significant, risk of leukemia in physicians employed for at least 6 months in a department where patients were treated with antineoplastic drugs (NIOSH, 2004). An association was also found in subjects potentially exposed to antineoplastic drugs at the employment and an elevated risk of breast and rectal cancer (Moretti *et al.*, 2011).

Past and current evidence indicates that workplace settings where anticancer drugs are prepared and administered to patients are themselves contaminated with the very drugs that have been used (Connor, 2006). To minimize the risk of occupational exposure, several guidelines for the handling of antineoplastic drugs and safety recommendations have been issued by national and international agencies. Despite the adoption of such guidelines in health care institutions, reports in current literature suggest that some healthcare workers do not follow the standards established by their employers, putting themselves at risk for mutagenicity, alterations in fertility, and long-term effects caused by chemotherapy agents (Kopjar *et al.*, 2009).

III. METHODOLOGY

CHAPTER 1 – PROCESSUAL ASPECTS

1. SAMPLES

The biological sampling took place at pathology anatomy laboratories and in pharmacy and nursing units at hospitals in Lisbon and the Tagus Valley region and also at Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL). The laboratory work was conducted in the laboratories of ESTeSL.

This research involves two different occupational settings, one where workers are exposed to formaldehyde and another where exposure is to cytostatics. In both settings the risk of nuclear anomalies is evaluated by a case-control blinded study design, therefore four separated samples were formed comprising a sample of exposed and a sample of controls in regard to each occupational setting. The risk of anomalies would be assessed by comparing the frequency of pre-established markers in those exposed with the frequency in controls by means of conventional statistical procedures (e. g. Schlesselman, 1982; Woodward, 2004).

The working places with exposure to formaldehyde were six laboratories of anatomic pathology, where a sample of 56 exposed workers was formed. The control group was formed by 85 subjects who have not been exposed to formaldehyde, namely students, teachers, and administrative staff of ESTeSL. As for exposure to cytostatics, the sample of cases comprised 46 workers which have been exposed in two pharmacy laboratories and three nursing hospitals. The control group was formed by 46 subjects who have not been exposed to cytostatics, namely students, teachers, and administrative staff of ESTeSL. The samples distribution for both occupational settings is schematized below (Figure 8):

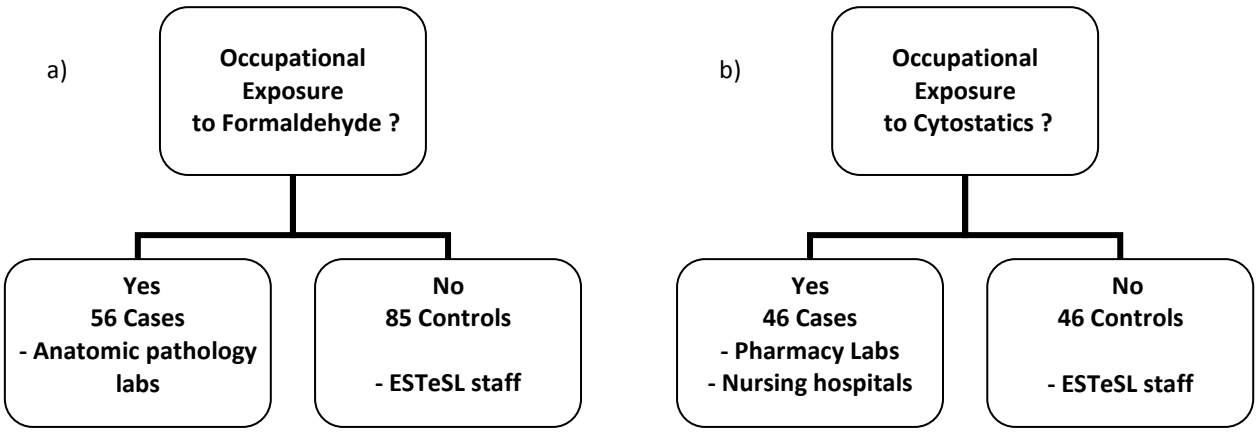


Figure 8 – Schematization of samples distribution for a) Occupational exposure to formaldehyde, and b) Occupational exposure to cytostatics.

The sampling method could be described as convenience cluster sampling, whereby two laboratories were selected based on our knowledge that occupational exposure takes place therein. All workers in the laboratories were invited and agreed to participate in this study. Controls were selected based on not having been exposed to formaldehyde or cytostatics and, simultaneously, for being statistically comparable to the subjects exposed in regard to variables usually suspected of confounding effects, like age and sex.

2. VARIABLES

The variables in this study are systematized in Table 1 (occupational exposure to formaldehyde) and in Table 2 (occupational exposure to cytostatics).

Table 1 – Variables in the study of association between occupational exposure to formaldehyde and genotoxicicity biomarkers, genetic polymorphisms and micronutrients.

Variables	Type of measure	Scale	Type of variable
Age	Quantitative	Discrete	Independent
Gender	Qualitative	Binary	Independent
Tobacco habits	Qualitative	Binary	Independent
Alcohol consumption	Qualitative	Binary	Independent
Formaldehyde exposure	Qualitative	Binary	Independent
ADH5 polymorphisms	Qualitative	Categorical	Independent
XRCC3 polymorphisms	Qualitative	Categorical	Independent
VDR polymorphisms	Qualitative	Categorical	Independent
Vitamins A, D and E	Quantitative	Discrete	Independent
Micronuclei in lymphocytes	Quantitative	Discrete	Dependent
Nucleoplasmic bridges	Quantitative	Discrete	Dependent
Nuclear buds	Quantitative	Discrete	Dependent
Micronucleus in buccal cells	Quantitative	Discrete	Dependent

Table 2 – Variables in the study of association between occupational exposure to cytostatics and genotoxicicity biomarkers, genetic polymorphisms and micronutrients.

Variables	Type of measure	Scale	Type of variable
Age	Quantitative	Discrete	Independent
Gender	Qualitative	Binary	Independent
Tobacco habits	Qualitative	Binary	Independent
Alcohol consumption	Qualitative	Binary	Independent
Cytostatics exposure	Qualitative	Binary	Independent
OGG1 polymorphisms	Qualitative	Categorical	Independent
Vitamins A and E	Quantitative	Discrete	Independent
FFQ intakes: retinol, vitamins B12, D and E, folate, iron, selenium	Quantitative	Discrete	Independent
Micronuclei in lymphocytes	Quantitative	Discrete	Dependent
Nucleoplasmic bridges	Quantitative	Discrete	Dependent
Nuclear buds	Quantitative	Discrete	Dependent
% DNA in tail	Quantitative	Discrete	Dependent
DNA oxidative damage (FPG)	Quantitative	Discrete	Dependent

3. DATA COLLECTION INSTRUMENTS

The data collection regarding these variables was derived from two main sources. First, individual questionnaires filled-in by all subjects provided information on age, sex, habits, and other personal traits. Second, microscopic visualization and laboratory procedures carried out provided information on nuclear anomalies and polymorphisms. Further details on these sources of information are presented next.

3.1. PERSONAL QUESTIONNAIRE

The study participants filled-in a questionnaire on individual characteristics and working practices. The questionnaire included questions regarding age, gender, tobacco and alcohol consumption habits, medication, hereditary diseases, exposure to formaldehyde, cytostatics

or other chemical agents, characterization of professional activity, usage of individual and collective protection equipment and hobbies. The questionnaire (Annex I) was validated by application of a pre-test in a group of subjects which was used as a pilot-sample at the beginning of the study.

3.2. FOOD-FREQUENCY QUESTIONNAIRE

Dietary intake was assessed using a self-administered Food Frequency Questionnaire (FFQ) (Lopes, 2000; Lopes *et al.,* 2007). The FFQ included type and quantity of food intake, namely some food items, which allowed for the quantification of different macronutrients and micronutrients. The FQQ is a 3-page booklet including a list of 92 common food and beverage items and questions relating to food preparation and dietary habits (Annex II). Participants were required to indicate how often each food and beverage was usually consumed per month, week, or day. Average daily consumption was based on the participants' reports on how often a specified serving size of each food or beverage item was consumed. This information, along with the nutrient composition of the food item/unit weight taken from 92 selected items, allowed participants' daily micronutrient and macronutrient intake to be calculated using the FREQUAN dietary analysis program (Baghurst and Record, 1984). The FFQ can be consulted in Annex II.

3.3. OBSERVATION LIST

A list of endpoint items were recorded during the microscopic visualization of slides obtained by the CBMN assay, micronucleus test, and comet assay. For the CBMN endpoints, the list included the following items: number of visualized cells, micronuclei in binuclear, mononuclear and multinuclear lymphocytes, nucleoplasmic bridges, and nuclear buds, and other observations than can be considered important (e.g. apoptosis and necrosis). The criteria of classification of these endpoints have been established and validated by the HUman MicroNucleus (HUMN) International Collaborative Project available in http://www.humn.org and in Fenech *et al.* (1999b).

For the MNT, the list was organized into number of visualized cells, micronuclei, and other important observations, such as karyorrhectic, pyknotic and karyolitic cells according to the criteria for scoring nuclear abnormalities in the buccal cells described by Tolbert *et al.* (1991) and Thomas *et al.* (2009).

For the comet assay test, the observation of 50 randomly chosen comets from each gel, totalizing 100 comets by slide, was performed by Comet Assay IV software from Perceptive Instruments®, measuring parameters such as: number of scored comets, head intensity, tail intensity, and % DNA in tail. These parameters are described in Collins (2002).

CHAPTER 2 – LABORATORY PROCEEDINGS

1. EXPOSURES ASSESSMENT

1.1. FORMALDEYDE EXPOSURE ASSESSMENT

The assessment of exposure to formaldehyde was based on two techniques of air monitoring conducted simultaneously. First, environmental samples were obtained by sampling the air with low flow pumps for 6 to 8 hours, during a typical working day. Formaldehyde levels were measured by Gas Chromatography (GC) analysis and time-weighted average (TWA_{8h}) was estimated according to the NIOSH method (NIOSH 2541).

The second method was aimed at measuring ceiling values of formaldehyde using Photo Ionization Detection (PID) equipment (11.7 eV lamps) with simultaneous video recording. Instantaneous values for formaldehyde concentration were obtained on a per second basis in both methods. A relationship can thus be established between worker activities and ceiling values, as well as to reveal the main exposure sources (McGlothlin, 2005; Viegas *et al.*, 2010). Measurements and sampling were performed in a macroscopic room, provided with fume hoods, always near workers breath.

1.2. CYTOSTATICS EXPOSURE ASSESSMENT

Surfaces contamination was investigated in the two hospitals by wipe sampling in areas where antineoplastic drugs were administered, as recommended by Hedmer *et al.* (2004, 2008). The cytostatics studied were considered suitable indicators for occupational exposure to antineoplastic drugs because they are frequently used in preparations and have been used in high amounts in both hospitals considered (Castiglia *et al.,* 2008). Sensitive analytical methods are already established for these drugs. In both hospitals, sampling took place in two different days. Regarding antineoplastic drug administration, sampling days were indicated by workers and services as being normal working days. Before wiping, gauzes were moistened with ethyl acetate. Sampling was performed by consecutive wiping to cover an area of 10x10 cm. The areas sampled were preparation tables, drug administration devices, chairs for drug administration, worktops, treatment registration tables and protection devices such as gloves and masks. All wipe samples were extracted as described by (Schmaus *et al.*, 2002). The analysis of the samples was blinded and performed by HPLC with Diode Array Detection

(HPLC-DAD) with a quantification limit (LOQ) of 10 ng/cm^2, in the same conditions described by Schmaus *et al.* (2002).

2. LABORATORY PROCEEDINGS

For each study sample – cases and controls – two biological matrixes were collected: peripheral blood obtained by venipuncture; and exfoliated epithelial cells obtained by scrapping the buccal mucosa with endobrush®. Epithelial cells were used exclusively for the MNT, whereas peripheral blood was used in many procedures, namely: CBMN assay (micronuclei, nucleoplasmic bridges and nuclear buds), comet assay (DNA damage and oxidative damage), Real-Time PCR (study of polymorphisms), HPLC (quantification of serum vitamins A and E), and enzyme immunoassay (quantification of serum vitamin D).

Whole blood and exfoliated cells (buccal mucosa cells) were collected between 10 a.m. and 12 p.m. from every subject and were processed for testing. As a considerable number of samples were collected in a short period of time and it was not feasible to process all samples at once, appropriate storage methods were required for preservation. Therefore, the whole blood was divided by three tubes: (i) blood tube to performed CBMN and comet assay; (ii) blood tube to centrifuge and separate serum; (iii) blood tube to study polymorphisms. These tubes where stored immediately at -20°C. Regarding the first type of tubes, once the lymphocytes were isolated, CBMN was performed in the same day that sampling took place; and 1mL of the isolated lymphocytes from each sample was cryopreserved at -80°C to perform comet assay. All samples were coded and analyzed under blind conditions.

2.1. CYTOKINESIS-BLOCKED MICRONUCLEUS ASSAY (CBMN)

The peripheral blood was obtained by venipuncture from all participating subjects and was divided by three tubes as follows: 10 mL of peripheral blood to 15 mL Falcon tubes with heparin (10U/ml blood, Sigma®), 3 mL to Kabevette serum tubes and 2 mL to Kabevette EDTA tubes.

The 10 mL freshly collected blood was directly used for the CBMN assay. Lymphocytes were isolated using Ficoll-Paque (Amersham Biosciences) gradient and placed in RPMI 1640 culture medium with L-glutamine and red phenol added with 10% inactivated fetal calf serum, 50 ug/ml streptomycin + 50U/mL penicillin, and 10 ug/mL phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37°C in a humidified 5% CO_2 incubator for 44h, and cytochalasin-b 6 ug/mL was added to the cultures in order to prevent cytokinesis. After 28h incubation, cells were spun onto microscope slides using a cytocentrifuge (Cyto-Tek®

Sakura). Smears were air-dried and double stained with May-Grünwald-Giemsa (Merck®) and mounted with Entellan®. Visualization was made with a Leica DM500 microscope with immersion oil and 1000x amplification by a single observer according to the criterion of scoring explained above. The figures concerning to these endpoints are presentend in chapter 5, for micronuclei in section 1.1, for nucleoplasmic bridges in section 1.2, and for nuclear buds in section 1.3.

2.1.1. CRITERION OF SCORING OF CBMN

The criteria used for scoring nuclear anomalies – micronuclei, nucleoplasmic bridges, and nuclear buds, are described in (Fenech *et al.*, 2003; Fenech, 2007) as follows.

The **criteria for scoring micronuclei** are: the diameter of micronuclei in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/126 and 1/9 of the area of one of the main nuclei in a binucleated cell, respectively; micronuclei are round or oval shape; micronuclei are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles; micronuclei are not linked or connected to the main nuclei; micronuclei may touch but not overlap the nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary; micronuclei usually have the same staining intensity as the main nuclei but occasionally may be more intense.

The **criteria for scoring nucleoplasmic bridges** are: nucleoplasmic bridges are a continuous nucleoplasmic link between the nuclei in a binucleated cell; the width of a nucleoplasmic bridges may vary considerable but usually does not exceed one-fourth of the diameter of the nuclei within the cell; nucleoplasmic bridges should have the same staining characteristics of the main nuclei; on rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell; a binucleated cell with a nucleoplasmic bridges may or may not contain one or more micronuclei.

The **criteria for scoring nuclear buds** are: nuclear buds are similar to micronuclei in appearance, except that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process; nuclear buds usually have the same staining intensity as micronuclei; occasionally, nuclear buds may appear to be located within a vacuole adjacent to the nucleus.

2.2. COMET ASSAY

Isolated lymphocytes were cryopreserved following the protocols of Duthie *et al.* (2002) and Singh & Lai, (2009). Briefly, isolated lymphocytes suspended in RPMI medium with L-glutamine were either centrifuged (600g, 10 min) and ressuspended in freezing mix (90% v/v heat-inactivated fetal calf serum and 10% v/v DMSO), frozen at -1°C/min in polystyrene and stored at -80°C.

For analysis of DNA damage and oxidative damage a modification of the comet assay (originally described by (Singh *et al.*, 1988) was used to measure the basal level of DNA oxidation in lymphocytes (Collins, 2009). The aliquots were rapidly thawed at 37°C and transferred to a 15 mL Falcon tube with 2 mL of PBS (Dulbecco's PBS, Sigma), and immediately centrifuged at 1800 g for 5 min to remove freezing mix. The pellets were ressuspended in PBS and cells were counted using a Neubauer Improved Haemocytometer.

Thirty microliters of the cell suspension ($2.0x10^4$ cells/mL) was mixed with 140 µl of 1% low melting-point agarose (LM Pronadisa) in a microcentrifuge tube and added to a slide previously pre-coated with 1% agarose (SeaKem®), two gels per slide. The gels were covered with a cover slip (22x22x1.0mm) and allowed to set on a cold plate. The cover slips were removed and the slides immersed in lysis solution [2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TRIS, 1% Triton® X-100 (pH 10)] for 60 min. Following lysis, the slides were immersed in two changes of Buffer F [40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA and 0.2 mg/L BSA (pH 8.0)] for 5 min, each time at 4°C. FPG [kindly donated by Prof. Andrew Collins (Department of Nutrition, University of Oslo, Norway)] was added to the gel previously diluted in Buffer F. Incubation of the slides with FPG and with Buffer F was performed in a humid chamber at 37ºC for 30 min. The reaction was stopped by placing them at 4°C.

The cover slips were removed and all the slides – lysis, Buffer F and FPG treatment - were placed on an electrophoresis platform, covered with electrophoresis buffer [1 mM Na_2EDTA, 0.3 M NaOH (pH 13)] and DNA was allow to unwind for 20 min before electrophoresis at 1.14 V/cm, 300 mA for a further 20 min. DNA unwinding and electrophoresis was performed in a cold unit at 4°C. The slides were transferred to a Coplin jar and immersed in PBS, the in distilled water, both for 10 min at 4°C. After, dehydrate the slides in increasing ethanol concentrations (70%, 96% and 100%), 5 min each. The slides were dried at room temperature, stained with 25 µl DAPI (1 µg/mL) and visualized. Slides were scored using Zeiss AxioScope.A1 fluorescence microscope and Comet Assay IV capture system (Perceptive Instruments) and 50 nucleoids were scored per gel. The tail intensity, defined as the percentage of DNA migrated from the head of the comet into the tail, was measured from each comet scored. Tail moment

was not used for data analysis, as it has no recognized units. In addition, the equation used to calculate tail moment uses tail length, which tends to increase rapidly with concentration at low levels of damage.

2.2.1. IMAGE ANALYSIS AND CRITERION OF SCORING

There are three main scoring methods of comet assay results: visual scoring, semi-automated image analysis and automated image analysis. Results from a study performed by Azqueta *et al.* (2011) verified that all three approaches can be regarded as trustworthy and – to a large extent – interchangeable.

The most important parameters to measure in comet assay are: tail length, relative fluorescence intensity of head and tail, normally expressed as % of DNA in tail, and tail moment (Collins, 2002).

The percentage DNA in the tail is considered the parameter that can be best compared among laboratories. The consensus in the International Workshop on Genotoxicity Test Procedures was that image analysis is preferred but not required and that the parameter % tail DNA appeared to be the most linearly related to dose and the easiest to intuitively understand (Kumaravel & Jha, 2006; Hartmann & Speit, 2009).

The % tail DNA values are constrained to a maximum of 100 and a minimum of 0 with no variability at the extremes and a maximum variability at intermediate values such as 50%. The % tail DNA has the advantage that it can be 'standardized' over studies while tail length and moment, although consistent within the study, may not be comparable across studies (Lovell & Omori, 2008). Therefore, relative tail intensity is the most useful parameter, as it bears a linear relationship to break frequency, is relatively unaffected by threshold settings, and allows discrimination of damage over the widest possible range. It also gives a very clear indication of what the comets actually looked like (Collins, 2002; Lovell & Omori, 2008). It is important to refer that a satisfactory condition for the assay is that untreated control cells should have a background level of breaks (i.e.≈ 10% DNA in tail) and there are suggestions that negative control cells should have between 0 and 20% DNA as described by Lovell & Omori, 2008.

The figure concerning to comets image is presented in chapter 5 in section 3.1.

2.3. GENETIC POLYMORPHISMS

In order to study genetic polymorphisms in genes *XRCC3, ADH5, OGG1* and *VDR* it is necessary to extract DNA from peripheral blood before using Real Time PCR to quantify the DNA in

study. The techniques used were: phenol-chloroform and by blood spot. It was also made a treatment with heparinase of the DNA of the samples from the formaldehyde study because they were preserved in heparin (anticoagulant) that inhibits the PCR reaction. All procedures are described below.

2.3.1. DNA EXTRACTION

2.3.1.1. DNA EXTRACTION FROM PERIPHERAL BLOOD BY PHENOL-CHLOROFORM TECHNIQUE

Four hundred µl of peripheral blood was drawn from each sample and placed in a microtube with equal volume of lysis buffer (100 mM Tris-HCL pH 8; 10 mM EDTA; 100 mM NaCl; 0.1% SDS) plus 5 µl of proteinase K [20 mg/mL], being then incubated overnight at 56ºC. It was centrifuged (13.000g, 10 minutes) with phenol, phenol/chloroform and chloroform, respectively, with supernatant transfer to a new microtube. Finally, it was added 40 µl of Sodium acetate 3M pH 5.0 and 800 µl of cold absolute ethanol and refrigerated at -80°C for 15 minutes. After centrifugation (13.000g, 15 minutes) the supernatant was discarded and the pellet was washed with ethanol 70% and centrifuged at 4ºC (13.000g, 10 minutes). The pellet was dried in a coven over at 37°C, ressuspended in 100 µl of ultrapure water (MiliQ), and stored at -20°C.

2.3.1.2. DNA EXTRACTION FROM BLOOD SPOT

The whole blood previously stored at -20°C was defrosted and 200 µl were dropped in 3 MM chromatography paper (Watman™) and air dried. Two samples of each biological sample were taken with a perforator and put in a microtube with 500 µl of ultrapure water (MiliQ). The perforator was disinfected between samples with ethanol 70%. Each microtube was placed in the vortex and kept at room temperature for 10 min, and next they were centrifuged at 16.000 g for 2 min. The sobrenatant was eliminated and 200 µl of Chelex at 6% were added in the microtube and mixed in vortex. The microtubes were put at 56°C for 10 min and then, after vortex, went for 10 more minutes at 100ºC. Finally, the microtubes were centrifugated at 6000 g for 2 min and stored at -20°C.

2.3.2. TREATMENT WITH HEPARINASE I

There are many substances that can strongly inhibit the PCR reaction, namely: proteinase K, phenol, quelants (EDTA), haemoglobin and other erythrocyte proteins, elevated concentrations of salts, and heparin.

In the formaldehyde occupational exposure study, heparin was used as anticoagulant in blood samples collected by venipuncture and it was necessary to provide a treatment with heparinase I. Heparin interferes with DNA polymerase during DNA transcription and with the reverse transcription of RNA. The treatment with heparinase I consists in its dissolution in a buffer solution (20 mM Tris-HCL, pH 7.5, 50 mM NaCl, 4 mM $CaCl_2$ and 0.01% BSA) and the addition of 0.83 µl of this solution plus 2 µl of ddH20 for each 7.2 µl of DNA extracted, for 2h at room temperature of 25°C.

2.3.3. STUDY OF THE POLYMORPHISMS BY REAL TIME PCR

The genotype of the polymorphisms was studied by Real Time PCR using the *iCycler iQ® Multicolor Real-Time PCR Detection System* (BIO-RAD). The polymorphisms under examination were: *XRCC3* Thr241Met, *ADH5* Val309Ile, *ADH5* Asp353Glu, *OGG1* Ser241Arg, *VDR BsmI*, according to the information provided in Table 3.

Table 3 – Polymorphisms studied, with the corresponding SNP ID and TaqMan SNP.

Polymorphism	SNP ID	TaqMan SNP
XRCC3 Thr241Met	rs861539	C_8901525_10
ADH5 Val309Ile	rs28730628	C_61623349_10
ADH5 Asp353Glu	rs16996593	C_33249205_20
OGG1 Ser326Cys	rs1052133	C_3095552_1
VDR BsmI	rs1544410	C_8716062_10

The programme for Real Time PCR of the polymorphisms under study is specified in Table 4.

Table 4 – Programme used to study polymorphisms by Real Time PCR.

Stage	Temperature (°C)	Time	Repeats
1º	50	2 min	-
2º	95	30 s	-
3º	95	30 s	2
4º	95	10 min	-
5º	92 60	15 s 1 min	50
6º	4	-	-

The PCR reaction mixture was constituted by: 10 µl de *TaqMan Universal PCR Master Mix* and 1 µl of specific primers for the polymorphisms under study, 5 µl of distilled water and 4 µl of DNA in study in a total volume of 20 µl. The primers and the *TaqMan Universal PCR Master Mix* were kept on ice during the preparation of the reaction solution. All reagents in the reaction solution were stored at -20°C, except for the *TaqMan Universal PCR Master Mix* which was stored at 4°C.

2.4. EPITHELIAL CELLS

Buccal cavity cells are obtained by scraping the cheeks with a tooth brush, wooden spatula, or a tongue depressor (Majer *et al.*, 2001; Holland *et al.*, 2008). The sampling of epithelial cells was performed by scraping the inside of both cheeks using a different brush for sampling left and right areas of the mouth to maximize cell sampling and to eliminate any unknown biases that may be caused by sampling one cheek only. It is important to note that repeated vigorous brushing of the same area can lead to increased collection of cells from the less differentiated basal layer (Thomas *et al*., 2009). For that purpose, buccal mucosa cells were collected with an endobrush®, a cytological brush; followed by a smear on two slides. The smears were fixed by pulverization with Mercofix®, a methanol fixative. The slides were stained by the Feulgen technique without counterstain and air dried. This technique allows for a highly selective demonstration of DNA. The reaction consists on an acid hydrolysis with nitric acid 5M aimed at selectively separating the purines (adenine and guanine) of the DNA molecule. The aldehydes groups formed in this stage stained pink by the Schiff's reagent action. For each subject, two slides were done. Visualization was made in the Leica DM500 microscope with immersion oil and 1000x amplification by a single observer according with the observation list explained as follows.

2.4.1. CRITERION OF SCORING

The criterion for scoring is originally based on the description by Tolbert *et al.* (1992), intended for classifying buccal cells into categories that distinguish between "normal" cells and cells that are considered "abnormal" on the basis of cytological and nuclear features, which are indicative of DNA damage, cytokinetic failure or cell death. Only cells free smearing, clumping or overlapping and those containing intact nuclei should be included in the scoring. Therefore, some definitions of the cytological findings are (Thomas *et al.,* 2009) as follows.

Normal "differentiated" cells have a uniformly stained nucleus, which is oval or round in shape. They are distinguished from basal cells by their larger size and by their smaller nucleus-

to-cytoplasm ratio. No other DNA-containing structures apart from the nucleus are observed in these cells, being considered to be terminally differentiated relative to basal cells, as no mitotic cells are observed in this population.

Normal differentiated cells should fulfil the following parameters for being scored: (i) have an intact cytoplasm and relatively flat cell position on the slide; (ii) little or no overlap with adjacent cells; (iii) little or no debris; and (iv) nucleus normal and intact, nuclear perimeter smooth and distinct. The suggested criteria for identifying micronuclei are: (i) chromatin intensity and staining pattern are similar to that of the main nucleus, (ii) the borders are distinctly recognizable indicating the presence of a nuclear membrane, (iii) the objects are round in the same optical plane with that of the main nucleus, (iv) and when they are contained within the same cytoplasm with the main nucleus (Tolbert *et al.*, 1992). Baseline frequencies for micronucleated cells in the buccal mucosa are usually within the 0.5-2.5 micronuclei /1000 cells range (Thomas *et al.,* 2009). The latter study suggested a minimum of 2000 differentiated cells to be scored. The figure concerning to this endpoint is presented in chapter 5 in section 2.2.

2.5. VITAMINS QUANTIFICATION

2.5.1. VITAMINS A AND E QUANTIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Vitamins A and E were quantified in serum samples according to Jaworowska & Bazylak (2008). Calibration curves were constructed from stock solutions of vitamin A (100 mM in methanol), vitamin E (100 mM in chloroform) and tocopheryl acetate (TA - 100 mM in methanol, internal standard).

At the outset, calibration curves for each vitamin were built from successive dilutions showed in Table 5 for vitamins A, E and tocopheryl acetate.

Table 5 – Range concentrations of vitamins A, E, and tocopheryl acetate to build the calibration curves.

Level	Vit. A (mM)	Vit. E (mM)	TA (mM)
Blank	0.000	0.000	0.25
1	0.050	0.050	0.25
2	0.125	0.125	0.25
3	0.250	0.250	0.25
4	0.375	0.375	0.25
5	0.425	0.425	0.25
6	0.500	0.500	0.25

The serum samples were thawed and analysed by HPLC-DAD. Before chromatography an extraction protocol was conducted, consisting in the addition of 400 µl of 62.5 µM TA (in methanol) to 200 µl of each serum sample and 330 µl n-hexane, followed by a 5-minute vortex homogenisation and centrifugation (13.400 g, 10 minutes).

The upper phase was collected in a HPLC vial and the lower phase was re-extracted twice. The n-hexane extracts were pooled together and evaporated under a nitrogen stream at 37°C. The dried extract was dissolved in 100 µl methanol and 25 µl were injected in triplicate. The chromatographic conditions are outlined in Table 6.

Table 6 – Chromatographic conditions for quantification of vitamins A and E and realizations of the respective calibration curves.

Conditions	Description
Column	Hypersil-BDS C18
Pre-column	Javelin BDS C8
Mobile phase	100% methanol (isocratic)
Injection volume	25 µl (no waste mode)
Needle wash between injections	Yes with methanol
Run time	20 minutes
Detection Wavelenght	285 nm, Tocopheryl acetate 290 nm, Vitamin E 325 nm, Vitamin A
Retention Times	2.91 min, Vitamin A 5.82 min, Vitamin E 8.47 min, Tocopheryl acetate
Total Scan	200 – 600 nm

Data of the calibration curves obtained for vitamins A and E are showed in Table 7, with the respective Limit of Detection (LOD) and Limit of Quantification (LOD) and Linearity (R).

Table 7 – Calibration curves data and LOD, LOQ, and Linearity (R) parameters.

	[Vit A]/µM	[Vit E]/µM
Equation	y = 605357x + 11002	Y=670317x + 2568
R^2	0.9999	0.9999
LOD (nmol)	0.0154	0.01
LOQ (nmol)	0.0468	0.04

2.5.2. VITAMIN D QUANTIFICATION BY IDS 25-HYDROXY VITAMIN D EIA KIT

The IDS 25-Hydroxy Vitamin D EIA kit (Immunodiagnostic Systems Ltd) is an enzyme immunoassay for the quantification of 25-hydroxyvitamin D and other hydroxylated metabolites in human serum or plasma. 25 µl of the samples were added to a calibrator and a control and to 1 mL of 25-D biotin solution; 200 µl of each sample were incubated in the appropriate wells with antibody coated microplate in duplicate. The plate was covered with an adhesive plate sealer for 2h at room temperature. Later it was washed three times with 250 µl of wash solution (PBS containing Tween). Then 200 µl of enzyme conjugate (TMB - PBS containing avidin linked to horseradish peroxidase) was added and the plate was covered for 30 minutes at room temperature. The wash step was repeated. An amount of 200 µl of TMB substrate was added to all wells for 30 minutes and 100 µl of HCL, in order to stop the reaction. The measure of the absorbance of each well was made at 450 nm (reference 650 nm) using a microplate reader within 30 minutes after adding the stop solution.

3. STATISTICAL PROCEDURES

The deviation of variables from the normal distribution was evaluated by the Shapiro-Wilk goodness-of-fit test. Rejection of the null hypothesis of underlying normality usually led us to proceed with non-parametric procedures to compare means and check associations. However, parametric procedures were also commony used for descriptive purposes, some of them being known for their robustness to deviations from normality assumptions. For example, means and standard deviations were commonly used.

The statistical procedures were aimed at investigating the association between genotoxicity biomarkers, herein conceptualized as dependent variables, and a selection of possible risk factors conceptualized as independent (explanatory) variables (Tables 1 and 2). The biomarkers were dichotomized (absent/present) and considered the dependent variable in multiple regression models, namely binary multiple logistic regression, where exposures were treated as independent variables. Odds ratios were computed to evaluate the risk of biomarkers presence and their significance was assessed.

The biomarkers were nuclear alterations given by the endpoints studied – micronuclei, nucleoplasmic bridges, nuclear buds and comets. The risk factors were tobacco and alcohol consumption habits, diet, genetic polymorphisms (susceptibility biomarkers), vitamin serum levels, and dietary intakes assessed by FFQ. Spearman correlation, Mann-Whitney and Kruskal-Wallis tests were also used to compare groups. Multiple regression analysis was used solely to identify potential risk factors and not with a predictive explanatory character.

The analysis of genotype and allele frequency and Fisher's exact test was made with the GenPop version 4.0.10 software, and all the other statistical procedures were made by using the Statistical Package for the Social Sciences (SPSS) version 21.0.

4. ETHICAL CONSIDERATIONS

This research project was done with the authorization of the presidents of the administration council, the ethic commission, and the directors of the laboratories and units where the study was conducted, to whom a letter explaining the study and its main objectives was addressed, as well as the pleading for authorization (Annex III).

All participants in the study did it free-willingly and their rights to privacy were guaranteed by assuring confidentiality of the data collected. Before biological samples were collected, participants signed a written informed consent (Annex IV), where anonymity and data confidentiality was granted, as required by ethical and deontological principles.

IV. RESULTS

CHAPTER 1 – FORMALDEYDE OCCUPATIONAL SETTING

1. SAMPLES

Two samples were formed - the group of those occupationally exposed to formaldehyde and the non-exposed group (controls). The characteristics of each group regarding gender, age, years of exposure, tobacco, and alcohol consumption, are presented in Table 8.

Table 8 – Characteristics of the samples regarding gender, age, years of exposure, tobacco and alcohol consumption.

	Control group	**Exposed group**
Number of subjects	85	56
Gender Females Males	 54 (64%) 31 (36%)	 37 (66%) 19 (34%)
Age **(mean ± standard deviation, in years)** Range	 32.42 ± 8.1 20-53	 39.45 ± 11.5 20-61
Years of exposure **(mean ± standard deviation, in years)** Range	n.a.	 14.5 1-33
Tobacco consumption Non-smokers Smokers	 60 (70.6%) 25 (29. 4%)	 45 (80.4%) 11 (19.6%)
Alcohol consumption Non-drinkers Drinkers	 19 (22.4%) 66 (77.6%)	 19 (33.9%) 37 (66.1%)

n.a. - non applicable

2. FORMALDEHYDE EXPOSURE ASSESSMENT

Results of formaldehyde exposure were determined using the two methods described – the NIOSH 2541 method (NIOSH, 1994) for average concentrations (TWA_{8h}) and the PID method for ceiling concentrations. For the first exposure metric, the formaldehyde mean level of the 56 individuals exposed was 0.16 ppm (0.04 – 0.51 ppm), a value lying below the OSHA critical

reference of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (0.18 – 2.93 ppm), a value well above the reference of 0.3 ppm established by the ACGIH for ceiling concentrations. The ceiling values varied among the different tasks developed in histopathology laboratories. The highest formaldehyde concentration was identified during macroscopic specimens' exam (Table 9). This task involves a careful observation and grossing of the biological specimen preserved in formaldehyde, by the pathologist or pathology anatomy technician, being prone to a direct and prolonged contact with formaldehyde vapors. Another task, jar filling, is the substitution of formaldehyde with a fresh solution in the recipients where it was used. The third task with high ceiling values was specimen wash, the washing of biological samples to remove residues that can affect macroscopy exam. Another task, the biopsy, is the collection of a small biological sample, usually containing the complete lesion and for this task the ceiling value was lower than for the macroscopic specimen exam (Table 9). Finally, disposal of specimen and used solutions consists in the removal of formaldehyde to the proper waste, and the disposal of the biological speciemens for incineration.

Table 9 - Formaldehyde ceiling values (ppm) by task in the macroscopy room.

Tasks	**Ceiling Values** (ppm)
Macroscopic specimen's exam	2.93
Disposal of specimen and used solutions	0.95
Jar filling	2.51
Specimen wash	2.28
Biopsy exam	1.91

3. GENOTOXICITY ASSESSMENT

For all genotoxicity biomarkers under study, workers exposed to formaldehyde had significantly higher mean values than the controls (Table 10).

In peripheral blood lymphocytes, significant differences (Mann-Whitney test, $p<0.001$) were observed between subjects exposed and non-exposed to formaldehyde, namely in mean MN (respectively, 3.96±0.53 *vs* 0.81±0.17), NPB (3.04±0.52 *vs* 0.18±0.06), and NBUD (0.98±0.27 *vs* 0.07±0.03). In buccal mucosa cells, the MN mean was also significantly higher ($p=0.002$) in exposed subjects (0.96±0.28) than in controls (0.16±0.06).

The odds ratios (OR) indicate an increased risk for the presence of biomarkers in those exposed to formaldehyde, compared to non-exposed (Table 10) and they were all significant ($p<0.001$).

Table 10 – Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD in the studied population (mean ± standard error of the mean, range), p-value of the Mann-Whitney test and results of binary logistic regression concerning the association between exposure and genotoxicity biomarkers, as evaluated by the odds ratio (OR) and their confidence intervals.

	MN in lymphocytes Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	3.96±0.53 (0-14)	3.04±0.52 (0-15)	0.98±0.27 (0-13)	0.96±0.28 (0-9)
Controls	0.81±0.17 (0-7)	0.18±0.06 (0-3)	0.07±0.03 (0-1)	0.16±0.06 (0-2)
p-value[1]	<0.001	<0.001	<0.001	0.002
OR	9.665	11.97	9.631	3.990
OR CI 95%	3.81-24.52	4.59-31.20	3.12-29.70	1.38-11.58
p-value[2]	<0.001	<0.001	<0.001	0.011

[1] Mann-Whitney test
[2] Binary logistic regression

Regarding the impact of the duration of exposure to formaldehyde, the mean values of MN in lymphocytes and in buccal cells tended to increase with years of exposure (Table 11) but the association was not statistically significant ($p>0.05$).

Table 11 –Descriptive statistics in the exposed group of MN in lymphocytes and buccal cells, NPB, and NBUD (mean ± standard error of the mean, range) by years of exposure to formaldehyde.

Years of exposure	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
<5	8	2.75±0.94 (0-8)	5.13±1.38 (0-10)	1.38±0.50 (0-3)	0.63±0.63 (0-5)
6-10	19	3.05±0.78 (0-12)	2.42±0.67 (0-9)	1.53±0.73 (0-13)	0.63±0.33 (0-6)
11 – 20	12	5.50±1.32 (0-14)	3.33±1.44 (0-14)	0.33±0.19 (0-2)	0.83±0.46 (0-5)
≥ 21	15	5.00±1.15 (0-13)	2.33±1.04 (0-15)	0.73±0.25 (0-2)	1.20±0.80 (0-9)

4. DEMOGRAPHIC AND LIFESTYLE HABITS

Age and gender are considered the most important demographic variables affecting the MN index. However, the mean of all genotoxicity biomarkers (Table 12) did not differ significantly between men and women either in the exposed or the controls (Mann-Whitney test, $p > 0.05$).

Table 12 –Descriptive statistics of MN in lymphocytes and buccal cells, NPB, and NBUD means by gender and exposure (mean ± standard error of the mean, range). There were no significant differences between means of the two genders either within the exposed or the controls.

Groups	Gender	N	MN in lymphocytes Mean ± S.E. (range)	NPB Mean ± S.E. (range)	NBUD Mean ± S.E. (range)	MN in buccal cells Mean ± S.E. (range)
Exposed	Females	37	4.43±0.68 (0-14)	3.03±0.70 (0-15)	1.34±0.42 (0-13)	1.14±0.35 (0-8)
	Males	19	3.47±0.88 (0-13)	2.95±0.82 (0-14)	0.42±0.16 (0-2)	0.74±0.50 (0-9)
Controls	Females	54	0.87±0.23 (0-7)	0.22±0.08 (0-3)	0.11±0.04 (0-1)	0.11±0.06 (0-2)
	Males	31	0.71±0.26 (0-6)	0.10±0.07 (0-2)	0.00	0.26±0.12 (0-2)

In order to examine the effect of age on biomarkers, individuals were split into exposed and non-exposed and, within each group, the association between age and biomarker frequency was studied by simple regression analysis, yielding the results in Table 13. There is a significant association between MN in lymphocytes and age in the exposed group, with the number of MNs tending to increase with age ($R^2 = 0.206$, $p<0.001$) as shown in Figure 9a. The regression coefficient (B in Table 13) indicates that a 10-year increase in age corresponds to an average increase of 1.55 MNs in lymphocytes. Age does not significantly account for variation in any other biomarker (Table 13) though.

Table 13 - Results of simple regression analysis of age on biomarkers by group (exposed/controls). R^2 is the coefficient of determination; A and B are, respectively, the ordinate and the slope in the regression line, and p is the likelihood of B in case of no association between age and the biomarker; ** signals a highly significant p.

Biomarker		R^2	A	B	p
MN in BN lymphocytes	**Exposed**	0.206	-2.143	0.155	<0.001**
	Controls	0.009	1.486	-0.019	0.395
NPB	**Exposed**	0.000	0.705	0.000	0.999
	Controls	0.001	0.384	-0.004	0.778
NBUD	**Exposed**	0.013	0.015	0.009	0.403
	Controls	0.001	0.107	-0.001	0.740
MN in Buccal	**Exposed**	0.003	0.278	-0.003	0.671
	Controls	0.003	0.241	0.013	0.637

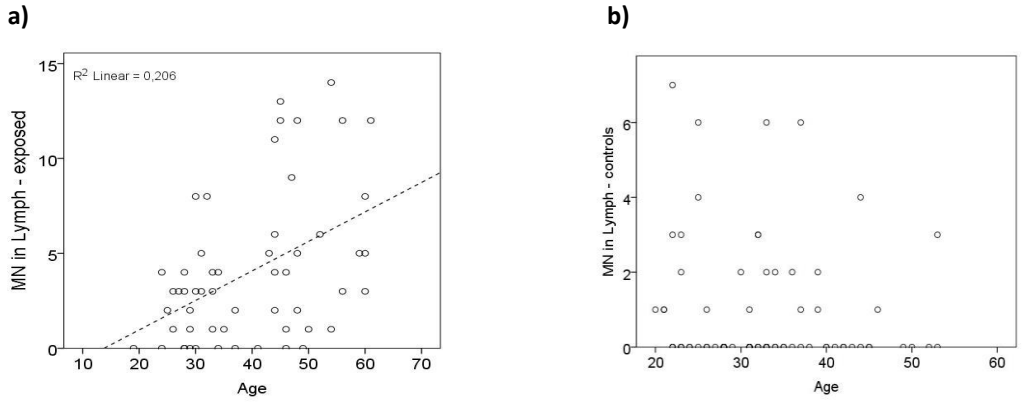


Figure 9 - Scatter plots of the number of MN in lymphocytes against age in the exposed (a) and control (b) groups. The slope of the regression line (dashed) in the exposed is statistically significant.

Although the association between years of exposure and genotoxicity biomarkers, namely MN in lymphocytes, is not statistically significant, exposed workers with higher age tend to have more years of exposure, an association that was not observed in the control group.

Descriptive statistics of every biomarker were decomposed by three age groups (20-30, 31-40, and ≥ 41 years old) in the exposed and control groups, allowing for a qualitative view of the same relationships (Table 14).

Table 14 – Age effects on descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± standard error of the mean, range).

Groups	Age	N	MN in lymphocytes Mean ± S.E. (range)	NPB Mean ± S.E. (range)	NBUD Mean ± S.E. (range)	MN in buccal cells Mean ± S.E. (range)
Exposed	**20-30**	18	2.19±0.53 (0-8)	3.56±0.93 (0-10)	1.63±0.82 (0-13)	0.75±0.47 (0-6)
	31-40	11	3.00±0.78 (0-8)	1.20±0.47 (0-4)	0.50±0.22 (880-2)	0.40±0.22 (0-2)
	≥ 41	27	5.54±0.88 (0-14)	3.00±0.88 (0-15)	0.69±0.23 (0-5)	1.46±0.50 (0-9)
Controls	**20-30**	36	0.47±0.16 (0-3)	0.14±0.07 (0-2)	0.08±0.05 (0-1)	0.19±0.96 (0-2)
	31-40	35	1.14±0.33 (0-7)	0.20±0.01 (0-3)	0.06±0.04 (0-1)	0.14±0.83 (0-2)
	≥ 41	14	0.86±0.50 (0-6)	0.21±0.16 (0-2)	0.07±0.71 (0-1)	0.14±0.14 (0-2)

In order to examine if gender adds a significant contribution to explain variability in genotoxicity biomarkers, regression analysis was repeated but now adding gender as an independent variable and examining whether it would improve upon the values of R^2 already presented for age (Table 15).

Table 15 - Results of multiple regression analysis of age and gender on biomarkers by group (exposed/controls). R^2, new R^2, and ΔR^2 are, respectively, the coefficients of determination of the model with only age, age and gender, and the difference between them. The significance of the addition of gender is shown by the value of p, none being significant.

		Age	Age and gender		
Biomarker	**Group**	**R^2**	**New R^2**	**ÄR^2**	**p**
MN in BN lymphocytes	**Exposed**	0.206	0.207	0.001	0.828
	Controls	0.009	0.014	0.005	0.574
NPB	**Exposed**	0.000	0.006	0.006	0.597
	Controls	0.001	0.014	0.013	0.325
NBUD	**Exposed**	0.013	0.040	0.027	0.244
	Controls	0.001	0.002	0.001	0.945
MN in Buccal	**Exposed**	0.003	0.013	0.010	0.488
	Controls	0.003	0.006	0.003	0.610

The results (Table 15) show that the increase in R^2 due to the presence of gender in the model was never significant, thus gender does not help to account for the frequency of genotoxicity biomarkers. Table 16 allows for a further examination of descriptive statistics of biomarkers by groups and gender.

Table 16 - Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means by exposition to formaldehyde, gender and age (mean ± standard error of the mean).

Groups		**N**	**MN in lymphocytes** Mean ± S.E.	**NPB** Mean ± S.E.	**NBUD** Mean ± S.E.	**MN in buccal cells** Mean ± S.E.
Exposed	**Females 20-30**	12	2.42±0.67	4.17±1.22	2.00±1.07	1.00±0.62
	Females 31-40	7	2.71±0.68	0.86±0.46	0.57±0.3	0.29±0.18
	Females >41	18	6.00±1.12	3.22±1.09	1.06±0.39	1.44±0.55
	Males 20-30	6	1.00±0.52	3.50±1.02	0.50±0.34	0.00±0.0
	Males 31-40	4	3.00±1.78	1.50±0.96	0.25±0.25	0.50±0.50
	Males >41	9	5.33±1.45	3.22±1.56	0.44±0.24	1.33±1.01
Controls	**Females 20-30**	23	0.43±0.20	0.13±0.70	0.13±0.7	0.13±0.10
	Females 31-40	22	1.32±0.44	0.27±0.15	0.09±0.06	0.14±0.10
	Females >41	9	0.89±0.68	0.33±0.24	0.11±0.11	0.00±0.00
	Males 20-30	13	0.54±0.27	0.15±0.15	0.00±0.00	0.31±0.21
	Males 31-40	13	0.85±0.48	0.08±0.08	0.00±0.00	0.15±0.15
	Males >41	5	0.80±0.80	0.00±0.00	0.00±0.00	0.40±0.40

The distribution of the genotoxicity biomarkers regarding tobacco consumption is presented in Table 17. Regarding smoking habits, a non-parametric analysis rejected the null hypothesis that biomarkers are the same for the four categories (control smokers and non-smokers, exposed smokers and non-smokers) (Kruskal-Wallis test, $p<0.001$). However, the analysis of the interactions between formaldehyde exposure and tobacco smoke between exposed and controls (Mann-Whitney test) showed that formaldehyde exposure, rather than tobacco, has a preponderant effect upon the determination of biomarker frequencies. In the control group,

non-smokers had slightly higher MN means in buccal cells in comparison with smokers; although the result did not reach statistical significance (Mann-Whitney test, p> 0.05).

Table 17 –Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means (mean ± standard error of the mean, range) by exposition to formaldehyde and tobacco habits.

Groups	**Tobacco consumption**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**Non-smokers**	44	4.39±0.60 (0-12)	0.72±0.23 (0-6)	0.37±0.14 (0-2)	0.16±0.08 (0-8)
	Smokers	11	2.55±1.08 (0-14)	0.64±0.24 (0-15)	0.36±0.24 (0-13)	0.18±0.18 (0-9)
Controls	**Non-smokers**	57	0.88±0.22 (0-6)	0.33±0.14 (0-3)	0.09±0.05 (0-1)	0.77±0.29 (0-2)
	Smokers	24	0.75±0.33 (0-7)	0.13±0.13 (0-2)	0.00	0.33±0.25 (0-2)

As for alcohol consumption, because uptake reported in enquires may differ considerably from real consumption, all consumers were gathered into a single entity, in contrast with non-consumers. Nevertheless, no one acknowledged having “heavy drink habits” in the questionnaires. The distribuition of the genotoxicity biomarkers regarding alcohol consumption is presented in Table 18.

Table 18 – Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD (mean ± standard error of the mean, range) by alcohol consumption and exposure to formaldehyde.

Groups	**Alcohol consumption**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**Non-drinkers**	19	4.00±0.83 (0-12)	3.58±0.94 (0-15)	1.00±0.33 (0-5)	0.79±0.31 (0-5)
	Drinkers	35	3.95±0.68 (0-14)	2.76±0.63 (0-14)	0.97±0.38 (0-13)	1.05±0.39 (0-9)
Controls	**Non-drinkers**	18	0.21±0.21 (0-4)	0.11±0.07 (0-1)	0.00±0.00	0.11±0.11 (0-2)
	Drinkers	63	0.98±0.21 (0-7)	0.20±0.07 (0-3)	0.09±0.04 (0-1)	0.18±0.07 (0-2)

Overall, biomarkers in both groups – exposed and controls, did not exhibit very different mean frequencies among alcohol consumers and non-consumers, and these differences were indeed not statistically significant (Mann-Whitney test, $p>0.05$).

The interaction between alcohol consumption and smoking habits was statistically significant (Kruskal-Wallis test, $p=0.043$), as subjects that do not smoke and do not drink tend to have lower frequencies of MN in buccal cells than those who drink and smoke, with a gradient of frequencies in between.

5. INDIVIDUAL SUSCEPTIBILITY

The frequencies of genotypes and alleles of the polymorphisms studied – *XRCC3* Met241Thr, *ADH5* Val309Ile and Asp353Glu, and *VDR BsmI* - in the two groups are shown in Table 19. No significant differences were observed between groups in genotype and allele frequencies for the four polymorphisms analyzed (Fisher's exact tests, $p > 0.05$).

Table 19 – Frequency of genotypes and alleles of *XRCC3* Met241Thr, *ADH5* Val309Ile and Asp353Glu, and *VDR BsmI* polymorphisms overall and by exposition group, with p-value of the Fishers's exact test.

Genes	Genotypes	All (%)	Exposed (%)	Controls (%)	p-value
XRCC3	Met/Met	33 (24.3)	13 (24.1)	20 (24.4)	0.660
	Met/Thr	49 (36.0)	22 (40.7)	27 (32.9)	
	Thr/Thr	54 (39.7)	19 (35.2)	35 (42.7)	
	Met	115	48 (0.4)	67 (41)	0.628
	Thr	157	60 (0.6)	97 (59)	
ADH5	Val/Val	50 (36.5)	21 (38.2)	29 (35.4)	0.856
	Val/Ile	87 (63.5)	34 (61.8)	53 (64.6)	
	Val	187	76 (69.1)	111 (67.7)	0.896
	Ile	87	34 (30.9)	53 (32.3)	
ADH5	Asp/Asp	59 (43.1)	24 (43.6)	35 (42.7)	0.999
	Asp/Glu	78 (56.9)	31 (56.4)	47 (57.3)	
	Asp	196	79 (71.8)	117 (71.3)	0.999
	Glu	78	31 (28.2)	47 (39.7)	
VDR	TT	3 (2.2)	3 (5.5)	0 (0.0)	0.042
	CT	85 (63.0)	25 (45.5)	60 (75.0)	
	CC	47 (34.8)	27 (49.0)	20 (25.0)	
	T	91	31 (28.2)	60 (37.5)	0.116
	C	179	79 (71.8)	100 (62.5)	

5.1. *XRCC3* MET241THR POLYMORPHISMS

Results of binary logistic regression for both groups provided evidence for a statistically significant association between *XRCC3* polymorphisms and NBUD. Specifically, *XRCC3* Met/Met (OR=3.975, $CI_{95\%}$ 1.053-14.998, p = 0.042) and *XRCC3* Thr/Met (OR=5.632, $CI_{95\%}$ 1.673-18.961, p = 0.005) are risk factors for NBUD in comparison with *XRCC3* Thr/Thr. As shown in Table 20, lower means of NBUD were found in carriers of Thr/Thr polymorphism in both exposed and controls.

Table 20 - Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD (mean ± standard error of the mean, range) by *XRCC3* Met241Thr polymorphisms and exposure, and p-value of the Kruskal-Wallis test.

Groups	**XRCC3**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**Met/Met**	13	2.92±0.93 (0-12)	2.00±1.14 (0-15)	0.38±0.18 (0-2)	1.00±0.71 (0-9)
	Thr/Met	22	5.05±0.98 (0-14)	3.91±0.84 (0-13)	1.50±0.33 (0-2)	1.05±0.38 (0-5)
	Thr/Thr	17	3.88±0.85 (0-12)	2.82±0.94 (0-13)	0.24±0.95 (0-2)	1.06±0.49 (0-8)
p-value			0.372	0.156	0.002*	0.733
Controls	**Met/Met**	20	1.15±0.46 (0-7)	0.25±0.12 (0-2)	0.2±0.09 (0-1)	0.25±0.14 (0-2)
	Thr/Met	27	0.70±0.3 (0-6)	0.15±0.12 (0-3)	0.04±0.04 (0-1)	0.11±0.82 (0-2)
	Thr/Thr	35	0.74±0.23 (0-6)	0.14±0.07 (0-2)	0.03±0.29 (0-1)	0.17±0.01 (0-2)
p-value			0.621	0.450	0.045*	0.664

The Kruskal-Wallis analysis corroborates the results from binary logist regression, confirming statistical significant differences regarding NBUDs in both groups. In the exposed group, Kruskal Wallis multiple comparisons showed that Thr/Met genotype differs significantly from the two homozygotes ($p<0.05$). Also in controls, Met/Met genotype differs significantly from the Thr/Thr genotype ($p=0.045$), presenting the latter lower means of NBUDs.

5.2. *ADH5* VAL309ILE AND ASP353GLU POLYMORPHISMS

Descriptive statistics of the genotoxicity biomarkers by the two *ADH5* polymorphisms studied are shown in Tables 21 and 22. There were no individuals with homozygous genotypes (Ile/Ile and Glu/Glu) for the variant allele of the two *ADH5* polymorphisms investigated. Results of binary logistic regression did not show statistically significant associations between *ADH5* polymorphisms and the genotoxicity biomarkers studied. However, a borderline significant association (p = 0.06) was found with NBUD, as the Asp/Asp genotype had lower means than the Asp/Glu genotype. There was a statistically significant difference between Val/Val and Val/Ile genotypes for the *ADH5* Val309Ile polymorphism in MN in lymphocytes in the exposed group (Kruskal-Wallis test, p = 0.024) with carriers of the heterozygote genotype having higher mean values than the homozygotes (Table 21).

Table 21 - Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD (mean ± standard error of the mean, range) by *ADH5* Val309Ile polymorphisms and exposure, and p-values of Mann-Whitney test.

Groups	**ADH5**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**Val/Val**	20	2.57±0.65 (0-11)	3.19±0.89 (0-14)	0.62±0.28 (0-5)	0.95±0.41 (0-6)
	Val/Ile	32	4.91±0.72 (0-14)	3.00±0.67 (0-15)	0.85±0.21 (0-5)	1.00±0.38 (0-9)
p-value			0.024*	0.957	0.274	0.713
Controls	**Val/Val**	29	0.97±0.28 (0-6)	0.17±0.07 (0-1)	0.00±0.00 (0)	0.14±0.10 (0-2)
	Val/Ile	53	0.75±0.23 (0-7)	0.17±0.08 (0-3)	0.11±0.04 (0-1)	0.19±0.08 (0-2)
p-value			0.176	0.370	0.061	0.546

There were no significant associations between the genotoxicity biomarkers and the two genotypes available for analysis (Table 22).

Table 22 - Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means (mean ± standard error of the mean, range) by *ADH5* Asp353Glu polymorphisms and exposure, and p-values of Mann-Whitney test.

Groups	**ADH5**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**Asp/Asp**	21	4.08±0.90 (0-14)	4.21±0.96 (0-15)	0.71±0.23 (0-3)	0.92±0.37 (0-6)
	Asp/Glu	31	3.97±0.65 (0-12)	2.19±0.55 (0-14)	0.81±0.24 (0-5)	1.03±0.41 (0-9)
p-value			0.700	0.217	0.740	0.983
Controls	**Asp/Asp**	35	0.86±0.23 (0-6)	0.29±0.12 (0-3)	0.06±0.04 (0-1)	0.29±0.12 (0-2)
	Asp/Glu	47	0.81±0.26 (0-7)	0.09±0.04 (0-1)	0.09±0.04 (0-1)	0.09±0.05 (0-2)
p-value			0.211	0.204	0.633	0.202

5.3. VITAMIN D RECEPTOR *BSMI* POLYMORPHISMS

The three possible *VDR BsmI* genotypes in the exposed group were regrouped in two genotypes, since there were only 3 carriers of the TT genotype. There were no significant differences between the serum concentrations of vitamin D by genotype (Mann-Whitney test, $p>0.05$).

Concerning genotoxicity biomarkers, a significant association was found in the exposed group between genotype and MN in lymphocytes (Mann Whitney test, $p=0.041$), as carriers of the CT+T genotype presented higher MN means than those with CC genotype (Table 23).

Table 23 - Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± standard error of the mean, range) by *VDR BmsI* polymorphisms and exposure, and p-value of Mann-Whitney test.

Groups	**VDR**	**N**	**MN in lymphocytes** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in buccal cells** Mean ± S.E. (range)
Exposed	**TT+CT**	25	5.11±0.83 (0-14)	0.93±0.29 (0-6)	0.37±0.12 (0-2)	0.07±0.07 (0-2)
	CC	27	2.89±0.59 (0-12)	0.48±0.25 (0-6)	0.37±0.21 (0-5)	0.26±0.13 (0-2)
p-value			0.041*	0.088	0.284	0.168
Controls	**CT**	60	0.75±0.19 (0-7)	0.23±0.11 (0-6)	0.07±0.04 (0-2)	0.65±0.25 (0-9)
	CC	20	1.15±0.44 (0-6)	0.40±0.24 (0-4)	0.05±0.05 (0-1)	0.60±0.47 (0-9)
p-value			0.350	0.649	0.988	0.616

6. MICRONUTRIENTS

All subjects had vitamin levels within the normal range (Table 24), no significant differences existed in the serum levels of vitamins in the formaldehyde exposed group compared with the controls.

Table 24 – Quantification of vitamins A, D and E in human serum in the studied population (mean ± standard error of the mean, range) and the biological normal limits.

Vitamins	Groups	N	Mean± Std. Error of Mean	Normal range limits
A (µmol/L)	**Exposed**	46	2.21±0.51 (1.11 – 3.17)	1.05 – 3.32 µmol/L
	Controls	75	1.79±0.29 (1.09 – 3.03)	
D (nmol/L)	**Exposed**	55	66.18±4.05 (20.8 – 141.1)	25 – 137 nmol/L
	Controls	81	80.56±4.99 (15.9 – 175.2)	
E (µmol/L)	**Exposed**	46	21.83±1.26 (7.12 – 39.03)	12 - 46 µmol/L
	Controls	78	20.06±0.77 (2.48 – 41.21)	

The distribution of the vitamin D serum values according to the *VDR* genotypes is presented in Table 25. No statistical significant differences were found between the two genotypes in each exposure group.

Table 25 – Distribution of the vitamin D serum values by the two *VDR* genotypes (mean ± standard error of the mean, range).

Groups	VDR	N	**Vitamin D** (nmol/L) Mean ± S.E. (range)
Exposed	**TT+CT**	25	83.80±6.21 (15.9 – 175.2)
	CC	27	72.80±8.07 (20.5 – 125.1)
p-value			0.606
Controls	**CT**	60	64.30±5.90 (25.6 - 135.3)
	CC	20	68.00±5.64 (20.8 – 141.1)
p-value			0.404

6.1. GENOTOXICITY BIOMARKERS AND VITAMINS A, D AND E

In order to examine if there was any association between the frequency of biomarkers and any of the three vitamins under study, multiple linear regression was conducted by group (exposed/controls), with the frequency of each biomarker being the dependent variable and the vitamin levels the independent set of explanatory variables. The results indicate that none of the three vitamins associate with biomarkers, to the exception of NPBs in the exposed group (Table 26). Indeed, altogether the three vitamins account for 34.1% of variability in NPB in the exposed, which is highly significant (p=0.001).

Table 26 - Results of multiple regression of biomarker frequency against the levels of a set of three vitamins (A, D, E) in the exposed and control groups. R^2 are the coefficients of determination for each regression and p indicates statistical significance of the model.

Biomarker		R^2	p
MN in lymphocytes	**Exposed**	0.106	0.191
	Controls	0.042	0.456
NPB	**Exposed**	0.341	0.001**
	Controls	0.053	0.348
NBUD	**Exposed**	0.119	0.144
	Controls	0.036	0.530
MN in Buccal cells	**Exposed**	0.042	0.614
	Controls	0.025	0.680

A closer examination of regression coefficients in the model of NPB in the exposed against vitamins, shows that vitamin A is by and large the major responsible for the statistical significance found (Table 27). Its regression coefficient (B=0.58) indicates that 1 unit increase in Vitamin A should, on average, correspond to an 0.58 unit increase in NPBs. Vitamin E is also marginally accountable for variation in NPB (p=0.17, once the effect of the other vitamins have been adjusted) but unlike Vitamin A, the correlation between Vitamin E and NPBs is negative (regression coefficient, B=-0.023). These results were confirmed by bivariate Spearman correlations (Table 28).

Table 27 - Decomposition of the model of multiple regression of NPB in the exposed. The B's are the regression coefficients and p indicates its significance.

NPB in the exposed		
Explanatory variables	**B**	**p**
Vitamin A	0.580	<0.001**
Vitamin D	-0.001	0.848
Vitamin E	-0.023	0.170

Table 28 – Spearman correlations between genotoxicity biomarkers (MN in lymphocytes and buccal cells, NPB, and NBUD) and vitamins A, D and E in the exposed (left) and the control group (right); Significant correlations are signaled by ** and *, respectively, p<0.01 and p<0.05.

a) Exposed

Biomarkers	**VitA**	**VitD**	**VitE**
MN lymphocytes	0.266	0.124	0.029
NPB	0.557**	-0.048	0.039
NBUD	-0.076	-0.106	-0.297*
MN BC	0.121	0.018	0.191

b) Controls

Biomarkers	**VitA**	**VitD**	**VitE**
MN lymphocytes	-0.110	0.044	0.035
NPB	-0.082	-0.107	-0.170
NBUD	-0.157	0.069	-0.109
MN BC	-0.042	0.008	-0.109

CHAPTER 2 – CYTOSTATICS DRUGS OCCUPATIONAL SETTING

1. SAMPLES

Two samples were formed - the group of those occupationally exposed to cytostatics and the non-exposed group (controls). Sample characteristics such as gender distribution, age, years of exposure, tobacco and alcohol consumption for the control and exposed groups are shown in Table 29.

Table 29 - Characteristics of the samples regarding gender, age, years of exposure, tobacco and alcohol consumption.

	Control group	**Exposed group**
Number of subjects	46	46
Gender		
Females	34 (73.9%)	40 (87.0%)
Males	12 (26.1%)	6 (13.0%)
Age		
(mean ± standard error of mean, in years)	39.26±1.42	33.85±1.21
Range	20-61	24-58
Years of exposure		
(mean ± standard error of mean, in years)	n.a.	6.62±0.94
Range		0.17 - 30
Tobacco consumption		
Non-smokers	34 (77.3%)	42 (91.3%)
Smokers	10 (22.7%)	4 (8.7%)
Alcohol consumption		
Non-drinkers	32 (72.7%)	34 (73.9%)
Drinkers	12 (27.3%)	12 (26.1%)

n.a. – non-applicable

2. CYTOSTATICS EXPOSURE ASSESSMENT

The analytic data from exposure assessment to cystostatic drugs, namely cyclophosphamide (CP), 5-fluorouracil (5-FU), and paclitaxel (PTX) is presented in Table 30.

Table 30 – Number of samples regarding surface contamination with cyclophosphamide (CP), 5-fluorouracil (5-FU), paclitaxel (PTX), and respective limits of detection (LOD) and quantification (LOQ).

Hospitals	CP	5-FU	PTX	Samples with contamination	Contamination with more than 1 drug
A	1/67 (1.5%)	17/67 (25.4%)	17/67 (25.4%)	21/67 (31.3%)	13/67 (19.4%)
B	14/260 (5.4%)	18/260 (6.9%)	54/260 (27.3%)	100/260 (38.5%)	15/260 (5.8%)
Totals	**15/327 (4.6%)**	**35/327 (10.7%)**	**71/327 (21.7%)**	**121/327 (37%)**	**28/327 (8.6%)**
LOD (μg/cm^2)	0.10	3.30	0.167		
LOQ (μg/cm^2)	0.30	10.00	0.50		

From the total of 327 analysed samples of both hospitals, 121 (37%) were positive. A sample was regarded as positive, when at least one of the three surrogate markers was detected. Considering hospital A, from the 67 samples, 21 (31.3%) were positive and 13 of them (19.4%) presented contamination from more than one drug. In hospital B, 100 (38.5%) out of 260 samples were positive and 15 (5.8%) showed contamination from more than one drug.

3. GENOTOXICITY ASSESSMENT

3.1. CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY

For all genotoxicity biomarkers under study, workers occupationally exposed to cytostatics had significantly higher means in comparison with controls (Table 31).

Significant differences (Mann-Whitney test, $p < 0.05$) were observed between subjects exposed and non-exposed to cytostatics, namely in mean MN in binucleated lymphocytes (respectively, 9.83±1.28 *vs* 5.09±0.89), NPB (0.65±0.14 *vs* 0.11±0.05), and NBUD (2.43±0.37 *vs* 1.37±0.32), MN in mononuclear lymphocytes (1.35±0.32 *vs* 0.41±0.11), multinuclear lymphocytes (4.09±0.78 *vs* 1.46±0.22). The odds ratios of binary logistic regression indicate an significant increased risk for the presence of biomarkers in those exposed to cytostatics,

compared to non-exposed (Table 31), and they were significant ($p<0.05$) for MN in binucleated cells, NPB, and NBUDs. The means MN in mono and multinucleated lymphocytes are increased in exposed comparing with controls, however did not reach statistical significance ($p=0.139$; $p=0.819$, respectively)

Table 31 – Descriptive statistics of MN, NPB and NBUD in the two groups (mean ± standard error of the mean, range), p-value of the Mann-Whitney test, and results of binary logistic regression concerning the association between exposure and genotoxicity biomarkers, as evaluated by the odds ratio (OR).

	MN in BN Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in MONO** Mean ± S.E. (range)	**MN in MULTI** Mean ± S.E. (range)
Exposed	9.83±1.28 (1-58)	0.65±0.14 (0-3)	2.43±0.37 (0-11)	1.35±0.32 (0-9)	4.09±0.78 (0-21)
Controls	5.09±0.89 (0-34)	0.11±0.05 (0-1)	1.37±0.32 (0-13)	0.41±0.11 (0-3)	1.46±0.22 (0-6)
p-value[1]	<0.001	0.001	0.006	0.027	0.044
OR	6.667	5.770	2.893	1.894	1.111
OR CI 95%	2.369-18.76	1.924-17.307	1.135-7.373	0.813-4.412	0.452-2.726
p-value[2]	<0.001	0.002	0.026	0.139	0.819

[1] Mann-Whitney test
[2] Binary logistic regression

In what concerns the influence of the duration of exposure to cytostatics, no association could be found between years of exposure and the presence of any of the biomarkers measured by CBMN assay (regression analysis slope, $p>0.05$).

3.2. COMET ASSAY

The mean of DNA damage (% DNA Tail) and oxidative DNA damage (FPG) in exposed and non-exposed samples is shown in Table 32. No statiscally significant differences (Mann-Whitney test, $p>0.05$) were found between subjects with and without exposure.

Table 32 – Descriptive statistics of % DNA in tail and FPG in the studied population (mean ± standard error of the mean, and range), and p-value of the Mann-Whitney test.

Groups	Statistics	% DNA in tail	FPG
Exposed	**Mean**	15.18	5.32
	Std. Error	1.40	0.54
	Range	1.79 – 44.5	0.19 - 13.91
Controls	**Mean**	12.41	4.59
	Std. Error	1.24	0.59
	Range	2.48 - 30.43	0.02 - 14.46
p-value		0.136	0.229

3.3. CORRELATION BETWEEN THE GENOTOXICITY ASSAYS

There were positive significant correlations between endpoints evaluated by the same technique.

Regarding CBMN assay results, there was a positive correlation between MN in binucleated lymphocytes and MN in mononuclear lymphocytes ($r=0.435$, $p=0.002$), and multinuclear lymphocytes ($r =0.670$, $p<0.001$) the same between NPB and NBUDs ($r=0.362$, $p=0.013$). As for the comet assay, % DNA in the tail and FPG to measure oxidative damage, were correlated ($r = 0.325$, $p=0.002$). However, correlations across the two assays – CBMN and comet assays – were not significant.

4. DEMOGRAPHIC AND LIFESTYLE HABITS

Age, gender, tobacco, and alcohol habits are possible confounding variables that can affect genotoxicity measurement and whose effect can be investigated by multiple regression analysis within the exposed and the control groups. The analysis shows that exposure to cytostatics was the only variable significantly affecting the DNA damage measured by CBMN assay, to the exception to MN in mononuclear and multinuclear lymphocytes. Gender, tobacco and alcohol consumption did not account for significant results ($p>0.05$).

As Table 33 shows, genotoxicity biomarkers did not differ significantly between men and women within the exposed and the controls (Mann-Whitney test, $p> 0.05$) for the biomarkers measured by CBMN assay.

Table 33 –Descriptive statistics of MN, NPB, and NBUD by gender and exposition (mean ± standard error of the mean, range). There were no significant differences between means of the two genders either within the exposed or the controls.

Groups	**Gender**	**N**	**MN in BN** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in MONO** Mean ± S.E. (range)	**MN in MULTI** Mean ± S.E. (range)
Exposed	**Females**	40	9.75±1.44 (1-58)	0.73±0.16 (0-3)	2.48±0.41 (0-11)	1.40±0.35 (0-9)	3.90±0.87 (0-21)
	Males	6	10.33±1.94 (6-18)	0.17±0.17 (0-1)	2.17±0.79 (0-5)	1.00±0.68 (0-4)	5.33±1.65 (0-12)
Controls	**Females**	34	5.56±1.11 (0-34)	0.12±0.06 (0-1)	1.38±0.41 (0-13)	0.38±0.11 (0-3)	1.56±0.24 (0-6)
	Males	12	3.75±1.27 (0-15)	0.08±0.08 (0-1)	1.33±0.43 (0-5)	0.50±0.26 (0-3)	1.17±0.47 (0-6)

In what concerns DNA damage and DNA oxidative damage measured by comet assay, the mean of these biomarkers also did not differ significantly between genders within the exposed and the controls, to the exception of % DNA in tail in the control group (Table 34).

Table 34 –Descriptive statistics of % DNA in tail and FPG means by gender and exposure (mean ± standard error of the mean, range).

Groups	**Gender**	**N**	**% DNA in tail** Mean ± S.E. (range)	**FPG** Mean ± S.E. (range)
Exposed	**Females**	40	14.84±1.38 (1.79-31.64)	5.31±0.61 (0.19-13.91)
	Males	6	17.43±5.96 (4.61-44.50)	5.39±1.09 (2.38-9.65)
Controls	**Females**	34	14.15±1.49 (2.48-30.43)	4.57±0.74 (0.02-14.46)
	Males	12	7.46±1.54 (2.79-18.49)	4.65±0.89 (0.10-9.61)

In order to examine the effect of age on biomarkers, individuals were split into exposed and non-exposed and, within each group, the association between age and biomarker frequency was studied by simple regression analysis, yielding the results in Table 35.

There is a significant association between MN in binucleated lymphocytes and age in the exposed group, with the number of MN tending to increase with age (R^2= 0.120, p=0.018) as shown in Figure 10a, and in the control group (R^2= 0.186, p=0.003) as shown in Figure 10b. Also, the MN in mononuclear lymphocytes were significantly associated with age in the exposed (R^2= 0.087, p=0.047) and in the controls (R^2= 0.164, p=0.005), as shown in Figures 11a and 11b, respectively.

Table 35 - Results of simple regression analysis of age on biomarkers by group (exposed/controls). R^2 is the coefficient of determination; A and B are, respectively, the ordinate and the slope in the regression line, and p is the likelihood of B in case of no association between age and the biomarker; ** signals a significant p-value.

Biomarker		R^2	A	B	p-value
MN in BN lymphocytes	**Exposed**	0.120	-2.586	0.367	0.018**
	Controls	0.186	-5.451	0.268	0.003**
NPB	**Exposed**	0.005	0.916	-0.008	0.657
	Controls	0.003	0.177	-0.002	0.724
NBUD	**Exposed**	0.032	4.290	-0.055	0.233
	Controls	0.016	2.487	-0.028	0.402
MN in MONO lymphocytes	**Exposed**	0.087	-1.277	0.078	0.047**
	Controls	0.164	-0.767	0.030	0.005**
MN in MULTI lymphocytes	**Exposed**	0.001	4.845	-0.022	0.819
	Controls	0.055	0.048	0.036	0.115
% DNA in tail	**Exposed**	0.029	21.863	-0.197	0.259
	Controls	0.022	7.375	0.128	0.330
FPG	**Exposed**	0.023	7.646	-0.069	0.312
	Controls	0.001	4.226	0.009	0.882

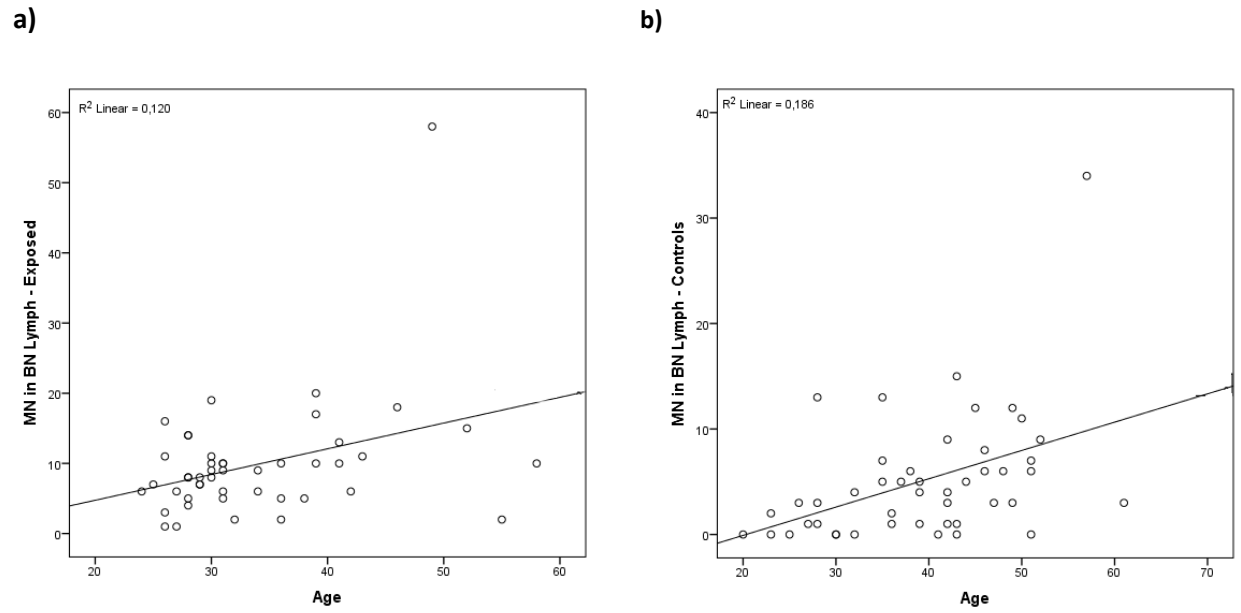


Figure 10 - Scatter plots of the number of MN in binucleated lymphocytes against age in the exposed (a) and control (b) groups. The slope of the regression line (dashed) in both groups is statistically significant.

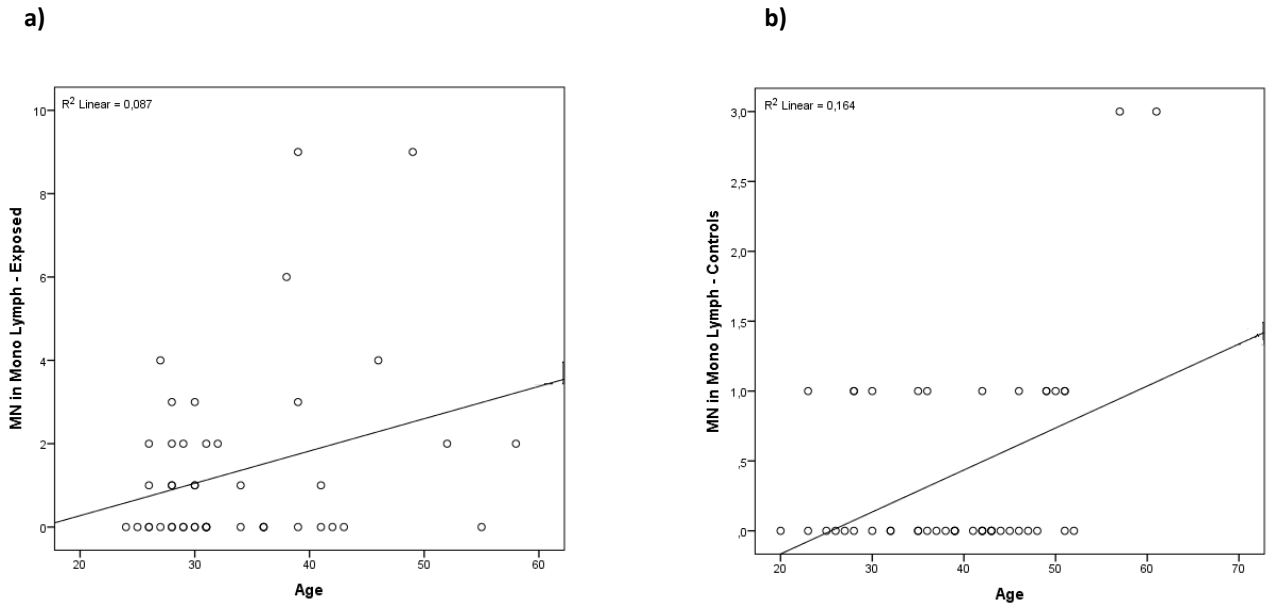


Figure 11 - Scatter plots of the number of MN in mononuclear lymphocytes against age in the exposed (a) and control (b) groups. The slope of the regression line (dashed) in both groups is statistically significant.

Descriptive statistics of every biomarker were stratified by three age groups (20-30, 31-40, and ≥ 41 years old) in the exposed and control groups, allowing for a qualitative view of the same relationships (Tables 36 and 37).

Table 36 –Descriptive statistics of MN, NPB, NBUD, MN in mono and multinuclear lymphocytes by age group in exposed and controls (mean ± standard error of the mean, range).

Groups	**Age**	**N**	**MN in BN** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in MONO** Mean ± S.E. (range)	**MN in MULTI** Mean ± S.E. (range)
Exposed	**20-30**	22	8.32±0.97 (1-19)	0.68±0.21 (0-3)	2.68±0.57 (0-13)	0.95±0.26 (0-4)	3.23±0.68 (0-10)
	31-40	15	8.40±1.29 (2-20)	0.67±0.27 (0-3)	2.73±0.70 (0-2)	1.53±0.69 (0-9)	5.40±1.90 (0-21)
	≥ 41	9	15.89±5.49 (2-58)	0.56±0.24 (0-2)	1.33±0.44 (0-5)	2.00±0.99 (0-9)	4.00±1.84 (0-17)
Controls	**20-30**	11	2.09±1.15 (0-13)	0.18±0.12 (0-1)	1.91±1.14 (0-13)	0.36±0.15 (0-1)	0.73±0.24 (0-2)
	31-40	12	4.42±1.00 (0-13)	0.17±0.11 (0-1)	1.42±0.47 (0-5)	0.17±0.11 (0-1)	1.83±0.39 (0-4)
	≥ 41	23	6.87±1.52 (0-34)	0.04±0.04 (0-1)	1.09±0.27 (0-5)	0.57±0.19 (0-3)	1.61±0.35 (0-6)

Table 37 –Descriptive statistics of % DNA in tail and FPG by age group in the exposed and controls (mean ± standard error of the mean, range).

Groups	**Age**	**N**	**% DNA in tail** Mean ± S.E. (range)	**FPG** Mean ± S.E. (range)
Exposed	**20-30**	22	18.76±2.16 (2.89-44.50)	5.90±0.73 (1.03-13.91)
	31-40	15	11.36±1.73 (1.79-24.04)	5.21±1.09 (0.19-12.90)
	≥ 41	9	12.79±3.17 (3.33-30.74)	4.09±1.15 (0.27-9.65)
Controls	**20-30**	11	12.23±2.59 (3.78-23.53)	5.24±1.37 (0.02-12.01)
	31-40	12	9.88±2.73 (2.48-30.43)	3.48±0.89 (0.32-11.61)
	≥ 41	23	13.81±1.63 (2.79-28.19)	4.86±0.87 (0.09-14.46)

In order to examine if gender adds a significant contribution to explain variability in genotoxicity biomarkers, regression analysis was repeated but now adding gender as an independent variable and examining whether it would improve upon the values of R^2 already presented for age (Table 38).

Table 38 - Results of multiple regression analysis of age and gender on biomarkers by group (exposed/controls). R^2, new R^2, and ΔR^2 are, respectively, the coefficients of determination of the model with only age, age and gender, and the difference between them. The significance of the addition of gender is shown by the value of p, ** signals a significant p-value.

		Age	Age and gender		
Biomarker	**Groups**	**R^2**	**New R^2**	**ΔR^2**	**p**
MN in BN lymphocytes	**Exposed**	0.120	0.122	0.002	0.061
	Controls	0.186	0.193	0.007	0.010**
NPB	**Exposed**	0.005	0.047	0.042	0.358
	Controls	0.003	0.006	0.003	0.880
NBUD	**Exposed**	0.032	0.035	0.003	0.465
	Controls	0.016	0.017	0.001	0.697
MN in MONO lymphocytes	**Exposed**	0.087	0.089	0.002	0.135
	Controls	0.164	0.179	0.015	0.014**
MN in MULTI lymphocytes	**Exposed**	0.001	0.009	0.008	0.817
	Controls	0.055	0.063	0.008	0.244
% DNA in Tail	**Exposed**	0.029	0.036	0.007	0.459
	Controls	0.022	0.135	0.113	0.044*
FPG	**Exposed**	0.023	0.023	0.000	0.604
	Controls	0.001	0.001	0.000	0.986

A closer examination of regression coefficients in the model of MN in binucleated lymphocytes in controls against age and gender, shows that age is the major responsible for the statistical significance found (Table 39). Its regression coefficient (B=0.262) indicates that 1 year increase in age should, on average, correspond to an 0.262 unit increase in MN in binucleated lymphocytes. The regression coefficient of gender (B=1.096) was not statistically significant. Figure 12a and Figure 12b show the distribution of the MN in binucleated lymphocytes by age and gender, for exposed and controls, respectively.

Table 39 - Regression coefficients (B) of age and gender in the model of multiple regression of MN in binucleated lymphocytes in the controls; p indicates their significance. The full model accounts for 19,3% of variability in the number of MN.

MN in BN lymphocytes in controls		
Explanatory variables	**B**	**p**
Age	0.262	0.004**
Gender	1.096	0.560

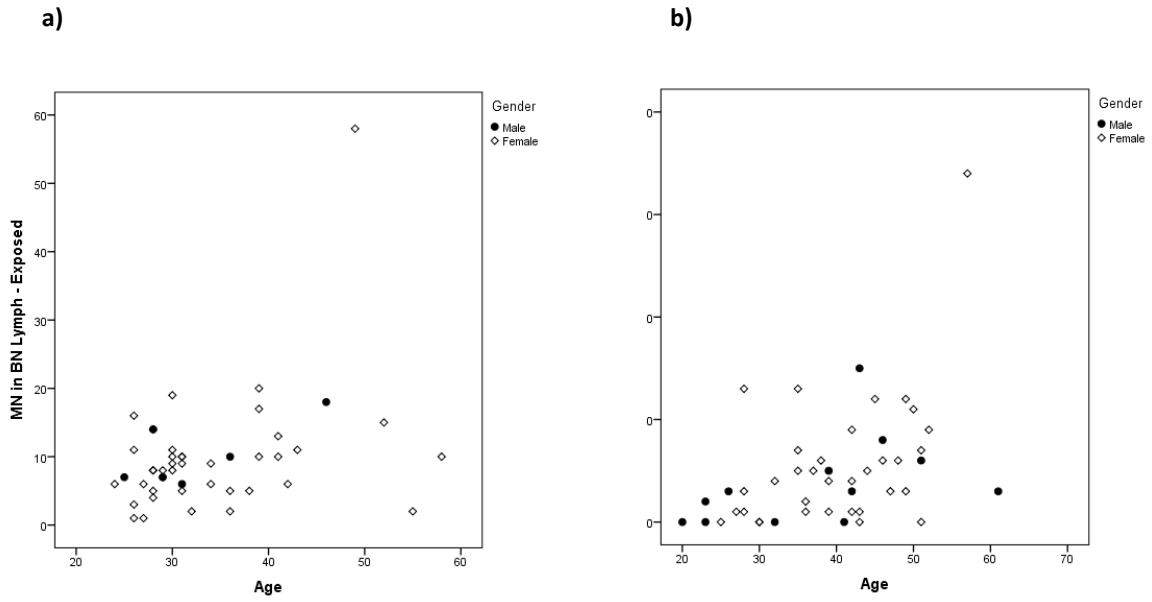


Figure 12 - Scatter plots of the number of MN in binucleated lymphocytes against age and gender in the exposed (a) and control (b) groups.

Examination of regression coefficients in the model of MN in mononuclear lymphocytes in the controls against age and gender shows that age is again the major responsible for the statistical significance found (Table 40). Its regression coefficient (B=0.031) indicates that 1 year increase should, on average, correspond to an 0.031 unit increase in MN in mononuclear lymphocytes. Figure 13a and Figure 13b show the distribution of the MN in mononuclear lymphocytes by age and gender, for exposed and controls, respectively.

Table 40 - Regression coefficients (B) of age and gender in the model of multiple regression of MN in mononuclear lymphocytes in the controls. The B's are the regression coefficients and p indicates their significance. The full model accounts for 17,9% of variability in the number of MN.

MN in MONO lymphocytes in controls		
Explanatory variables	**B**	**p**
Age	0.031	0.004**
Gender	-0.203	0.373

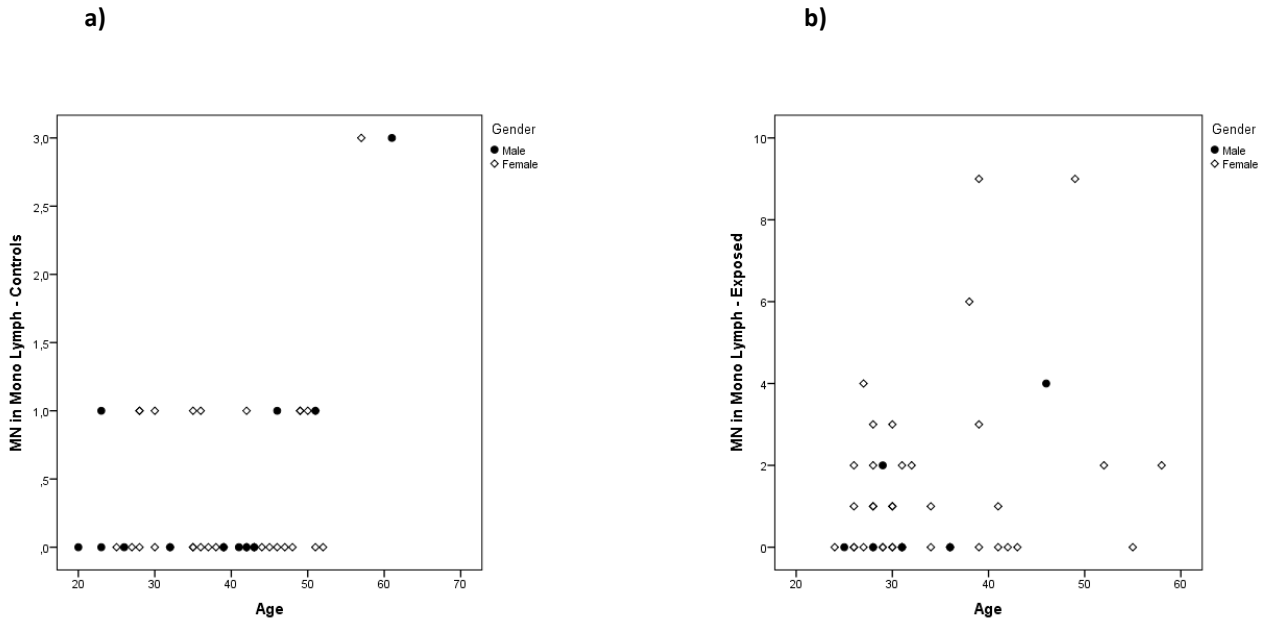


Figure 13 - Scatter plots of the number of MN in mononuclear lymphocytes against age and gender in the exposed (a) and control (b) groups.

The examination of regression coefficients in the model of % DNA in tail in the controls against age and gender shows that gender is the major responsible for the statistical significance found (Table 41). Its regression coefficient (B=6.439) indicates that being woman increases, on average, 6.4 the % of DNA in tail in the control group. Figure 14a and Figure 14b show the distribution of the % DNA in tail by age and gender, for exposed and controls, respectively.

Table 41 - Decomposition of the model of multiple regression of % DNA in tail the controls. The B's are the regression coefficients and p indicates its significance.

% DNA in Tail in controls		
Explanatory variables	**B**	**p**
Age	0.091	0.469
Gender	6.439	0.022*

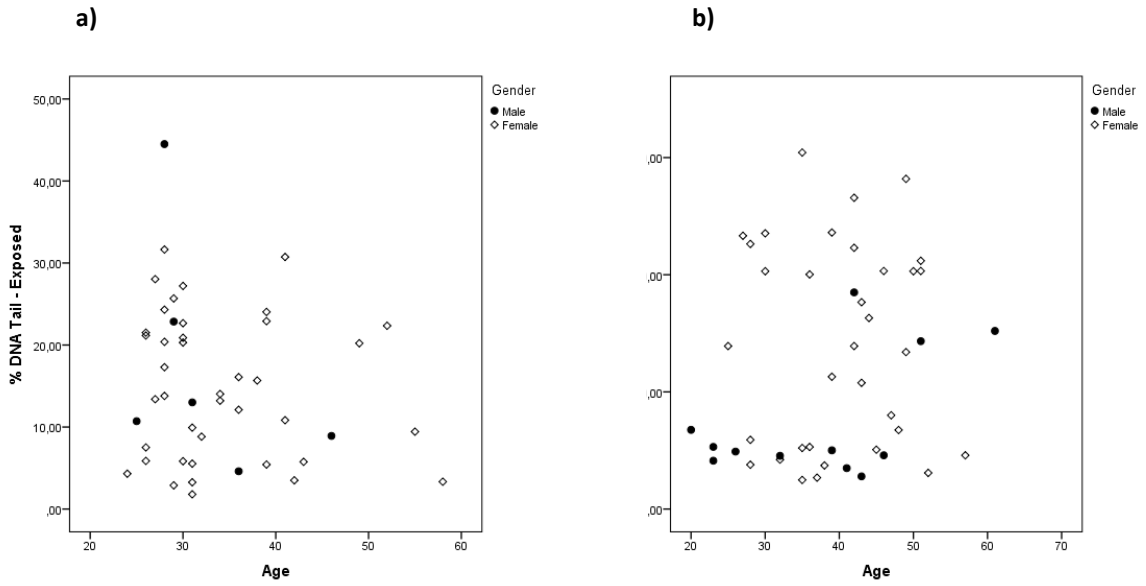


Figure 14 - Scatter plots of % DNA in tail against age and gender in the exposed (a) and control (b) groups.

Alcohol consumption and tobacco habits did not show statistically significant associations within genotoxicity biomarkers measured by CBMN assay for both the exposed and non exposed groups. In what concerns the comet assay, it was observed a positive association between alcohol consumption and % DNA in tail ($r = 0.266$, $p=0.037$) in the exposed group, indicating that alcohol consumption is associated with higher DNA damage.

5. INDIVIDUAL SUSCEPTIBILITY

5.1. *OGG1* SER326CYS POLYMORPHISMS

The frequencies of genotypes and alleles of the *OGG1* Ser326Cys polymorphisms studied in the two groups are shown in Table 42. No significant differences were observed in genotypic and allelic frequencies in *OGG1* polymorphisms under study between exposed and controls (Fisher's exact test, $p>0.05$).

Table 42 – Frequency of genotypes and alleles of *OGG1 Ser326Cys* polymorphisms in the study sample, p-value of Fisher's exact test.

Gene	Genotypes	All (%)	Exposed (%)	Controls (%)	p-value
OGG1	Cys/Cys	9 (9.8)	7 (15.2)	2 (4.4)	0.446
	Ser/Cys	32 (34.8)	14 (30.4)	18 (39.1)	
	Ser/Ser	51 (55.4)	25 (54.4)	26 (56.5)	
	Cys	50	31 (30.4)	22 (23.9)	0.409
	Ser	134	79 (69.6)	70 (76.1)	

The descriptive statistics concerning the relationship between genotoxicity biomarkers provided by CBMN and comet assay and *OGG1* polymorphisms studied are shown in Tables 43 and 44. For both, there was no consistent trend regarding the variation of biomarkers with *OGG1* polymorphisms.

In what concerns the genotoxicity biomarkers measured by CBMN, the Kruskal-Wallis test did not reject the null hypothesis of equal means between the *OGG1* polymorphisms.

Table 43 – Descriptive statistics of MN, NPB, and NBUD (mean ± standard error of the mean, and range) by *OGG1* Ser326Cys polymorphisms and exposure, p-value of the Kruskal-Wallis test.

Groups	**OGG1**	**N**	**MN in BN** Mean ± S.E. (range)	**NPB** Mean ± S.E. (range)	**NBUD** Mean ± S.E. (range)	**MN in MONO** Mean ± S.E. (range)	**MN in MULTI** Mean ± S.E. (range)
Exposed	**Cys/Cys**	7	7.14±1.87 (2 – 15)	0.71±0.47 (0 – 3)	0.86±0.26 (0 – 2)	1.00±0.49 (0 – 3)	2.57±1.41 (0 – 8)
	Ser/Cys	14	12.86±3.75 (1 – 58)	0.71±0.24 (0 – 3)	3.14±0.94 (0 – 11)	2.50±0.86 (0 – 9)	4.79±1.51 (0 – 18)
	Ser/Ser	25	8.88±0.87 (1 – 18)	0.60±0.18 (0 – 3)	2.48±0.39 (0 – 8)	0.80±0.25 (0 – 4)	4.12±1.11 (0 – 21)
p-value			0.535	0.828	0.100	0.185	0.530
Controls	**Cys/Cys**	2	1.50±1.50 (0 – 3)	0.00±0.00 (0 – 0)	1.50±0.50 (1 - 2)	0.50±0.50 (0 – 1)	1.00±0.00 (1 – 1)
	Ser/Cys	18	4.72±1.00 (0 – 13)	0.11±0.08 (0 – 1)	0.89±0.31 (0 – 5)	0.28±0.11 (0 – 1)	1.39±0.28 (0 – 4)
	Ser/Ser	26	5.62±1.40 (0 – 34)	0.12±0.06 (0 – 1)	1.69±0.52 (0 – 13)	0.50±0.17 (0 – 13)	1.54±0.33 (0 – 6)
p-value			0.506	0.882	0.313	0.744	0.939

Also in the results provided by comet assay, no consistent trend is observed (Table 44). The Kruskal Wallis test did not reject the null hypothesis of equality among *OGG1* polymorphisms in regard to the means of the two comet assay parameters.

Table 44 - Descriptive statistics of % DNA Tail and FPG means in the studied population (mean ± standard error of the mean, and range) by *OGG1* Ser326Cys polymorphisms and exposure, p-value of Kruskal-Wallis test.

Groups	**OGG1**	**N**	**% DNA Tail** Mean ± S.E. (range)	**FPG** Mean ± S.E. (range)
Exposed	**Cys/Cys**	7	13.11±2.80 (1.79 – 23.53)	5.03±1.48 (0.27 – 12.0)
	Ser/Cys	14	13.23±1.42 (2.48 – 28.19)	4.97±0.72 (0.10 – 14.46)
	Ser/Ser	25	14.26±1.38 (2.68 – 44.50)	4.94±0.51 (0.02 – 12.90)
p-value			0.777	0.647
Controls	**Cys/Cys**	2	14.22±9.31 (4.91 – 23.53)	7.39±4.61 (2.78 – 12.00)
	Ser/Cys	18	12.04±1.86 (2.48 – 28.19)	4.69±1.00 (0.10 – 14.46)
	Ser/Ser	26	12.52±1.74 (2.68 – 30.43)	4.30±0.73 (0.02 – 12.69)
p-value			0.906	0.682

6. MICRONUTRIENTS

Most study subjects had vitamin levels within the normal range (Table 45), and no significant differences existed between the serum levels of vitamins in the group exposed to cytostatics compared to controls (Mann-Whitney test, $p>0.05$).

Table 45 – Quantification of vitamins A and E in human serum in the studied population (mean ± standard error of the mean, range) and the biological lower limits (p-value of Mann-Whitney test).

Vitamins	Groups	Mean ± Std. Deviation (range)	Normal range limits
A	Controls	2.36±0.82 (1.37 – 4.08)	1.05 – 3.32 µmol/L
	Exposed	3.11±0.31 (1.06 – 11.52)	
p-value		0.204	
E	Controls	26.46±1.10 (12.18 – 43.92)	12.00 – 46.00 µmol/L
	Exposed	26.58±1.10 (13.83 – 51.27)	
p-value		0.793	

6.1. GENOTOXICITY BIOMARKERS AND VITAMINS A AND E IN SERUM

In order to examine if there was any association between the frequency of biomarkers and the serum vitamins A and E, multiple linear regression was conducted by group (exposed/controls), with the frequency of each biomarker being the dependent variable and the vitamin levels the independent set of explanatory variables. The results indicate that vitamins influence the number of NPB in the exposed, as well as NBUDs and FPG biomarkers in the control group (Table 46).

Table 46 - Results of multiple regression of biomarker frequency against the levels of a set of vitamins A and E in the exposed and control groups. R^2 are the coefficients of determination for each regression and p indicates statistical significance of the model.

Biomarker		R^2	p
MN in BN lymphocytes	**Exposed**	0.001	0.978
	Controls	0.001	0.987
NPB	**Exposed**	0.195	0.010**
	Controls	0.031	0.505
NBUD	**Exposed**	0.081	0.169
	Controls	0.161	0.023**
MN in MONO lymphocytes	**Exposed**	0.006	0.879
	Controls	0.013	0.762
MN in MULTI lymphocytes	**Exposed**	0.049	0.347
	Controls	0.007	0.861
% DNA in tail	**Exposed**	0.001	0.988
	Controls	0.075	0.187
FPG	**Exposed**	0.055	0.305
	Controls	0.250	0.002**

Table 47 shows statistically significant regression coefficients of the regression of biomarkers against the levels of the vitamins. In the model of NPB in the exposed against vitamins, both vitamins A and E significantly account for the variability in number of NPBs. Vitamin A coefficient (B=0.173) indicates that 1 unit increase in vitamin A should, on average, correspond to an 0.173 unit increase in NPBs in the exposed group. Vitamin E also accounts for variation in NPB (p=0.003), but unlike vitamin A, the correlation between vitamin E and NPBs is negative (B= -0.066). In what concerns NBUD in the control group, the vitamin E coefficient (B=0.096) indicates that 1 unit increase in vitamin E should, on average, correspond to an 0.096 unit increase in NBUDs in the control group. Finally, in the control group, the vitamin A coefficient (B=3.571) indicates that 1 unit increase in vitamin A should, on average, correspond to an 3.571 unit increase of DNA oxidative damage (FPG) in the control group.

Table 47 - Regression coefficients in the models of multiple regression of NPB in the exposed, and NBUDs and FPG in controls. The B's are the regression coefficients and p indicates their significance.

Biomarkers	*Explanatory variables*	B	p
NPB in Exposed	VitA	0.173	0.028**
	VitE	-0.066	0.003**
NBUD in Controls	VitA	0.837	0.131
	VitE	0.096	0.023**
FPG in Controls	VitA	3.571	>0.001**
	VitE	-0.022	0.755

In the exposed group, there is a significant negative correlation between vitamin E and NPB (Spearman's correlation r =-0.311, $p<0.05$), meaning that higher serum vitamin E levels are associated with lower mean NPB (Table 48A).

Vitamin E and NBUD are significantly correlated (r= 0.339, $p<0.05$) and so are vitamin A and DNA oxidative damage (r= 0.498, $p<0.01$) in the control group (Table 48B), meaning that higher levels of these vitamins are correlated with higher means of these genotoxicity biomarkers.

Table 48 – Spearman correlations between genotoxicity biomarkers MN, NPB, and NBUD and vitamins A and E in (A) exposed group and (B) the control group.

A – Exposed

	Vit A	Vit E
MN BN	-0.032	-0.013
NPB	0.096	-0.311*
NBUD	0.263	0.052
MN MONO	0.062	-0.006
MN MULTI	-0.116	-0.222
% DNA TAIL	-0.023	-0.017
FPG	0.165	0.229

B – Controls

	Vit A	Vit E
MN BN	-0.021	0.010
NPB	0.013	0.177
NBUD	0.229	0.339*
MN MONO	-0.080	0.076
MN MULTI	-0.083	-0.012
% DNA TAIL	0.228	0.161
FPG	0.498**	-0.020

** Spearman Correlation $p<0.01$

* Spearman Correlation $p<0.05$

6.2. GENOTOXICITY BIOMARKERS AND MICRONUTRIENTS MEASURED BY FFQ

The following nutritional items were selected from the Food Frequency Questionnaire (FFQ) for analysis: calories, retinol, vitamin B12, folate, vitamins D and E, iron, and selenium. The quantification of the dietary parameters for these items is shown in Table 49.

Table 49 –Dietary parameters (calories, retinol, vitamin B12, folate, vitamins D and E, iron, and selenium) by FFQ (mean intake per day ± standard deviation) and respective dietary reference intakes.

Parameters	Groups	Mean± Std. Deviation (daily nutrient intake)	Dietary References Intakes (Food and Nutrition Board, Institute of Medicine, National Academies) d= day
Calories	**Exposed**	2652.55±188.71	Variable by age and gender (kcal)
	Controls	2527.40±123.07	
Retinol	**Exposed**	996.82±157.24	Females: 500 µg/d Males: 625 µg/d
	Controls	776.51±70.10	
Vit B12	**Exposed**	13.86±1.54	2.0 µg/d
	Controls	12.31±0.78	
Folate	**Exposed**	461.25±42.22	320 µg/d
	Controls	401.21±26.21	
Vit D	**Exposed**	5.08±0.46	10 µg/d
	Controls	4.67±0.35	
Vit E	**Exposed**	12.82±1.14	12 mg/d
	Controls	11.80±0.67	
Iron	**Exposed**	20.58±1.65	Females: 8.1 mg/d Males: 6 mg/d
	Controls	18.82±1.07	
Selenium	**Exposed**	131.51±9.34	45 µg/d
	Controls	138.67±8.58	

In order to investigate the association between genotoxicity biomarkers and nutritional items, multiple linear regression was conducted by group (exposed/controls), with the frequency of

each biomarker being the dependent variable and the nutritional items ~~as~~ the independent set of explanatory variables. The results indicate that a significant percentage of the variability in % DNA in tail in the exposed group, and in FPG biomarker in the controls can be accounted by nutrition (Table 50).

Table 50 - Results of multiple regression of biomarker frequency against a set of itens measured by the FFQ (calories, retinol, vitamin B12, folate, vitamins D and E, folate, iron, and selenium) in the exposed and control groups. R^2 are the coefficients of determination for each regression and p indicates the statistical significance of the model.

Biomarker		R^2	p
MN in BN lymphocytes	**Exposed**	0.122	0.736
	Controls	0.112	0.787
NPB	**Exposed**	0.097	0.849
	Controls	0.095	0.860
NBUD	**Exposed**	0.081	0.907
	Controls	0.129	0.702
MN in MONO lymphocytes	**Exposed**	0.147	0.611
	Controls	0.213	0.296
MN in MULTI lymphocytes	**Exposed**	0.170	0.490
	Controls	0.077	0.921
% DNA in tail	**Exposed**	0.384	0.013**
	Controls	0.272	0.124
FPG	**Exposed**	0.142	0.633
	Controls	0.364	0.021**

The model of % DNA in tail in the exposed against nutritional items showed that calories, folate, vitamin E and iron are responsible for the statistical significance found (Table 51). Calories (B=-0.011) and folate (B=-0.078) decrease DNA damage (% DNA in tail) indicates that 1 unit increase of calories and folate intake should, on average, correspond to an 0.011 unit and 0.078 decrease, respectively, in % DNA in tail in the exposed group. Vitamin E and iron are also accountable for variation in % DNA in tail (p=0.002 and p<0.001, respectively), but unlike calories and folate, the correlation between vitamin E and iron and % DNA in tail is positive. Vitamin E coefficient (B=1.912) indicates that 1 unit increase in Vitamin E should, on average, correspond to an 1.912 unit increase of % DNA in tail in the exposed group. Same positive correlation for iron (B=2.345), meaning that 1 unit increase of iron should, on average, correspond to an 2.345 unit increase of % DNA in tail in the exposed group.

Table 51 – Regression coefficients (B) and their significance (p) in the model of multiple regression of % DNA in tail in the exposed group.

Biomarkers	***Explanatory variables***	**B**	**p**
% DNA in Tail in Exposed	Calories	-0.011	0.013
	Folate	-0.078	<0.001
	Vitamin E	1.912	0.002
	Iron	2.345	<0.001

Regarding DNA oxidative damage (FPG) in the control group, the model showed that calories, retinol, and vitamin B12 are responsible for the statistical significance found (Table 52). Calories (B=-0.006) and vitamin B12 (B=-0.589) decrease DNA oxidative damage (FPG) as 1 unit increase of calories and vitamin B12 intake should, on average, correspond to an 0.006 unit and 0.589 decrease, respectively, in FPG in the control group.

Table 52 - Regression coefficients (B) and their significance (p) in the model of multiple regression of FPG in the control group.

Biomarkers	***Explanatory variables***	**B**	**p**
FPG in Controls	Calories	-0.006	0.002
	Retinol	0.004	0.020
	Vitamin B12	-0.589	0.006

Bivariate Spearman correlations do not show strong associations inside both the exposed and non exposed group, or between groups. In the non exposed, the majority of correlations between biomarkers and nutritional items were negative but with no statistical significance. A significant negative correlation ($p<0.05$) between calories intake and DNA damage (% DNA in tail) was found, suggesting that greater intake of calories decrease DNA damage. No statistical significant correlations were found in the exposed group for any genotoxicity biomarkers under study (Table 53A). In controls, higher vitamin E values associate with higher mean MN present in mononucleated lymphocytes (Table 53B).

Table 53 – Spearman correlations between MN, NPB, and NBUD and dietary parameters (calories, retinol, vitamin B12, folate, vitamins D and E, folate, iron, and selenium) in the exposed (A) and the control (B) groups; Significant correlations are signaled by * for p<0.05.

A – Exposed

	Calories	Retinol	Vit B12	Folate	Vit D	Vit E	Iron	Selenium
MN lymp	0.004	0.135	0.136	-0.063	0.027	-0.020	-0.083	-0.053
NPB	0.106	-0.007	-0.020	0.043	-0.021	0.069	0.065	-0.037
NBUD	-0.131	0.102	0.024	-0.102	-0.014	-0.160	-0.100	-0.148
MN MONO	0.078	0.281	0.161	0.100	-0.053	0.007	0.073	0.006
MN MULTI	0.012	-0.086	-0.029	-0.009	-0.001	-0.013	-0.055	-0.005
% DNA TAIL	0.154	0.254	0.153	0.111	0.034	0.139	0.188	0.073
FPG	-0.057	0.161	0.089	-0.161	0.028	-0.100	-0.131	-0.069

B- Non exposed

	Calories	Retinol	Vit B12	Folate	Vit D	Vit E	Iron	Selenium
MN BN	-0.70	-0.255	-0.176	-0.135	-0.081	0.036	-0.130	-0.096
NPB	-0.005	0.080	-0.090	-0.071	-0.122	-0.033	-0.030	-0.143
NBUD	-0.149	-0.180	-0.216	-0.172	-0.187	-0.261	-0.138	-0.063
MN MONO	0.175	-0.090	0.104	0.168	0.243	0.349*	0.181	0.188
MN MULTI	-0.149	-0.196	-0.093	-0.084	-0.017	-0.018	-0.158	-0.114
% DNA TAIL	-0.370*	-0.042	-0.186	-0.129	-0.138	-0.162	-0.256	-0.222
FPG	0.013	0.203	-0.086	0.187	-0.019	0.131	0.171	0.074

The proportion of subjects who were below and above the dietary reference intake (DRI) per nutritional item does not appear to confound the results, as there are no significant differences between the exposed and the control groups in regard to such proportions (Table 54).

Table 54 – Count of absolute number of subjects with measurements above dietary reference intake (≥ DRI) and below (< DRI), split by exposed and control group, p is from the Fisher's exact test and were not computable for iron and selenium as all subjects were above DRI.

Parameters	Groups	≥ DRI	< DRI	p-value
Retinol	**Exposed**	32	14	0.412
	Controls	30	16	
Vit B12	**Exposed**	45	1	0.500
	Controls	46	0	
Folate	**Exposed**	32	14	0.067
	Controls	24	22	
Vit D	**Exposed**	3	43	0.308
	Controls	1	45	
Vit E	**Exposed**	16	30	0.334
	Controls	19	27	
Iron	**Exposed**	46	0	n.a.
	Controls	46	0	
elenium	**Exposed**	46	0	n.a.
	Controls	46	0	

n.a. - non-applicable

6.3. CORRELATION BETWEEN TECNHIQUES

Vitamins A and E concentrations in serum were measured by HPLC, and different nutritional items, such as: calories, retinol, vitamin B12, folate, vitamins D and E, iron, and selenium were assessed by a FFQ. Therefore, information on vitamins A (retinol) and E were available from both methods. Comparing the results, no statistical significant correlations were found between measurements from the two methods. Correlation between vitamin A measured by HPLC and retinol intake measured by FFQ was positive (r=0.184), but did not reach statistical significance (p=0.08). Also, correlation between vitamin E measured by HPLC and intake measured by FFQ was positive (r=0.145) but not statistically significant (p=0.169). Scatterplots with the results from both measurements are shown in Figure 15a and 15b, respectively for vitamin A and E.

a) **b)**

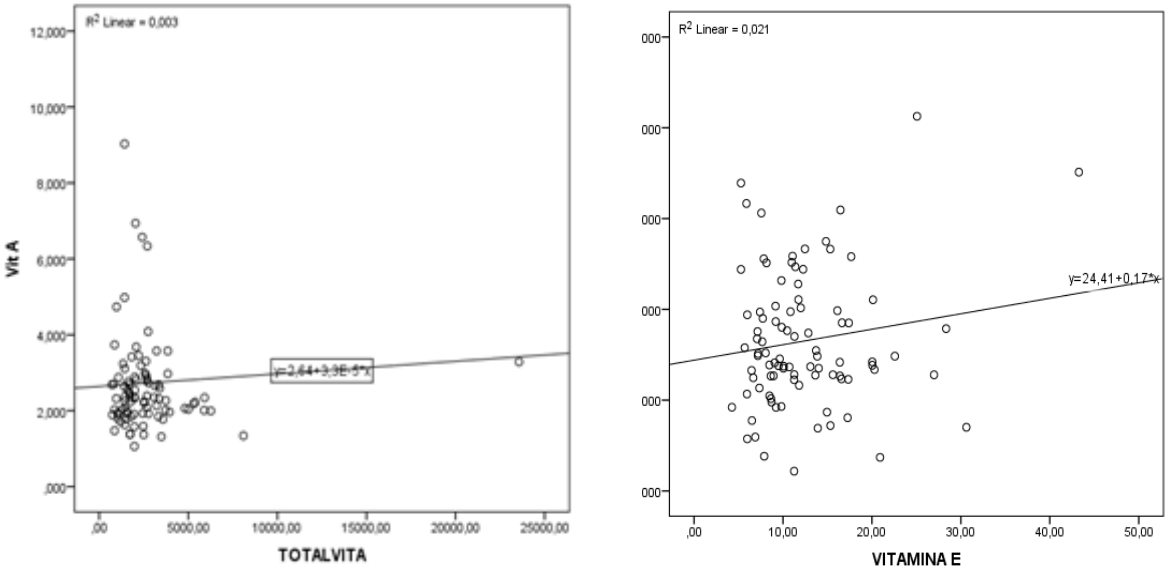


Figure 15 - Scatter plots of the correlation between vitamin A measured by HPLC (Vit A) and vitamin A measured by FFQ (TotalvitA) (a) and vitamin E measured by HPLC (Vit E) and vitamin E measured by FFQ (Vitamina E) (b).

V. DISCUSSION

CHAPTER 1 – FORMALDEHYDE OCCUPATIONAL SETTING

1. FORMALDEHYDE EXPOSURE ASSESSMENT

Long exposures to formaldehyde to which some workers are subjected for occupational reasons, are suspected to be associated with genotoxic effects that can be evaluated by biomarkers (Conaway *et al.*, 1996; IARC, 2006; Viegas & Prista, 2007; Zhang *et al.*, 2009b). Formaldehyde exposure can cause a wide range of toxic effects such as the formation of DNA-protein crosslinks, cytotoxicity, immune activation, and sensory irritation. Formaldehyde toxicity is thought to be mediated by the activation of free radical producing enzymes and also by the inhibition of free radical scavenging systems, thereby enhancing the accumulation of ROS (Gulec *et al.,* 2006), well-known DNA damaging compounds. The health effects (mostly cancer) which associate with formaldehyde exposure seems to be more related with peaks of high concentrations than with long time exposure at low levels (IARC, 2006; Pyatt *et al.,* 2008). Moreover, the choice of exposure metric should be based on the mode of action of the chemical agent (Preller *et al.*, 2004). These two factors contribute to explain why the genotoxicity biomarkers showed high values when we obtained low values for the time-weighted exposure metric. Previously, Pyatt *et al.* (2008) pointed out that an important limitation in most previous epidemiological studies was the lack of data regarding exposure to peak concentrations. In those studies, health effects resulting from occupational exposure to formaldehyde are normally associated to exposure exclusively based on TWA concentrations. Until 2004 only two studies (Hauptmann *et al.*, 2004; Pinkerton *et al.,* 2004) presented data on exposure to ceiling concentrations and, as a result, obtained higher values for the relative risk of nasopharyngeal cancer. Hauptmann *et al.* (2009) found out that mortality rate from leukemia also increases significantly not just with number of years of activity, in this case embalming, but also with the increase in peak values.

In this study the results suggest that workers in histopathology laboratories are exposed to formaldehyde levels that exceed recommended exposure limits (chapter 1 of results, 2. formaldehyde exposure assessment section). Macroscopic specimens' exam, in particular, is the task that involves higher exposure, because it requires a greater proximity to anatomical species impregnated with formaldehyde, corroborating the studies of (Goyer *et al.,* 2004; Orsière *et al.,* 2006).

2. GENOTOXICITY ASSESSMENT

It has been shown that a higher micronuclei frequency is directly associated with decreased efficiency of DNA repair and increased genome instability (Kirsch-Volders *et al.*, 2006; Orsiére *et al.*, 2006). The data has shown a significant increase of micronuclei in lymphocytes in the exposed group. This can be explained in light of genomic instability, understood as an increased amount of mutations and/or chromosomal aberrations that cytogenetically translate into a greater frequency of changes in chromosome number and/or structure and in the formation of micronuclei (Zietkiewicz *et al.*, 2009).

A statistically significant association was found between formaldehyde exposure and biomarkers of genotoxicity, namely micronuclei in lymphocytes and buccal cells, nucleoplasmic bridges, and nuclear buds limits (chapter 1 of results, 3. genotoxicity assessment section). Chromosome damage and effects upon lymphocytes arise because formaldehyde escapes from sites of direct contact, such as nose or mouth, originating nuclear alterations in the lymphocytes of those exposed (Goyer *et al.,* 2004; Orsière *et al.*, 2006; Zhang *et al.*, 2009b; Speit *et al.*, 2010). Our results thus corroborate previous reports (He *et al.*, 1998) that lymphocytes can be damaged by long term exposure to formaldehyde. Moreover, the changes in peripheral lymphocytes indicate that the cytogenetic effects triggered by formaldehyde can reach tissues faraway from the site of initial contact (Ye *et al*., 2005). Long term exposures to high concentrations of formaldehyde indeed appear to have a potential for DNA damage; these effects were well demonstrated in previous experimental studies with animals, where local genotoxic effects followed formaldehyde exposure, namely DNA-protein cross links and chromosome damage (IARC, 2006). In humans, formaldehyde exposure is also associated with an increase in the frequency of micronuclei in buccal epithelial cells (Suruda *et al.*, 1993; Burgaz *et al.*, 2002; Speit *et al.*, 2007), as corroborated by the results presented here.

Suruda *et al.* (1993) claim that although changes in oral and nasal epithelial cells and peripheral blood cells do not indicate a direct mechanism leading to carcinogenesis, they present evidence that DNA alteration took place. It thus appears reasonable to conclude that formaldehyde is a cancer risk factor for those who are occupationally exposed in histopathology laboratories (IARC, 2006).

3. DEMOGRAPHIC AND LIFESTYLE HABITS

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking and alcohol consumption, upon the association between disease and exposure (Bonassi *et al.,* 2001; Iarmarcovai *et al.*, 2008). Concerning gender, studies realized by Fenech *et al.* (1999b and Wojda *et al.* (2007) reported that biomarker frequencies were greater in females than in males by a factor of 1.2 to 1.6 depending on the age group. To the exception of micronuclei in the buccal cells of controls, the results presented here point to females having higher frequencies than males in all genotoxicity biomarkers, although the differences usually lacked statistical significance (chapter 1 of results, 4. demographic and lifestyle habits section). Such trend is concordant with previous studies that reported higher micronuclei frequency in lymphocytes in females and a slightly higher micronuclei frequency in buccal cells in males (Holland *et al.*, 2008) and that can be explained by preferential aneugenic events involving the X-chromosome. A possible explanation is the micronucleation of the X chromosome, which has been shown to occur in lymphocytes in females, both *in vitro* and *in vivo*, and that can be accounted for by the presence of two X chromosomes. This finding might explain the preferential micronucleation of the inactive X (Catalán *et al.*, 1998, 2000a, 2000b).

Aging in humans appears to be associated with genomic instability. Cytogenetically, ageing is associated with a number of gross cellular changes, including altered size and morphology, genomic instability and changes in expression and proliferation (Bolognesi *et al.,* 1999; Zietkiewicz *et al.,* 2009). The involvement of micronucleation in age-related chromosome loss has been supported by several studies showing that the rate of micronuclei formation increases with age, especially in women (Catalán *et al.,* 1998).

This study provides evidence that age and gender interact to determine the frequency of micronuclei in the lymphocytes of exposed subjects (chapter 1 of results, 4. demographic and lifestyle habits section). The higher incidence of micronuclei in both genders is more manifest in older age groups and the effect of gender becomes more pronounced as age increases. Several reports link this observation to an elevated loss of X chromosomes (Battershill *et al.,* 2008).

Tobacco smoke has been epidemiologically associated to a higher risk of cancer development, especially in the oral cavity, larynx, and lungs, as these are places of direct contact with the carcinogenic tobacco's compounds. In this study, smoking habits did not influence the frequency of the genotoxicity biomarkers; moreover, the frequencies of micronuclei in buccal cells were unexpectedly higher in exposed non-smokers than in exposed smokers, though the

difference was not statistically significant. In most reports, the results about the effect of tobacco upon the frequency of micronuclei in human lymphocytes were negative as in many instances smokers had lower micronuclei frequencies than non-smokers (Bonassi *et al.*, 2003). In the current study, the analysis of the interaction between formaldehyde exposure and smoking habits indicates that exposure is preponderant in determining the frequency of biomarkers (chapter 1 of results, 4. demographic and lifestyle habits section). Nevertheless, the effect of smoking upon biomarkers remains controversial. Some studies (El-Zein *et al.,* 2006, 2008) reported an increased frequency of micronuclei in lymphocytes, nucleoplasmic bridges, and nuclear buds as a consequence of the tobacco-specific nicotine derived nitrosamino ketone (NNK). Still in this study no associations were observed between tobacco and nuclear abnormalities.

As for alcohol consumption, it did not appear to influence the frequency of genotoxicity biomarkers under study, to the exception of micronuclei in lymphocytes in controls (Mann-Whitney, p=0.011), with drinkers having higher means. Alcohol is definitely a recognized genotoxic agent, being cited as able to potentiate the development of carcinogenic lesions (Ramirez & Saldanha, 2002). In our study, drinkers in the control group had higher mean frequencies of all biomarkers than non-drinkers, but the differences were only significant for micronuclei in lymphocytes. Stich & Rosin (1983) study on alcoholic individuals, reported absence of significant differences concerning micronuclei frequencies in buccal cells. The same study (Stich & Rosin, 1983) concluded that neither alcohol nor smoking, alone, increase micronuclei frequency in buccal cells, but a combination of both resulted in a significant elevation in micronucleated cells in the buccal mucosa. However, the synergism between alcohol consumption and tobacco has not been observed to act upon all biomarkers and, in several studies of lifestyle factors, it was difficult to differentiate the effect of alcohol from that of smoking (Holland *et al.,* 2008).

The CBMN assay is a simple, practical, low cost screening technique that can be used for clinical prevention and management of workers subjected to occupational carcinogenic risks, namely exposure to a genotoxic agent such as formaldehyde. The results obtained in this study provide unequivocal evidence of association between occupational exposure to formaldehyde in histopathology laboratory workers and the presence of nuclear changes.

Given these results, preventive actions must prioritize safety conditions for those who perform macroscopic exams. In general, reduction of exposure to formaldehyde in this occupational setting may be achieved by the use of adequate local exhaust ventilation and by keeping biological specimen containers closed during the macroscopic exam. Individual

equipment, namely masks with proper formaldehyde filters, should be provided and used by health care workers that handle this chemical agent.

4. INDIVIDUAL SUSCEPTIBILITY

Exposure to formaldehyde in occupational settings is often prolonged enough to lead to the accumulation of DNA damage and increased mutation risk (Mateuca *et al.*, 2006). Previous studies have suggested that genetic polymorphisms in specific genes affect susceptibility to chromosome damage associated with environmental exposure to genotoxic agents (Umegaki & Fenech, 2000). Genetic polymorphisms are potentially important in micronuclei formation, depending on the level of exposure, biological matrix studied and ethnicity of the studied population (Umegaki & Fenech, 2000). Chromosomal instability and impaired cell viability have been correlated with *XRCC3* mutations and several other genes known or thought to be involved in HR (Bolognesi *et al.,* 1999; Brenneman *et al.,* 2000), being this pathway required in processing DNA damage induced by formaldehyde (Zhang *et al.*, 2010).

In this study, we report a statistically significant association between *XRCC3* Thr241Met polymorphism and nuclear buds, biomarkers of gene amplification (chapter 1 of results, 5.1. *XRCC3* Met241Thr polymorphisms section). The carriers of the *XRCC3* Met/Met and Thr/Met genotypes had higher nuclear buds frequencies than their Thr/Thr genotype counterparts. Gene amplification plays a crucial role on the malignant transformation of human cells as it mediates the activation of oncogenes or the acquisition of drug resistance (Utani *et al.,* 2007). Excess DNA may be expelled from the nucleus by the formation of nuclear buds and subsequent micronucleation (Lindberg *et al.,* 2007). Previous studies have described *in vivo* budding of nuclear material in cell lines where changes in chromosomal numbers were occurring, and the spontaneous formation of nuclear buds structures was seen as a possible mechanism for the loss of chromosomes and for the generation of micronuclei (Fenech *et al.* 2011). Therefore, nuclear buds should also be considered genotoxic biomarkers with an origin comparable to that of micronuclei (Serrano-García & Montero-Montoya, 2001).

Previous studies have shown that carriers of the *XRCC3* heterozygous genotype had increased levels of chromatid breaks and sister-chromatid exchanges in smokers and increased DNA adducts in lymphocytes (Fenech *et al.,* 1999a) suggesting that this polymorphism is associated with low DNA repair capacity and may increase the risk of many types of cancer (Benhamou *et al.*, 2004; Han *et al.*, 2006; Battershill *et al.*, 2008). Yoshihara *et al.* (2004) and Lindh *et al.* (2006) suggested that *XRCC3* Thr241Met variants contribute to the induction of micronuclei

arising from chromosome loss. Carriers of the Met/Met alleles would present higher micronuclei frequencies than their wild-type Thr/Thr allele counterparts (Mateuca *et al.,* 2008). A significant increase of micronuclei frequency in the Thr/Met genotype of *XRCC3* was reported in workers exposed to oil, indicating that this polymorphism must be taken into account in chronic exposure scenarios (Pérez-Cadahía *et al.*, 2008).

However, other studies did not find evidence for the influence of *XRCC3* genotype in the micronuclei basal frequency (Iarmarcovai *et al.,* 2006). The functional differences between the *XRCC3* alleles are not entirely understood. The amino acid substitution of a threonine by a methionine has the potential to affect protein structure and integrity (Dhillon *et al.,* 2011). Variants leading to diminished *XRCC3* function may be predicted to confer an increased risk of cancer due to accumulated levels of DNA damage. As many genes are involved in the repair of DNA damage, there is also the possibility that these polymorphisms might be in linkage disequilibrium with other causative factors (Figueiredo *et al.,* 2004).

Shen *et al.* (2002) suggested that the Met/Met genotype may contribute to a subset of squamous cell carcinoma of the head and neck and Figueiredo *et al.* (2004) found that both carriers of Met/Met and Thr/Met genotypes have an increased risk for breast cancer. The Met/Met genotype may cause genetic instability and lead to an increased susceptibility to various cancers due to the inability of genotype carriers to complement the centrosome amplification defect and to a decrease of apoptotic rates (Lindt *et al.* 2006), factors that may prevent aberrant cells from entering apoptosis.

A better understanding of micronuclei induction driven by genetic polymorphism affecting DNA repair and/or genome stability, in particular *XRCC3* Thr241Met, requires larger scale studies and the assessment of other relevant polymorphism interacting with individual DNA repair capacity (Mateuca *et al.,* 2008).

Our study did not provide conclusive evidence that some *ADH5* polymorphisms may influence the carrier's capacity to protect against DNA damage (chapter 1 of results, 5.2. ADH5 Bal309Ile and Asp353Glu polymorphisms). A borderline association ($p = 0.06$) was found between the frequency of nuclear buds and the homozygous Asp/Asp genotype, as compared to the Asp/Glu heterozygous genotype. These individuals may be more prone to nuclear alterations following a possible alteration in formaldehyde metabolism and adduct formation. Another interesting result was the statistically significant difference in carriers of the Val/Ile genotype in comparison with Val/Val genotype of the *ADH5* Val309Ile polymorphism in micronuclei in lymphocytes in the exposed group. The carriers of the heterozygous genotype showed higher means of micronuclei in lymphocytes in the exposed group but not in the control group

suggesting that the carriers of Val/Ile genotype metabolize poorly formaldehyde and present more DNA damage. Our results are in agreement with the findings of Just *et al.* (2011), who investigated three different polymorphisms in the transcribed regions of *ADH5* for inter-individual differences against the genotoxicity of formaldehyde in the German population and found no biologically relevant variants. The biological significance of *ADH5* polymorphisms in relation to disease remains uncertain.

Regarding the *VDR BsmI* polymorphisms, in the exposed group we have found a significant association between micronuclei in lymphocytes and the genotypes studied (chapter 1 of results, 5.3. Vitamin D receptor *BmsI* polymorphisms). Carriers of the CT+T genotype have higher mean micronuclei in lymphocytes than CC carriers. The *BsmI* polymorphisms exhibit a heterogeneous geographical distribution. For instance, a study of Caucasian women reported increased risk of breast cancer with the *BsmI bb* (TT) genotype and low levels of vitamin D; whereas, *BsmI BB* (CC) genotype was associated to breast cancer among Hispanic and Taiwanese women (Lowe *et al.,* 2005; Raimondi *et al.,* 2009; Shahbazi *et al.,* 2013). Studies corroborate the role of the TT *BsmI* genotype as a risk factor for a variety of pathologies, such as nephrolithiases, high blood pressure (Valdivielso & Fernandez, 2006), melanoma (Denzer *et al.*, 2011; Orlow *et al.,* 2012), higher levels of antinuclear antibodies related with systemic lupus erythematosus (Kaleta *et al.*, 2013), severe coronary artery disease (Schooten et al., 1998), lower levels of calcium and more frequent type 2 diabetes mellitus (Al-Daghri *et al.,* 2012), lower levels of 25(OH)D and prostate cancer (Taylor *et al.*, 1996; Ma *et al.,* 1998; Lowe *et al.,* 2005).

Qin *et al.* (2013) reported, in a subgroup analysis, that the TC + TT genotypes were risk factors in ovarian cancer compared to CC genotype, however Mostowska *et al.*, (2013) concluded that the CC genotype might be a moderate risk factor for ovarian cancer development in the Polish population.

The association between SNPs in relevant genes and the frequency of micronuclei in lymphocytes is a valuable tool for this purpose, as the latter is one of the best-validated DNA damage biomarker known to be sensitive to a wide range of endogenous, environmental, and lifestyle factors that can harm the genome (Dhillon *et al.,* 2011). Some genetic polymorphisms of xenobiotic-metabolizing enzymes have been observed to influence the level of genotoxic damage in humans. This may facilitate the identification of risk groups and increase the sensitivity of biomarkers in biomonitoring (Norppa, 2001). However, studies that report an association between genotypes and biomarkers, such as micronuclei, have some limitations in design and analysis. Common limitations are group sample size, usually too small to evaluate

rare polymorphisms, and the wide range of allele frequency variation for each genotype in different ethnic populations. The statistical analysis is often plagued with problems of lack of power (due to insufficient sample size) and confounding can seldom be precluded given the amount of potential factors involved that have not been measured (Hunter, 2005; Chung *et al.*, 2010).

Our results showed a significant statistical association between *XRCC3* Thr241Met polymorphism and NBUD. *ADH5* polymorphisms did not show significant association with the genotoxicity biomarkers studied, and the carriers of the CT+T genotype of the *VDR BsmI* polymorphisms have higher mean micronuclei in lymphocytes than CC carriers. Several association studies have recently addressed the link between DNA repair polymorphism and micronuclei induction, but the evidence that DNA repair polymorphisms influence micronuclei frequencies remains limited (Mateuca *et al.,* 2008). Haplotype analysis – whereby all possible genotypes would be checked against all biomarkers – was not performed given the lack of association in the more global analysis and because of insufficient sample size. Any further sample slitting by genotype would lead to reduction in statistical power too drastic to proceed.

5. MICRONUTRIENTS

Vitamins, essential minerals and other components are required in small quantities in the human diet for efficient metabolism. However, there is no consensus regarding the level of micronutrients necessary to prevent DNA damage in humans. Individual characterization of ideal dietary intakes in order to prevent DNA damage is a fundamental goal, because the amount of micronutrients which prove to be protective against genome damage varies according to food types and a cautious choice is needed if we are to design dietary patterns optimized for genome health maintenance (Prado *et al.,* 2010).

Experimental studies reported by Bonassi e*t al.* (2007) and Fenech (2010) support the critical role played by micronutrients in the preservation of genomic integrity (Lal & Ames, 2011), namely in the decrease of micronuclei frequencies when the subjects consume vitamin-antioxidant mixtures (Gaziev *et al.,* 1996).

In the non-exposed group, there was a strong positive correlation among the genotoxicity biomarkers under study, namely micronuclei in lymphocytes and nucleoplasmic bridges with nuclear buds, and nucleoplasmic bridges and micronuclei in buccal mucosa cells. These results corroborate the study of Fenech & Crott (2002), where a strong cross-correlation between micronuclei, nucleoplasmic bridges and nuclear buds frequency suggested a common

mechanism. Analogous models via other molecular pathways, such as DNA repair, can be developed to explain the generation of micronuclei, nucleoplasmic bridges and nuclear buds induction by exposure to specific genotoxic agents such as ionising radiation or other agents that cause double-stranded DNA breaks (Preller *et al.,* 2004; IARC, 2006; Pyatt *et al.*, 2008; Fenech, 2010).

Our results showed a positive correlation between vitamin A and nucleoplasmic bridges, meaning that higher value of vitamin A increases this biomarker (chapter 1 of results, 6.1. genotoxicity biomarkers and vitamins A,D, and E). As nucleoplasmic bridges are biomarkers of chromosome rearrangement, this suggests that vitamin A promotes genomic instability, acting as a risk factor instead of a protective one.

De Flora *et al.* (1999) verified that retinol was ineffective and some *in vitro* results even suggested it enhanced DNA damage, point mutations of differential specificity, numerical and structural chromosomal alterations, and impairment of DNA repair mechanisms, modulated by β-carotene or vitamin A. The apparently protective effect of increased retinol may be due to its one-step conversion to retinoic acid, which has been shown to suppress the inappropriate expression of NFκβ, which leads to inflammation, increased cell proliferation and inhibition of apoptosis, all of which may lead to an increased micronuclei expression in lymphocytes (Fenech *et al.,* 2005). This result is conflicting with the findings of Gaziev *et al.* (1996) who reported that vitamin A and β-carotene enhance the antioxidant activity of cells and are able to reduce micronuclei frequency in human lymphocytes; however they supplemented subjects with a multivitamin during a 4 month period.

Studies by (Stich *et al.,* 1984a, 1984b) reported considerable reduction of micronuclei in exfoliated buccal mucosa cells following the administration of a vitamin A plus beta-carotene regime. Benner *et al.,* (1994) demonstrated a decrease in micronuclei frequencies after alpha-tocopherol treatment. Overall, the measurement of micronuclei in exfoliated cells is suitable to monitor specific health risk arising from various kinds of exposure to carcinogenic hazards (Majer *et al.,* 2001), our results did not find significant associations between micronuclei in exfoliated buccal cells and the vitamins under study. Our results can probably be explained by the fact that the buccal mucosal tissue cells are rapidly dividing and vitamin levels in desquamated buccal mucosal cells may be a poor indicator of vitamin status in the dividing basal cell layer where micronuclei formation occurs (Piyathilake *et al.*, 1995).

A considerable body of evidence indicates that vitamin E, the most potent lipid peroxyl radical scavenger, significantly decreases free radical induced chromosomal damages (Claycombe & Meydani, 2001). There are many studies that showed no effects of vitamin E upon

genotoxicity biomarkers, namely no correlation between micronuclei levels and vitamin E (Stich *et al.,* 1984a, 1984b; Benner *et al.,* 1994; Piyathilake *et al.,* 1995; Fenech *et al.,* 2005).

Gulec *et al.* (2006) suggested that formaldehyde exposure of experimental animals causes depression in their antioxidant status due to increased lipid peroxidation, and formation of free radicals. Therefore, concluding that vitamin E prevents biochemical changes in the liver tissue and plasma of rats due to oxidative damage and cytotoxicity; it also has a protective effect against the administration of mutagenic chemical compounds (Awodele *et al.,* 2010) and a radioprotection effect, measured by micronuclei in bone marrow and exfoliated cells (Konopacka *et al.*, 1998). Schneider *et al.* (2001) reported a distinctive decrease in micronuclei frequency after diet supplementation with vitamin E. All these studies reported on micronuclei levels, whereas we have found a significant association between vitamin E and nuclear buds ($p<0.05$) in the exposed group.

Current evidence suggesting that vitamin D prevents DNA damage and regulates the cell cycle has been limited to studies in cultured cells and experimental animal models (Raimondi *et al.,* 2009). Our study did not show any association between this vitamin and the genotoxicity biomarkers evaluated. We also did not find significant associations between vitamin D and A or E. However, Sarkar *et al.* (2000) showed that vitamin D analogs alone, and particularly in combination with retinoid acid, exert antitumor effects by means of induction of cell differentiation, inhibition of proliferation and angiogenesis. Jones *et al.*, (2011) also showed that vitamins D and E influence micronuclei frequencies significantly.

There are some limitations, concerning the study of the association between genotoxicity biomarkers and vitamins, namely the small sampling size, which limits the power of statistical tests. Moreover, vitamin measurements were only done once in the study, and the temporal variation of the serum concentrations can influence the observed results. Determining the intake levels of micronutrients required to maintain genome stability is an essential step in the definition of optimal diets for the prevention of cancer and other diseases caused by genome damage (Fenech *et al.,* 2005; Thomas *et al.,* 2011). Although the presence of micronuclei is a strong indicator of chromosomal damage resulting from either whole chromosome loss or breakage, the other genotoxicity biomarkers provided by the CBMN assay should not be ignored because they are indicative of genomic instability. As genome damage is considered the most fundamental of all disease pathologies, it is essential to determine which micronutrients are necessary to maintain optimal genome health and who is likely to benefit (Thomas *et al.,* 2011). In interpreting the data from this study, it is important to note that micronutrients usually exhibit metabolic dose-response effects in which both deficiency and

excess can be deleterious and it is probable that in a specific mixed diet, depending on the intake level of an individual, some of the micronutrients may be outside the intake range that is optimal for the prevention of genome instability (Fenech *et al.,* 2005).

The fact that both vitamin deficiency and excess can increase carcinogenesis is supported by several studies and highlights the acute need for better knowledge of dose-response relationships between micronutrient intake and genome health (Fenech *et al.,* 2005).

In conclusion, our study seems to indicate that vitamin levels may modulate direct signs of genotoxicity and the fact that we have measured them directly, rather than indirectly from a dietary questionnaire, adds strength to our results. However, is necessary to increase the size of the sample in order to test these and other associations.

CHAPTER 2 – CYTOSTATICS DRUGS OCCUPATIONAL SETTING

1. CYTOSTATICS EXPOSURE ASSESSMENT

Healthcare workers handling antineoplastic drugs usually implement collective and individual protective measures. However, contamination of the work environment is still possible, and the safety measures employed can be insufficient to prevent exposure. In addition, workers may not apply all the safety measures required for handling such substances.

Considering surfaces contamination results, positive samples were found for all surrogate markers in both hospitals. These results raise concern because health effects associated to exposure to carcinogenic, mutagenic, and teratogenic substances usually do not depend on a minimum dose but rather on a prolonged exposure (Fucic, 1998; Sessink & Bos, 1999; Bouraoui, 2011; Buschini, 2013). Therefore, it can be concluded that there is no safety threshold dose concerning exposure to these drugs being appropriate to apply the ALARA principle: keep exposure/contamination levels "As Low As Reasonably Achievable" (Hon *et al.,* 2013). Widespread contamination was also observed in other studies, despite the implementation of safety procedures for handling antineoplastic drugs (Schmaus *et al.*, 2002; Connor *et al.*, 2006; Castiglia *et al.,* 2008).

Our results showed that 36% of the samples were contaminated with one cytostatic drug, and 8.6% of the surfaces were contaminated with more than one antineoplastic drug, being most of these samples from the administration units (chapter 2 of results, 2. cytostatics exposure assessment). These results can be explained once again by the more strict safety and hygiene rules in preparation units when compared with administration units, eventually stemming from the high number of organizations that research on this field and continuously develop new rules and safety measures (NIOSH 2004; ISOPP 2007, among others). In this case, inappropriate cleaning combined with incorrect working procedures probably are contributing to the contamination found, a good example being the telephone handling without taking off the gloves.

Our results showed that the amount of contaminated surfaces varied with the drugs considered. For example, it was possible to observe fewer surfaces contaminated with cyclophosphamide (4.6%) than with 5-fluorouracil and paclitaxel (10.7% and 26.9%, respectively). This can nevertheless be explained by the lower sensitivity of the cyclophosphamide detection method, unable to measure values below 30 µg, as compared to

5-fluorouracil (LOQ=1000 ng) and paclitaxel (LOQ=50 ng) (Viegas *et al.*, 2014). Additionally, it seems that cyclophosphamide is rapidly degraded at room temperature disappearing more easily from the environment (Hedmer *et al.,* 2004). Taking this into account, probably the results only represent the sampling of a one-day contamination and not what might be resulting from other working days. Paclitaxel, on the other hand, presented the highest number of contaminated surfaces (21.7%) as compared to the other two drugs (4.6% for cyclophosphamide and 10.7% for 5-fluorouracil) (chapter 2 of results, 2. cytostatics exposure assessment). Paclitaxel is characterized by low aqueous solubility and high physicochemical stability. These two factors combined can explain the results because this drug is probably more persistent on surfaces, resisting to environmental conditions and cleaning (Kopjar *et al.,* 2007). This brings up another important aspect that should be considered for risk assessment in hospital settings and alike, exposure is not to one single drug but rather to different antineoplastic drugs and the health effects of such mixtures are unpredictable (Fucic *et al.*, 1998; Cavallo *et al.,* 2005; Kopjar *et al.,* 2009).

The contamination of various surfaces by antineoplastic drugs in the workplaces implies an increased risk for health care workers to become dermally exposed (Hedmer & Wohlfart, 2012).

2. GENOTOXICITY ASSESSMENT

2.1. CBMN ASSAY

As newly developed antineoplastic drugs are designed and introduced, in order to attack specific intracellular targets, their harmful effects could easily “escape” from detection by most standard endpoints. The CBMN should be used to accurately evaluate cytogenetic outcomes of such exposures (Kopjar *et al.,* 2009). Its value in the assessment of genotoxic damage among occupationally exposed personnel was also confirmed in this study. The data obtained has shown a significant increase of micronuclei in lymphocytes in the exposed group in comparison with controls, on account of genomic instability, as an increased amount of mutations and/or chromosomal aberrations that cytogenetically translate into a greater frequency of changes in chromosome and in the formation of micronuclei (Zietkiewicz *et al.,* 2009).

In what concerns genotoxicity assessment, our results showed statistical significant higher means in all genotoxicity biomarkers in the exposed as compared to control groups ($p<0.05$) (chapter 2 of results, 3.1. cytokinesis-block micronucleus assay). These results, namely those

concerning micronuclei frequency, are corroborated by many others studies (Fucic *et al.,* 1998; Deng *et al.,* 2005; Cavallo *et al.,* 2007; Cornetta *et al.,* 2008; Kopjar *et al.*, 2009; Bouraoui *et al.,* 2011; El-Ebiary *et al.,* 2011) which found significant increases of micronuclei frequency in workers handling antineoplastic drugs.

The results of cytogenetic studies are often ambiguous. For example, in studies performed in Austria (Pilger *et al.,* 2000) and in Sweden (Thiringer *et al.,* 1991) no significant differences in micronuclei in hospital pharmacy personnel and unexposed controls were found. Also Maluf *et al.* (2000), Hessel *et al.* (2001), Cavallo *et al.* (2005), Laffon *et al.* (2005) showed no significant micronuclei increase in those exposed to antineoplastic drugs as compared to non-exposed. Thus it appears that research on the genotoxicity of antineoplastic drugs displays conflicting results; such inconsistency could be attributed to differences in the antineoplastic drugs handled, or the protective measures available and used effectively. Moreover, based on genetic bases, the human response to genotoxic xenobiotics may vary due to the presence of individual differences in DNA damage repairing capacity (Rekhadevi *et al.,* 2007, El-Ebiary *et al.,* 2011).

In what concerns other endpoints measured by the CBMN assay, Kopjar *et al.* (2007) reported an increase of nucleoplasmic bridges and nuclear buds in those exposed to irinotecan, an antineoplastic drug. Our results also showed higher means of these biomarkers in the exposed group.

For micronuclei in mono and multinucleated lymphocytes, results form binary logistic regression suggested that exposure can not be considered a risk factor for these two endpoints, however there were statistical significant differences between the exposed group and the controls (Mann-Whitney test, $p<0.05$).

It is actually unknown to what extent mutagen exposure either leads to the formation of micronuclei already *in vivo* or to the formation of micronuclei *ex vivo* during cell culture as consequence of DNA damage which is not repaired *in vivo* or *in vitro* and persists until lymphocytes divide in culture (Martelli *et al.,* 2000; Arsoy *et al.,* 2009). Therefore, it has been postulated that micronuclei induced *in vivo* is observed in mononuclear lymphocytes, and *in vitro* damage is observed in binucleated lymphocytes. Increased frequencies in micronuclei can only be expected if lymphocytes with persistent damage are obtained and cultured (Arsoy *et al.*, 2009).

As for the presence of a high number of multinucleated lymphocytes, because antineoplastic drugs act at a diversity of cellular levels, and some groups of these drugs cause abnormalities

of the mitotic spindle, the presence of a considerable number of multinucleated lymphocytes with micronuclei was to be expected.

We have not found evidence that the amount of years of exposure to cytostatics influenced the frequency of micronuclei. This result is corroborated by the studies of Thiringer *et al.* (1991), Hessel *et al.* (2001), Bouraoui *et al.* (2011), El-Ebiary *et al.* (2011), and Villarini *et al.* (2012) who also did not find association between years of exposure to antineoplastic drugs and micronuclei increase. Once again, there are controversial results regarding this matter, as studies by Kevekordes *et al.* (1998), Kasuba *et al.* (1999), Laffon *et al.* (2005), Cavallo *et al.* (2007), Rekhadevi *et al.* (2007), and Kopjar *et al.* (2009) showed an association between the years of exposure and the increase in micronuclei. The study by Laffon *et al.* (2005) reported this influence following 10 years of cumulative exposure.

The present study confirmed surface contamination in the workplaces considered and the cytogenetic endpoint studied (CBMN) showed signs of a relationship with exposure. Since genotoxicity may be due to combined effects of all or some of the antineoplastic drugs, it is not possible to attribute damage to any particular agent. Results of this study as well as previous investigations on subjects occupationally exposed to antineoplastic drugs using different genotoxicity endpoints suggest that antineoplastic drugs in long-term occupational exposure may act as clastogens on the DNA molecule of somatic cells (Rekhadevi *et al.,* 2007).

2.2. COMET ASSAY

The comet assay identifies injuries which are still reparable, such as single and double-strand DNA breaks, alkali labile lesions that are converted to strand breaks under alkaline conditions and single-strand breaks associated with incomplete excision repair sites (Villarini *et al.,* 2012), thus providing information about recent exposures (Laffon *et al.,* 2005). In particular, the comet assay with the use of enzymes, which recognizes and cuts specifically oxidized DNA bases allows for the evaluation of oxidative DNA damage (Collins *et al.,* 1999). It is one of the most used methods in biomonitoring studies of genotoxicity on blood lymphocytes (Cavallo *et al.*, 2009), and is widely used to evaluate the genotoxic effects of exposure to specific antineoplastic drugs in several *in vitro* and *in vivo* studies (Digue *et al.,* 1999; Blasiak *et al.,* 2000; Brahnam *et al.,* 2004).

In what concerns comet assay, our findings suggest that occupational exposure to antineoplastic drugs in healthcare workers induces DNA damage but the increase compared to controls was not significant. The results showed higher mean DNA damage, measured by %

DNA in tail and oxidative DNA damage (FPG), in the lymphocytes of the exposed subjects compared to controls, although without reaching statistical significance (chapter 2 of results, 3.2. comet assay). Our results are in line with studies by Ursini *et al.*, (2006) and Buchini *et al.* (2013), which used the alkaline comet assay of peripheral blood lymphocytes to evaluate biomonitoring of subjects exposed to antineoplastic drugs, and also did not reach a statistically significant difference between exposed and controls, or the weak significant trend reported by (Mader *et al.,* 2008). Sasaki *et al.* (2008) evaluated DNA damage by measuring comet tail moment, and there was no significant difference between exposed and control subjects either. A possible explanation may have to do with comet assay predominantly detecting single-strand breaks and alkali-labile sites, which are induced by antineoplastic drugs (Kopjar *et al.,* 2009). Since both types of DNA damage are continuously and efficiently repaired, the measured damage level is a result of equilibrium between the amount of DNA damage inflicted and the speed of repair (Kopjar & Garaj-Vrhovac, 2001). Generally, the type, level and persistence of DNA damage in lymphocytes of exposed populations depend on the kind of antineoplastic drugs used as well as on the concentrations of drugs producing the mutagenic response (Kopjar & Garaj-Vrhovac, 2001). Also, antineoplastic drugs are well-known cross-linking agents, which can increase the effective molecular weight of DNA, are thereby are known to reduce the ability of DNA containing strand breaks to migrate in an electric field. The presence of a cross-linking agent could have hidden an increase in DNA migration associated with the induction of DNA strand breaks by other genotoxic agents, with a higher effect in terms of DNA tail mobility (Villarini *et al.,* 2011).

Contrary to these findings, other researchers evaluating DNA damage in healthcare workers handling antineoplastic drugs were able to show a statistically significant increase in DNA damage on the exposed group with respect to controls with the comet assay (Ündeğer *et al.*, 1999; Maluf & Erdtmann, 2000; Kopjar & Garaj-Vrhovac, 2001; Yoshida *et al.*, 2006; Kopjar *et al.*, 2007; Rekhadevi *et al.,* 2007; Cornetta *et al.,* 2008; Izdes *et al.,* 2009; Kopjar *et al.,* 2009; Rombaldi *et al.*, 2009; Villarini *et al.*, 2011).

The comet assay and the CBMN assay detect genotoxic effects caused by different mechanisms. The comet assay identifies still reparable injuries such as single and double-strand DNA breaks, alkali labile lesions that are converted to strand breaks under alkaline conditions and single-strand breaks associated with incomplete excision repair sites; whereas the CBMN assay detects injuries that survive at least one mitotic cycle and reflect unrepaired fixed DNA damage (Villarini *et al.*, 2012). It is considered that, for chronic exposures,

micronuclei test express cumulative facts whereas comet assay provides information on recent exposures (Laffon *et al.*, 2005).

Comet assay used in biomonitoring studies reflect the current exposure (over the previous few weeks) and the actual levels of DNA damage present in white blood cells at the moment of blood sampling. The comet assay is able to sensitively reveal early, still repairable, moderate DNA damage, and can therefore furnish useful information on early effects induced by occupational exposure to low doses of xenobiotics (Kopjar *et al.,* 2009) being recommended to monitor population chronically exposed to genotoxic agents combined with CBMN assay (Maluf & Erdtmann, 2000; Kopjar & Garaj-Vrhovac, 2001; Rekhadevi *et al.,* 2007; Cornetta *et al.,* 2008; Cavallo *et al.,* 2009; Kopjar *et al.,* 2009). A combination of cytogenetic tests and the comet assay in biomonitoring studies makes it possible to compare the relative sensitivities of the two test systems and, therefore, gives us a possible clue about the fraction of the DNA damage detected by the comet assay that will lead to fixed mutations (Milic *et al.,* 2010).

Our results concerning of positive findings by micronuclei and non significant ones by comet assay, are corroborated by Deng *et al.* (2005) study performed in workers occupationally exposed to methotrexate, also a cytostatic drug. According to Cavallo *et al.* (2009), the comet assay seems to be more suitable for the prompt evaluation of the genotoxic effects, for instance, of polycyclic aromatic hydrocarbons mixtures containing volatile substances, whereas the micronucleus test seems more appropriate to evaluate the effects of exposure to antineoplastic agents. However, there are studies that observed an increase in both the comet assay and the micronucleus test in nurses handling antineoplastic drugs, although statistical significance was only seen in the comet assay, quite the opposite of our results (Maluf & Erdtmann, 2000; Laffon *et al.* 2005).

3. DEMOGRAPHIC AND LIFESTYLE HABITS

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking and alcohol consumption, upon the association between disease and exposure (Fenech *et al.*, 1999; Bonassi *et al.,* 2001). Concerning gender, studies by Fenech *et al.* (1999) and Ladeira *et al.* (2011) reported that biomarker frequencies in lymphocytes were greater in females in comparison with males. In our study (chapter 2 of results, 4. demographic and lifestyle habits), gender was not associated with an increase in the frequency of micronuclei, or other of the biomarkers measured by CBMN assay, either in the exposed or control group, a result in agreement with Villarini *et al.* (2012). As for the

comet assay, it was found a moderate positive association in the control group, indicating that females have higher DNA damage. Regarding the possible influence of gender in the basal DNA damage measured using comet assay, studies in humans that used comet assay also produced divergent results. Some authors observed higher levels of basal genetic damage in males than in females (Bajpayee *et al.*, 2002; Lam *et al.,* 2002; Manikantan *et al.*, 2009), whereas others have found no difference between genders (Diem *et al.,* 2002).

Cytogenetically, ageing is associated with a number of gross cellular changes, including altered size and morphology, genomic instability and changes in expression and proliferation (Bolognesi *et al.,* 1999; Zietkiewicz *et al.,* 2009). It has been shown that higher micronuclei frequency is directly associated with decreased efficiency in DNA repair and increased genome instability (Kirsch-Volders *et al.*, 2006; Orsière *et al.,* 2006), and it also affects the level of induced and basal DNA damage detected by comet assay in mammalian cells (Heuser *et al.,* 2008). The data obtained has shown a significant increase of micronuclei in binucleated and mononucleated lymphocytes in both groups, showing that micronuclei frequencies tended to rise with age, like in the results by Hessel *et al.* (2001) and Kopjar *et al.* (2009). The analysis of age and gender together corroborate the previous results, being age the factor that influence micronuclei in binucleated and mononucleated lymphocytes, and gender in DNA damage (chapter 2 of results, 4. demographic and lifestyle habits). When using the comet assay, our results did not reach statistical significance, unlike previous studies (Goukassian *et al.*, 2002; López-Diazguerrero *et al.*, 2005; Rossi *et al.,* 2007; Heuser *et al.,* 2008), where DNA damage, detected by comet assay, showed an increase with age. However, Manikantan *et al.* (2009) also failed to find a significant association between DNA damage and age.

Tobacco smoke has been epidemiologically associated to a higher risk of cancer development; in the present study smoking habits did not affect any of the genotoxicity biomarkers measured by CBMN assay, either in the exposed or in the control subjects (chapter 2 of results, 4. demographic and lifestyle habits) being this result corroborated by Villarini *et al.* (2012).

In the present study, smoking habits did not increase the levels of DNA damage measured by comet assay significantly, in either the control or the exposed subjects. Our results, which are in agreement with findings by Hellman *et al.* (1997, 1999), Wojewódzka *et al.*, (1999) and Garaj-Vrhovac & Kopjar (2003) indicate that cigarette smoking is not a very potent confounding factor on the comet parameters measured. Some authors observed a connection between cigarette smoking and increased migration of human lymphocyte DNA during alkaline comet assay (Fuchs *et al.*, 1995; Moller *et al,* 2000; Speit *et al.*, 2003; Hininger *et al.*,

2004; Manikantan *et al.,* 2009) but our results were not significant in this regard. A possible explanation is that comet assay measurements may reflect both individual repair ability and DNA damage level. Because the damage level measured is the result of the equilibrium between damage infliction and repair, a low damage level as assessed experimentally in an individual may be the result of an actual low number of lesions or of a high efficiency of repair (Wojewódzka *et al.,* 1999).

No associations between genotoxicity assessment and alcohol consumptions were found in what concerns biomarkers measured by CBMN assay (chapter 2 of results, 4. demographic and lifestyle habits). However, a positive association in the exposed group was found, indicating that alcohol consumption is associated with higher DNA damage. The study from Manikantan *et al.* (2009) showed that alcoholic users had a significantly greater amount of DNA damage than non-users in the exposed group. Ethanol is mutagenic via its first-metabolite, acetaldehyde, the latter being associated with chromosomal aberrations, sister-chromatid exchanges and cross-links between DNA strands (Obe & Ristow, 1979). However, Philips (2001) found no significant evidence that ethanol is a genotoxic hazard according to the criteria normally applied for the purpose of classification and labelling of industrial chemicals.

4. INDIVIDUAL SUSCEPTIBILITY

OGG1 is considered to be the main enzyme responsible for the removal of 8-OHdG in humans, believed to play an important role in carcinogenesis because it is abundant and highly mutagenic. Epidemiological studies have previously related the Ser326Cys on *OGG1* polymorphism and the risk of different types of cancer, namely esophageal (Xing *et al.,* 2001), orolaryngeal (Elahi *et al.*, 2002) , lung cancer (Hu & Ahrendt, 2005), larynx colon (Pawlowska *et al.*, 2009), colon cancer (Kim *et al.,* 2003), and gastric cancer (Tekezaki *et al.,* 2002).

The genetic polymorphism of *OGG1* at codon 326 was shown to encode serine and cystein aminoacids showing OGG1 Ser/Ser higher repair activity toward 8-OHdG than the OGG1 Cys/Cys (Chen *et al.*, 2003). In general, our results (chapter 2 of results, 5.1. *OGG1* Ser326Cys polymorphisms) did not show a consistent trend regarding the variation of biomarkers with *OGG1* polymorphism, and there were not statistical significant results. However, in what concerns micronuclei, the Ser/Ser genotype carriers presented higher means of that genotoxicity biomarker, unlike the study by Chen *et al.* (2003). Mateuca *et al.,* (2008) observed also significantly lower micronuclei frequencies in carriers of the Cys/Cys genotype compared to the wild-type Ser/Ser carriers, being these result in line with our findings. Hu &

Ahrendt (2005) verified that Ser/Ser genotype was significantly associated with an increase in the frequency of *p53* mutations among patients with non small cell lung cancer.

As for the comet assay, the association obtained, although not statistically significant, was that Cys/Cys carriers presented higher levels of oxidative DNA damage in the control group. However, that association was not observed in the exposed group, where the Cys/Cys genotype revealed the lower results due to oxidative damage. Many studies reported that the DNA repair activity of the mutant *OGG1* Cys/Cys protein is lower than that of the wild-type Ser/Ser protein being 8-OHdG levels in lymphocyte DNA significantly higher in homozygotes for the Cys326 allele than for individuals with other *OGG1* genotypes.

Aka *et al.* (2004) and Pawlowska *et al.* (2009) verified that Cys/Cys and Ser/Cys *OGG1* genotypes have less DNA repair capacity compared to the Ser/Ser *OGG1* genotype. Kohno *et al.* (1998) reported that mean 8-OHdG levels were similar in peripheral leukocytes expressing either Ser/Ser or Cys/Cys.

These results suggest that the effect of Ser326Cys polymorphisms on DNA repair capacity may differ with the type and strength of the DNA-damaging exposures and may be influenced by the interaction between the *OGG1* polymorphism and other genetic polymorphisms (Mateuca *et al.,* 2008). The polymorphism of the *OGG1* gene is worth investigation, inasmuch as a population with decreased enzyme activity of the OGG1 protein would be at risk of accumulating 8-OHdG in nuclear DNA, because of the incomplete repair of oxidatively damaged DNA (Tarng *et al.,* 2001). It is important to refer that the codon 326 polymorphism may be in linkage disequilibrium with other functional polymorphisms in cancer-related genes (Hu & Ahrendt, 2005).

Once again, the small size of our sample hampered the finding of a possible association, let alone a causality relationship.

5. MICRONUTRIENTS

Micronutrient deficiency or excess can have modifying effects on genomic integrity. Our results showed significant correlations between serum vitamins A and E and the genotoxicity biomarkers studied (chapter 2 of results, 6.1. genotoxicity biomarkers and vitamins A and E in serum).

Vitamin A was positively correlated with nucleoplasmic bridges in the exposed group, and with nuclear buds and FPG in controls. As in the study by van Helden *et al.* (2009) on how beta-carotene affects oxidative stress-related DNA damage in lung epithelial cells, we have also found an association: vitamin A can act as pro-oxidant or antioxidant, depending on the type

of radicals involved, and may lead to DNA oxidative damage (Alakhras *et al.,* 2011). The study by van Helsen *et al.* (2009) demonstrated that vitamin A enhances OH radical formation in the Fenton reaction. Azqueta & Collins (2012) clearly distinguished between effects of vitamin A, pro-vitamin A carotenoids, and non-vitamin A carotenoids; being the latter group almost invariably reported to protect against DNA damage, whether endogenous or induced by exogenous agents, the pro-vitamin A carotenoids show a wider spectrum of effects, sometimes protecting and sometimes enhancing DNA damage.

Alakhras *et al.* (2011) investigated the genotoxicity of all-trans retinoic acid and its steroidal analogue EA-4, concluding that the retinoids affected chromosome orientation during metaphase by inducing bipolar metaphases with non-congressed genetic material, which may give rise to micronuclei.

Nucleoplasmic bridges occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase and frequently fail to segregate in an orderly manner, instead forming bridges between the two forming nucleus. Alakhras *et al.* (2011) concluded that retinoic acid exerts cytotoxic and genotoxic activities, the mechanism of its action being clastogenicity and also its ability to provoke chromosome delay by defects in microtubule network and mitotic spindle integrity. As previously mentioned, these results are conflicting with the findings by Gaziev *et al.* (1996), where vitamin A was reported to lower micronuclei frequencies.

Our results derived from the food frequency questionnaire, where retinol (vitamin A) was assess, found also a positive correlation between retinol and oxidative DNA damage (FPG).

Giovannelli *et al.* (2002) found that a high intake of selected antioxidants (vitamin A, C, E and β-carotene) tended to be positively associated with oxidative DNA damage, with a borderline statistical significance for vitamin E, in disagreement with several studies (mostly intervention studies) which showed a protective effect of antioxidants with respect to DNA damage. In what concerns serum vitamin E, our results showed a negative significant correlation with nucleoplasmic bridges in the exposed group. Most studies aimed at establishing an association between vitamin E and genotoxicity biomarkers measured by CBMN evaluated micronuclei. Some report a decrease of this biomarker after vitamin E supplementation (Schneider *et al.,* 2001), whereas others were quite inconclusive (Stich *et al.,* 1984a, 1984b; Benner *et al.,* 1994; Piyathilake *et al.,* 1995; Fenech *et al.,* 2005).

Dietary habits are recognized to be an important modifiable environmental factor influencing cancer risk and tumour behaviour. Our data gathered by the FFQ led to four significant positive correlations (chapter 2 of results, 6.2. genotoxicity biomarkers and micronutrients

measured by FFQ). Vitamin E was found to be positively correlated with % DNA in tail and micronuclei in mononuclear lymphocytes. Watters *et al.* (2007) also found a positive association of vitamin E and oxidative DNA damage in a healthy, non-smoking population of young adults. A possible explanation for this result stems from some evidence that in the presence of copper or in smokers with a fat rich diet, vitamin E can act as a strong pro-oxidant, nevertheless it remains an unexpected result. Duthie *et al.* (1996) found a positive correlation between DNA strand breaks and vitamin E, but the result was not interpreted as indicating any deleterious effect by the vitamin.

Both iron deficiency and excess are known to contribute to oxidative stress and DNA damage. Our results found a positive correlation between iron and DNA damage (% DNA in tail), meaning that higher intake of iron associates with higher DNA damage. Oxidative lesions, and more specifically 8-OHdG, is one of the most prevalent lesions induced by iron containing substances (Prá *et al.,* 2012), however the FPG biomarker was not statistically associated with iron. Iron can be mutagenic by different mechanisms, mostly involving base oxidation. There is sound evidence that iron deficiency increases genome instability, among other mechanisms, by impairing enzymes involved in antioxidant and nuclei acid metabolism (Prá *et al.,* 2012). Being a transition metal, iron and its ionic forms are prone to participate in one-electron transfer reactions. This capacity also enables iron to generate free radicals. Under conditions of increased hydrogen peroxide production by activated phagocytes in the presence of Fe^{2+}, very reactive hydroxyl radicals may be formed via the Fenton reaction. This can potentiate oxidative DNA damage (Tarng *et al.,* 2001).

Our results found that the amount of calories ingested was negatively correlated with both biomarkers assessed by comet assay, namely % DNA in tail and FPG. This was somewhat unexpected, as calorie restriction reduces metabolic rate and oxidative stress, meaning that lower calories ingestion decreases DNA damage and DNA oxidative damage (Hart *et al.,* 1999; Heilbronn & Ravussin, 2003).

A significant negative correlation was found between folate and % DNA in tail. Courtemanche *et al.* (2004) also found that folate deficiency leads to increased DNA damage in primary lymphocytes, and that deficiency in the physiological level of folate caused more DNA damage than low-dose radiation in primary T lymphocytes. On the contrary, Kapiszewska *et al.* (2005) found no correlation between (serum) folate levels and strand breaks measured using standard alkaline comet assay. Folate deficiency is associated with the expression of chromosomal fragile sites, chromosome breaks, micronucleus formation and mitochondrial DNA deletions (Duthie & Hawdon, 1998; Fenech, 2001; Fergurson & Fenech, 2012), increased

DNA strand breakage, misincorporated uracil, and reduced DNA repair efficiency (Duthie & McMillan, 1997; Duthie & Hawdon, 1998); however, no correlations were found between folate and micronuclei, as in the results of (Fenech & Rinaldi, 1998).

We have found a significant negative correlation between vitamin B12 and DNA oxidative damage (FPG), suggesting that vitamin B12 acts like a protective factor (Ames, 2001; Ames & Wakimoto, 2002; Ames, 2006). Minnet *et al.* (2011) also found a negative correlation between DNA damage and vitamin B12 levels, meaning that higher levels of vitamin B12 decrease DNA damage, in good agreement with our results. However, our findings are specifically on DNA oxidative damage and not DNA damage measured by alkaline comet assay for strand breaks. Milic *et al.* (2010) did not find correlations between folate and vitamin B12, on the one hand, and DNA damage measured by comet assay on the other hand; however vitamin B12 showed a significant positive correlation with micronuclei, such as in the Everson *et al.* (1988) study where blood cell micronuclei were elevated in subjects with low levels of blood folate and vitamin B12. Other studies (Glória *et al.*, 1997; Fenech *et al.* 1998, Fenech 1999) found significant negative correlations between serum vitamin B12 baseline levels and micronuclei frequency. As for micronuclei, our study did not find any significant correlation with vitamin B12.

Comet assay allows for the study of the effects of nutrients with known anti- or pro-oxidant capacities on different cell types and in different concentrations. These studies have revealed an apparent paradox, or at least an hormetic effect, whereby many of these antioxidant compounds seem to protect against DNA damage at low doses while actually causing DNA damage at higher doses (Wasson *et al.*, 2008). There are several possible reasons why significant associations are difficult to find. First, samples usually comprise mostly healthy persons; second, it is possible that a synergistic effect exists involving all antioxidants which is not seen for each individual nutrient (Watters *et al.*, 2007). Third, it is plausible that associations between some of the antioxidants examined and oxidative DNA damage may be better captured using other measures of oxidative DNA damage. Fourth, it is possible that the range of antioxidant concentrations and/or oxidative DNA damage in this study was not wide enough to detect associations or that the associations simply do not exist (Watters *et al.,* 2007). Previous studies have suggested a significant moderating effect of long-term antioxidant supplementation on endogenous and exogenous oxidative DNA damage in lymphocytes, supporting the hypothesis that dietary antioxidants may protect against cancer. The ability to demonstrate such clear differences between subjects on placebo and on

supplements confirms that the comet assay is a useful tool for screening populations for genotoxic effects (Duthie *et al.,* 1996).

In conclusion, the results of this study confirm that the alkaline comet assay is applicable for detection of genotoxic effects induced *in vivo* by occupational exposure to various mutagens. The relative simplicity and rapidity of the method, combined with the important practical factor that few cells are required for the analysis, makes it attractive for biomonitoring purposes in human populations (Kopjar & Garaj-Vrhovac, 2001). As genotoxicity may be due to combined effects of all or some of the antineoplastic drugs, it is usually not possible to attribute the damage to any particular agent (Rekhadevi *et al.,* 2007). The results obtained in our investigation, as well as studies by other authors, suggest that genotoxic damage is likely to occur in workers occupationally handling antineoplastic drugs. The professionals who are continuously exposed to these agents need to be monitored for risk behaviour, so that such hazardous compounds are properly managed by hospital staff (Rombaldi *et al.,* 2009).

We failed to find significant correlations between measurements by HPLC and FFQ. There are various possible explanations for lack of correlation between the measurement of vitamins A and E in serum by HPLC, a highly sensitive method, and the assessment of the intake values of these vitamins by FFQ. For instance, the FFQ was self-administered, increasing the degree of subjectivity to which questionnaires are usually prone. Other possible explanation is that the FFQ assesses the intake of vitamins based on the food items selected and their doses; the HPLC, on the other hand, measures vitamins in serum after metabolic processes took place, and the bioavailability of the vitamins differs since their intake. Both methods are valid, and widely used in nutritional research.

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

1. CONCLUSIONS

Molecular epidemiology and laboratory methods, used in close association, turn out to be a powerful tool in the investigation of cancer related epidemiological questions.

The use of biomarkers, in particular, allow for the identification of environmental exposures related to lifestyle, occupation, or ambient pollution, which are prone to promote cancer development (Vainio, 1998; Portier & Bell, 1999; Bartsch, 2000; Dusinska & Collins, 2008; Spitz & Bondy, 2010). When developing a human biomonitoring programme, it is important to use a combination of different biomarkers and to study a range of variables that can act as confounders and influence the evidence of risk from carcinogenic exposure, namely ethnicity, age or stage of development, gender, pre-existing health impairment and nutritional factors. Also, individual susceptibility should not be discarded in biomonitoring endeavours (Perera, 2000; Perera & Weinstein, 2000; Weis *et al.,* 2005).

Biomarkers supply information on the various stages of the multistep process towards putative disease. Markers of exposure have an important role in environmental and occupational health; some reflect progression along a causal pathway to disease, and others reflect innate or acquired susceptibility to the effects of etiological agents. If unequivocal biomarkers of effects and biomarkers of susceptibility could be developed, the identification of groups of individuals at increased risk would then be possible and very helpful to preventive medicine (Vainio, 1998).

The present study simulated a human biomonitoring programme in two different occupational settings, using biomarkers to assess genotoxic damage, and investigating possible associations to individual susceptibility and dietary habits.

For both occupational settings – workers exposed to formaldehyde and workers exposed to cytostatics drugs – there was strong evidence for an increase of genotoxicity biomarkers in association with occupational exposure, revealing that both types of chemical agents studied promoted DNA damage. The DNA damage detected could have been caused cumulatively, as previously explained, and is a clear indicator of exposure to the chemical. Our results suggest that the safety measures adopted by workers at both occupational settings were not enough to avoid exposure/contamination and to prevent the health consequences of handling chemical agents.

Age, gender, tobacco and alcohol habits are important variables that should be included in a biomonitoring study we found evidence that some of these variables can have an influence on the biomarkers studied, however not as dramatically as exposure to the xenobiotics in study.

In order to cope with confounding factors, a biomonitoring programme is also to assess susceptibility biomarkers and dietary habits. We provide here some evidence for the putative role of these variables but, like behavioural habits, their association to genotoxic biomarkers seems more complex and controversial than the genotoxic drugs by themselves.
In general, our findings address the need for regular biomonitoring of personnel occupationally exposed to formaldehyde and cytostatics. Although biological monitoring in many circumstances is complicated with assay sensitivity, costs and interpretation of results, it should be a necessary requirement at least in cases of accidental exposure (Kopjar *et al.,* 2009). The risk to health is influenced by the extent of the exposure and the potency and toxicity of the hazardous. To provide maximum protection to workers, employers should assure that workers implement safety procedures and use proper protective equipment for handling hazardous drugs (NIOSH, 2004).

Given the constant introduction of an increasing variety of chemical and physical agents in occupational settings, it is reasonable to anticipate that the risks for workers will be even higher in the future. However, the spectrum and specificity of biomarkers available also increased considerably in the recent years, allowing for a more efficient and accurate prediction of individual risk (Kopjar *et al.,* 2009). To our knowledge, the current practice in Portuguese hospitals does not include regular monitoring of the workplace, but to ensure maximal occupational safety for those daily exposed, periodic biomonitoring is recommended.
Biomonitoring studies will continue to build an understanding of the consequences of people's exposure to toxic environmental chemicals. Nonetheless these data will not obviate the need to collect other kinds of relevant information – to monitor sources of pollution, to conduct surveys of toxic substances in the environment, and to study human activities and behaviours that contribute to exposure. Moreover, further research in toxicology and epidemiology is necessary before specialists can interpret the health significance of exposure biomarkers for the majority of environmental chemicals. As detection methods improve – enabling investigators to measure lower concentrations of more chemicals from smaller samples at less cost – scientific understanding of what the body does to the chemical (and vice versa) must keep pace with new environmental challenges. If this effort is successful, a full screen of exposure biomarkers may be a part of every routine physical exam in the not-too-distant future (Sexton *et al.,* 2004). A challenge for toxicology is to utilize the available knowledge of the extensive genetic variation in individual susceptibility by integrating it in the evaluation of exposure related risk (Mohrenweiser, 2004).

The main conclusion of this investigation is that it is possible and necessary to implement human biomonitoring programmes in occupational exposure contexts. The biomonitoring programme should include biomarkers of exposure, effect, and susceptibility. Known possible confounding factors should also be addressed, namely diet and habits, into a whole integrated package aimed at preventing disease.

2. LIMITATIONS

Common limitations are sample size, usually too small to evaluate rare polymorphisms, and the wide range of allele frequency variation for each *loci* in different ethnic populations. The statistical analysis is often plagued with problems of lack of power (due to insufficient sample size) and confounding can seldom be precluded given the amount of potential factors involved that have not been measured (Hunter, 2005; Chung *et al.,* 2010).

The self-reported dietary data used here was subject to both random and systematic bias and because blood was collected at only one point in time, seasonal variability in antioxidant intakes could not be assessed. Nonetheless, the results using self-reported and biological measures of diet were comparable, and the coefficients of variation for the laboratory assays were within acceptable standards.

The capacity for DNA repair activity was not measured; thus these estimates represent the oxidative DNA damage level only at time of collection.

Although several covariates were controlled, residual confounding is still a concern. The fact that our study population consisted of generally healthy volunteers, may limit generalization. Finally, due to the cross-sectional nature of this study, it was not possible to examine changes in oxidative DNA damage over time and no conclusions concerning causality could be drawn.

3. FUTURE PERSPECTIVES

Molecular biomonitoring provides an opportunity to address certain issues of exposure, susceptibility, and risk in diet-associated human carcinogenesis (Strickland & Goopman, 1995). Molecular epidemiologic approaches, coupled with bioinformatics, will provide important evidence for the role of specific dietary mutagens in certain human cancers. Optimizing nutritional approaches towards the reduction of mutagenesis will require the innovative application of many of the newer technologies which are becoming available. Nutrition and food carcinogens continue to be a most challenging subject for research towards cancer

control (Sugimura, 2000). The interaction between diet and risk of cancer is a hugely important research area, and even a brief review of the literature shows that there are thousands of foodstuffs, micronutrients, phytochemicals and other dietary factors being investigated for their effect on carcinogenesis. Yet in many cases, the molecular mechanisms through which various nutrients might enhance or protect against carcinogenesis are still unknown. In this respect, the development and optimization of biomarkers suitable for use to investigate the molecular effects of dietary factors in human trials, animal studies and *in vitro* studies is of great importance (Ferguson & Philpott, 2008; Wasson *et al.,* 2008).

Although it is not possible to entirely avoid mutagenic food components or certain dietary regimes, rational developments of antimutagens as chemopreventive agents, coupled with technologies appropriate to nutrigenomics, lead to an optimistic outlook for the future drawing of personalized nutrition plans, aimed at protecting against diet-related mutagenesis (Ferguson & Philpott, 2008).

Prevention of DNA damage and/or enhanced DNA repair activity by dietary agents constitutes an important strategy to prevent mutations and consequently inhibit the carcinogenic process (Ramos *et al.,* 2011). With more research, we will develop a better understanding of the mechanisms by which specific micronutrients regulate normal cell function, and how their deficiencies can alter normal metabolism. “Tuning-up” human metabolism, which varies with genetic constitution and changes with age, could prove to be a simple and inexpensive way to minimize DNA damage, prevent cancer, improve health and prolong a healthy lifespan (Ames & Wakimoto, 2002).

VII. References

Afman, L. & Müller, M. (2006). Nutrigenomics: from molecular nutrition to prevention of disease. *Journal of the American Dietetic Association*. 106 (4). p.pp. 569–576.

Aka, P., Mateuca, R., Buchet, J.P., Thierens, H. & Kirsch-Volders, M. (2004). Are genetic polymorphisms in OGG1, XRCC1 and XRCC3 genes predictive for the DNA strand break repair phenotype and genotoxicity in workers exposed to low dose ionising radiations? *Mutation Research* 556 (1-2) p.pp. 169 – 181.

Alakhras, R.S., Stephanou, G., Demopoulos, N.A. & Nikolaropoulos, S.S. (2011). Genotoxicity of all-trans retinoic acid (ATRA) and its steroidal analogue EA-4 in human lymphocytes and mouse cells in vitro. *Cancer Lett*ers. 306 (1). p.pp. 15 – 26.

Albertini, R.J., Nicklas, J.A. & O'Neill, J.P. (1996). Future research directions for evaluating human genetic and cancer risk from environmental exposures. *Environmental Health Perspectives*. 104 (Suppl 3). p.pp. 503–510.

Al-Daghri, N.M., Al-Attas, O., Alokail, M.S., Alkharfy, K.M., Draz, H.M., Agliardi, C., Mohammed, A.K., Guerini, F.R. & Clerici, M. (2012). Vitamin D Receptor Gene Polymorphisms and HLA DRB1*04 Cosegregation in Saudi Type 2 Diabetes Patients. *The Journal of Immunology*. 188 (3). p.pp. 1325–1332.

Al-Sharif, M. (2012). Studies on the Genotoxic Effects of Anticancer Drug Paclitaxel (Taxol) in Mice. *World Applied Sciences Journal*. 16 (7). p.pp. 989 – 997.

Ames, B.N. (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science (New York, N.Y.)*. 221 (4617). p.pp. 1256–1264.

Ames, B.N. (1998). Micronutrients prevent cancer and delay aging. *Toxicology letters*. 102-103. p.pp. 5–18.

Ames, B.N. (2001). DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutation research*. 475 (1-2). p.pp. 7–20.

Ames, B.N. (2004). Supplements and Tuning Up Metabolism. *The Journal of Nutrition*. 134 (11). p.p. 3164S–3168S.

Ames, B.N. (2006). Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage. *Proceedings of the National Academy of Sciences of the United States of America*. 103 (47). p.pp. 17589–17594.

Ames, B.N. & Wakimoto, P. (2002). Are vitamin and mineral deficiencies a major cancer risk? *Nature reviews. Cancer*. 2 (9). p.pp. 694–704.

Anand, P., Kunnumakkara, A.B., Kunnumakara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B. & Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical research*. 25 (9). p.pp. 2097–2116.

Angerer, J., Ewers, U. & Wilhelm, M. (2007). Human biomonitoring: state of the art. *International journal of hygiene and environmental health*. 210 (3-4). p.pp. 201–228.

Anon (1995). Guiding principles for the use of biological markers in the assessment of human exposure to environmental factors: an integrative approach of epidemiology and toxicology. Report on a WHO consultation. *WHO Regional Office for Europe.* 101 (1-2). p.pp. 1–10.

Araujo, F.D., Pierce, A.J., Stark, J.M. & Jasin, M. (2002). Variant XRCC3 implicated in cancer is functional in homology-directed repair of double-strand breaks. *Oncogene*. 21 (26). p.pp. 4176–4180.

Arsoy, N.S. Neuss S., Wessendorf S., Bommer, M., Viardot, A., Schütz, P. & Speit, G. (2009). Micronuclei in peripheral blood from patients after cytostatic therapy mainly arise ex vivo from persistent damage. *Mutagenesis.* 24 (4). p.pp 351-357.

Au, W.W. (2007). Usefulness of biomarkers in population studies: From exposure to susceptibility and to prediction of cancer. *International Journal of Hygiene and Environmental Health*. 210 (3-4). p.pp. 239–246.

Au, W.W., Cajas-Salazar, N. & Salama, S. (1998). Factors contributing to discrepancies in population monitoring studies. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 400 (1). p.pp. 467–478.

Au, W.W., Navasumrit, P. & Ruchirawat, M. (2004). Use of biomarkers to characterize functions of polymorphic DNA repair genotypes. *International journal of hygiene and environmental health*. 207 (4). p.pp. 301–313.

Awodele, O., Olayemi, S.O., Alimba, C.G., Egbejiogu, C. & Akintonwa, A. (2010). Protective effect of vitamin C and or vitamin E on micronuclei induction by rifampicin in mice. *Tanzania Journal of Health Research*. 12 (2). p.pp. 150–154.

Ayyad, S.B.A., Israel, E., El-Setouhy, M., Nasr, G.R., Mohamed, M.K. & Loffredo, C.A. (2006). Evaluation of Papanicolaou stain for studying micronuclei in buccal cells under field conditions. *Acta cytologica*. 50 (4). p.pp. 398–402.

Azqueta, A. & Collins, A.R. (2012). Carotenoids and DNA damage. *Mutation Research*. 733 (1-2). p.pp. 4–13.

Azqueta A., Shaposhnikov S. & Collins A. (2009). Detection of oxidised DNA using DNA repair enzymes. In: *The Comet Assay in Toxicology*. [Online]. Royal Society of Chemistry, pp. 58–63. Available from: www.rsc.org.

Azqueta, A., Meier, S., Priestley, C., Gutzkow, K.B., Brunborg, G., Sallette, J., Soussaline, F. & Collins, A. (2011). The influence of scoring method on variability in results obtained with the comet assay. *Mutagenesis*. 26 (3). p.pp. 393–399.

Baan, R., Grosse, Y., Straif, K., Secretan, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Freeman, C., Galichet, L. & Cogliano, V. (2009). A review of human carcinogens—Part F: Chemical agents and related occupations. *The Lancet Oncology*. 10 (12). p.pp. 1143–1144.

Baghurst, K. & Record, S. (1984). A computerised dietary analysis system for use with diet diaries on food freuqency questionnaires. *Australian and New Zealand Journal of Public Health.* 1(8). P.pp. 11 – 18.

Bajic, V., Stanimirovic, Z., Stevanovic, J., Milicevic, Z., Zivkovic, L. & Spremo-Potparevic, B. (2010). The effect of paclitaxel alone and in combination with cycloheximide on the frequency of premature centromere division in vitro. *Archives of Biological Sciences*. 62 (1). p.pp. 63–74.

Bajpayee, M., Dhawan, A., Parmar, D., Pandey, A.K., Mathur, N. & Seth, P.K. (2002). Gender-related differences in basal DNA damage in lymphocytes of a healthy Indian population using the alkaline Comet assay. *Mutation Research*. 520 (1-2). p.pp. 83–91.

Bao, Y., Ng, K., Wolpin, B.M., Michaud, D.S., Giovannucci, E. & Fuchs, C.S. (2010). Predicted vitamin D status and pancreatic cancer risk in two prospective cohort studies. *British Journal of Cancer*. 102 (9). p.pp. 1422–1427.

Barrett, J.C., Vainio, H., Peakall, D. & Goldstein, B.D. (1997). 12th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals: susceptibility to environmental hazards. *Environmental HealthPperspectives*. 105 Suppl 4. p.pp. 699–737.

Bartsch, H. (2000). Studies on biomarkers in cancer etiology and prevention: a summary and challenge of 20 years of interdisciplinary research. *Mutation Research-Reviews in Mutation Research*. 462 (2-3). p.pp. 255–279.

Bartsch, H. & Gerhäuser, C. (2009). Molecular Mechanisms of Cancer Induction and Chemoprevention. In: S. Knasmüller, D. M. DeMarini, I. Johnson, & C. Gerhäuser (eds.). *Chemoprevention of Cancer and DNA Damage by Dietary Factors*. [Online]. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 1–19.

Battershill, J.M., Burnett, K. & Bull, S. (2008). Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. *Mutagenesis*. 23 (6). p.pp. 423–437.

Bayat, Nejatpour & Mahdizadeu (2011). Theoretical study on physicochemical properties of paclitaxel and its complexes in order to understanding of the biological and anti cancer activities of them. *J. Chem. Pharm. Res*. (3). p.pp. 940 – 946.

Beilby, J., Ambrosini, G.L., Rossi, E., de Klerk, N.H. & Musk, A.W. (2010). Serum levels of folate, lycopene, β-carotene, retinol and vitamin E and prostate cancer risk. *European journal of clinical nutrition*. 64 (10). p.pp. 1235–1238.

Bender, D. (2006). The antioxidant paradox: damage and defence. *The Biochemist*. p.pp. 9 – 12.

Benhamou, S., Tuimala, J., Bouchardy, C., Dayer, P., Sarasin, A. & Hirvonen, A. (2004). DNA repair gene XRCC2 and XRCC3 polymorphisms and susceptibility to cancers of the upper aerodigestive tract. *International Journal of Cancer. Journal International Du Cancer*. 112 (5). p.pp. 901–904.

Benner, S.E., Wargovich, M.J., Lippman, S.M., Fisher, R., Velasco, M., Winn, R.J. & Hong, W.K. (1994). Reduction in oral mucosa micronuclei frequency following alpha-tocopherol treatment of oral leukoplakia. *Cancer Epidemiology Biomarkers & Prevention*. 3 (1). p.pp. 73–76.

Bertazzi, P.A. & Mutti, A. (2008). Biomarkers, Disease Mechanisms and their Role in Regulatory Decisions. In: C. Wild, P. Vineis, & S. Garte (eds.). *Molecular Epidemiology of Chronic Diseases*. [Online]. John Wiley & Sons, Ltd, pp. 243–254.

Berwick, M. & Albertini, R.J. (2008). Biomarkers of Mutation and DNA Repair Capacity. In: C. Wild, P. Vineis, & S. Garte (eds.). *Molecular Epidemiology of Chronic Diseases*. [Online]. John Wiley & Sons, Ltd, pp. 127–139.

Berwick, M. & Vineis, P. (2000). Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. *Journal of the National Cancer Institute*. 92 (11). p.pp. 874–897.

Blasiak, J., Kowalik, J., Małecka-Panas, E., Drzewoski, J. & Wojewódzka, M. (2000). DNA damage and repair in human lymphocytes exposed to three anticancer platinum drugs. *Teratogenesis, Carcinogenesis, and Mutagenesis*. 20 (3). p.pp. 119–131.

Boiteux, S. & Radicella, J.P. (1999). Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress. *Biochimie*. 81 (1-2). p.pp. 59–67.

Bolognesi, C., Knasmueller, S., Nersesyan, A., Thomas, P. & Fenech, M. (2013). The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay - an update and expanded photogallery. *Mutation Research*. 753 (2). p.pp. 100–113.

Bolognesi, C., Lando, C., Forni, A., Landini, E., Scarpato, R., Migliore, L. & Bonassi, S. (1999). Chromosomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. *Age and Ageing*. 28 (4). p.pp. 393–397.

Bolt, H.M., Degen, G.H. & Hengstler, J.G. (2010). The carcinogenicity debate on formaldehyde: How to derive safe exposure limits? *Archives of Toxicology*. 84 (6). p.pp. 421–422.

Bonassi, S. & Au, W.W. (2002). Biomarkers in molecular epidemiology studies for health risk prediction. *Mutation Research*. 511 (1). p.pp. 73–86.

Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., Kirsh-Volders, M., Knasmueller, S., Zeiger, E., Carnesoltas, D., Cavallo, D., da Silva, J., de Andrade, V.M., Demircigil, G.C., Odio, A.D., Donmez-Altuntas, H., Gattas, G., Giri, A., Giri, S., Gómez-Meda, B., Gómez-Arroyo, S., Hadjidekova, V., Haveric, A., Kamboj, M., Kurteshi, K., Martino-Roth, M.G., Montoya, R.M., Nersesyan, A., Pastor-Benito, S., Salvadori, D.M.F., Shaposhnikova, A., Stopper, H., Thomas, P., Torres-Bugarín, O., Yadav, A.S., González, G.Z. & Fenech, M. (2011). The HUman MicroNucleus project on eXfoLiated buccal cells (HUMNXL): The role of life-style, host factors, occupational exposures, health status, and assay protocol. *Mutation Research/Reviews in Mutation Research*. 728 (3). p.pp. 88–97.

Bonassi, S., Fenech, M., Lando, C., Lin, Y.P., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L.R., Fucic, A., Lima, O.G., Hrelia, P., Krishnaja, A.P., Lee, T.K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Müller, W.U., Odagiri, Y., Scarffi, M.R., Szabova, E., Vorobtsova, I., Vral, A. & Zijno, A. (2001). HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environmental and Molecular Mutagenesis*. 37 (1). p.pp. 31–45.

Bonassi, S., Neri, M., Lando, C., Ceppi, M., Lin, Y., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Fenech, M. & HUMN collaborative group (2003). Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. *Mutation Research*. 543 (2). p.pp. 155–166.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H. & Fenech, M. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*. 28 (3). p.pp. 625–631.

Bosetti, C., McLaughlin, J.K., Tarone, R.E., Pira, E. & Vecchia, C.L. (2008). Formaldehyde and cancer risk: a quantitative review of cohort studies through 2006. *Annals of Oncology*. 19 (1). p.pp. 29–43.

Bouraoui, S., Brahem, A., Tabka, F., Mrizek, N., Saad, A. & Elghezal, H. (2011). Assessment of chromosomal aberrations, micronuclei and proliferation rate index in peripheral lymphocytes from Tunisian nurses handling cytotoxic drugs. *Environmental Toxicology and Pharmacology*. 31 (1). p.pp. 250–257.

Branham, M.T., Nadin, S.B., Vargas-Roiq, L.M. & Ciocca, D.R. (2004). DNA damage induced by paclitaxel and DNA repair capability of peripheral blood lymphocytes as evaluated by the alkaline comet assay. *Mutation Research*. 560 (1). p.pp. 11 – 17.

Brenneman, M.A., Weiss, A.E., Nickoloff, J.A. & Chen, D.J. (2000). XRCC3 is required for efficient repair of chromosome breaks by homologous recombination. *Mutation Research*. 459 (2). p.pp. 89–97.

Brewer, M. s. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety*. 10 (4). p.pp. 221–247.

Brooks, L.D. (2003). SNPs: Why Do We Care? In: P.-Y. K. MD (ed.). *Single Nucleotide Polymorphisms*. Methods in Molecular Biology™. [Online]. Springer New York, pp. 1–14.

Bull, C. & Fenech, M. (2008). Genome-health nutrigenomics and nutrigenetics: nutritional requirements or 'nutriomes' for chromosomal stability and telomere maintenance at the individual level. *The Proceedings of the Nutrition Society*. 67 (2). p.pp. 146–156.

Burgaz, S., Erdem, O., Cakmak, G., Erdem, N., Karakaya, A. & Karakaya, A.E. (2002). Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. *Biomarkers: biochemical indicators of exposure, response, and susceptibility to chemicals*. 7 (2). p.pp. 151–161.

Burgaz, S., Karahalil, B., Bayrak, P., Taşkin, L., Yavuzaslan, F., Bökesoy, I., Anzion, R.B., Bos, R.P. & Platin, N. (1999). Urinary cyclophosphamide excretion and micronuclei frequencies in peripheral lymphocytes and in exfoliated buccal epithelial cells of nurses handling antineoplastics. *Mutation Research*. 439 (1). p.pp. 97–104.

Buschini, A., Villarini, M., Feretti, D., Mussi, F., Dominici, L., Zerbini, I., Moretti, M., Ceretti, E., Bonfiglioli, R., Carrieri, M., Gelatti, U., Rossi, C., Monarca, S. & Poli, P. (2013). Multicentre study for the evaluation of mutagenic/carcinogenic risk in nurses exposed to antineoplastic drugs: assessment of DNA damage. *Occupational and Environmental Medicine*. 70 (11). p.pp. 789–794.

Carmona, B., Guerreiro, C., Cravo, M., Nobre-Leitão, C. & Brito, M. (2008). 5' and 3' UTR thymidylate synthase polymorphisms modulate the risk of colorectal cancer independently of the intake of methyl group donors. *Molecular Medicine Reports*. 1 (5). p.pp. 747–752.

Castiglia, L., Miraglia, N., Pieri, M., Simonelli, A., Basilicata, P., Genovese, G., Guadagni, R., Acampora, A., Sannolo, N. & Scafarto, M.V. (2008). Evaluation of occupational exposure to antiblastic drugs in an Italian hospital oncological department. *Journal of Occupational Health*. 50 (1). p.pp. 48–56.

Catalán, J., Autio, K., Kuosma, E. & Norppa, H. (1998). Age-dependent inclusion of sex chromosomes in lymphocyte micronuclei of man. *American Journal of Human Genetics*. 63 (5). p.pp. 1464–1472.

Catalán, J., Falck, G.C. & Norppa, H. (2000a). The X chromosome frequently lags behind in female lymphocyte anaphase. *American Journal of Human Genetics*. 66 (2). p.pp. 687–691.

Catalán, J., Surrallés, J., Falck, G.C., Autio, K. & Norppa, H. (2000b). Segregation of sex chromosomes in human lymphocytes. *Mutagenesis*. 15 (3). p.pp. 251–255.

Cavallo, D., Ursini, C., Perniconi, B., Francesco, A., Giglio, M., Rubino, F., Marinaccio, A. & Iavicoli, S. (2005). Evaluation of genotoxic effects induced by exposure to antineoplastic drugs in lymphocytes and exfoliated buccal cells of oncology nurses and pharmacy employees. *Mutation Research* 587: 45 – 51.

Cavallo, D., Ursini, C.L., Omodeo-Salè, E. & Iavicoli, S. (2007). Micronucleus induction and FISH analysis in buccal cells and lymphocytes of nurses administering antineoplastic drugs. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 628 (1). p.pp. 11–18.

Cavallo, D., Ursini, C.L., Rondinone, B. & Iavicoli, S. (2009). Evaluation of a suitable DNA damage biomarker for human biomonitoring of exposed workers. *Environmental and Molecular Mutagenesis*. 50 (9). p.pp. 781–790.

Cerqueira, E. M. M. & Meireles, J. R. C. (2012). The use of the Micronucleus Test to monitoring individuals at risk for oral cancer. In: *The Research and Biology of Cancer*. Hong Kong: iConcept Press Ltd.

Cheng, T.-Y.D. & Neuhouser, M.L. (2012). Serum 25-hydroxyvitamin D, vitamin A, and lung cancer mortality in the US population: a potential nutrient-nutrient interaction. *Cancer Causes & Control: CCC*. 23 (9). p.pp. 1557–1565.

Chen, S.-K., Hsieh, W.A., Tsai, M.-H., Chen, C.-C., Hong, A.I., Wei, Y.-H. & Chang, W.P. (2003). Age-associated decrease of oxidative repair enzymes, human 8-oxoguanine DNA glycosylases (hOgg1), in human aging. *Journal of Radiation Research*. 44 (1). p.pp. 31–35.

Chung, C.C., Magalhaes, W.C.S., Gonzalez-Bosquet, J. & Chanock, S.J. (2010). Genome-wide association studies in cancer current and future directions. *Carcinogenesis*. 31 (1). p.pp. 111–120.

Chu, W.C., Hon, C.-Y., Danyluk, Q., Chua, P.P. & Astrakianakis, G. (2011). Pilot assessment of the antineoplastic drug contamination levels in British Columbian hospitals pre- and post-cleaning. *Journal of Oncology Pharmacy Practice*. 18 (1). p.pp. 46–51.

Claycombe, K.J. & Meydani, S.N. (2001). Vitamin E and genome stability. *Mutation Research*. 475 (1-2). p.pp. 37–44.

Cole, B.F., Baron, J.A., Sandler, R.S., Haile, R.W., Ahnen, D.J., Bresalier, R.S., McKeown-Eyssen, G., Summers, R.W., Rothstein, R.I., Burke, C.A., Snover, D.C., Church, T.R., Allen, J.I., Robertson, D.J., Beck, G.J., Bond, J.H., Byers, T., Mandel, J.S., Mott, L.A., Pearson, L.H., Barry, E.L., Rees, J.R., Marcon, N., Saibil, F., Ueland, P.M., Greenberg, E.R. & Polyp Prevention Study Group (2007). Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA: the journal of the American Medical Association*. 297 (21). p.pp. 2351–2359.

Collins, A.R. (1999). Oxidative DNA damage, antioxidants, and cancer. *BioEssays: news and reviews in molecular, cellular and developmental biology*. 21 (3). p.pp. 238–246.

Collins, A.R. (2002). The comet assay. Principles, applications, and limitations. *Methods in Molecular Biology (Clifton, N.J.)*. 203. p.pp. 163–177.

Collins, A.R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular biotechnology*. 26 (3). p.pp. 249–261.

Collins, A.R. (2009). Investigating oxidative DNA damage and its repair using the comet assay. *Mutation Research*. 681 (1). p.pp. 24–32.

Collins A. & Dusinska M. (2009). Applications of the Comet Assay in Human Biomonitoring. In: *The Comet Assay in Toxicology*. [Online]. Royal Society of Chemistry, pp. 201 – 202. Available from: www.rsc.org.

Collins, A.R., Dusinská, M., Horváthová, E., Munro, E., Savio, M. & Stětina, R. (2001). Inter-individual differences in repair of DNA base oxidation, measured *in vitro* with the comet assay. *Mutagenesis*. 16 (4). p.pp. 297–301.

Collins, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslová, K. & Vaughan, N. (1997). Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environmental and Molecular Mutagenesis*. 30 (2). p.pp. 139–146.

Collins, A.R. & Ferguson, L.R. (2004). Nutrition and carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 551 (1-2). p.pp. 1–8.

Collins, J.J. & Lineker, G.A. (2004). A review and meta-analysis of formaldehyde exposure and leukemia. *Regulatory Toxicology and Pharmacology*. 40 (2). p.pp. 81–91.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C. & Stetina, R. (2008). The comet assay: topical issues. *Mutagenesis*. 23 (3). p.pp. 143–151.

Conaway, C.C., Whysner, J., Verna, L.K. & Williams, G.M. (1996). Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. *Pharmacology & therapeutics*. 71 (1-2). p.pp. 29–55.

Connor, T.H. (2006). Hazardous anticancer drugs in health care: environmental exposure assessment. *Annals of the New York Academy of Sciences*. 1076. p.pp. 615–623.

Cornetta, T., Padua, L., Testa, A., Ievoli, E., Festa, F., Tranfo, G., Baccelliere, L. & Cozzi, R. (2008). Molecular biomonitoring of population of nurses handling antineoplastic drugs. *Mutation Research.* 638. p.pp. 75 – 82.

Cooke, M.S., Evans, M.D., Dizdaroglu, M. & Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 17 (10). p.pp. 1195–1214.

Cooke, M.S., Evans, M.D., Mistry, N. & Lunec, J. (2002). Role of dietary antioxidants in the prevention of *in vivo* oxidative DNA damage. *Nutrition Research Reviews*. 15 (1). p.pp. 19–42.

Cook, L.S., Neilson, H.K., Lorenzetti, D.L. & Lee, R.C. (2010). A systematic literature review of vitamin D and ovarian cancer. *American journal of obstetrics and gynecology*. 203 (1). p.pp. 70.e1–8.

Courtemanche, C., Huang, A.C., Elson-Schwab, I., Kerry, N., Ng, B.Y. & Ames, B.N. (2004). Folate deficiency and ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 18 (1). p.pp. 209–211.

Couto, E., Boffetta, P., Lagiou, P., Ferrari, P., Buckland, G., Overvad, K., Dahm, C.C., Tjønneland, A., Olsen, A., Clavel-Chapelon, F., Boutron-Ruault, M.-C., Cottet, V., Trichopoulos, D., Naska, A., Benetou, V., Kaaks, R., Rohrmann, S., Boeing, H., von Ruesten, A., Panico, S., Pala, V., Vineis, P., Palli, D., Tumino, R., May, A., Peeters, P.H., Bueno-de-Mesquita, H.B., Büchner, F.L., Lund, E., Skeie, G., Engeset, D., Gonzalez, C.A., Navarro, C., Rodríguez, L., Sánchez, M.-J., Amiano, P., Barricarte, A., Hallmans, G., Johansson, I., Manjer, J., Wirfärt, E., Allen, N.E., Crowe, F., Khaw, K.-T., Wareham, N., Moskal, A., Slimani, N., Jenab, M., Romaguera, D., Mouw, T., Norat, T., Riboli, E. & Trichopoulou, A. (2011). Mediterranean dietary pattern and cancer risk in the EPIC cohort. *British Journal of Cancer*. 104 (9). p.pp. 1493–1499.

Couto, E., Sandin, S., Löf, M., Ursin, G., Adami, H.-O. & Weiderpass, E. (2013). Mediterranean Dietary Pattern and Risk of Breast Cancer. *PLoS ONE*. 8 (2). p.p. e55374.

Crawford, D.C., Akey, D.T. & Nickerson, D.A. (2005). The patterns of natural variation in human genes. *Annual Review of Genomics and Human Genetics*. 6 (1). p.pp. 287–312.

Cui, Y. & Rohan, T.E. (2006). Vitamin D, calcium, and breast cancer risk: a review. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 15 (8). p.pp. 1427–1437.

Cunha, K.S., Reguly, M.L., Graf, U. & Andrade, H.H.R. de (2001). Taxanes: the genetic toxicity of paclitaxel and docetaxel in somatic cells of *Drosophila melanogaster*. *Mutagenesis*. 16 (1). p.pp. 79–84.

Davis, C.D. & Milner, J. (2004). Frontiers in nutrigenomics, proteomics, metabolomics and cancer prevention. *Mutation Research*. 551 (1-2). p.pp. 51–64.

Davis, C.D. & Milner, J.A. (2007). Biomarkers for diet and cancer prevention research: potentials and challenges. *Acta pharmacologica Sinica*. 28 (9). p.pp. 1262–1273.

Davis, J., McLauchlan, R. & Connor, T.H. (2011). Exposure to hazardous drugs in Healthcare: An issue that will not go away. *Journal of Oncology Pharmacy Practice*. 17 (1). p.pp. 9–13.

DeCaprio, A.P. (1997). Biomarkers: Coming of Age for Environmental Health and Risk Assessment. *Environmental Science & Technology*. 31 (7). p.pp. 1837–1848.

DeCaprio, A. (1999). Biomarkers of exposure and susceptibility. In: *General and Applied Toxicology*. Stockton Press.

Deeb, K.K., Trump, D.L. & Johnson, C.S. (2007). Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nature Reviews. Cancer*. 7 (9). p.pp. 684–700.

Deng, H., Zhang, M., He, J., Wu, W., Jin, L., Zheng, W., Lou, J. & Wang, B. (2005). Investigating genetic damage in workers occupationally exposed to methotrexate using three genetic endpoints. *Mutagenesis* 20, 351 – 357.

Denzer, N., Vogt, T. & Reichrath, J. (2011). Vitamin D receptor (VDR) polymorphisms and skin cancer. *Dermato-endocrinology*. 3 (3). p.pp. 205–210.

Dhillon, V.S., Thomas, P., Iarmarcovai, G., Kirsch-Volders, M., Bonassi, S. & Fenech, M. (2011). Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. *Mutagenesis*. 26 (1). p.pp. 33–42.

Diem, E., Ivancsits, S. & Rüdiger, H.W. (2002). Basal levels of DNA strand breaks in human leukocytes determined by comet assay. *Journal of Toxicology and Environmental Health. Part A*. 65 (9). p.pp. 641–648.

Digue, L., Orsière, T., De Méo, M., Mattéi, M.G., Depetris, D., Duffaud, F., Favre, R. & Botta, A. (1999). Evaluation of the genotoxic activity of paclitaxel by the in vitro micronucleus test in combination with fluorescent in situ hybridization of a DNA centromeric probe and the alkaline single cell gel electrophoresis technique (comet assay) in human T-lymphocytes. *Environmental and Molecular Mutagenesis*. 34 (4). p.pp. 269–278.

Dizdaroglu, M., Jaruga, P., Birincioglu, M. & Rodriguez, H. (2002). Free radical-induced damage to DNA: mechanisms and measurement. *Free Radical Biology & Medicine*. 32 (11). p.pp. 1102–1115.

Djuric, Z. & Kritschevsky, D. (1993). Modulation of oxidative DNA damage levels by dietary fat and calories. *Mutation Research*. 295 (4-6). p.pp. 181–190.

Dusinska, M. & Collins, A.R. (2008). The comet assay in human biomonitoring: gene–environment interactions. *Mutagenesis*. 23 (3). p.pp. 191–205.

Duthie, S.J. & Hawdon, A. (1998). DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes *in vitro*. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 12 (14). p.pp. 1491–1497.

Duthie, S.J., Ma, A., Ross, M.A. & Collins, A.R. (1996). Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Research*. 56 (6). p.pp. 1291–1295.

Duthie, S.J. & McMillan, P. (1997). Uracil misincorporation in human DNA detected using single cell gel electrophoresis. *Carcinogenesis*. 18 (9). p.pp. 1709–1714.

Duthie, S.J., Narayanan, S., Brand, G.M., Pirie, L. & Grant, G. (2002). Impact of Folate Deficiency on DNA Stability. *The Journal of Nutrition*. 132 (8). p.p. 2444S–2449S.

Elahi, A., Zheng, Z., Park, J., Eyring, K., McCaffrey, T. & Lazarus, P. (2002). The human OGG1 DNA repair enzyme and its association with orolaryngeal cancer risk. *Carcinogenesis*. 23 (7). p.pp. 1229–1234.

El-Ebiary, A.A., Abuelfadl, A.A. & Sarhan, N.I. (2013). Evaluation of genotoxicity induced by exposure to antineoplastic drugs in lymphocytes of oncology nurses and pharmacists. *Journal of Applied Toxicology*. 33 (3). p.pp. 196–201.

Elliott, R. & Ong, T.J. (2002). Nutritional genomics. *BMJ: British Medical Journal*. 324 (7351). p.pp. 1438–1442.

El-Zein, R.A., Fenech, M., Lopez, M.S., Spitz, M.R. & Etzel, C.J. (2008). Cytokinesis-blocked micronucleus cytome assay biomarkers identify lung cancer cases amongst smokers. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*. 17 (5). p.pp. 1111–1119.

El-Zein, R.A., Schabath, M.B., Etzel, C.J., Lopez, M.S., Franklin, J.D. & Spitz, M.R. (2006). Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Research*. 66 (12). p.pp. 6449–6456.

El-Zein, R., Vral, A. & Etzel, C.J. (2011). Cytokinesis-blocked micronucleus assay and cancer risk assessment. *Mutagenesis*. 26 (1). p.pp. 101–106.

Engeland, K., Höög, J.O., Holmquist, B., Estonius, M., Jörnvall, H. & Vallee, B.L. (1993). Mutation of Arg-115 of human class III alcohol dehydrogenase: a binding site required for formaldehyde dehydrogenase activity and fatty acid activation. *Proceedings of the National Academy of Sciences of the United States of America*. 90 (6). p.pp. 2491–2494.

Epstein, J.B. & Gorsky, M. (1999). Topical application of vitamin A to oral leukoplakia: A clinical case series. *Cancer*. 86 (6). p.pp. 921–927.

Ersson, C. (2011). *International validation of the comet assay and a human intervention study*. Stockholm: Karolinska Institutet.

European Standards Committee on Oxidative DNA Damage (ESCODD). (2003). Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free radical biology & medicine*. 34 (8). p.pp. 1089–1099.

Everson, R.B., Wehr, C.M., Erexson, G.L. & MacGregor, J.T. (1988). Association of marginal folate depletion with increased human chromosomal damage in vivo: demonstration by analysis of micronucleated erythrocytes. *Journal of the National Cancer Institute*. 80 (7). p.pp. 525–529.

Fairbairn, D.W., Olive, P.L. & O'Neill, K.L. (1995). The comet assay: a comprehensive review. *Mutation Research*. 339 (1). p.pp. 37–59.

Fenech, M. (1997). The advantages and disadvantages of the cytokinesis-block micronucleus method. *Mutation Research*. 392 (1-2). p.pp. 11–18.

Fenech, M. (1998). Important variables that influence base-line micronucleus frequency in cytokinesis-blocked lymphocytes—a biomarker for DNA damage in human populations. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 404 (1–2). p.pp. 155–165.

Fenech, M. (2000). The *in vitro* micronucleus technique. *Mutation Research*. 455 (1-2). p.pp. 81–95.

Fenech, M. (2001). The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 475 (1–2). p.pp. 57–67.

Fenech, M. (2005). The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. *Mutagenesis*. 20 (4). p.pp. 255–269.

Fenech, M. (2006). Cytokinesis-block micronucleus assay evolves into a 'cytome' assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research*. 600 (1-2). p.pp. 58–66.

Fenech, M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols*. 2 (5). p.pp. 1084–1104.

Fenech, M. (2010). Dietary reference values of individual micronutrients and nutriomes for genome damage prevention: current status and a road map to the future. *The American Journal of Vlinical Nutrition*. 91 (5). p.p. 1438S–1454S.

Fenech, M., Aitken, C. & Rinaldi, J. (1998). Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis*. 19 (7). p.pp. 1163–1171.

Fenech, M., Baghurst, P., Luderer, W., Turner, J., Record, S., Ceppi, M. & Bonassi, S. (2005). Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, β-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability—results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis*. 26 (5). p.pp. 991–999.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E. & HUman MicronNucleus project (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*. 534 (1-2). p.pp. 65–75.

Fenech, M., Crott, J., Turner, J. & Brown, S. (1999a). Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured simultaneously within the cytokinesis-block micronucleus assay: description of the method and results for hydrogen peroxide. *Mutagenesis*. 14 (6). p.pp. 605–612.

Fenech, M. & Crott, J.W. (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *MutationRresearch*. 504 (1-2). p.pp. 131–136.

Fenech, M. & Ferguson, L.R. (2001). Vitamins/minerals and genomic stability in humans. *Mutation Research*. 475 (1-2). p.pp. 1–6.

Fenech, M., Holland, N., Chang, W.P., Zeiger, E. & Bonassi, S. (1999b). The HUman MicroNucleus Project—An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 428 (1–2). p.pp. 271–283.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D. & Thomas, P. (2011). Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 26 (1). p.pp. 125–132.

Fergurson, L. (2008). Biomarkers as endpoints in intervention studies. In: *Molecular Epidemiology of chronic diseases*. John Wiley & Sons, Ltd.

Ferguson, L.R. (2002). Natural and human-made mutagens and carcinogens in the human diet. *Toxicology*. 181-182. p.pp. 79–82.

Ferguson, L.R. & Fenech, M.F. (2012). Vitamin and minerals that influence genome integrity, and exposure/intake levels associated with DNA damage prevention. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 733 (1–2). p.pp. 1–3.

Ferguson, L.R., Karunasinghe, N., Zhu, S. & Wang, A.H. (2012). Selenium and its' role in the maintenance of genomic stability. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 733 (1–2). p.pp. 100–110.

Ferguson, L.R. & Philpott, M. (2008). Nutrition and Mutagenesis. *Annual Review of Nutrition*. 28 (1). p.pp. 313–329.

Figueiredo, J.C., Knight, J.A., Briollais, L., Andrulis, I.L. & Ozcelik, H. (2004). Polymorphisms XRCC1-R399Q and XRCC3-T241M and the risk of breast cancer at the Ontario site of the Breast Cancer Family Registry. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*. 13 (4). p.pp. 583–591.

De Flora, S., Bagnasco, M. & Vainio, H. (1999). Modulation of genotoxic and related effects by carotenoids and vitamin A in experimental models: mechanistic issues. *Mutagenesis*. 14 (2). p.pp. 153–172.

Floyd, R.A. (1990). The role of 8-hydroxyguanine in. *Carcinogenesis*. 11 (9). p.pp. 1447–1450.

Franks, S.J. (2005). A mathematical model for the absorption and metabolism of formaldehyde vapour by humans. *Toxicology and Applied Pharmacology*. 206 (3). p.pp. 309–320.

Fransman, W., Roeleveld, N., Peelen, S., de Kort, W., Kromhout, H. & Heederik, D. (2007). Nurses with dermal exposure to antineoplastic drugs: reproductive outcomes. *Epidemiology (Cambridge, Mass.)*. 18 (1). p.pp. 112–119.

Fransman, W., Vermeulen, R. & Kromhout, H. (2005). Dermal exposure to cyclophosphamide in hospitals during preparation, nursing and cleaning activities. *International Archives of Occupational and Environmental Health*. 78 (5). p.pp. 403–412.

Fransman, W., Vermeulen, R. & Kromhout, H. (2004). Occupational dermal exposure to cyclophosphamide in Dutch hospitals: a pilot study. *The Annals of occupational hygiene*. 48 (3). p.pp. 237–244.

Freeman, L.E.B., Blair, A., Lubin, J.H., Stewart, P.A., Hayes, R.B., Hoover, R.N. & Hauptmann, M. (2009). Mortality From Lymphohematopoietic Malignancies Among Workers in Formaldehyde Industries: The National Cancer Institute Cohort. *Journal of the National Cancer Institute*. 101 (10). p.pp. 751–761.

Frei, B. (1999). Molecular and biological mechanisms of antioxidant action. *The FASEB Journal*. 13 (9). p.pp. 963–964.

Friedberg, E.C., Walker, G., Siede, Wolfram, Wood, Richard, Schultz, Roger & Ellenburger, Tom (2006). *DNA repair and mutagenesis*. Washington, D.C.: ASM Press.

Fritz, H., Kennedy, D., Fergusson, D., Fernandes, R., Doucette, S., Cooley, K., Seely, A., Sagar, S., Wong, R. & Seely, D. (2011). Vitamin A and retinoid derivatives for lung cancer: a systematic review and meta analysis. *PloS one*. 6 (6). p.p. e21107.

Froneberg, B. (2006). National and International Response to Occupational Hazards in the Healthcare Sector. *Annals of the New York Academy of Sciences*. 1076 (1). p.pp. 607–614.

Fuchs, J., Hengstler, J.G., Jung, D., Hiltl, G., Konietzko, J. & Oesch, F. (1995). DNA damage in nurses handling antineoplastic agents. *Mutation Research*. 342 (1-2). p.pp. 17–23.

Fucic, A., Jazbec, A., Mijic, A., Šešo-Šimic, đ & Tomek, R. (1998). Cytogenetic consequences after occupational exposure to antineoplastic drugs. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 416 (1-2). p.pp. 59–66.

Garaj-Vrhovac, V. & Kopjar, N. (2003). The alkaline Comet assay as biomarker in assessment of DNA damage in medical personnel occupationally exposed to ionizing radiation. *Mutagenesis*. 18 (3). p.pp. 265–271.

Garte, S. (2008). Individual Susceptibility and Gene–Environment Interaction. In: C. Wild, P. Vineis, & S. Garte (eds.). *Molecular Epidemiology of Chronic Diseases*. [Online]. John Wiley & Sons, Ltd, pp. 55–69.

Gaziano, J.M., Glynn, R.J., Christen, W.G., Kurth, T., Belanger, C., MacFadyen, J., Bubes, V., Manson, J.E., Sesso, H.D. & Buring, J.E. (2009). Vitamins E and C in the prevention of prostate and total cancer in men: the Physicians' Health Study II randomized controlled trial. *JAMA: the journal of the American Medical Association*. 301 (1). p.pp. 52–62.

Gaziev, A.I., Sologub, G.R., Fomenko, L.A., Zaichkina, S.I., Kosyakova, N.I. & Bradbury, R.J. (1996). Effect of vitamin-antioxidant micronutrients on the frequency of spontaneous and in vitro gamma-ray-induced micronuclei in lymphocytes of donors: the age factor. *Carcinogenesis*. 17 (3). p.pp. 493–499.

Gedik, C.M., Boyle, S.P., Wood, S.G., Vaughan, N.J. & Collins, A.R. (2002). Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis*. 23 (9). p.pp. 1441–1446.

Giovannelli, L., Saieva, C., Masala, G., Testa, G., Salvini, S., Pitozzi, V., Riboli, E., Dolara, P. & Palli, D. (2002). Nutritional and lifestyle determinates of DNA oxidative damage: a study in a Mediterranean population. *Carcinogenesis.* 23 (9). p.pp. 1483 – 1489.

Glória, L., Cravo, M., Camilo, M.E., Resende, M., Cardoso, J.N., Oliveira, A.G., Leitão, C.N. & Mira, F.C. (1997). Nutritional deficiencies in chronic alcoholics: relation to dietary intake and alcohol consumption. *The American Journal of Gastroenterology*. 92 (3). p.pp. 485–489.

Gonzalez, C.F., Proudfoot, M., Brown, G., Korniyenko, Y., Mori, H., Savchenko, A.V. & Yakunin, A.F. (2006). Molecular Basis of Formaldehyde Detoxification: characterization of two s-formylglutathione hydrolases from *Escherichia coli*, FrmB AND YeiG. *Journal of Biological Chemistry*. 281 (20). p.pp. 14514–14522.

Goukassian, D.A., Bagheri, S., el-Keeb, L., Eller, M.S. & Gilchrest, B.A. (2002). DNA oligonucleotide treatment corrects the age-associated decline in DNA repair capacity. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 16 (7). p.pp. 754–756.

Go, V.L.W., Butrum, R.R. & Wong, D.A. (2003). Diet, nutrition, and cancer prevention: the postgenomic era. *The Journal of Nutrition*. 133 (11 Suppl 1). p.p. 3830S–3836S.

Goyer, N., Beaudry, C., Bégin, D., Bouchard, M., Buissonet, S., Carrier, G., Gely, O., Gérin, M., Lavoué, L., Lefebvre, P., Noisel, N., Perraut, G. & Roberge, B. (2004). *Impacts d'un abaissement de la valeur d'exposition admissible au formaldéhyde : industries de fabrication de formaldéhde et de résines à base de formaldéhyde*. Móntreal: Institut de Recherche Robert-Sauvé en Santé et Securité du Travail.

de Graaf, B., Clore, A. & McCullough, A.K. (2009). Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks. *DNA Repair*. 8 (10). p.pp. 1207–1214.

Grant, W.B. (2010). Good evidence exists that solar ultraviolet-B and vitamin D reduce the risk of ovarian cancer. *American Journal of Obstetrics and Gynecology*. 203 (5). p.p. e10.

Greim, H., Csanády, G., Filser, J.G., Kreuzer, P., Schwarz, L., Wolff, T. & Werner, S. (1995). Biomarkers as tools in human health risk assessment. *Clinical Chemistry*. 41 (12). p.pp. 1804–1808.

De Groot, A.C., Flyvholm, M.-A., Lensen, G., Menné, T. & Coenraads, P.-J. (2009). Formaldehyde-releasers: relationship to formaldehyde contact allergy. Contact allergy to formaldehyde and inventory of formaldehyde-releasers. *Contact dermatitis*. 61 (2). p.pp. 63–85.

Gross, L. & Dreyfuss, Y. (1990). Prevention of spontaneous and radiation-induced tumors in rats by reduction of food intake. *Proceedings of the National Academy of Sciences*. 87 (17). p.pp. 6795–6797.

Guarnieri, S., Loft, S., Riso, P., Porrini, M., Risom, L., Poulsen, H.E., Dragsted, L.O. & Møller, P. (2008). DNA repair phenotype and dietary antioxidant supplementation. *The British journal of nutrition*. 99 (5). p.pp. 1018–1024.

Guerreiro, C.S., Cravo, M.L., Brito, M., Vidal, P.M., Fidalgo, P.O. & Leitão, C.N. (2007). The D1822V APC polymorphism interacts with fat, calcium, and fiber intakes in modulating the risk of colorectal cancer in Portuguese persons. *The American Journal of Clinical Nutrition*. 85 (6). p.pp. 1592–1597.

Gulec, M., Gurel, A. & Armutcu, F. (2006). Vitamin E protects against oxidative damage caused by formaldehyde in the liver and plasma of rats. *Molecular and Cellular Biochemistry*. 290 (1-2). p.pp. 61–67.

Gulten, T., Evke, E., Ercan, I., Evrensel, T., Kurt, E. & Manavoglu, O. (2011). Lack of genotoxicity in medical oncology nurses handling antineoplastic drugs: effect of work environment and protective equipment. *Work (Reading, Mass.)*. 39 (4). p.pp. 485–489.

Hagmar, L., Bonassi, S., Strömberg, U., Mikoczy, Z., Lando, C., Hansteen, I.L., Montagud, A.H., Knudsen, L., Norppa, H., Reuterwall, C., Tinnerberg, H., Brogger, A., Forni, A., Högstedt, B., Lambert, B., Mitelman, F., Nordenson, I., Salomaa, S. & Skerfving, S. (1998). Cancer predictive value of cytogenetic markers used in occupational health surveillance programs: a report from an ongoing study by the European Study Group on Cytogenetic Biomarkers and Health. *Mutation Research*. 405 (2). p.pp. 171–178.

Hall, A., Harrington, J.M. & Aw, T.C. (1991). Mortality study of British pathologists. *American Journal of Industrial Medicine*. 20 (1). p.pp. 83–89.

Han, S., Zhang, H.-T., Wang, Z., Xie, Y., Tang, R., Mao, Y. & Li, Y. (2006). DNA repair gene XRCC3 polymorphisms and cancer risk: a meta-analysis of 48 case-control studies. *European journal of human genetics: EJHG*. 14 (10). p.pp. 1136–1144.

Hamid, O.O.A. (2010). Antioxidants: Its medicinal and pharmacological applications. *African Journal of Pure and Applied Chemistry*. 4. p.pp. 142–151.

Harrison, B.R. (2006). Comparison of surface contamination with cyclophosphamide and fluorouracil using a closed-system drug transfer device versus standard preparation techniques. *American Journal of Health-System Pharmacy*. 63 (18). p.pp. 1736–1744.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., Tice, R.R. & 4th International Comet Assay Workshop (2003). Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis*. 18 (1). p.pp. 45–51.

Hartmann A & Speit Guenter (2009). Comet assay – protocols and testing strategies. In: *The Comet Assay in Toxicology*. [Online]. Royal Society of Chemistry, p. 378. Available from: www.rsc.org.

Hart, R.W., Dixit, R., Seng, J., Turturro, A., Leakey, J.E., Feuers, R., Duffy, P., Buffington, C., Cowan, G., Lewis, S., Pipkin, J. & Li, S.Y. (1999). Adaptive role of caloric intake on the degenerative disease processes. *Toxicological Sciences: An Official Journal of the Society of Toxicology*. 52 (2 Suppl). p.pp. 3–12.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B. & Blair, A. (2004). Mortality from solid cancers among workers in formaldehyde industries. *American journal of epidemiology*. 159 (12). p.pp. 1117–1130.

Hauptmann, M., Stewart, P.A., Lubin, J.H., Freeman, L.E.B., Hornung, R.W., Herrick, R.F., Hoover, R.N., Fraumeni, J.F., Blair, A. & Hayes, R.B. (2009). Mortality From Lymphohematopoietic Malignancies and Brain Cancer Among Embalmers Exposed to Formaldehyde. *Journal of the National Cancer Institute*. [Online]. Available from: http://jnci.oxfordjournals.org/content/early/2009/11/20/jnci.djp416.

Hayes, R.B., Blair, A., Stewart, P.A., Herrick, R.F. & Mahar, H. (1990). Mortality of U.S. Embalmers and funeral directors. *American Journal of Industrial Medicine*. 18 (6). p.pp. 641–652.

Heck, H. & Casanova, M. (1999). Pharmacodynamics of formaldehyde: applications of a model for the arrest of DNA replication by DNA-protein cross-links. *Toxicology and Applied pharmacology*. 160 (1). p.pp. 86–100.

Heck, H. d'A. & Casanova, M. (2004). The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. *Regulatory Toxicology and Pharmacology*. 40 (2). p.pp. 92–106.

Hedberg, J. (2001). *Function, expression and polymorphism of human alcohol dehydrogenase 3/glutathionedependent formaldehyde dehydrogenase*. [Online].

Hedmer, M., Jönsson, B.A.G. & Nygren, O. (2004). Development and validation of methods for environmental monitoring of cyclophosphamide in workplaces. *Journal of Environmental Monitoring*. 6 (12). p.pp. 979–984.

Hedmer, M., Tinnerberg, H., Axmon, A. & Jönsson, B. (2008). Environmental and biological monitoring of antineoplastic drugs in four workplaces in a Swedish hospital. *International Archives of Occupational and Environmental Health*. 81 (7). p.pp. 899–911.

Hedmer, M. & Wohlfart, G. (2012). Hygienic guidance values for wipe sampling of antineoplastic drugs in Swedish hospitals. *Journal of Environmental Monitoring*. 14 (7). p.pp. 1968–1975.

Heidelberger, C. (1965). Fluorinated Pyrimidines. In: J.N. Davidson and Waldo E. Cohn (ed.). *Progress in Nucleic Acid Research and Molecular Biology*. [Online]. Academic Press, pp. 1–50.

Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven, E. & Scheiner, J. (1957). Fluorinated Pyrimidines, A New Class of Tumour-Inhibitory Compounds. *Nature*. 179 (4561). p.pp. 663–666.

Heilbronn, L.K. & Ravussin, E. (2003). Calorie restriction and aging: review of the literature and implications for studies in humans. *The American Journal of Clinical Nutrition*. 78 (3). p.pp. 361–369.

He, J.L., Jin, L.F. & Jin, H.Y. (1998). Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. *Biomedical and environmental sciences: BES*. 11 (1). p.pp. 87–92.

Hedmer, M. & Wohlfart, G. (2012). Hygienic guidance values for wipe sampling of antineoplastic drugs in Swedish hospitals *J Environ Monit,* 14: 1968-1975.

Hessel, H., Radon, K., Pethran, A., Maisch, B., Gröbmair, S., Sautter, I. & Fruhmann, G. (2001). The genotoxic risk of hospital, pharmacy and medical personnel occupationally exposed to cytostatic drugs – evaluation by the micronucleus assay. *Mutation Research* 497: 101 – 109.

Heuser, V.D., Andrade, V.M., Peres, A., Gomes de Macedo Braga, L.M. & Bogo Chies J.A. (2008). Influence of age and sex on the spontaneous DNA damage detected by micronucleus test and comet assay in mice peripheral blood cells. *Cell Biol Int.* 32 (10). p.pp. 1223 – 1229.

Hellman, B., Friis, L., Vaghef, H. & Edling, C. (1999). Alkaline single cell gel electrophoresis and human biomonitoring for genotoxicity: a study on subjects with residential exposure to radon. *Mutation Research*. 442 (2). p.pp. 121–132.

Hellman, B., Vaghef, H., Friis, L. & Edling, C. (1997). Alkaline single cell gel electrophoresis of DNA fragments in biomonitoring for genotoxicity: an introductory study on healthy human volunteers. *International Archives of Occupational and Environmental Health*. 69 (3). p.pp. 185–192.

Hines, R.N., Sargent, D., Autrup, H., Birnbaum, L.S., Brent, R.L., Doerrer, N.G., Cohen Hubal, E.A., Juberg, D.R., Laurent, C., Luebke, R., Olejniczak, K., Portier, C.J. & Slikker, W. (2010a). Approaches for Assessing Risks to Sensitive Populations: Lessons Learned from Evaluating Risks in the Pediatric Population. *Toxicological Sciences*. 113 (1). p.pp. 4–26.

Hines, S.L., Jorn, H.K.S., Thompson, K.M. & Larson, J.M. (2010b). Breast cancer survivors and vitamin D: a review. *Nutrition (Burbank, Los Angeles County, Calif.)*. 26 (3). p.pp. 255–262.

Hininger, I., Chollat-Namy, A., Sauvaigo, S., Osman, M., Faure, H., Cadet, J., Favier, A. & Roussel, A.-M. (2004). Assessment of DNA damage by comet assay on frozen total blood: method and evaluation in smokers and non-smokers. *Mutation Research*. 558 (1-2). p.pp. 75–80.

Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature*. 411 (6835). p.pp. 366–374.

Hoffelder, D.R., Luo, L., Burke, N.A., Watkins, S.C., Gollin, S.M. & Saunders, W.S. (2004). Resolution of anaphase bridges in cancer cells. *Chromosoma*. 112 (8). p.pp. 389–397.

Holland, N., Bolognesi, C., Kirschvolders, M., Bonassi, S., Zeiger, E., Knasmueller, S. & Fenech, M. (2008). The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps. *Mutation Research/Reviews in Mutation Research*. 659 (1-2). p.pp. 93–108.

Hon, C., P. P. Chua, Danyluk, Q. & Astrakianakis, G. (2013). Examining factors that influence the effectiveness of cleaning antineoplastic drugs from drug preparation surfaces: a pilot study. *J Oncol Pharm Pract.*

Huang, Y., Fenech, M. & Shi, Q. (2011). Micronucleus formation detected by live-cell imaging. *Mutagenesis*. 26 (1). p.pp. 133–138.

Hunter, D.J. (2005). Gene–environment interactions in human diseases. *Nature Reviews Genetics*. 6 (4). p.pp. 287–298.

Hur, M.W. & Edenberg, H.J. (1992). Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase. *Gene*. 121 (2). p.pp. 305–311.

Hursting, S.D., Lavigne, J.A., Berrigan, D., Perkins, S.N. & Barrett, J.C. (2003). Calorie restriction, aging, and cancer prevention: mechanisms of action and applicability to humans. *Annual review of medicine*. 54. p.pp. 131–152.

Husgafvel-Pursiainen, K. (2002). Molecular biomarkers in studies on environmental cancer. *Journal of Epidemiology and Community Health*. 56 (10). p.pp. 730–731.

Hussain, S.P. & Harris, C.C. (1998). Molecular epidemiology of human cancer. *Toxicology letters*. 102-103. p.pp. 219–225.

Hu, Y.C. & Ahrendt, S.A. (2005). hOGG1 Ser326Cys polymorphism and G:C-to-T:A mutations: no evidence for a role in tobacco-related non small cell lung cancer. *International Journal of Cancer. Journal International Du Cancer*. 114 (3). p.pp. 387–393.

Hwang, E.-S. & Bowen, P.E. (2007). DNA damage, a biomarker of carcinogenesis: its measurement and modulation by diet and environment. *Critical reviews in food science and nutrition*. 47 (1). p.pp. 27–50.

IARC (1981). *International Agency for Research on Cancer (IARC) - Summaries & Evaluations*. 26.

IARC (1987). *IARC Supplement 7*. [Online]. Available from: www.iarc.fr.

IARC (1989). *Report of an IARC Working Group to Review the Approaches and Processes Used to Evaluate the Carcinogenicity of Mixtures and Groups of Chemicals. IARC Internal Technical Report No. 89/004*. [Online]. Available from: www.iarc.fr.

IARC (1995). Wood dust and formaldehyde. *IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer*. 62. p.pp. 35–215.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2006). Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol. *IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer*. 88. p.pp. 1–478.

IARC (2012). *A Review of Human Carcinogens: Chemical Agents and Related Occupations*. 100F.

Iarmarcovai, G., Bonassi, S., Botta, A., Baan, R.A. & Orsière, T. (2008). Genetic polymorphisms and micronucleus formation: a review of the literature. *Mutation research*. 658 (3). p.pp. 215–233.

Iarmarcovai, G., Sari-Minodier, I., Orsière, T., De Méo, M., Gallice, P., Bideau, C., Iniesta, D., Pompili, J., Bergé-Lefranc, J.L. & Botta, A. (2006). A combined analysis of XRCC1, XRCC3, GSTM1 and GSTT1 polymorphisms and centromere content of micronuclei in welders. *Mutagenesis*. 21 (2). p.pp. 159–165.

Ishibe, N. & Kelsey, K.T. (1997). Genetic susceptibility to environmental and occupational cancers. *Cancer causes & control: CCC*. 8 (3). p.pp. 504–513.

Izdes, S., Sardas, S., Kadioglu, E., Kaymak, C. & Ozcagli, E. (2009). Assessment of genotoxic damage in nurses occupationally exposed to anaesthetic gases or antineoplastic drugs by the comet assay. *J Occup Health* . 52 (3). p.pp. 283 – 286.

Jackson, M.A., Stack, H.F. & Waters, M.D. (1996). Genetic activity profiles of anticancer drugs. *Mutation Research*. 355 (1-2). p.pp. 171–208.

Jaruga, P., Speina, E., Gackowski, D., Tudek, B. & Olinski, R. (2000). Endogenous oxidative DNA base modifications analysed with repair enzymes and GC/MS technique. *Nucleic Acids Research*. 28 (6). p.pp. E16.

Jaworowska, A. & Bazylak, G. (2008). HPLC Assessment and Multivariate Predictability of Serum Retinol and α-Tocopherol Concentrations in Adult Female Subjects. *The Open Chemical and Biomedical Methods Journal*. 1 (1). p.pp. 11–21.

Jenab, M., Salvini, S., van Gils, C.H., Brustad, M., Shakya-Shrestha, S., Buijsse, B., Verhagen, H., Touvier, M., Biessy, C., Wallström, P., Bouckaert, K., Lund, E., Waaseth, M., Roswall, N., Joensen, A.M., Linseisen, J., Boeing, H., Vasilopoulou, E., Dilis, V., Sieri, S., Sacerdote, C., Ferrari, P., Manjer, J., Nilsson, S., Welch, A.A., Travis, R., Boutron-Ruault, M.C., Niravong, M., Bueno-de-Mesquita, H.B., van der Schouw, Y.T., Tormo, M.J., Barricarte, A., Riboli, E., Bingham, S. & Slimani, N. (2009). Dietary intakes of retinol, beta-carotene, vitamin D and vitamin E in the European Prospective Investigation into Cancer and Nutrition cohort. *European Journal of Clinical Nutrition*. 63 Suppl 4. p.pp. S150–178.

Jiang, S., Yu, L., Cheng, J., Leng, S., Dai, Y., Zhang, Y., Niu, Y., Yan, H., Qu, W., Zhang, C., Zhang, K., Yang, R., Zhou, L. & Zheng, Y. (2010). Genomic damages in peripheral blood lymphocytes and association with polymorphisms of three glutathione S-transferases in workers exposed to formaldehyde. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 695 (1-2). p.pp. 9–15.

Jiao, X., Huang, J., Wu, S., Lv, M., Hu, Y., Jianfu, null, Su, X., Luo, C. & Ce, B. (2007). hOGG1 Ser326Cys polymorphism and susceptibility to gallbladder cancer in a Chinese population. *International Journal of Cancer. Journal International Du Cancer*. 121 (3). p.pp. 501–505.

Johansson, C., Møller, P., Forchhammer, L., Loft, S., Godschalk, R.W.L., Langie, S.A.S., Lumeij, S., Jones, G.D.D., Kwok, R.W.L., Azqueta, A., Phillips, D.H., Sozeri, O., Routledge, M.N., Charlton, A.J., Riso, P., Porrini, M., Allione, A., Matullo, G., Palus, J., Stepnik, M., Collins, A.R. & Möller, L. (2010). An ECVAG trial on assessment of oxidative damage to DNA measured by the comet assay. *Mutagenesis*. 25 (2). p.pp. 125–132.

Johnson, R.D. & Jasin, M. (2001). Double-strand-break-induced homologous recombination in mammalian cells. *Biochemical Society transactions*. 29 (Pt 2). p.pp. 196–201.

Jones, K.H., York, T.P., Juusola, J., Ferreira-Gonzalez, A., Maes, H.H. & Jackson-Cook, C. (2011). Genetic and environmental influences on spontaneous micronuclei frequencies in children and adults: a twin study. *Mutagenesis*. 26 (6). p.pp. 745–752.

Just, W., Zeller, J., Riegert, C. & Speit, G. (2011). Genetic polymorphisms in the formaldehyde dehydrogenase gene and their biological significance. *Toxicology Letters*. 207 (2). p.pp. 121–127.

Kaiser, R., Holmquist, B., Vallee, B.L. & Jörnvall, H. (1991). Human class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase. *Journal of Protein Chemistry*. 10 (1). p.pp. 69–73.

Kaleta, B., Bogaczewicz, J., Robak, E., Sysajedrzejowska, A., Wrzosek, M., Szubierajska, W., Mroz, P., Jaceklukaszkiewicz, J., Wozniacka, A. (2013). Vitamin D Receptor Gene BsmI Polymorphism in Polish Patients with Systemic Lupus Erythematosus. *ISRN Endocrinology*. 2013. p.p. e427818.

Kállay, E., Bareis, P., Bajna, E., Kriwanek, S., Bonner, E., Toyokuni, S. & Cross, H.S. (2002). Vitamin D receptor activity and prevention of colonic hyperproliferation and oxidative stress. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association*. 40 (8). p.pp. 1191–1196.

Kapiszewska, M., Kalemba, M., Wojciech, U. & Milewicz, T. (2005). Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B12 concentrations and methylenetetrahydrofolate reductase polymorphisms. A pilot study. *The Journal of Nutritional Biochemistry*. 16 (8). p.pp. 467–478.

Kashyap, B. & Reddy, P.S. (2012). Micronuclei assay of exfoliated oral buccal cells: means to assess the nuclear abnormalities in different diseases. *Journal of Cancer Research and Therapeutics*. 8 (2). p.pp. 184–191.

Kastner, P.E., Casset, A. & Pons, F. (2011). Formaldehyde interferes with airway epithelium integrity and functions in a dose- and time-dependent manner. *Toxicology Letters*. 200 (1-2). p.pp. 109–116.

Kasuba, V., Rozgaj, R. & Garaj-Vrhovac, V. (1999). Analysis of sister chromatid exchange and micronuclei in peripheral blood lymphocytes of nurses handling cytostatics drugs. *Journal of Applied Toxicology* 19: 401 – 404.

Ketelslegers, H.B., Gottschalk, R.W.H., Koppen, G., Schoeters, G., Baeyens, W.F., van Larebeke, N.A., van Delft, J.H.M. & Kleinjans, J.C.S. (2008). Multiplex Genotyping as a Biomarker for Susceptibility to Carcinogenic Exposure in the FLEHS Biomonitoring Study. *Cancer Epidemiology Biomarkers & Prevention*. 17 (8). p.pp. 1902–1912.

Kevekordes, S., Gebel, T.W., Hellwig, M., Dames, W. & Dunkelberg, H. (1998). Human effect monitoring in cases of occupational exposure to antineoplastic drugs: a method comparison. *Occup Environ Med* 55: 145 - 149.

Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A. & Travis, R.C. (2004). Diet, nutrition and the prevention of cancer. *Public Health Nutrition*. 7 (1A). p.pp. 187–200.

Khanna, K.K. & Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nature Genetics*. 27 (3). p.pp. 247–254.

Kiffmeyer, T. & Hadtstein, C. (2007). Handling of chemotherapeutic drugs in the or: hazards and safety considerations. *Cancer Treatment and Research*. 134. p.pp. 275–290.

Kiffmeyer, T.K., Tuerk, J., Hahn, M., Stuetzer, H., Hadtstein, C., Heinemann, A. & Eickmann, U. (2012). Application and Assessment of a Regular Environmental Monitoring of the Antineoplastic Drug Contamination Level in Pharmacies - The MEWIP Project. *Annals of Occupational Hygiene*. 57 (4). p.pp. 444–455.

Kim, K.-H., Jahan, S.A. & Lee, J.-T. (2011). Exposure to Formaldehyde and Its Potential Human Health Hazards. *Journal of Environmental Science and Health, Part C*. 29 (4). p.pp. 277–299.

Kim, Y.-I. (2004). Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. *Environmental and Molecular Mutagenesis*. 44 (1). p.pp. 10–25.

Kirsch-Volders, M., Bonassi, S., Knasmueller, S., Holland, N., Bolognesi, C. & Fenech, M.F. (2014). Commentary: Critical questions, misconceptions and a road map for improving the use of the lymphocyte cytokinesis-block micronucleus assay for in vivo biomonitoring of human exposure to genotoxic chemicals-A HUMN project perspective. *Mutation Research*. 759. p.pp. 49 – 58.

Kirsch-Volders, M. & Fenech, M. (2001). Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes. *Mutagenesis*. 16 (1). p.pp. 51–58.

Kirsch-Volders, M., Mateuca, R.A., Roelants, M., Tremp, A., Zeiger, E., Bonassi, S., Holland, N., Chang, W.P., Aka, P.V., Deboeck, M., Godderis, L., Haufroid, V., Ishikawa, H., Laffon, B., Marcos, R., Migliore, L., Norppa, H., Teixeira, J.P., Zijno, A. & Fenech, M. (2006). The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 15 (5). p.pp. 1038–1042.

Klein, E.A., Thompson, I.M., Jr, Tangen, C.M., Crowley, J.J., Lucia, M.S., Goodman, P.J., Minasian, L.M., Ford, L.G., Parnes, H.L., Gaziano, J.M., Karp, D.D., Lieber, M.M., Walther, P.J., Klotz, L., Parsons, J.K., Chin, J.L., Darke, A.K., Lippman, S.M., Goodman, G.E., Meyskens, F.L., Jr & Baker, L.H. (2011). Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA: the journal of the American Medical Association*. 306 (14). p.pp. 1549–1556.

De Klerk, N.H., Musk, A.W., Ambrosini, G.L., Eccles, J.L., Hansen, J., Olsen, N., Watts, V.L., Lund, H.G., Pang, S.C., Beilby, J. & Hobbs, M.S. (1998). Vitamin A and cancer prevention II: comparison of the effects of retinol and beta-carotene. *International journal of cancer. Journal international du cancer*. 75 (3). p.pp. 362–367.

Knudsen, L.E. & Hansen, A.M. (2007). Biomarkers of intermediate endpoints in environmental and occupational health. *International Journal of Hygiene and Environmental Health*. 210 (3-4). p.pp. 461–470.

Kohno, T., Shinmura, K., Tosaka, M., Tani, M., Kim, S.R., Sugimura, H., Nohmi, T., Kasai, H. & Yokota, J. (1998). Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. *Oncogene*. 16 (25). p.pp. 3219–3225.

Kolmodin-Hedman, B., Hartvig, P., Sorsa, M. & Falck, K. (1983). Occupational handling of cytostatic drugs. *Archives of Toxicology*. 54 (1). p.pp. 25–33.

Konopacka, M., Widel, M. & Rzeszowska-Wolny, J. (1998). Modifying effect of vitamins C, E and beta-carotene against gamma-ray-induced DNA damage in mouse cells. *Mutation Research*. 417 (2-3). p.pp. 85–94.

Kopjar, N., Garaj-Vrhovac, V., Kašuba, V., Rozgaj, R., Ramić, S., Pavlica, V. & Želježić, D. (2009). Assessment of genotoxic risks in Croatian health care workers occupationally exposed to cytotoxic drugs: A multi-biomarker approach. *International Journal of Hygiene and Environmental Health*. 212 (4). p.pp. 414–431.

Kopjar, N., Milas, I., Garaj-Vrhovac, V. & Gamulin, M. (2006). Alkaline comet assay study with breast cancer patients: evaluation of baseline and chemotherapy-induced DNA damage in non-target cells. *Clinical and Experimental Medicine*. 6 (4). p.pp. 177–190.

Kopjar, N., Želježić, D., Vrdoljak, A.L., Radić, B., Ramić, S., Milić, M., Gamulin, M., Pavlica, V. & Fučić, A. (2007). Irinotecan Toxicity to Human Blood Cells in vitro: Relationship between Various Biomarkers. *Basic & Clinical Pharmacology & Toxicology*. 100 (6). p.pp. 403–413.

Kopp, B., Crauste-Manciet, S., Guibert, A., Mourier, W., Guerrault-Moro, M.-N., Ferrari, S., Jomier, J.-Y., Brossard, D. & Schierl, R. (2013). Environmental and Biological Monitoring of Platinum-Containing Drugs in Two Hospital Pharmacies Using Positive Air Pressure Isolators. *Annals of Occupational Hygiene*. 57 (3). p.pp. 374–383.

Kristal, A.R. (2004). Vitamin A, retinoids and carotenoids as chemopreventive agents for prostate cancer. *The Journal of Urology*. 171 (2 Pt 2). p.pp. S54–58.

Kromhout, H., Hoek, F., Uitterhoeve, R., Huijbers, R., Overmars, R.F., Anzion, R. & Vermeulen, R. (2000). Postulating a dermal pathway for exposure to anti-neoplastic drugs among hospital workers. Applying a conceptual model to the results of three workplace surveys. *The Annals of occupational hygiene*. 44 (7). p.pp. 551–560.

Kumaravel, T.S. & Jha, A.N. (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutation Research*. 605 (1-2). p.pp. 7–16.

Kumar, S. (2011). *Free radicals and antioxidants: human and food system.* 2 (1). p.p. 129.

Kusnetz, E. & Condon, M. (2003). Acute effects from occupational exposure to antineoplastic drugs in a para-professional health care worker. *American Journal of Industrial Medicine*. 44 (1). p.pp. 107–109.

Kyrtopoulos, S. (2006). Biomarkers in environmental carcinogenesis research: Striving for a new momentum. *Toxicology Letters*. 162 (1). p.pp. 3–15.

Ladeira, C., Viegas, S., Carolino, E., Gomes, M.C. & Brito, M. (2013). The influence of genetic polymorphisms in XRCC3 and ADH5 genes on the frequency of genotoxicity biomarkers in workers exposed to formaldehyde. *Environmental Molecular Mutagens* 54: 213 – 221.

Ladeira, C., Viegas, S., Carolino, E., Prista, J., Gomes, M.C. & Brito, M. (2011). Genotoxicity biomarkers in occupational exposure to formaldehyde – The case of histopathology laboratories. *Mutation Research* 721: 15-20.

Laffon, B., Teixeira, J.P., Silva, S., Loureiro, J., Torres, J., Pásaro, E., Méndez, J. & Mayan, O. (2005). Genotoxic effects in a population of nurses handling antineoplastic drugs, and relationship with genetic polymorphisms in DNA repair enzymes. *American Journal of Industrial Medicine*. 48 (2). p.pp. 128–136.

Lagopoulos, L. & Stalder, R. (1987). The influence of food intake on the development of diethylnitrosamine-induced liver tumours in mice. *Carcinogenesis*. 8 (1). p.pp. 33–37.

Lal, A. & Ames, B.N. (2011). Association of chromosome damage detected as micronuclei with hematological diseases and micronutrient status. *Mutagenesis*. 26 (1). p.pp. 57–62.

Lam, T.H., Zhu, C.Q. & Jiang, C.Q. (2002). Lymphocyte DNA damage in elevator manufacturing workers in Guangzhou, China. *Mutation Research*. 515 (1-2). p.pp. 147–157.

Larson, R.R., Khazaeli, M.B. & Dillon, H.K. (2003). A new monitoring method using solid sorbent media for evaluation of airborne cyclophosphamide and other antineoplastic agents. *Applied Occupational and Environmental Hygiene*. 18 (2). p.pp. 120–131.

Lee, S.-L., Wang, M.-F., Lee, A.-I. & Yin, S.-J. (2003). The metabolic role of human ADH3 functioning as ethanol dehydrogenase. *FEBS letters*. 544 (1-3). p.pp. 143–147.

Li, G-Y., Lee, H.Y., Shin, Ho-Sang, S., Kim, H.Y., Lim, C-H., Lee, B-H. (2007). Indentification of gene markers for formaldehyde exposure in humans. *Environ Health Perspect.* 115 (10). p.pp. 1460 – 1466.

Lindberg, H.K., Wang, X., Järventaus, H., Falck, G.C.-M., Norppa, H. & Fenech, M. (2007). Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. *Mutation Research*. 617 (1-2). p.pp. 33–45.

Lindh, A.R., Rafii, S., Schultz, N., Cox, A. & Helleday, T. (2006). Mitotic defects in XRCC3 variants T241M and D213N and their relation to cancer susceptibility. *Human MolecularGgenetics*. 15 (7). p.pp. 1217–1224.

Lippman, S.M., Klein, E.A., Goodman, P.J., Lucia, M.S., Thompson, I.M., Ford, L.G., Parnes, H.L., Minasian, L.M., Gaziano, J.M., Hartline, J.A., Parsons, J.K., Bearden, J.D., 3rd, Crawford, E.D., Goodman, G.E., Claudio, J., Winquist, E., Cook, E.D., Karp, D.D., Walther, P., Lieber, M.M., Kristal, A.R., Darke, A.K., Arnold, K.B., Ganz, P.A., Santella, R.M., Albanes, D., Taylor, P.R., Probstfield, J.L., Jagpal, T.J., Crowley, J.J., Meyskens, F.L., Jr, Baker, L.H. & Coltman, C.A., Jr (2009). Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA: the journal of the American Medical Association*. 301 (1). p.pp. 39–51.

Liteplo, R., World Health Organization, International Labour Organisation, United Nations Environment Programme & Inter-Organization Programme for the Sound Management of Chemicals (2002). *Formaldehyde*. Geneva: World Health Organization.

Liu, N., Lamerdin, J.E., Tebbs, R.S., Schild, D., Tucker, J.D., Shen, M.R., Brookman, K.W., Siciliano, M.J., Walter, C.A., Fan, W., Narayana, L.S., Zhou, Z.Q., Adamson, A.W., Sorensen, K.J., Chen, D.J., Jones, N.J. & Thompson, L.H. (1998). XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Molecular Cell*. 1 (6). p.pp. 783–793.

Lloyd, D.R. & Phillips, D.H. (1999). Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. *Mutation Research*. 424 (1-2). p.pp. 23–36.

Loft, S., Høgh Danielsen, P., Mikkelsen, L., Risom, L., Forchhammer, L. & Møller, P. (2008). Biomarkers of oxidative damage to DNA and repair. *Biochemical Society Transactions*. 36 (5). p.p. 1071. Available from: [Accessed: 10 April 2014].

Lonn, E., Bosch, J., Yusuf, S., Sheridan, P., Pogue, J., Arnold, J.M.O., Ross, C., Arnold, A., Sleight, P., Probstfield, J., Dagenais, G.R. & HOPE and HOPE-TOO Trial Investigators (2005). Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA: the journal of the American Medical Association*. 293 (11). p.pp. 1338–1347.

Lopes, C. (2000). Reproducibility and validation of a food frequency questionnaire. In: Diet and myocardial infarction: a community-based case-control study. A population-based case control study. (PhD thesis in portuguese). University of Porto, pp. 79 – 115.

Lopes, C., Aro, A., Azevedo, A., Ramos, E. & Barros, H. (2007). Intake and adipose tissue composition of fatty acids and risk of myocardial infartion in a male Portuguese community sample. *Journal of American Dietetic Association.* 107(2). P.pp. 276 – 286.

López-Diazguerrero, N.E., Luna-López, A., Gutiérrez-Ruiz, M.C., Zentella, A. & Königsberg, M. (2005). Susceptibility of DNA to oxidative stressors in young and aging mice. *Life Sciences*. 77 (22). p.pp. 2840–2854.

Lotan, Y., Goodman, P.J., Youssef, R.F., Svatek, R.S., Shariat, S.F., Tangen, C.M., Thompson, I.M., Jr & Klein, E.A. (2012). Evaluation of vitamin E and selenium supplementation for the prevention of bladder cancer in SWOG coordinated SELECT. *The Journal of Urology*. 187 (6). p.pp. 2005–2010.

Lovell, D.P. & Omori, T. (2008). Statistical issues in the use of the comet assay. *Mutagenesis*. 23 (3). p.pp. 171–182.

Lowe, L.C., Guy, M., Mansi, J.L., Peckitt, C., Bliss, J., Wilson, R.G. & Colston, K.W. (2005). Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population. *European journal of cancer (Oxford, England: 1990)*. 41 (8). p.pp. 1164–1169.

Lü, J.-M., Lin, P.H., Yao, Q. & Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal of cellular and molecular Medicine*. 14 (4). p.pp. 840–860.

Ma, J., Stampfer, M.J., Gann, P.H., Hough, H.L., Giovannucci, E., Kelsey, K.T., Hennekens, C.H. & Hunter, D.J. (1998). Vitamin D receptor polymorphisms, circulating vitamin D metabolites, and risk of prostate cancer in United States physicians. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 7 (5). p.pp. 385–390.

Macpherson, P., Barone, F., Maga, G., Mazzei, F., Karran, P. & Bignami, M. (2005). 8-Oxoguanine incorporation into DNA repeats in vitro and mismatch recognition by MutSα. *Nucleic Acids Research*. 33 (16). p.pp. 5094–5105.

Mader, R.M., Kokalj, A., Kratochvil, E., Pilger, A. & Rüdiger, H.W. (2009). Longitudinal biomonitoring of nurses handling antineoplastic drugs. *Journal of Clinical Nursing*. 18 (2). p.pp. 263–269.

Maluf, S. & Erdtmann, B. (2000). Follow-up study of the genetic damage in lymphocytes of pharmacists and nurses handling antineoplastic drugs evaluated by cytokinesis-block micronuclei analysis and single cell gel electrophoresis. *Mutation Research* 471: 21 – 27.

Mahboob, M., Rahman, M.F., Rekhadevi, P.V., Sailaja, N., Balasubramanyam, A., Prabhakar, P.V., Singh, S.P., Reddy, U.A., Rao, G.S. & Grover, P. (2012). Monitoring of Oxidative Stress in Nurses Occupationally Exposed to Antineoplastic Drugs. *Toxicology International*. 19 (1). p.pp. 20–24.

Mahendrakumar Chandrasekharappa Banakar, S.K.P. (2004). 1alpha, 25-dihydroxyvitamin D3 prevents DNA damage and restores antioxidant enzymes in rat hepatocarcinogenesis induced by diethylnitrosamine and promoted by phenobarbital. *World Journal of Gastroenterology : WJG*. 10 (9). p.pp. 1268–75.

Majer, B.J., Laky, B., Knasmüller, S. & Kassie, F. (2001). Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. *Mutation Research*. 489 (2-3). p.pp. 147–172.

Manikantan, P., Balachandar, V., Sasikala, K., Mohanadevi, S. & Lakshmankumar, B. (2009). DNA Damage in workers occupationally exposed to photocopying machines in Coimbatore south India, using comet assay. *The Internet Journal of Toxicology*. 7 (2).

Manno, M., Viau, C., in collaboration with, Cocker, J., Colosio, C., Lowry, L., Mutti, A., Nordberg, M. & Wang, S. (2010). Biomonitoring for occupational health risk assessment (BOHRA). *Toxicology letters*. 192 (1). p.pp. 3–16.

Manuguerra, M., Saletta, F., Karagas, M.R., Berwick, M., Veglia, F., Vineis, P. & Matullo, G. (2006). XRCC3 and XPD/ERCC2 single nucleotide polymorphisms and the risk of cancer: a HuGE review. *American Journal of Epidemiology*. 164 (4). p.pp. 297–302.

Di Marco, P., Priestly, B. & Buckett, K. (1998). Carcinogen risk assessment. Can we harmonise? *Toxicology Letters*. 102-103. p.pp. 241–246.

Marnett, L.J. (2000). Oxyradicals and DNA damage. *Carcinogenesis*. 21 (3). p.pp. 361–370.

Marsh, G.M. & Youk, A.O. (2004). Reevaluation of mortality risks from leukemia in the formaldehyde cohort study of the National Cancer Institute. *Regulatory Toxicology and Pharmacology*. 40 (2). p.pp. 113–124.

Marsh, G.M., Youk, A.O., Morfeld, P., Collins, J.J. & Symons, J.M. (2010). Incomplete follow-up in the National Cancer Institute's formaldehyde worker study and the impact on subsequent reanalyses and causal evaluations. *Regulatory Toxicology and Pharmacology*. 58 (2). p.pp. 233–236.

Martelli, A., Robbiano, L., Cosso, M., Perrone, C., Tagliazucchi, A., Giuliano, L., Aresca, G.G. & Brambilla, G. (2000). Comparison of micronuclei frequencies in mono-, bi- and polynucleated lymphocytes from subjects of a residential suburb and subjects living near a metallurgical plant. *Mutation Research.* 470 (2). p.pp. 211 – 219.

Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., Suzuki, H., Akino, K., Ohe-Toyota, M., Maruyama, Y., Tatsumi, H., Imai, K., Shinomura, Y. & Tokino, T. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Research*. 66 (9). p.pp. 4574–4583.

Masoro, E.J. (2005). Overview of caloric restriction and ageing. *Mechanisms of Ageing and Development*. 126 (9). p.pp. 913–922.

Mateuca, R.A., Roelants, M., Iarmarcovai, G., Aka, P.V., Godderis, L., Tremp, A., Bonassi, S., Fenech, M., Bergé-Lefranc, J.-L. & Kirsch-Volders, M. (2008). hOGG1(326), XRCC1(399) and XRCC3(241) polymorphisms influence micronucleus frequencies in human lymphocytes in vivo. *Mutagenesis*. 23 (1). p.pp. 35–41.

Mateuca, R., Lombaert, N., Aka, P.V., Decordier, I. & Kirsch-Volders, M. (2006). Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie*. 88 (11). p.pp. 1515–1531.

Matullo, G., Palli, D., Peluso, M., Guarrera, S., Carturan, S., Celentano, E., Krogh, V., Munnia, A., Tumino, R., Polidoro, S., Piazza, A. & Vineis, P. (2001). XRCC1, XRCC3, XPD gene polymorphisms, smoking and (32)P-DNA adducts in a sample of healthy subjects. *Carcinogenesis*. 22 (9). p.pp. 1437–1445.

McDiarmid, M.A. (2006). Chemical Hazards in Health Care. *Annals of the New York Academy of Sciences*. 1076 (1). p.pp. 601–606.

McGlothlin JD (2005). *Occupational exposure assessment and control using video exposure monitoring in the pharmaceutical industry*. International Scientific Conference (IOHA 2005), 6, 19–23 September 2005, Pilanesberg National Park North West Province, South Africa. Pilanesberg, IOHA–International Occupational Hygiene. MVS–Mine Ventilation of South Africa.

Mecocci, P., Polidori, M.C., Cherubini, A., Ingegni, T., Mattioli, P., Catani, M., Rinaldi, P., Cecchetti, R., Stahl, W., Senin, U. & Beal, M.F. (2002). Lymphocyte oxidative DNA damage and plasma antioxidants in Alzheimer disease. *Archives of Neurology*. 59 (5). p.pp. 794–798.

Medeiros, R., Morais, A., Vasconcelos, A., Costa, S., Pinto, D., Oliveira, J. & Lopes, C. (2002). The role of vitamin D receptor gene polymorphisms in the susceptibility to prostate cancer of a southern European population. *Journal of Human Genetics*. 47 (8). p.pp. 413–418.

Milić, M., Rozgaj, R., Kašuba, V., Oreščanin, V., Balija, M. & Jukić, I. (2010). Correlation between folate and vitamin B_{12} and markers of DNA stability in healthy men: preliminary results. *Acta Biochimica Polonica*. 57 (3). p.pp. 339–345.

Miller, E.R., 3rd, Pastor-Barriuso, R., Dalal, D., Riemersma, R.A., Appel, L.J. & Guallar, E. (2005). Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine*. 142 (1). p.pp. 37–46.

Minnet, C., Koc, A., Aycicek, A. & Kocyigit, A. (2011). Vitamin B12 treatment reduces mononuclear DNA damage. *Pediatrics International: Official Journal of the Japan Pediatric Society*. 53 (6). p.pp. 1023–1027.

Minozzo, R., Deimling, L.I. & Santos-Mello, R. (2010). Cytokinesis-blocked micronucleus cytome and comet assays in peripheral blood lymphocytes of workers exposed to lead considering folate and vitamin B12 status. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 697 (1–2). p.pp. 24–32.

Miyazaki, M., Doi, Y., Ikeda, F., Ninomiya, T., Hata, J., Uchida, K., Shirota, T., Matsumoto, T., Iida, M. & Kiyohara, Y. (2012). Dietary vitamin A intake and incidence of gastric cancer in a general Japanese population: the Hisayama Study. *Gastric cancer: official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association*. 15 (2). p.pp. 162–169.

Mohrenweiser, H. (2004). Genetic Variation and Exposure Related Risk Estimation: Will Toxicology Enter a New Era? DNA Repair and Cancer as a Paradigm. *Toxicologic Pathology*. 32 (6). p.pp. 136–145.

Moller, P., Knudsen, L.E., Loft, S. & Wallin, H. (2000). The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*. 9 (10). p.pp. 1005–1015.

Moretti, M., Bonfiglioli, R., Feretti, D., Pavanello, S., Mussi, F., Grollino, M.G., Villarini, M., Barbieri, A., Ceretti, E., Carrieri, M., Buschini, A., Appolloni, M., Dominici, L., Sabatini, L., Gelatti, U., Bartolucci, G.B., Poli, P., Stronati, L., Mastrangelo, G. & Monarca, S. (2011). A study protocol for the evaluation of occupational mutagenic/carcinogenic risks in subjects exposed to antineoplastic drugs: a multicentric project. *BMC Public Health*. 11. p.p. 195.

Mostowska, A., Sajdak, S., Pawlik, P., Lianeri, M. & Jagodzinski, P.P. (2013). Vitamin D receptor gene BsmI and FokI polymorphisms in relation to ovarian cancer risk in the Polish population. *GeneticTtesting and MolecularBbiomarkers*. 17 (3). p.pp. 183–187.

Mutti, A. (1999). Biological monitoring in occupational and environmental toxicology. *Toxicology letters*. 108 (2-3). p.pp. 77–89.

National Research Council (1987). Biological markers in environmental health research. Committee on Biological Markers of the National Research Council. *Environmental Health Perspectives*. 74. p.pp. 3–9.

Nersesyan, A., Kundi, M., Atefie, K., Schulte-Hermann, R. & Knasmuller, S. (2006). Effect of Staining Procedures on the Results of Micronucleus Assays with Exfoliated Oral Mucosa Cells. *Cancer Epidemiology Biomarkers & Prevention*. 15 (10). p.pp. 1835–1840.

Neuss, S., Moepps, B. & Speit, G. (2010). Exposure of human nasal epithelial cells to formaldehyde does not lead to DNA damage in lymphocytes after co-cultivation. *Mutagenesis*. 25 (4). p.pp. 359–364.

Neuss, S. & Speit, G. (2008). Further characterization of the genotoxicity of formaldehyde in vitro by the sister chromatid exchange test and co-cultivation experiments. *Mutagenesis*. 23 (5). p.pp. 355–357.

Nielsen, G.D., Larsen, S.T. & Wolkoff, P. (2013). Recent trend in risk assessment of formaldehyde exposures from indoor air. *Archives of Toxicology*. 87 (1). p.pp. 73–98.

Niki, E. (2000). Free radicals in the 1900's: from in vitro to in vivo. *Free Radical Research*. 33 (6). p.pp. 693–704.

NIOSH (2004). *Manual of analytical methods*. 4th Ed. DHHS (NIOSH) Publication.

NIOSH (1994). *Manual of analytical methods*. DHHS (NIOSH) Publication.

Norliana, S., Abdulamir, A.S., Bakar, F.A. & Salleh, A.B. (2009). The Health Risk of Formaldehyde to Human Beings. *American Journal of Pharmacology and Toxicology*. 4 (3). p.pp. 98–106.

Norppa, H. (2001). Genetic polymorphisms and chromosome damage. *International Journal of Hygiene and Environmental Health*. 204 (1). p.pp. 31–38.

Norppa, H. & Falck, G.C.-M. (2003). What do human micronuclei contain? *Mutagenesis*. 18 (3). p.pp. 221–233.

NTP (2011). *Report on Carcinogens, Twelfth Edition.*

Obe, G. & Ristow, H. (1979). Mutagenic, cancerogenic and teratogenic effects of alcohol. *Mutation Research/Reviews in Genetic Toxicology*. 65 (4). p.pp. 229–259.

Olden, K. & Guthrie, J. (2001). Genomics: implications for toxicology. *Mutation Research*. 473 (1). p.pp. 3–10.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Jr, Valanis, B., Williams, J.H., Jr, Barnhart, S., Cherniack, M.G., Brodkin, C.A. & Hammar, S. (1996). Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *Journal of the National Cancer Institute*. 88 (21). p.pp. 1550–1559.

Ordovas, J.M. & Corella, D. (2004). Nutritional Genomics. *Annual Review of Genomics and Human Genetics*. 5 (1). p.pp. 71–118.

Orlow, I., Roy, P., Reiner, A.S., Yoo, S., Patel, H., Paine, S., Armstrong, B.K., Kricker, A., Marrett, L.D., Millikan, R.C., Thomas, N.E., Gruber, S.B., Anton-Culver, H., Rosso, S., Gallagher, R.P., Dwyer, T., Kanetsky, P.A., Busam, K., From, L., Begg, C.B., Berwick, M. & GEM Study Group (2012). Vitamin D receptor polymorphisms in patients with cutaneous melanoma. *International journal of cancer. Journal international du cancer*. 130 (2). p.pp. 405–418.

Orsière, T., Sari-Minodier, I., Iarmarcovai, G. & Botta, A. (2006). Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 605 (1-2). p.pp. 30–41.

Padjas, A., Lesisz, D., Lankoff, A., Banasik, A., Lisowska, H., Bakalarz, R., Góźdź, S. & Wojcik, A. (2005). Cytogenetic damage in lymphocytes of patients undergoing therapy for small cell lung cancer and ovarian carcinoma. *Toxicology and Applied Pharmacology*. 209 (2). p.pp. 183–191.

Pala, M., Ugolini, D., Ceppi, M., Rizzo, F., Maiorana, L., Bolognesi, C., Schilirò, T., Gilli, G., Bigatti, P., Bono, R. & Vecchio, D. (2008). Occupational exposure to formaldehyde and biological monitoring of Research Institute workers. *Cancer Detection and Prevention*. 32 (2). p.pp. 121–126.

Panchagnula, R. (1998). Pharmaceutical aspects of paclitaxel. *International Journal of Pharmaceutics*. 172 (1–2). p.pp. 1–15.

Park, E.Y., Pinali, D., Lindley, K. & Lane, M.A. (2012). Hepatic vitamin A preloading reduces colorectal cancer metastatic multiplicity in a mouse xenograft model. *Nutrition and Cancer*. 64 (5). p.pp. 732–740.

Pasquali, M.A., Gelain, D.P., Zeidán-Chuliá, F., Pires, A.S., Gasparotto, J., Terra, S.R. & Moreira, J.C.F. (2013). Vitamin A (retinol) downregulates the receptor for advanced glycation endproducts (RAGE) by oxidant-dependent activation of p38 MAPK and NF-kB in human lung cancer A549 cells. *Cellular Signalling*. 25 (4). p.pp. 939–954.

Patterson, R.E., Kristal, A.R. & Neuhouser, M.L. (1999). Vitamin supplements and cancer risk: epidemiologic research and recommendations. In: *Preventive Nutrition II*. New Jersey: Bendich, A., Deckelbaum, R.J., p. 21.

Pawlowska, E., Janik-Papis, K., Rydzanicz, M., Zuk, K., Kaczmarczyk, D., Olszewski, J., Szyfter, K., Blasiak, J. & Morawiec-Sztandera, A. (2009). The Cys326 allele of the 8-oxoguanine DNA N-glycosylase 1 gene as a risk factor in smoking- and drinking-associated larynx cancer. *The Tohoku Journal of Experimental Medicine*. 219 (4). p.pp. 269–275.

Perera, F.P. (1996). Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. *Journal of the National Cancer Institute*. 88 (8). p.pp. 496–509.

Perera, F.P. (2000). Molecular Epidemiology: On the Path to Prevention? *Journal of the National Cancer Institute*. 92 (8). p.pp. 602–612.

Perera, F.P. & Weinstein, I.B. (2000). Molecular epidemiology: recent advances and future directions. *Carcinogenesis*. 21 (3). p.pp. 517–524.

Pérez-Cadahía, B., Laffon, B., Valdiglesias, V., Pásaro, E. & Méndez, J. (2008). Cytogenetic effects induced by Prestige oil on human populations: the role of polymorphisms in genes involved in metabolism and DNA repair. *Mutation Research*. 653 (1-2). p.pp. 117–123.

Persinger, R.L., Melamede, R., Bespalov, I., Wallace, S., Taatjes, D.J. & Janssen-Heininger, Y. (2001). Imaging techniques used for the detection of 8-oxoguanine adducts and DNA repair proteins in cells and tissues. *Experimental Gerontology*. 36 (9). p.pp. 1483–1494.

Pilger, A., Köhler, I., Stettner, H., Mader, R.M., Rizovski, B., Terkola, R., Diem, E., Franz-Hainzl, E., Konnaris, C., Valic, E. & Rüdiger, H.W. (2000). Long-term monitoring of sister chromatid exchanges and micronucleus frequencies in pharmacy personnel occupationally exposed to cytostatic drugs. *Int Arch Occup Environ Health* 73: 442-448.

Pilger, A. & Rüdiger, H.W. (2006). 8-Hydroxy-2′-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures. *International Archives of Occupational and Environmental Health*. 80 (1). p.pp. 1–15. Pinkerton, L.E., Hein, M.J. & Stayner, L.T. (2004). Mortality among a cohort of garment workers exposed to formaldehyde: an update. *Occupational and Environmental Medicine*. 61 (3). p.pp. 193–200.

Pirkle, J.L., Sampson, E.J., Needham, L.L., Patterson, D.G. & Ashley, D.L. (1995). Using biological monitoring to assess human exposure to priority toxicants. *Environmental Health Perspectives*. 103 (Suppl 3). p.pp. 45–48.

Piyathilake, C.J., Macaluso, M., Hine, R.J., Vinter, D.W., Richards, E.W. & Krumdieck, C.L. (1995). Cigarette smoking, intracellular vitamin deficiency, and occurrence of micronuclei in epithelial cells of the buccal mucosa. *Cancer Epidemiology Biomarkers & Prevention*. 4 (7). p.pp. 751–758.

Polidori, M.C. & Stahl, W. (2009). Carotenoids and Vitamin A. In: S. Knasmüller, D. M. DeMarini, I. Johnson, & C. Gerhäuser (eds.). *Chemoprevention of Cancer and DNA Damage by Dietary Factors*. [Online]. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 369–383.

Pollak, M. (2000). Insulin-like growth factor physiology and cancer risk. *European Journal of Cancer (Oxford, England: 1990)*. 36 (10). p.pp. 1224–1228.

Portier, C.J. & Bell, D.A. (1998). Genetic susceptibility: significance in risk assessment. *Toxicology Letters*. 102-103. p.pp. 185–189.

Potischman, N. & Freudenheim, J.L. (2003). Biomarkers of Nutritional Exposure and Nutritional Status: An Overview. *The Journal of Nutrition*. 133 (3). p.p. 873S–874S.

Poulsen, H.E., Prieme, H. & Loft, S. (1998). Role of oxidative DNA damage in cancer initiation and promotion. *European journal of cancer prevention: the official journal of the European Cancer Prevention Organisation (ECP)*. 7 (1). p.pp. 9–16.

Prá, D., Bortoluzzi, A., Müller, L.L., Hermes, L., Horta, J.A., Maluf, S.W., Henriques, J.A.P., Fenech, M. & Franke, S.I.R. (2011). Iron intake, red cell indicators of iron status, and DNA damage in young subjects. *Nutrition (Burbank, Los Angeles County, Calif.)*. 27 (3). p.pp. 293–297.

Prá, D., Franke, S.I.R., Henriques, J.A.P. & Fenech, M. (2012). Iron and genome stability: An update. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 733 (1–2). p.pp. 92–99.

Prado, R.P., dos Santos, B.F., Pinto, C.L. de S., de Assis, K.R.C., Salvadori, D.M.F. & Ladeira, M.S.P. (2010). Influence of diet on oxidative DNA damage, uracil misincorporation and DNA repair capability. *Mutagenesis*. 25 (5). p.pp. 483–487.

Preller, L., Burstyn, I., Pater, N.D. & Kromhout, H. (2004). Characteristics of Peaks of Inhalation Exposure to Organic Solvents. *Annals of Occupational Hygiene*. 48 (7). p.pp. 643–652.

Proia, N.K. (2006). Smoking and Smokeless Tobacco-Associated Human Buccal Cell Mutations and Their Association with Oral Cancer--A Review. *Cancer Epidemiology Biomarkers & Prevention*. 15 (6). p.pp. 1061–1077.

Pyatt, D., Natelson, E. & Golden, R. (2008). Is inhalation exposure to formaldehyde a biologically plausible cause of lymphohematopoietic malignancies? *Regulatory Toxicology and Pharmacology: RTP*. 51 (1). p.pp. 119–133.

Qin, X., Lu, Y., Qin, A., Chen, Z., Peng, Q., Deng, Y., Xie, L., Wang, J., Li, R., Zeng, J., Li, S. & Zhao, J. (2013). Vitamin D receptor BsmI polymorphism and ovarian cancer risk: a meta-analysis. *International journal of gynecological cancer: official journal of the International Gynecological Cancer Society*. 23 (7). p.pp. 1178–1183.

Raby, B.A., Lazarus, R., Silverman, E.K., Lake, S., Lange, C., Wjst, M. & Weiss, S.T. (2004). Association of vitamin D receptor gene polymorphisms with childhood and adult asthma. *American Journal of Respiratory and Critical Care Medicine*. 170 (10). p.pp. 1057–1065.

Raimondi, S., Johansson, H., Maisonneuve, P. & Gandini, S. (2009). Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk. *Carcinogenesis*. 30 (7). p.pp. 1170–1180.

Ramirez, A. & Saldanha, P.H. (2002). Micronucleus investigation of alcoholic patients with oral carcinomas. *Genetics and Molecular Research: GMR*. 1 (3). p.pp. 246–260.

Ramos, A., Lima, C. & Pereira-Wilson, C. (2011). DNA damage protection and induction of repair by dietary phytochemicals and cancer prevention: what do we know? *Selected Topics in DNA Repair*. Chen, C. (ed). University of California, San Diego, USA. ISBN: 978-953-307-606-5.

Rekhadevi, P.V., Sailaja, N., Chandrasekhar, M., Mahboob, M., Rahman, M.F. & Grover, P. (2007). Genotoxicity assessment in oncology nurses handling anti-neoplastic drugs. *Mutagenesis*. 22 (6). p.pp. 395–401.

Rhomberg, L.R., Bailey, L.A., Goodman, J.E., Hamade, A.K. & Mayfield, D. (2011). Is exposure to formaldehyde in air causally associated with leukemia?—A hypothesis-based weight-of-evidence analysis. *Critical Reviews in Toxicology*. 41 (7). p.pp. 555–621.

Rodriguez, C., Jacobs, E.J., Mondul, A.M., Calle, E.E., McCullough, M.L. & Thun, M.J. (2004). Vitamin E Supplements and Risk of Prostate Cancer in U.S. Men. *Cancer Epidemiology Biomarkers & Prevention*. 13 (3). p.pp. 378–382.

Rombaldi, F., Cassini, C., Salvador, M., Saffi, J. & Erdtmann, B. (2008). Occupational risk assessment of genotoxicity and oxidative stress in workers handling anti-neoplastic drugs during a working week. *Mutagenesis*. 24 (2). p.pp. 143–148.

Rossi, D.J., Seita, J., Czechowicz, A., Bhattacharya, D., Bryder, D. & Weissman, I.L. (2007). Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. *Cell Cycle (Georgetown, Tex.)*. 6 (19). p.pp. 2371–2376.

Rothman, N., Wacholder, S., Caporaso, N.E., Garcia-Closas, M., Buetow, K. & Fraumeni, J.F., Jr (2001). The use of common genetic polymorphisms to enhance the epidemiologic study of environmental carcinogens. *Biochimica et Biophysica Acta*. 1471 (2). p.pp. C1–10.

Rudolph, K.L., Millard, M., Bosenberg, M.W. & DePinho, R.A. (2001). Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. *Nature Genetics*. 28 (2). p.pp. 155–159.

Saito, Y., Nishio, K., Yoshida, Y. & Niki, E. (2005). Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. *Toxicology*. 210 (2-3). p.pp. 235–245.

Salisbury, B.A., Pungliya, M., Choi, J.Y., Jiang, R., Sun, X.J. & Stephens, J.C. (2003). SNP and haplotype variation in the human genome. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 526 (1-2). p.pp. 53–61.

Santos-Burgoa, C. (2006). Beyond Managing Healthcare Risks. *Annals of the New York Academy of Sciences*. 1076 (1). p.pp. 624–627.

Sarkar, A., Saha, B.K., Basak, R., Mukhopadhyay, I., Karmakar, R. & Chatterjee, M. (2000). Anticlastogenic potential of 1alpha,25-dihydroxyvitamin D3 in murine lymphoma. *Cancer Letters*. 150 (1). p.pp. 1–13.

Sasaki, M., Dakeishi, M., Hoshi, S., Ishii, N. & Murata, K. (2008). Assessment of DNA Damage in Japanese Nurses Handling Antineoplastic Drugs by the Comet Assay. *Journal of Occupational Health*. 50 (1). p.pp. 7–12.

Schierl, R., Böhlandt, A. & Nowak, D. (2009). Guidance Values for Surface Monitoring of Antineoplastic Drugs in German Pharmacies. *Annals of Occupational Hygiene*. 53 (7). p.pp. 703–711.

Schlesselman, JJ (1982). *Case-Control Studies : Design, Conduct, Analysis: Design, Conduct, Analysis*. Oxford University Press.

Schlosser, P.M. (1999). Relative roles of convection and chemical reaction for the disposition of formaldehyde and ozone in nasal mucus. *Inhalation Toxicology*. 11 (10). p.pp. 967–980.

Schmaus, G., Schierl, R. & Funck, S. (2002). Monitoring surface contamination by antineoplastic drugs using gas chromatography-mass spectrometry and voltammetry. *American Journal of Health-System Pharmacy*. 59 (10). p.pp. 956–961.

Schneider, M., Diemer, K., Engelhart, K., Zankl, H., Trommer, W.E. & Biesalski, H.K. (2001). Protective effects of vitamins C and E on the number of micronuclei in lymphocytes in smokers and their role in ascorbate free radical formation in plasma. *Free Radical Research*. 34 (3). p.pp. 209–219.

Schooten, F.J.V., Hirvonen, A., Maas, L.M., Mol, B.A.D., Kleinjans, J.C.S., Bell, D.A. & Durrer, J.D. (1998). Putative susceptibility markers of coronary artery disease: association between VDR genotype, smoking, and aromatic DNA adduct levels in human right atrial tissue. *The FASEB Journal*. 12 (13). p.pp. 1409–1417.

Schulte, P. & Mazzuckelli, L.F. (1991). Validation of biological markers for quantitative risk assessment. *Environmental Health Perspectives*. 90. p.pp. 239–246.

Sedelnikova, O.A., Redon, C.E., Dickey, J.S., Nakamura, A.J., Georgakilas, A.G. & Bonner, W.M. (2010). Role of oxidatively induced DNA lesions in human pathogenesis. *Mutation Research/Reviews in Mutation Research*. 704 (1-3). p.pp. 152–159.

Serrano-García, L. & Montero-Montoya, R. (2001). Micronuclei and chromatid buds are the result of related genotoxic events. *Environmental and Molecular Mutagenesis*. 38 (1). p.pp. 38–45.

Sessink, P.J., Boer, K.A., Scheefhals, A.P., Anzion, R.B. & Bos, R.P. (1992). Occupational exposure to antineoplastic agents at several departments in a hospital. Environmental contamination and excretion of cyclophosphamide and ifosfamide in urine of exposed workers. *International Archives of Occupational and Environmental Health*. 64 (2). p.pp. 105–112.

Sessink, P.J. & Bos, R.P. (1999). Drugs hazardous to healthcare workers. Evaluation of methods for monitoring occupational exposure to cytostatic drugs. *Drug safety: an international journal of medical toxicology and drug experience*. 20 (4). p.pp. 347–359.

Sessink, P.J.M., Kroese, E.D., Kranen, H.J. van & Bos, R.P. (1995). Cancer risk assessment for health care workers occupationally exposed to cyclophosphamide. *International Archives of Occupational and Environmental Health*. 67 (5). p.pp. 317–323.

Sexton, K., Needham, L. & Pirkle, J. (2004). Human Biomonitoring of Environmental Chemicals. *American Scientist*. 92 (1). p.p. 38.

Shahbazi, S., Alavi, S., Majidzadeh-A, K., Ghaffarpour, M., Soleimani, A. & Mahdian, R. (2013). BsmI but not FokI polymorphism of VDR gene is contributed in breast cancer. *Medical Oncology (Northwood, London, England)*. 30 (1). p.p. 393.

Shen, H., Sturgis, E.M., Dahlstrom, K.R., Zheng, Y., Spitz, M.R. & Wei, Q. (2002). A variant of the DNA repair gene XRCC3 and risk of squamous cell carcinoma of the head and neck: a case-control analysis. *International Journal of Cancer.* 99 (6). p.pp. 869–872.

Shimizu, N. (2011). Molecular mechanisms of the origin of micronuclei from extrachromosomal elements. *Mutagenesis*. 26 (1). p.pp. 119–123.

Shimizu, N., Itoh, N., Utiyama, H. & Wahl, G.M. (1998). Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase. *The Journal of Cell Biology*. 140 (6). p.pp. 1307–1320.

Shimizu, N., Shimura, T. & Tanaka, T. (2000). Selective elimination of acentric double minutes from cancer cells through the extrusion of micronuclei. *Mutation Research*. 448 (1). p.pp. 81–90.

Shimizu, N., Shingaki, K., Kaneko-Sasaguri, Y., Hashizume, T. & Kanda, T. (2005). When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. *Experimental Cell Research*. 302 (2). p.pp. 233–243.

Shojaei, A.H. (1998). Buccal mucosa as a route for systemic drug delivery: a review. *Journal of pharmacy & pharmaceutical sciences: a publication of the Canadian Society for Pharmaceutical Sciences, Société canadienne des sciences pharmaceutiques*. 1 (1). p.pp. 15–30.

Simpson, D. & Plosker, G.L. (2004). Paclitaxel: As Adjuvant or Neoadjuvant Therapy in Early Breast Cancer. *Drugs*. 64 (16). p.pp. 1839–1847.

Singh, N. & Lai, H. (2009). Methods for freezing blood samples at -80^{o}C for DNA damage analysis in human leukocytes. In: *The Comet Assay in Toxicology*. Chapter 5. [Online]. Royal Society of Chemistry, pp. 120 – 128. Available from: www.rsc.org.

Singh, N.P., McCoy, M.T., Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*. 175 (1). p.pp. 184–191.

Singh, U., Devaraj, S. & Jialal, I. (2005). Vitamin E, oxidative stress, and inflammation. *Annual Review of Nutrition*. 25. p.pp. 151–174.

Skov, T., Maarup, B., Olsen, J., Rørth, M., Winthereik, H. & Lynge, E. (1992). Leukaemia and reproductive outcome among nurses handling antineoplastic drugs. *British Journal of Industrial Medicine*. 49 (12). p.pp. 855–861.

Smith, C.C., O'Donovan, M.R. & Martin, E.A. (2006). hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis*. 21 (3). p.pp. 185–190.

Sorsa, M., Hämeilä, M. & Järviluoma, E. (2006). Handling Anticancer Drugs. *Annals of the New York Academy of Sciences*. 1076 (1). p.pp. 628–634.

Speit, G., Gelbke, H.-P., Pallapies, D. & Morfeld, P. (2010). Occupational Exposure to Formaldehyde, Hematotoxicity and Leukemia-Specific Chromosome Changes in Cultured Myeloid Progenitor Cells - Letter. *Cancer Epidemiology Biomarkers & Prevention*. 19 (7). p.pp. 1882–1884.

Speit, G., Ladeira, C., Linsenmeyer, R., Schütz, P. & Högel, J. (2012). Re-evaluation of a reported increased micronucleus frequency in lymphocytes of workers occupationally exposed to formaldehyde. *Mutation Research*. 744 (2). p.pp. 161–166.

Speit, G. & Schmid, O. (2006). Local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated epithelial cells. *Mutation Research/Reviews in Mutation Research*. 613 (1). p.pp. 1–9.

Speit, G., Schmid, O., Fröhler-Keller, M., Lang, I. & Triebig, G. (2007). Assessment of local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated buccal mucosa cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 627 (2). p.pp. 129–135.

Speit, G., Witton-Davies, T., Heepchantree, W., Trenz, K. & Hoffmann, H. (2003). Investigations on the effect of cigarette smoking in the comet assay. *Mutation Research*. 542 (1-2). p.pp. 33–42.

Speit, G., Zeller, J. & Neuss, S. (2011). The in vivo or ex vivo origin of micronuclei measured in human biomonitoring studies. *Mutagenesis*. 26 (1). p.pp. 107–110.

Spitz, M.R. & Bondy, M.L. (2010). The evolving discipline of molecular epidemiology of cancer. *Carcinogenesis*. 31 (1). p.pp. 127–134.

Stich, H.F. & Rosin, M.P. (1983). Quantitating the synergistic effect of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells. *International journal of cancer. Journal international du cancer*. 31 (3). p.pp. 305–308.

Stich, H.F., Rosin, M.P. & Vallejera, M.O. (1984a). Reduction with vitamin A and beta-carotene administration of proportion of micronucleated buccal mucosal cells in Asian betal nut and tobacco chewers. *Lancet*. 1 (8388). p.pp. 1204–1206.

Stich, H.F., Stich, W., Rosin, M.P. & Vallejera, M.O. (1984b). Use of the micronucleus test to monitor the effect of vitamin A, beta-carotene and canthaxanthin on the buccal mucosa of betel nut/tobacco chewers. *International journal of cancer. Journal international du cancer*. 34 (6). p.pp. 745–750.

Stover, D. & Achutan, C. (2011). Occupational exposures to antineoplastic drugs in an Oncology-Hematology Department. *Journal of Occupational and Environmental Hygiene*. 8 (1). p.pp. D1–6.

Strickland, P.T. & Groopman, J.D. (1995). Biomarkers for assessing environmental exposure to carcinogens in the diet. *The American Journal of Clinical Nutrition*. 61 (3 Suppl). p.p. 710S–720S.

Stücker, I., Caillard, J.F., Collin, R., Gout, M., Poyen, D. & Hémon, D. (1990). Risk of spontaneous abortion among nurses handling antineoplastic drugs. *Scandinavian Journal of Work, Environment & Health*. 16 (2). p.pp. 102–107.

Sugimura, T. (2000). Nutrition and dietary carcinogens. *Carcinogenesis*. 21 (3). p.pp. 387–395.

Sul, D., Kim, H., Oh, E., Phark, S., Cho, E., Choi, S., Kang, H.-S., Kim, E.-M., Hwang, K.-W. & Jung, W.-W. (2007). Gene expression profiling in lung tissues from rats exposed to formaldehyde. *Archives of Toxicology*. 81 (8). p.pp. 589–597.

Suruda, A., Schulte, P., Boeniger, M., Hayes, R.B., Livingston, G.K., Steenland, K., Stewart, P., Herrick, R., Douthit, D. & Fingerhut, M.A. (1993). Cytogenetic effects of formaldehyde exposure in students of mortuary science. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2 (5). p.pp. 453–460.

Sutandyo, N. (2010). Nutritional carcinogenesis. *Acta medica Indonesiana*. 42 (1). p.pp. 36–42.

Takezaki, T., Gao, C.M., Wu, J.Z., Li, Z.Y., Wang, J.D., Ding, J.H., Liu, Y.T., Hu, X., Xu, T.L., Tajima, K. & Sugimura, H. (2002). hOGG1 Ser(326)Cys polymorphism and modification by environmental factors of stomach cancer risk in Chinese. *Int J Cancer*. 99 (4). p.pp. 624 – 627.

Tanaka, T., Kohno, H. & Mori, H. (2001). Chemoprevention of Colon Carcinogenesis by Dietary Non-nutritive Compounds. *Asian Pacific journal of cancer prevention: APJCP*. 2 (3). p.pp. 165–177.

Tarng, D.-C., Tsai, T.-J., Chen, W.-T., Liu, T.-Y. & Wei, Y.-H. (2001). Effect of Human OGG1 1245C→G Gene Polymorphism on 8-Hydroxy-2′-Deoxyguanosine Levels of Leukocyte DNA among Patients Undergoing Chronic Hemodialysis. *Journal of the American Society of Nephrology*. 12 (11). p.pp. 2338–2347.

Taylor, J.A., Hirvonen, A., Watson, M., Pittman, G., Mohler, J.L. & Bell, D.A. (1996). Association of prostate cancer with vitamin D receptor gene polymorphism. *Cancer research*. 56 (18). p.pp. 4108–4110.

Teng, S., Beard, K., Pourahmad, J., Moridani, M., Easson, E., Poon, R. & O'Brien, P.J. (2001). The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. *Chemico-biological interactions*. 130-132 (1-3). p.pp. 285–296.

Thier, R., Brüning, T., Roos, P.H., Rihs, H.-P., Golka, K., Ko, Y. & Bolt, H.M. (2003). Markers of genetic susceptibility in human environmental hygiene and toxicology: The role of selected CYP, NAT and GST genes. *International Journal of Hygiene and Environmental Health*. 206 (3). p.pp. 149–171.

Thiringer, G., Granung, G., Holmén, A., Högstedt, B., Järvholm, B., Jönsson, D., Persson, L., Wahlström, J. & Westin, J. (1991). Comparison of methods for the biomonitoring of nurses handling antitumor drugs. *Scand J Work Environ Health* 17: 133 – 138.

Thomas, P. & Fenech, M. (2011). Buccal micronucleus cytome assay. *Methods in molecular biology (Clifton, N.J.)*. 682. p.pp. 235–248.

Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S. & Fenech, M. (2009). Buccal micronucleus cytome assay. *Nature Protocols*. 4 (6). p.pp. 825–837.

Thomas, P., Umegaki, K. & Fenech, M. (2003). Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. *Mutagenesis*. 18 (2). p.pp. 187–194.

Thomas, P., Wu, J., Dhillon, V. & Fenech, M. (2011). Effect of dietary intervention on human micronucleus frequency in lymphocytes and buccal cells. *Mutagenesis*. 26 (1). p.pp. 69–76.

Thompson, H.J. (2004). DNA Oxidation Products, Antioxidant Status, and Cancer Prevention. *The Journal of Nutrition*. 134 (11). p.p. 3186S–3187S.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. & Sasaki, Y.F. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*. 35 (3). p.pp. 206–221.

Timbrell, J.A. (1998). Biomarkers in toxicology. *Toxicology*. 129 (1). p.pp. 1–12.

Tinwell, H. & Ashby, J. (1994). Genetic toxicity and potential carcinogenicity of taxol. *Carcinogenesis*. 15 (8). p.pp. 1499–1501.

Tolbert, P.E., Shy, C.M. & Allen, J.W. (1991). Micronuclei and other nuclear anomalies in buccal smears: a field test in snuff users. *American journal of epidemiology*. 134 (8). p.pp. 840–850.

Tolbert, P.E., Shy, C.M. & Allen, J.W. (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutation Research/Environmental Mutagenesis and Related Subjects*. 271 (1). p.pp. 69–77.

Tomasetti, M., Gellert, N., Procopio, A. & Neuzil, J. (2004). A vitamin E analogue suppresses malignant mesothelioma in a preclinical model: a future drug against a fatal neoplastic disease? *International journal of cancer. Journal international du cancer*. 109 (5). p.pp. 641–642.

Toscano, W.A. & Oehlke, K.P. (2005). Systems Biology: New Approaches to Old Environmental Health Problems. *International Journal of Environmental Research and Public Health*. 2 (1). p.pp. 4–9.

Turci, R., Minoia, C., Sottani, C., Coghi, R., Severi, P., Castriotta, C., Del Bianco, M. & Imbriani, M. (2010). Occupational exposure to antineoplastic drugs in seven Italian hospitals: The effect of quality assurance and adherence to guidelines. *Journal of Oncology Pharmacy Practice*. 17 (4). p.pp. 320–332.

Uitterlinden, A.G., Fang, Y., Van Meurs, J.B.J., Pols, H.A.P. & Van Leeuwen, J.P.T.M. (2004). Genetics and biology of vitamin D receptor polymorphisms. *Gene*. 338 (2). p.pp. 143–156.

Umegaki, K. & Fenech, M. (2000). Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis*. 15 (3). p.pp. 261–269.

Ündeğer, Ü., Başaran, N., Kars, A. & Güç, D. (1999). Assessment of DNA damage in nurses handling antineoplastic drugs by the alkaline COMET assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 439 (2). p.pp. 277–285.

Ursini, C.L., Cavallo, D., Colombi, A., Giglio, M., Marinaccio, A. & Iavicoli, S. (2006). Evaluation of early DNA damage in healthcare workers handling antineoplastic drugs. *International Archives of Occupational and Environmental Health*. 80 (2). p.pp. 134–140.

U.S. Congress, Office of Technology Assessment (1990). *Genetic monitoring and screening in the workplace*.

US EPA, O. (n.d.). *Formaldehyde | Technology Transfer Network Air Toxics Web site | US EPA*. [Online]. Available from: http://www.epa.gov/ttn/atw/hlthef/formalde.html. [Accessed: 4 April 2014].

Utani, K., Kawamoto, J. & Shimizu, N. (2007). Micronuclei bearing acentric extrachromosomal chromatin are transcriptionally competent and may perturb the cancer cell phenotype. *Molecular cancer research: MCR*. 5 (7). p.pp. 695–704.

Utani, K., Kohno, Y., Okamoto, A. & Shimizu, N. (2010). Emergence of micronuclei and their effects on the fate of cells under replication stress. *PloS one*. 5 (4). p.p. e10089.

Vähäkangas, K. (2008). Molecular Epidemiology and Ethics: Biomarkers for Disease Susceptibility. In: C. Wild, P. Vineis, & S. Garte (eds.). *Molecular Epidemiology of Chronic Diseases*. [Online]. John Wiley & Sons, Ltd, pp. 281–297.

Vainio, H. (1998). Use of biomarkers — new frontiers in occupational toxicology and epidemiology. *Toxicology Letters*. 102-103. p.pp. 581–589.

van Helden, Y.G., Keijer, J., Knaapen, A.M., Heil, S.G., Briedé, J.J., van Schooten, F.J. & Godschalk, R.W. (2009). Beta-carotene metabolites enhance inflammation-induced oxidative DNA damage in lung epithelial cells. *Free Radic Biol Med.* 46 (2). p.pp. 299 – 304.

Valavanidis, A., Vlachogianni, T. & Fiotakis, C. (2009). 8-hydroxy-2′ -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *Journal of Environmental Science and Health, Part C*. 27 (2). p.pp. 120–139.

Valdivielso, J.M. & Fernandez, E. (2006). Vitamin D receptor polymorphisms and diseases. *Clinica Chimica Acta*. 371 (1–2). p.pp. 1–12.

Valverde, M. & Rojas, E. (2009). Environmental and occupational biomonitoring using the Comet assay. *Mutation Research*. 681 (1). p.pp. 93–109.

Valverde M & Rojas E (2009). The Comet assay in human biomonitoring. In: *The Comet Assay in Toxicology*. [Online]. Royal Society of Chemistry, pp. 227 – 251. Available from: www.rsc.org.

Viegas, S., Ladeira, C., Nunes, C., Malta-Vacas, J., Gomes, M., Brito, M., Mendonca, P. & Prista, J. (2010). Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. *Journal of occupational medicine and toxicology (London, England)*. 5 (1). p.p. 25.

Viegas, S., Pádua, M., Veiga, A.C., Carolino, E. & Gomes, M. (2014). Antineoplastic drugs contamination of workplace surfaces in two Portuguese hospitals. *Environmental Monitoring and Assessment*. [Online]. Available from: http://link.springer.com/10.1007/s10661-014-3969-1. [Accessed: 12 August 2014].

Viegas, S. & Prista, J. (2007). Cancro Nasofaríngeo e Exposição a Formaldeído: avaliação da história profissional em 63 casos registados. *Sociedade Portuguesa de Medicina do Trabalho*. 6. p.pp. 13 – 22.

Villarini, M., Dominici, L., Fatigoni, C., Muzi, G., Monarca, S. & Moretti, M. (2012). Biological effect monitoring in peripheral blood lymphocytes from subjects occupationally exposed to antineoplastic drugs: assessment of micronuclei frequency. *Journal of Occupational Health*. 54 (6). p.pp. 405–415.

Villarini, M., Dominici, L., Piccinini, R., Fatigoni, C., Ambrogi, M., Curti, G., Morucci, P., Muzi, G., Monarca, S. & Moretti, M. (2011). Assessment of primary, oxidative and excision repaired DNA damage in hospital personnel handling antineoplastic drugs. *Mutagenesis*. 26 (3). p.pp. 359–369.

Vineis, P. (1995). Use of biomarkers in epidemiology. The example of metabolic susceptibility to cancer. *Toxicology letters*. 77 (1-3). p.pp. 163–168.

Vineis, P. & Garte, S. (2008). Biomarker validation. In: *Molecular Epidemiology of chronic diseases*. John Wiley & Sons, Ltd.

Vineis, P. & Perera, F. (2007). Molecular Epidemiology and Biomarkers in Etiologic Cancer Research: The New in Light of the Old. *Cancer Epidemiology Biomarkers & Prevention*. 16 (10). p.pp. 1954–1965.

Vineis, P. & Porta, M. (1996). Causal thinking, biomarkers, and mechanisms of carcinogenesis. *Journal of Clinical Epidemiology*. 49 (9). p.pp. 951–956.

Volkovová, K., Dusinska Maria & Collins Andrew (2006). From oxidative DNA damage to molecular epidemiology. *Journal of Applied Biomedicine*. (4). p.pp. 39 – 43.

Vral, A., Fenech, M. & Thierens, H. (2011). The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure. *Mutagenesis*. 26 (1). p.pp. 11–17.

Walrath, J. & Fraumeni, J.F., Jr (1983). Mortality patterns among embalmers. *International journal of cancer. Journal international du cancer*. 31 (4). p.pp. 407–411.

Wanasundara, P.K.J.P.D. & Shahidi, F. (2005). Antioxidants: Science, Technology, and Applications. In: *Bailey's Industrial Oil and Fat Products*. [Online]. John Wiley & Sons, Inc. Available from: http://onlinelibrary.wiley.com/doi/10.1002/047167849X.bio002/abstract. [Accessed: 30 April 2014].

Wang, L.-L., Yang, A.-K., Li, Y., Liu, J.-P. & Zhou, S.-F. (2010). Phenotype prediction of deleterious nonsynonymous single nucleotide polymorphisms in human alcohol metabolism-related genes: a bioinformatics study. *Alcohol (Fayetteville, N.Y.)*. 44 (5). p.pp. 425–438.

Wang, Y., Liang, D., Spitz, M.R., Zhang, K., Dong, Q., Amos, C.I. & Wu, X. (2003). XRCC3 genetic polymorphism, smoking, and lung carcinoma risk in minority populations. *Cancer*. 98 (8). p.pp. 1701–1706.

Wasson, G.R., McKelvey-Martin, V.J. & Downes, C.S. (2008). The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis*. 23 (3). p.pp. 153–162.

Waterfield, C. & Timbrell, J. A. (1999). Biomarkers – An Overview. In: *General and Applied Toxicology*. Stockton Press.

Waters, M.D., Stack, H.F. & Jackson, M.A. (1999). Genetic toxicology data in the evaluation of potential human environmental carcinogens. *Mutation Research*. 437 (1). p.pp. 21–49.

Watson, W.P. & Mutti, A. (2004). Role of biomarkers in monitoring exposures to chemicals: present position, future prospects. *Biomarkers*. 9 (3). p.pp. 211–242.

Watters, J.L., Satia, J.A., Kupper, L.L., Swenberg, J.A., Schroeder, J.C. & Switzer, B.R. (2007). Associations of Antioxidant Nutrients and Oxidative DNA Damage in Healthy African-American and White Adults. *Cancer Epidemiology Biomarkers & Prevention*. 16 (7). p.pp. 1428–1436.

Weis, B.K., Balshaw, D., Barr, J.R., Brown, D., Ellisman, M., Lioy, P., Omenn, G., Potter, J.D., Smith, M.T., Sohn, L., Suk, W.A., Sumner, S., Swenberg, J., Walt, D.R., Watkins, S., Thompson, C. & Wilson, S.H. (2005). Personalized Exposure Assessment: Promising Approaches for Human Environmental Health Research. *Environmental Health Perspectives*. 113 (7). p.pp. 840–848.

Wild, C.P. (2009a). Environmental exposure measurement in cancer epidemiology. *Mutagenesis*. 24 (2). p.pp. 117–125.

Willett, W. & Giovannucci, E. (2006). Epidemiology of Diet and cancer risk. In: *Modern Nutrition in Health and Disease*. Lippincott Williams and Wilkins: M Shils, M., Shike, M., Ross, A., Caballer, B. and Cousins, R., p. 1627.

Winsey, S.L., Haldar, N.A., Marsh, H.P., Bunce, M., Marshall, S.E., Harris, A.L., Wojnarowska, F. & Welsh, K.I. (2000). A variant within the DNA repair gene XRCC3 is associated with the development of melanoma skin cancer. *Cancer Research*. 60 (20). p.pp. 5612–5616.

Wiseman, H. & Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *The Biochemical journal*. 313 (Pt 1). p.pp. 17–29.

Wojda, A., Zietkiewicz, E. & Witt, M. (2007). Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects. *Mutagenesis*. 22 (3). p.pp. 195–200.

Wojewódzka, M., Kruszewski, M., Iwanenko, T., Collins, A.R. & Szumiel, I. (1999). Lack of adverse effect of smoking habit on DNA strand breakage and base damage, as revealed by the alkaline comet assay. *Mutation Research*. 440 (1). p.pp. 19–25.

Woodward, M. (2004). *Epidemiology: Study Design and Data Analysis, Second Edition*. 2 edition. Boca Raton: Chapman and Hall/CRC.

Wright, M.E., Virtamo, J., Hartman, A.M., Pietinen, P., Edwards, B.K., Taylor, P.R., Huttunen, J.K. & Albanes, D. (2007). Effects of alpha-tocopherol and beta-carotene supplementation on upper aerodigestive tract cancers in a large, randomized controlled trial. *Cancer*. 109 (5). p.pp. 891–898.

Xing, D.-Y., Tan, W., Song, N. & Lin, D.-X. (2001). Ser326Cys polymorphism in hOGG1 gene and risk of esophageal cancer in a Chinese population. *International Journal of Cancer*. 95 (3). p.pp. 140–143.

Yamane, A., Kohno, T., Ito, K., Sunaga, N., Aoki, K., Yoshimura, K., Murakami, H., Nojima, Y. & Yokota, J. (2004). Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo. *Carcinogenesis*. 25 (9). p.pp. 1689–1694.

Yang, H., Jia, X., Chen, X., Yang, C.S. & Li, N. (2012). Time-selective chemoprevention of vitamin E and selenium on esophageal carcinogenesis in rats: the possible role of nuclear factor kappaB signaling pathway. *International Journal of Cancer.* 131 (7). p.pp. 1517–1527.

Ye, X., Yan, W., Xie, H., Zhao, M. & Ying, C. (2005). Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. *Mutation Research*. 588 (1). p.pp. 22–27.

Yoshida, J., Kosaka, H., Tomioka, K. & Kumagai, S. (2006). Genotoxic Risks to Nurses from Contamination of the Work Environment with Antineoplastic Drugs in Japan. *Journal of Occupational Health*. 48 (6). p.pp. 517–522.

Yoshihara, T., Ishida, M., Kinomura, A., Katsura, M., Tsuruga, T., Tashiro, S., Asahara, T. & Miyagawa, K. (2004). XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. *The EMBO journal*. 23 (3). p.pp. 670–680.

Zhang, L., Freeman, L.E.B., Nakamura, J., Hecht, S.S., Vandenberg, J.J., Smith, M.T. & Sonawane, B.R. (2009a). Formaldehyde and leukemia: Epidemiology, potential mechanisms, and implications for risk assessment. *Environmental and Molecular Mutagenesis*. p.p. NA–NA.

Zhang, L., Steinmaus, C., Eastmond, D.A., Xin, X.K. & Smith, M.T. (2009b). Formaldehyde exposure and leukemia: A new meta-analysis and potential mechanisms. *Mutation Research/Reviews in Mutation Research*. 681 (2-3). p.pp. 150–168.

Zhang, L., Tang, X., Rothman, N., Vermeulen, R., Ji, Z., Shen, M., Qiu, C., Guo, W., Liu, S., Reiss, B., Freeman, L.B., Ge, Y., Hubbard, A.E., Hua, M., Blair, A., Galvan, N., Ruan, X., Alter, B.P., Xin, K.X., Li, S., Moore, L.E., Kim, S., Xie, Y., Hayes, R.B., Azuma, M., Hauptmann, M., Xiong, J., Stewart, P., Li, L., Rappaport, S.M., Huang, H., Fraumeni, J.F., Smith, M.T. & Lan, Q. (2010). Occupational Exposure to Formaldehyde, Hematotoxicity, and Leukemia-Specific Chromosome Changes in Cultured Myeloid Progenitor Cells. *Cancer Epidemiology Biomarkers & Prevention*. 19 (1). p.pp. 80–88.

Zhang, X., Dai, B., Zhang, B. & Wang, Z. (2012). Vitamin A and risk of cervical cancer: a meta-analysis. *Gynecologic Oncology*. 124 (2). p.pp. 366–373.

Zietkiewicz, E., Wojda, A. & Witt, M. (2009). Cytogenetic perspective of ageing and longevity in men and women. *Journal of Applied Genetics*. 50 (3). p.pp. 261–273.

Zijno, A., Verdina, A., Galati, R., Leopardi, P., Marcon, F., Andreoli, C., Rossi, S. & Crebelli, R. (2006). Influence of DNA repair polymorphisms on biomarkers of genotoxic damage in peripheral lymphocytes of healthy subjects. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 600 (1-2). p.pp. 184–192.

Zingg, J.-M. & Azzi, A. (2004). Non-antioxidant activities of vitamin E. *Current medicinal chemistry*. 11 (9). p.pp. 1113–1133.

VIII. Annexes

Annex I

Personnal Questionnaire

QUESTIONÁRIO

Secção I – Identificação

1.1. Género: M ☐ F ☐

1.2. Idade:·☐

1.3. Nome: _____
(Apenas para identificação da exposição)

Secção II – Historial Social

2.1. Carga tabágica

- Fuma ou alguma vez fumou? Sim ☐ Não ☐
- Se sim, com que idade começou a fumar regularmente? ____ anos
- Continua a fumar? Sim ☐ Quantos cigarros por dia? ____
  Não ☐ Quando parou de fumar? ____

2.2. Consumo de álcool

Com que frequência consome álcool? _____

Qual a quantidade que consome? _____

Secção III – Historial Ocupacional

3.1. Presente Ocupação

Área de Trabalho: _____

Função exercida: _____

Função exercida há ____anos

Descrição do tipo de trabalho:

_____

_____

Tempo de actividade na empresa: _____ (anos)

Existe exposição ao formaldeído no seu posto de trabalho? Sim ☐ Não ☐

Se sim passe para o ponto 3.2.

Se não passe para o ponto 3.3.

3.2. Exposição ao formaldeído no local de trabalho

3.2.1. Ocupações ou actividades exercidas actualmente com exposição ao formaldeído.

	Função exercida	**Horas por dia**	**Dias por semana**
1			
2			
3			

3.2.2. Ocupações ou actividades exercidas anteriormente com exposição ao formaldeído

	Função exercida	**Horas por dia**	**Dias por semana**	**De… a …. (anos)**
1				
2				
3				
4				

3.3. Exposição a outros produtos no local de trabalho

Considera estar exposto a algum destes produtos?

- Fenol ☐
- Metanol ☐
- Ácido acético ☐
- Soda cáustica ☐
- Ácido clorídrico ☐
- Hipoclorito de sódio (lixívia) ☐
- Partículas ☐

Secção IV – Susceptibilidade individual

4.1. Tem, ou teve, alguma doença respiratória? Sim ☐ Não ☐

Se sim, qual? _____

Toma alguma medicação? Sim ☐ Não ☐

Se sim, qual? _____

4.2. Tem, ou teve, alguma dificuldade respiratória? Sim ☐ Não ☐

Se sim, qual? _____

Toma alguma medicação? Sim ☐ Não ☐

Se sim, qual? _____

4.3. Tem, ou teve, alguma doença oncológica? Sim ☐ Não ☐

Se sim, qual? _____

Toma alguma medicação? Sim ☐ Não ☐

Se sim, qual? _____

4.4 Existem doenças oncológicas em familiares directos?

Sim ☐ Não ☐

Se sim, quais? _____

4.5. Toma algum suplemento alimentar?

Sim ☐ Não ☐

Se sim, quais? _____

4.6. Toma presentemente algum dos seguintes medicamentos?

Sim ☐ Não ☐

Se sim, quais?

☐ Folifer

☐ Neurobion

☐ Folacin

☐ Outro_____

Secção V – Equipamento de Protecção Colectiva

Existem medidas de protecção colectiva? Sim ☐ Não ☐

Se sim, quais?

Sistema de exaustão de ar? Sim ☐ Não ☐

Sistema de insuflação de ar? Sim ☐ Não ☐

Sistema de climatização? Sim ☐ Não ☐

Secção VI – Equipamento de Protecção Individual

Utiliza equipamento de protecção individual? Sim ☐ Não ☐
Se sim, especifique:

Botas de protecção: Sim ☐ Não ☐

Luvas: Sim ☐ Não ☐

Máscara das vias respiratórias: Sim ☐ Não ☐

Óculos de protecção: Sim ☐ Não ☐

Vestuário adequado: Sim ☐ Não ☐

Protectores auriculares: Sim ☐ Não ☐

Capacete: Sim ☐ Não ☐

Secção VII – Tempos Livres

Que tipo de actividades desenvolve para além da sua actividade profissional?

_____

_____

_____

_____.

Annex II

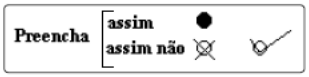
Food Frequency Questionnaire

Unidade de Epidemiologia Nutricional
Serviço de Higiene e Epidemiologia
Faculdade de Medicina do Porto

INSTRUÇÕES (PARA ENTREVISTADOR)

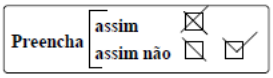
• As questões devem ser "neutras", isto é, não devem influenciar de qualquer forma o tipo de respostas

• O questionário pretende identificar o consumo de alimentos do ano anterior. Assim para cada alimento, deve assinalar, preenchendo o respectivo círculo, quantas vezes, em média, por dia, semana ou mês o inquirido consumiu cada um dos alimentos referidos nesta lista, **ao longo do último ano**. Não se esqueça de assinalar no círculo respectivo os alimentos que o inquirido nunca come, ou come menos de 1 vez por mês.



• Na coluna correspondente à quantidade assinale se a porção que habitualmente o inquirido come é igual, maior ou menor do que a referida como porção média.

• Para os alimentos que só são consumidos, em determinadas épocas do ano (por ex: cerejas, diospiros, etc.), assinale as vezes em que o inquirido consumiu o alimento nessa época, e coloque uma cruz (x) na última coluna (Sazonal).



• Não se esqueça de ter em conta as vezes que o alimento é consumido sozinho e aquelas em que é adicionado a outros alimentos ou pratos (ex: café com leite, os ovos das omeletas, etc).

• No grupo III - **Óleos e Gorduras** - pergunte apenas os que são **adicionados** em saladas, no prato, no pão, etc, e **não** aos utilizados para cozinhar

• No grupo VI - **Hortaliças e Legumes** - pergunte pensando nos que são consumidos no prato (cozidos ou em saladas) e **não** nos que entram na confecção da sopa.

• No item nº 86, anote a frequência com que o inquirido come sopa de legumes. No caso da sopa consumida ser caldo verde, canja ou sopa instantânea, com uma frequência de **pelo menos 1 vez por semana**, deve assinalar este consumo separadamente no quadro existente para outros alimentos, tendo o cuidado em o subtrair à frequência que foi referida anteriormente para a sopa de legumes.

• Se houver algum alimento não mencionado na lista de alimentos e que consuma pelo menos 1 vez por semana, assinale, no quadro que existe para **outros alimentos**, a respectiva frequência e indique ainda a porção média de consumo. ***Por ex: frutos tropicais, sumos de fruta natural, bebidas espirituosas, café de mistura, alheiras, farinheiras, frutos secos (figo, ameixa, damasco), produtos dietéticos, rebuçados, etc.***

30295

ID

Por favor, **antes de iniciar o questionário leia as instruções da página anterior.**
Pense durante o último ano quantas vezes por dia, semana ou mês, em média, consumiu cada um dos alimentos referidos. Na coluna referente à quantidade deverá assinalar se sua porção é igual, menor ou maior do que a referida como porção média. Para os alimentos consumidos só em determinadas épocas do ano, anote a frequência com que o alimento é consumido nessa época e assinale com uma cruz **(x)** na última coluna (Sazonal).

I. P. LÁCTEOS	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
1. Leite gordo	○	○	○	○	○	○	○	○	○	1 chávena = 250 ml	○	○	○	□
2. Leite meio-gordo	○	○	○	○	○	○	○	○	○	1 chávena = 250 ml	○	○	○	□
3. Leite magro	○	○	○	○	○	○	○	○	○	1 chávena = 250 ml	○	○	○	□
4. Iogurte	○	○	○	○	○	○	○	○	○	Um =125g	○	○	○	□
5.Queijo (de qualquer tipo incluindo queijo fresco e requeijão)	○	○	○	○	○	○	○	○	○	1 fatia = 30g	○	○	○	□
6. Sobremesas lácteas: pudim, aletria e leite creme , etc	○	○	○	○	○	○	○	○	○	Um ou 1 prato sobremesa	○	○	○	□
7. Gelados	○	○	○	○	○	○	○	○	○	Um ou 2 bolas	○	○	○	□

II. OVOS, CARNES E PEIXES	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
8.Ovos	○	○	○	○	○	○	○	○	○	Um	○	○	○	□
9.Frango	○	○	○	○	○	○	○	○	○	1 porção ou 2 peças=150g	○	○	○	□
10.Peru, coelho	○	○	○	○	○	○	○	○	○	1 porção ou 2 peças=150g	○	○	○	□
11.Carne vaca, porco,cabrito	○	○	○	○	○	○	○	○	○	1 porção =120g	○	○	○	□
12. Figado de vaca, porco, frango	○	○	○	○	○	○	○	○	○	1 porção = 120g	○	○	○	□
13 Língua, mão de vaca, tripas, chispe, coração, rim	○	○	○	○	○	○	○	○	○	1 porção =100g	○	○	○	□
14.Fiambre, chouriço, salpição, presunto, etc	○	○	○	○	○	○	○	○	○	2 fatias ou 3 rodelas =20g	○	○	○	□
15. Salsichas	○	○	○	○	○	○	○	○	○	3 médias	○	○	○	□
16. Toucinho, bacon	○	○	○	○	○	○	○	○	○	2 fatias=50g	○	○	○	□
17. Peixe gordo: sardinha, cavala, carapau, salmão,	○	○	○	○	○	○	○	○	○	1 porção =125g	○	○	○	□
18. Peixe magro: pescada, faneca, dourada, etc	○	○	○	○	○	○	○	○	○	1 porção =125g	○	○	○	□
19.Bacalhau	○	○	○	○	○	○	○	○	○	1 porção =125g	○	○	○	□
20.Peixe conserva: atum, sardinhas,etc	○	○	○	○	○	○	○	○	○	1 lata	○	○	○	□
21.Lulas, polvo	○	○	○	○	○	○	○	○	○	1 porção =100g	○	○	○	□
22.Camarão, amêijoas, mexilhão, etc	○	○	○	○	○	○	○	○	○	1 prato sobremesa =100g	○	○	○	□

III. Óleos e Gorduras	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
23. Azeite	○	○	○	○	○	○	○	○	○	1 colher sopa	○	○	○	□
24. Óleos: girassol, milho, soja	○	○	○	○	○	○	○	○	○	1 colher sopa	○	○	○	□
25. Margarina	○	○	○	○	○	○	○	○	○	1 colher chá	○	○	○	□
26. Manteiga	○	○	○	○	○	○	○	○	○	1 colher chá	○	○	○	□

30295

Unidade de Epidemiologia Nutricional
Serviço de Higiene e Epidemiologia - FMUP

ID

IV.PÃO, CEREAIS E SIMILARES	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
27. Pão branco ou tostas	○	○	○	○	○	○	○	○	○	Um ou 2 tostas = 40g	○	○	○	□
28. Pão (ou tostas), integral,centeio, mistura	○	○	○	○	○	○	○	○	○	Um ou 2 tostas= 50g	○	○	○	□
29. Broa, broa de avintes	○	○	○	○	○	○	○	○	○	1 fatia = 80g	○	○	○	□
30. Flocos cereais (muesli, corn-flakes, chocapic,etc.)	○	○	○	○	○	○	○	○	○	1 chávena =40g	○	○	○	□
31. Arroz	○	○	○	○	○	○	○	○	○	½ prato = 100g	○	○	○	□
32. Massas: esparguete, macarrão, etc.	○	○	○	○	○	○	○	○	○	½ prato = 100g	○	○	○	□
33. Batatas fritas caseiras	○	○	○	○	○	○	○	○	○	½ prato = 100g	○	○	○	□
34. Batatas fritas de pacote	○	○	○	○	○	○	○	○	○	1 pacote pequeno =30g	○	○	○	□
35. Batatas cozidas, assadas, estufadas e puré	○	○	○	○	○	○	○	○	○	2 batatas médias =160 g	○	○	○	□

V.DOCES E PASTÉIS	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
36. Bolachas tipo maria, água e sal ou integrais	○	○	○	○	○	○	○	○	○	3 bolachas	○	○	○	□
37. Outras bolachas ou biscoitos	○	○	○	○	○	○	○	○	○	3 bolachas	○	○	○	□
38. Croissant, pasteis, bolicao, doughnut ou bolos	○	○	○	○	○	○	○	○	○	Um; 1 fatia = 80g	○	○	○	□
39.Chocolate (tablete ou em pó)	○	○	○	○	○	○	○	○	○	3 quadrados; 1 colher sopa	○	○	○	□
40. Snacks de chocolate (Mars, Twix, Kit Kat, etc)	○	○	○	○	○	○	○	○	○	Um	○	○	○	□
41. Marmelada, compota, geleia, mel	○	○	○	○	○	○	○	○	○	1 colher sobremesa	○	○	○	□
42. Açúcar	○	○	○	○	○	○	○	○	○	1 colher sobremesa; 1 pacote	○	○	○	□

VI. HORTALIÇAS E LEGUMES	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
43. Couve branca, couve lombarda	○	○	○	○	○	○	○	○	○	½ chávena =75g	○	○	○	□
44. Penca, Tronchuda	○	○	○	○	○	○	○	○	○	½ chávena = 65g	○	○	○	□
45. Couve galega	○	○	○	○	○	○	○	○	○	½ chávena =65g	○	○	○	□
46. Brócolos	○	○	○	○	○	○	○	○	○	½ chávena =85g	○	○	○	□
47. Couve-flor, Couve-bruxelas	○	○	○	○	○	○	○	○	○	½ chávena =65g	○	○	○	□
48. Grelos, Nabiças, Espinafres	○	○	○	○	○	○	○	○	○	½ chávena =72g	○	○	○	□
49. Feijão verde	○	○	○	○	○	○	○	○	○	½ chávena =65g	○	○	○	□
50. Alface, Agrião	○	○	○	○	○	○	○	○	○	½ chávena =15g	○	○	○	□
51. Cebola	○	○	○	○	○	○	○	○	○	½ média=40g	○	○	○	□
52. Cenoura	○	○	○	○	○	○	○	○	○	1 média=80g	○	○	○	□
53. Nabo	○	○	○	○	○	○	○	○	○	1 médio=78g	○	○	○	□
54. Tomate fresco	○	○	○	○	○	○	○	○	○	½ médio=63g	○	○	○	□
55. Pimento	○	○	○	○	○	○	○	○	○	½ médio=68g	○	○	○	□
56. Pepino	○	○	○	○	○	○	○	○	○	¼ médio=50g	○	○	○	□
57. Leguminosas: feijão, grão de bico	○	○	○	○	○	○	○	○	○	1 chávena	○	○	○	□
58. Ervilha grão, Fava	○	○	○	○	○	○	○	○	○	½ chávena	○	○	○	□

Unidade de Epidemiologia Nutricional
Serviço de Higiene e Epidemiologia - FMUP

30295

ID

VII. FRUTOS	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
59. Maça, pêra	○	○	○	○	○	○	○	○	○	uma média	○	○	○	□
60. Laranja, Tangerinas	○	○	○	○	○	○	○	○	○	1 média; 2 médias	○	○	○	□
61. Banana	○	○	○	○	○	○	○	○	○	uma média	○	○	○	□
62. Kiwi	○	○	○	○	○	○	○	○	○	um médio	○	○	○	□
63. Morangos	○	○	○	○	○	○	○	○	○	1 chávena	○	○	○	□
64. Cerejas	○	○	○	○	○	○	○	○	○	1 chávena	○	○	○	□
65. Pêssego, Ameixa	○	○	○	○	○	○	○	○	○	1 médio; 3 médios	○	○	○	□
66. Melão, Melancia	○	○	○	○	○	○	○	○	○	1 fatia média = 150g	○	○	○	□
67. Diospiro	○	○	○	○	○	○	○	○	○	1 médio	○	○	○	□
68. Figo fresco, Nêsperas, Damascos	○	○	○	○	○	○	○	○	○	3 médios	○	○	○	□
69. Uvas frescas	○	○	○	○	○	○	○	○	○	1 cacho médio	○	○	○	□
70. Frutos conserva pêssego, ananás	○	○	○	○	○	○	○	○	○	2 metades ou rodelas	○	○	○	□
71. Amêndoas, avelãs, nozes, amendoins, pistachio, etc.	○	○	○	○	○	○	○	○	○	½ chávena (descascado)	○	○	○	□
72. Azeitonas	○	○	○	○	○	○	○	○	○	6 unidades	○	○	○	□

VIII. BEBIDAS E MISCELANEAS	Frequência alimentar									Quantidade				sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	A sua porção é: Menor	Igual	Maior	
73. Vinho	○	○	○	○	○	○	○	○	○	1 copo=125m	○	○	○	□
74. Cerveja	○	○	○	○	○	○	○	○	○	1 garrafa ou 1 lata=330 ml	○	○	○	□
75. Bebidas brancas: whisky, aguardente, brandy, etc	○	○	○	○	○	○	○	○	○	1 cálice = 40 ml	○	○	○	□
76. Coca-cola, pepsi-cola ou outras colas	○	○	○	○	○	○	○	○	○	1 garrafa ou 1 lata=330 ml	○	○	○	□
77. Ice-tea	○	○	○	○	○	○	○	○	○	1 garrafa ou 1 lata=330 ml	○	○	○	□
78.Outros refrigerantes, sumos de fruta ou néctares embalados	○	○	○	○	○	○	○	○	○	1 garrafa ou 1 copo = 250 ml	○	○	○	□
79.Café (incluindo pingo, meia de leite e outras bebidas com café)	○	○	○	○	○	○	○	○	○	1 chávena café	○	○	○	□
80. Chá preto e verde	○	○	○	○	○	○	○	○	○	1 chávena	○	○	○	□
81. Croquetes, rissóis, bolinhos de bacalhau, etc.	○	○	○	○	○	○	○	○	○	3 unidades	○	○	○	□
82. Maionese	○	○	○	○	○	○	○	○	○	1 colher sobremesa	○	○	○	□
83. Molho de tomate, ketchup	○	○	○	○	○	○	○	○	○	1 colher sopa	○	○	○	□
84. Pizza	○	○	○	○	○	○	○	○	○	Meia pizza-normal	○	○	○	□
85. Hambúrguer	○	○	○	○	○	○	○	○	○	Um médio	○	○	○	□
86. Sopa de legumes	○	○	○	○	○	○	○	○	○	1 prato	○	○	○	□

Existe algum alimento ou bebida que eu não tenha mencionado e que tenha consumido pelo menos 1 vez por semana mesmo em pequenas quantidades, ou numa época em particular. Por ex: **frutos tropicais, sumos de fruta natural, bebidas espirituosas, café de mistura, alheiras, farinheiras, frutos secos (figo, ameixa, damasco), produtos dietéticos, rebuçados, etc.**

Outros Alimentos	Frequência alimentar									Quantidade	sazonal
	Nunca ou <1 mês	1-3 por mês	1 por sem	2-4 por sem	5-6 por sem	1 por dia	2-3 por dia	4-5 por dia	6 + por dia	Porção Média	
	○	○	○	○	○	○	○	○	○		□
	○	○	○	○	○	○	○	○	○		□
	○	○	○	○	○	○	○	○	○		□

Unidade de Epidemiologia Nutricional
Serviço de Higiene e Epidemiologia - FMUP

30295

Annex III

Authorization Request

Exma. Senhor(a)

Dr. (a designar)

Director(a) de [redacted]

Centro Hospitalar de [redacted]

ASSUNTO: Exposição Profissional a Citostáticos: caracterização da exposição em Unidades Hospitalares Portuguesas.

A Escola Superior de Tecnologia de Saúde de Lisboa, em parceria com a Escola Nacional de Saúde Publica, encontra-se neste momento a desenvolver um estudo sobre a Exposição Profissional a Citostáticos, o qual é suportado pela Autoridade para as Condições do Trabalho.

Os citostáticos constituem um grupo farmacoterapêutico que interfere por vários mecanismos de acção com o DNA, levando à destruição celular. Estes agentes terapêuticos são preparados diariamente em Unidades Hospitalares Portuguesas, e posteriormente, usados no tratamento de várias doenças, nomeadamente neoplasias. A toxicidade destes medicamentos sobre o organismo dos indivíduos expostos manifesta-se a níveis diversos com gravidades distintas, incluindo efeitos adversos como a mutagénese, a teratogénese e a carcinogénese. Apesar da utilização de equipamentos de protecção, os operadores envolvidos na manipulação destes fármacos, nomeadamente os técnicos de farmácia, farmacêuticos e enfermeiros, podem estar expostos de forma significativa a este factor de risco.

Estudos realizados demonstraram que a maioria das superfícies das áreas de trabalho em que os citostáticos são manipulados está contaminada promovendo o contacto com a pele e eventual absorção. No que concerne à exposição por via aérea, esta tem sido pouco estudada, mas julga-se poder ser uma realidade e contribuir de forma significativa para a dose absorvida. Embora ainda insuficiente, a investigação desenvolvida recentemente tem-se centrado na necessidade de desenvolver conhecimento não só sobre os efeitos para a saúde mas também, a enfatizar a criação de programas de prevenção e vigilância da saúde.

Neste contexto, pretende-se realizar um estudo em Unidades Hospitalares Portuguesas, aprofundando 3 vertentes essenciais: a caracterização da exposição, os critérios de avaliação das repercussões sobre o organismo, e os processos de organização dos programas preventivos.

Este estudo que se propõe pretende contribuir para a caracterização da exposição a citostáticos num contexto profissional específico (salas limpas da Farmácia Hospitalar e Serviços de Internamento), identificando os factores que a condicionam e os eventuais efeitos para a saúde dos trabalhadores decorrentes dessa exposição.

É neste sentido que vimos por este meio solicitar a V. Ex.ª autorização para que o trabalho possa ser realizado nos Serviços [redacted] do Hospital [redacted], de cujo serviço V. Ex.ª é Directora, comprometendo-nos, naturalmente, a fornecer todos os resultados obtidos e as nossas próprias reflexões sobre eles, podendo os mesmos ser utilizados para os fins que se entenda convenientes. O estudo será ainda apresentado à Administração do Hospital bem como à Comissão de Ética.

Todos os indivíduos que participem serão informados dos objectivos do estudo, sendo explicado que têm a possibilidade de não participar. Será solicitado o consentimento informado por escrito para pesquisa dos meios biológicos propostos.

Os resultados obtidos serão facultados às Unidades Hospitalares na forma de relatório técnico onde constam os resultados das monitorizações ambientais e as propostas de medidas de eliminação e/ou controlo do risco.

Agradecendo antecipadamente a atenção dispensada a este assunto, enviamos os melhores cumprimentos,

P'la Equipa de Investigação

A Coordenadora

_____

Susana Viegas

(susana.viegas@estesl.ipl.pt)

Annex IV

Consent Informed

TERMO DE CONSENTIMENTO INFORMADO

A Escola Superior de Tecnologia da Saúde de Lisboa encontra-se a desenvolver um projecto de investigação com o tema: "Caracterização da Exposição ao Formaldeído e Conhecimento dos Eventuais Efeitos na Saúde".

Este projecto tem como objectivos primordiais a caracterização da exposição deste agente químico em diversas situações de trabalho, bem como a investigação de eventuais efeitos genotóxicos.

De modo à concretização dos nossos objectivos, pretendemos recolher amostras de sangue periférico e tecido epitelial do interior da cavidade bucal a indivíduos expostos profissionalmente a formaldeído.

A metodologia a utilizar é a referenciada por diversos autores, que tem como objectivo analisar detalhadamente a exposição dos trabalhadores ao formaldeído e, consequentemente, contribuir para minimizar essa exposição.

Acresce-se que a **privacidade** assim como a completa **confidencialidade** dos dados obtidos será assegurada.

Se tiver alguma dúvida poderá esclarece-la com as responsáveis pelo projecto. Obrigada pela atenção e disponibilidade.

Eu, _____ (preencha com o seu nome completo), dou o meu consentimento livre e informado, para participar na realização da colheita acima referidas, autorizando posterior uso e publicação dos dados.

Data ___/___/___

Assinatura

_____

Annex V

Publications

Mutation Research 721 (2011) 15–20

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxicity biomarkers in occupational exposure to formaldehyde—The case of histopathology laboratories

Carina Ladeira [a,b,*], Susana Viegas [a,b], Elisabete Carolino [a], João Prista [c], Manuel C. Gomes [d], Miguel Brito [a]

[a] Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa, Portugal
[b] Centro de Investigação e Estudos em Saúde Pública, Universidade Nova de Lisboa, Portugal
[c] Escola Nacional de Saúde Pública, Universidade Nova de Lisboa, Portugal
[d] Faculdade de Ciências, Universidade de Lisboa, Portugal

ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form 25 October 2010
Accepted 15 November 2010
Available online 20 January 2011

Keywords:
Formaldehyde
Occupational exposure
Pathology/anatomy workers
Genotoxicity biomarkers
Tobacco
Alcohol

ABSTRACT

Formaldehyde, classified by the IARC as carcinogenic in humans and experimental animals, is a chemical agent that is widely used in histopathology laboratories. The exposure to this substance is epidemiologically linked to cancer and to nuclear changes detected by the cytokinesis-block micronucleus test (CBMN). This method is extensively used in molecular epidemiology, since it provides information on several biomarkers of genotoxicity, such as micronuclei (MN), which are biomarkers of chromosomes breakage or loss, nucleoplasmic bridges (NPB), common biomarkers of chromosome rearrangement, poor repair and/or telomere fusion, and nuclear buds (NBUD), biomarkers of elimination of amplified DNA.

The aim of this study is to compare the frequency of genotoxicity biomarkers, provided by the CBMN assay in peripheral lymphocytes and the MN test in buccal cells, between individuals occupationally exposed and non-exposed to formaldehyde and other environmental factors, namely tobacco and alcohol consumption.

The sample comprised two groups: 56 individuals occupationally exposed to formaldehyde (cases) and 85 unexposed individuals (controls), from whom both peripheral blood and exfoliated epithelial cells of the oral mucosa were collected in order to measure the genetic endpoints proposed in this study.

The mean level of TWA_{8h} was 0.16 ± 0.11 ppm (<detection limit until 0.51 ppm) and the mean of ceiling values was 1.14 ± 0.74 ppm (0.18–2.93 ppm). All genotoxicity biomarkers showed significant increases in exposed workers in comparison with controls (Mann–Whitney test, $p < 0.002$) and the analysis of confounding factors showed that there were no differences between genders.

As for age, only the mean MN frequency in lymphocytes was found significantly higher in elderly people among the exposed groups ($p = 0.006$), and there was also evidence of an interaction between age and gender with regards to that biomarker in those exposed.

Smoking habits did not influence the frequency of the biomarkers, whereas alcohol consumption only influenced the MN frequency in lymphocytes in controls ($p = 0.011$), with drinkers showing higher mean values. These results provide evidence of the association between occupational exposure to formaldehyde and the presence of genotoxicity biomarkers.

1. Introduction

Formaldehyde (FA) is a reactive, flammable and colourless gas with a strong and very characteristic pungent odour. When combined with air, FA can form explosive mixtures. FA occurs as an endogenous metabolic product of N-, O- and S-demethylation reactions in most living systems. It is used mainly in the production of resins and their applications, such as adhesives and binders in wood product, pulp and paper, synthetic vitreous fibre industries, production of plastics, coatings, textile finishing, and also as an intermediate in the synthesis of other industrial chemical compounds. Common non-occupational sources of exposure to FA include vehicle emissions, particle boards and similar building materials, carpets, paints and varnishes, food and cooking, tobacco smoke and its use as a disinfectant [1–5].

Commercially, FA is manufactured as an aqueous solution called formalin, usually containing 37–40% by weight of dissolved FA [6], which is commonly used in histopathology laboratories as a cytological fixative to preserve the integrity of cellular architecture for diagnosis.

Exogenous FA can be absorbed following inhalation, dermal or oral exposure, the extent of absorption being dependent on the route of exposure. The International Agency for Research on Cancer

* Corresponding author at: Escola Superior de Tecnologia da Saúde de Lisboa–Instituto Politécnico de Lisboa, Portugal.
E-mail address: carina.ladeira@estesl.ipl.pt (C. Ladeira).

1383-5718/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2010.11.015

(IARC) reclassified FA as a human carcinogen (group 1) in June 2004 based on "*sufficient epidemiological evidence that FA causes nasopharyngeal cancer in humans*" [3,6]. In their review, IARC also concluded that there was "*strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to formaldehyde*" [6,7]. Subsequently, on the basis of additional information the IARC concluded in 2009 that there is sufficient evidence that formaldehyde causes nasopharyngeal cancer and leukaemia [8]. However, some studies have also led to mixed results and inconclusive evidence [4,9].

The inhalation of vapours can produce irritation to eyes, nose and the upper respiratory tract. Whilst occupational exposure to high FA concentrations may result in respiratory irritation and asthmatic reactions, it may also aggravate a pre-existing asthma condition. Skin reactions following exposure to FA are very common, because the chemical is both irritating and allergenic [2]. FA induces genotoxic and cytotoxic effects in bacteria and mammalian cells [10] and its carcinogenicity and genotoxicity have been proven, respectively, in epidemiological studies and in experimental studies that used proliferating cultured mammalian cells and human lymphocytes [2,11] to determine DNA–protein cross-links, chromosome aberrations, sister chromatid exchange, and micronuclei [6].

The cytokinesis-block micronucleus cytome assay (CBMN) is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. DNA-damage events are scored specifically in once-divided binucleated cells and comprise micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD). This assay has been applied successfully for bio-monitoring of *in vivo* exposure to genotoxins, *in vitro* genotoxicity testing, and in areas like nutrigenomics and pharmacogenomics [12–14].

MN originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division and are not included in the main daughter nuclei. Thus MN provide a measure of both chromosome breakage and loss and they have been shown to be at least as sensitive an indicator of chromosome damage as the classical metaphase chromosome analysis [13,15–17].

The analysis of NPB was validated as a biomarker of DNA damage in human WIL2-NS cells treated with hydrogen peroxide, superoxide or after co-incubation with activated human neutrophils [18]. NPB should be scored because they provide a measure of chromosome rearrangement, which is otherwise not assessed if only MN are scored [17,19]. This event occurs when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. NPB are therefore biomarkers of dicentric chromosomes resulting from telomere end-fusions or DNA mis-repair [19–22].

NBUD are characterized by the same morphology as MN, except that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmatic material, depending on the stage of the nuclear budding process. They are classified as biomarkers of the elimination of amplified DNA and/or DNA-repair complexes [19,21,22].

The goal of this study is to compare the frequencies of genotoxicity biomarkers, provided by the CBMN assay in peripheral lymphocytes and the MN test in buccal cells, between workers of histopathology laboratories exposed to formaldehyde and individuals not exposed to formaldehyde, and other environmental factors, i.e. tobacco and alcohol consumption.

2. Methods

2.1. Subjects

The study population consisted of 56 workers occupationally exposed to FA from six hospital-associated histopathology laboratories located in Portugal (Lisbon and Tagus Valley region), and 85 administrative staff without occupational exposure to FA. The characteristics of both groups are described in Table 1.

Ethical approval for this study was obtained from the institutional Ethical Board and the service directors of the participating hospitals, and all subjects gave informed consent to participate in this study. Every person completed a questionnaire aimed at identifying exclusion criteria like history of cancer, radio or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the past six months, intake of vitamins or other supplements like folic acid, as well as information related to working practices (such as years of employment and the use of protective measures). In this study, none of the participants were excluded.

Table 1
Characteristics of the sample.

	Control group	Exposed group
Number of subjects	85	56
Gender		
Females	54 (64%)	37 (66%)
Masculine	31 (36%)	19 (34%)
Age (mean ± standard deviation, in years)		
Range	32.42 ± 8.1 20–53	39.45 ± 11.5 20–61
Years of employment (mean ± standard deviation, in years)		
Range	–	14.5 1–33
Tobacco consumption		
Non-smokers	60 (70,6%)	45 (80,4%)
Smokers	25 (29, 4%)	11 (19,6%)
Alcohol consumption		
Non-drinkers	19 (22,4%)	19 (33,9%)
Drinkers	66 (77,6%)	37 (66,1%)

2.2. Environmental monitoring of FA exposure

Exposure assessment was based on two techniques of air-monitoring conducted simultaneously. First, environmental samples were obtained by air-sampling with low-flow pumps for 6–8 h, during a typical working day. FA levels were measured by gas chromatography analysis and the time-weighted average (TWA_{8h}) was estimated according to a method described by the National Institute of Occupational Safety and Health (NIOSH 2541) [23].

The second method was aimed at measuring ceiling values of FA using photoionization detection (PID) equipment (11.7-eV lamps) with simultaneous video recording. Instantaneous values for FA concentration were obtained on a per second basis in both methods. A relationship can thus be established between worker activities and ceiling values, and serve to reveal the main exposure sources [24,25].

Measurements and sampling were always performed in the workers' breathing zone, in a room that was equipped with fume hoods.

2.3. Cytokinesis-block micronucleus cytome assay

Evaluation of genotoxic effects was performed by applying the CBMN assay in peripheral blood lymphocytes and exfoliated cells from the buccal mucosa.

Whole blood and exfoliated cells (buccal mucosa cells) were collected from every subject between 10 a.m. and 12 p.m. and processed for testing. All samples were coded and analyzed under blind conditions. The criteria for scoring the nuclear abnormalities in lymphocytes and MN in the buccal cells were described by, respectively, Fenech et al. [17] and Tolbert et al. [26].

2.3.1. Peripheral blood lymphocytes

Heparinized blood samples were obtained by venipuncture from all subjects and freshly collected blood was directly used for the micronucleus test. Lymphocytes were isolated by use of a Ficoll-Paque gradient and placed in RPMI1640 culture medium with L-glutamine and phenol red added, with 10% inactivated fetal calf serum, 50 μg/ml streptomycin + 50 U/mL penicillin, and 10 μg/mL phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37 °C in a humidified 5% CO_2 incubator for 44 h, and cytochalasin-b (6 μg/mL) was added to the cultures in order to prevent cytokinesis. After 28 h of incubation, cells were spun onto microscope slides by use of a cytocentrifuge. Smears were air-dried and double-stained with May–Grünwald–Giemsa and mounted with Entellan®. One thousand cells were scored from each individual by two independent observers on a total of two slides. Each observer visualized 500 cells/individual.

2.3.2. Buccal mucosa cells

Cells from the buccal mucosa were sampled by endobrushing. Exfoliated cells were smeared onto the slides and fixed with Mercofix®. The standard protocol used was Feulgen staining without counterstain. Two thousand cells were scored from each individual by two independent observers on a total of two slides. Each observer visualized 1000 cells/individual. Only cells containing intact nuclei that were neither clumped nor overlapping were included in the analysis.

Table 2
FA ceiling values (ppm) by tasks in the macroscopy room.

Tasks	Ceiling values (ppm)
Macroscopic specimen's exam	2.93
Disposal of specimen and used solutions	0.95
Jar filling	2.51
Specimen wash	2.28
Biopsy exam	1.91

2.4. Statistical analysis

The deviation of variables from the normal distribution was evaluated by the Shapiro–Wilk goodness-of-fit test. The association between each of the genotoxicity biomarkers and occupational exposure to FA was evaluated by binary logistic regression. The biomarkers were dichotomized (absent/present) and considered as the dependent variable in regression models where exposure was an independent variable. Odd's ratios were computed to evaluate the risk of the biomarkers' presence and their significance was assessed. The non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests were also used to evaluate interactions involving confounding factors. All statistical analyses were performed with the SPSS package for Windows, version 15.0.

3. Results

3.1. FA exposure levels

FA exposure was determined with the two methods described—the NIOSH 2541 method [23] for average concentrations (TWA_{8h}) and the PID method for ceiling concentrations. For the first exposure metric, the FA mean exposure level of the 56 individuals studied was 0.16 ppm (0.04–0.51 ppm), a value that lies below the OSHA reference value of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (0.18–2.93 ppm), a value well above the reference of the American Conference of Governmental Industrial Hygienists (ACGIH) for ceiling concentrations (0.3 ppm). As for the different tasks developed in histopathology laboratories, the highest FA concentration was identified during macroscopic examination of specimens. This task involves careful observation and grossing of the specimen preserved in FA, which involves direct and prolonged contact of the observer with FA vapours (Table 2).

3.2. Genotoxicity biomarkers

For all genotoxicity biomarkers under study, the workers exposed to FA had significantly higher mean values than the controls (Table 3).

In peripheral blood lymphocytes, significant differences (Mann–Whitney test, $p < 0.001$) were observed between subjects exposed and those not exposed to FA, namely in mean MN (respectively, 3.96 ± 0.525 *vs* 0.81 ± 0.172), NPB (3.04 ± 0.523 *vs* 0.18 ± 0.056), and NBUD (0.98 ± 0.273 *vs* 0.07 ± 0.028). In buccal mucosa cells, the mean MN frequency was also significantly higher ($p = 0.002$) in exposed subjects (0.96 ± 0.277) than in controls (0.16 ± 0.058).

The odds ratios indicate an increased risk for the presence of biomarkers in those exposed to FA, compared to non-exposed (Table 3); all odd's ratios were significant ($p < 0.001$).

Table 3
Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD in the studied population (mean ± mean standard error, range), *p*-value of the Mann–Whitney test and results of binary logistic regression concerning the association between exposure and genotoxicity biomarkers, as evaluated by the odds ratio (OR).

	MN in lymphocytes Mean ± S.E. (range)	NPB Mean ± S.E. (range)	NBUD Mean ± S.E. (range)	MN in buccal cells Mean ± S.E. (range)
Controls	0.81 ± 0.172 (0–7)	0.18 ± 0.056 (0–3)	0.07 ± 0.028 (0–1)	0.16 ± 0.058 (0–2)
Exposed	3.96 ± 0.525 (0–14)	3.04 ± 0.523 (0–15)	0.98 ± 0.273 (0–13)	0.96 ± 0.277 (0–9)
p-value*	<0.001	<0.001	<0.001	0.002
OR	9.665	11.97	9.631	3.990
CI 95%	3.81–24.52	4.59–31.20	3.12–29.70	1.38–11.58
p-value**	<0.001	<0.001	<0.001	0.011

* Mann–Whitney test.
** Binary logistic regression.

Table 4
Descriptive statistics in the exposed group of MN in lymphocytes and buccal cells, NPB, and NBUD (mean ± mean standard error, range) by years of exposure to formaldehyde.

Years of exposure	*N*	MN in lymphocytes Mean ± S.E. (range)	NPB Mean ± S.E. (range)	NBUD Mean ± S.E. (range)	MN in buccal cells Mean ± S.E. (range)
<5	8	2.75 ± 0.940 (0–8)	5.13 ± 1.381 (0–10)	1.38 ± 0.498 (0–3)	0.63 ± 0.625 (0–5)
6–10	19	3.05 ± 0.775 (0–12)	2.42 ± 0.668 (0–9)	1.53 ± 0.731 (0–13)	0.63 ± 0.326 (0–6)
11–20	12	5.50 ± 1.317 (0–14)	3.33 ± 1.443 (0–14)	0.33 ± 0.188 (0–2)	0.83 ± 0.458 (0–5)
>21	15	5.00 ± 1.151 (0–13)	2.33 ± 1.036 (0–15)	0.73 ± 0.248 (0–2)	1.20 ± 0.8 (0–9)

Table 5
Descriptive statistics of MN in lymphocytes and buccal cells, NPB, and NBUD means by gender and exposition (mean ± mean standard error, range).

Groups	Exposed		Controls	
	Females[a] *N* = 37	Males[a] *N* = 19	Females[a] *N* = 54	Males[a] *N* = 31
MN in lymphocytes Mean ± S.E. (range)	4.43 ± 0.676 (0–14)	3.47 ± 0.883 (0–13)	0.87 ± 0.229 (0–7)	0.71 ± 0.255 (0–6)
NPB Mean ± S.E. (range)	3.03 ± 0.699 (0–15)	2.95 ± 0.818 (0–14)	0.22 ± 0.078 (0–3)	0.10 ± 0.071 (0–2)
NBUD Mean ± S.E. (range)	1.34 ± 0.418 (0–13)	0.42 ± 0.158 (0–2)	0.11 ± 0.043 (0–1)	0.00
MN in buccal cells Mean ± S.E. (range)	1.14 ± 0.353 (0–8)	0.74 ± 0.495 (0–9)	0.11 ± 0.057 (0–2)	0.26 ± 0.122 (0–2)

[a] Gender.

Table 6
Age effects on descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± mean standard error, range).

Groups	Age	N	MN in lymphocytes Mean ± S.E. (range)	NPB Mean ± S.E. (range)	NBUD Mean ± S.E. (range)	MN in buccal cells Mean ± S.E. (range)
Exposed	20–30	18	2.19 ± 0.526 (0–8)	3.56 ± 0.926 (0–10)	1.63 ± 0.816 (0–13)	0.75 ± 0.470 (0–6)
	31–40	11	3.00 ± 0.775 (0–8)	1.20 ± 0.467 (0–4)	0.50 ± 0.224 (880–2)	0.40 ± 0.221 (0–2)
	>41	27	5.54 ± 0.876 (0–14)	3.00 ± 0.879 (0–15)	0.69 ± 0.234 (0–5)	1.46 ± 0.503 (0–9)
Controls	20–30	36	0.47 ± 0.157 (0–3)	0.14 ± 0.071 (0–2)	0.08 ± 0.047 (0–1)	0.19 ± 0.96 (0–2)
	31–40	35	1.14 ± 0.326 (0–7)	0.20 ± 0.099 (0–3)	0.06 ± 0.040 (0–1)	0.14 ± 0.83 (0–2)
	>41	14	0.86 ± 0.501 (0–6)	0.21 ± 0.155 (0–2)	0.07 ± 0.71 (0–1)	0.14 ± 0.143 (0–2)

Regarding the impact of the duration of exposure to FA, the mean values of MN in lymphocytes and in buccal cells tended to increase with years of exposure (Table 4) but the association was not statistically significant.

Gender and age are considered the most important demographic variables affecting the MN index. However, Table 5 shows that the mean of all the genotoxicity biomarkers did not differ between men and women within the exposed and the controls ($p > 0.05$).

In order to examine the effect of age, exposed and non-exposed individuals were stratified by age groups: 20–30, 31–40, and ≥41 years old (Table 6). There was no consistent trend regarding the variation of biomarkers with age, the only exception being the MN frequency in lymphocytes in the exposed group (Kruskal–Wallis, $p = 0.006$), where the higher means where found in the older group. According to the Mann–Whitney test, there is a statistically significant difference between the younger and the older group (20–30 and >41 years old, $p = 0.02$), however the comparison between 20–30 and 31–40 groups ($p = 0.262$) and 31–40 and >41 groups ($p = 0.065$) did not reach statistical significance.

The interaction between age and gender in determining the frequencies of genotoxicity biomarkers was investigated and found to be significant only for MN in lymphocytes in exposed subjects (Kruskal–Wallis, $p = 0.04$). In general the MN tended to be more frequent in the >41 years old category in both genders; however women had the higher means (Table 7).

Regarding smoking habits, a non-parametric analysis rejected the null hypothesis that biomarkers are the same for the four categories (control smokers and non-smokers, exposed smokers and non-smokers) (Kruskall–Wallis, $p < 0.001$). However, the analysis of the interactions between FA exposure and tobacco smoke between exposed and controls (Mann–Whitney test) showed that FA exposure, rather than tobacco, has a preponderant effect upon the determination of biomarker frequencies. In the control group, non-smokers had slightly higher MN means in buccal cells in comparison with smokers; although the result did not reach statistical significance (Mann–Whitney, $p > 0.05$).

As for alcohol consumption, because uptake reported in enquires may differ considerably from real consumption, all consumers were gathered into a single entity, in contrast with non-consumers. Nevertheless, no one acknowledged having "heavy drinking habits" in the questionnaires.

Overall, biomarkers in controls exhibited higher mean frequencies among alcohol consumers than among non-consumers. Among those exposed, however, mean frequencies were slightly lower among drinkers, suggesting that exposure was the major predominant factor in determining the high biomarker frequencies of those who are exposed. Differences between drinkers and non-drinkers were not statistically significant, except with respect to the MN frequency in lymphocytes in controls (Mann–Whitney, $p = 0.011$), where drinkers have higher means. The interaction between alcohol consumption and smoking habits was statistically significant (Kruskal–Wallis, $p = 0.043$), as subjects that do not smoke and do not drink tend to have lower frequencies of MN in buccal cells than those who drink and smoke, with a gradient of frequencies in between.

4. Discussion

Long-term exposures to FA, such as those to which some workers are subjected for occupational reasons, are suspected to be associated with genotoxic effects, which can be evaluated by analysis of biomarkers [1,3,5,6]. In this study the results suggest that workers in histopathology laboratories are exposed to FA levels that exceed recommended exposure limits. In particular, macroscopic examination of specimens is the task that involves higher exposure, because it requires a greater proximity to anatomical preparations impregnated with FA, as supported by the studies of Goyer et al. [27] and Orsière et al. [28].

A statistically significant association was found between FA exposure and biomarkers of genotoxicity, namely micronuclei (MN) in lymphocytes, nucleoplasmic bridges, nuclear buds and MN in buccal cells. Chromosome damage and effects on lymphocytes arise because FA escapes from sites of direct contact, such as the mouth, causing nuclear alterations in the lymphocytes of those exposed [6,10,28,29]. Our results thus corroborate previous reports [30] that lymphocytes can be damaged by long-term FA exposure.

Table 7
Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means by exposition, gender and age (mean ± mean standard error, range).

Groups		N	MN lymphocytes Mean ± S.E.	NPB Mean± S.E.	NBUD Mean ± S.E.	MN buccal cells Mean ± S.E.
Exposed	Females 20–30	12	2.42 ± 0.67	4.17 ± 1.22	2.00 ± 1.07	1.00 ± 0.62
	Females 31–40	7	2.71 ± 0.68	0.86 ± 0.46	0.57 ± 0.3	0.29 ± 0.18
	Females >41	18	6.00 ± 1.12	3.22 ± 1.09	1.06 ± 0.39	1.44 ± 0.55
	Males 20–30	6	1.00 ± 0.52	3.50 ± 1.02	0.50 ± 0.34	0.00 ± 0.0
	Males 31–40	4	3.00 ± 1.78	1.50 ± 0.96	0.25 ± 0.25	0.50 ± 0.50
	Males >41	9	5.33 ± 1.45	3.22 ± 1.56	0.44 ± 0.24	1.33 ± 1.01
Controls	Females 20–30	23	0.43 ± 0.20	0.13 ± 0.70	0.13 ± 0.7	0.13 ± 0.10
	Females 31–40	22	1.32 ± 0.44	0.27 ± 0.15	0.09 ± 0.06	0.14 ± 0.10
	Females >41	9	0.89 ± 0.68	0.33 ± 0.24	0.11 ± 0.11	0.00 ± 0.00
	Males 20–30	13	0.54 ± 0.27	0.15 ± 0.15	0.00 ± 0.00	0.31 ± 0.21
	Males 31–40	13	0.85 ± 0.48	0.08 ± 0.08	0.00 ± 0.00	0.15 ± 0.15
	Males >41	5	0.80 ± 0.80	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.40

Moreover, the changes in peripheral lymphocytes indicate that the cytogenetic effects triggered by FA can reach tissues far away from the site of initial contact [31]. Long-term exposures to high concentrations of FA indeed appear to have a potential for inducing DNA damage; these effects were well demonstrated in experimental studies with animals, in which local genotoxic effects following FA exposure were observed, i.e. DNA–protein cross-links and chromosome damage [3].

In humans, FA exposure is associated with an increase in the frequency of MN in buccal epithelium cells [32–34], as corroborated by the results presented here.

Suruda et al. claim that although changes in oral and nasal epithelial cells and peripheral blood cells do not indicate a direct mechanism leading to carcinogenesis, they present evidence that DNA alteration took place [31]. It thus appears reasonable to conclude that FA is a cancer risk factor for those who are occupationally exposed in histopathology laboratories [3].

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking and alcohol consumption, in the association between disease and exposure [17,35]. Concerning gender, studies realized by Fenech et al. and Wojda et al. reported that biomarker frequencies were higher in females than in males by a factor of 1.2–1.6, depending on the age group [17,36]. Except for MN in the buccal cells of controls, the results presented here point to females having higher frequencies than males in all genotoxicity biomarkers, although the differences usually lacked statistical significance. Such a trend is concordant with previous studies that reported higher MN frequency in lymphocytes in females and a slightly higher MN frequency in buccal cells in males [37], which can be explained by preferential aneugenic events involving the X-chromosome. A possible explanation is the micro-nucleation of the X-chromosome, which has been shown to occur in lymphocytes in females, both *in vitro* and *in vivo*, and which can be accounted for by the presence of two X chromosomes. This finding may explain the preferential micro-nucleation of the inactive X [38–40].

Ageing in humans appears to be associated with genomic instability. Cytogenetically, ageing is associated with a number of gross cellular changes, including altered size and morphology, genomic instability and changes in expression and proliferation [41,42]. It has been shown that a higher MN frequency is directly associated with decreased efficiency of DNA repair and increased genomic instability [28,43]. The data show a significant increase of the MN frequency in lymphocytes in the exposed group. This can be explained in the light of genomic instability, understood as an increased number of mutations and/or chromosomal aberrations, which cytogenetically translate into a higher frequency of changes in chromosome number and/or structure and in the formation of micronuclei [41]. The involvement of micro-nucleation in age-related chromosome loss has been supported by several studies showing that the rate of MN formation increases with age, especially in women [38].

This study provides evidence that age and gender interact to determine the frequency of MN in the lymphocytes of exposed subjects. The higher incidence of MN in both genders is more manifest in older age groups and the effect of gender becomes more pronounced as age increases. Several reports link this observation to an elevated loss of X chromosomes [44].

Tobacco smoke has been epidemiologically associated with a higher risk for cancer development, especially in the oral cavity, larynx, and lungs, as these are places of direct contact with the carcinogenic tobacco compounds. In this study, smoking habits did not influence the frequency of the genotoxicity biomarkers; moreover, the frequencies of MN in buccal cells were unexpectedly higher in exposed non-smokers than in exposed smokers, although the difference was not statistically significant. In most reports, the results about the effect of tobacco upon the frequency of MN in human lymphocytes were negative, as in many instances smokers had lower MN frequencies than non-smokers [45]. In the current study, the analysis of the interaction between FA exposure and smoking habits indicates that exposure is preponderant in determining the frequency of biomarkers. Nevertheless, the effect of smoking upon these biomarkers remains controversial. Some studies [46,47] reported an increased frequency of MN in lymphocytes, NPB, and NBUD as a consequence of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Still in this study no associations were observed between tobacco and nuclear abnormalities.

Alcohol consumption did not appear to influence the frequency of genotoxicity biomarkers in this study, except that of MN in lymphocytes in controls (Mann–Whitney, $p = 0.011$), with drinkers having higher means. Alcohol is definitely a recognized genotoxic agent, being cited as able to potentiate the development of carcinogenic lesions [48]. In our study, drinkers in the control group had higher mean frequencies of all biomarkers than non-drinkers, but the differences were only significant for MN in lymphocytes. Stich and Rosin [49] studied alcoholic individuals and reported the absence of significant differences concerning MN frequencies in buccal cells. This is important to corroborate our result, because of the lack of "heavy drinkers" in our study. The same study [49] concluded that neither alcohol nor smoking, alone, increased MN frequency in buccal cells, but a combination of both resulted in a significant elevation in micronucleated cells in the buccal mucosa. However, the synergism between alcohol consumption and tobacco has not been observed to act upon all biomarkers and, in several studies of lifestyle factors, it was difficult to differentiate the effect of alcohol from that of smoking [37].

The CBMN assay is a simple, practical, low-cost screening technique that can be used for clinical prevention and management of workers subject to occupational carcinogenic risks, namely through exposure to a genotoxic agent such as formaldehyde. The results obtained in this study provide unequivocal evidence of an association between occupational exposure to formaldehyde in histopathology laboratory workers and the presence of nuclear changes.

Given these results, preventive actions must prioritize safety conditions for those who perform macroscopic examination of specimens. In general, reduction of exposure to FA in this occupational setting may be achieved through adequate local exhaust ventilation and by keeping the biological specimen containers closed during the macroscopic examination.

Conflict of interest

None.

References

[1] S. Viegas, J. Prista, Cancro Nasofaríngeo e Exposição a Formaldeído: avaliação da história profissional em 63 casos registados, Soc. Port. Med. Trabalho 6 (2007) 13–22.
[2] M. Pala, D. Ugolini, M. Ceppi, F. Rizzo, L. Maiorana, C. Bolognesi, T. Schilirò, G. Gilli, P. Bigatti, R. Bono, D. Vecchio, Occupational exposure to formaldehyde and biological monitoring of Research Institute workers, Cancer Detect. Prev. 32 (2008) 121–126.
[3] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Human, volume 88: formaldehyde, 2-Butoxyethanol and 1-tert-Butoxy-2-propanol, World Health Organization, Lyon, 2006.
[4] S.J. Franks, A mathematical model for the absorption and metabolism of formaldehyde vapour by humans, Toxicol. Appl. Pharmacol. 206 (2005) 309–320.
[5] C. Conaway, J. Whysner, L. Verna, G. Williams, Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation, Pharmacol. Ther. 71 (1996) 29–55.

[6] L. Zhang, C. Steinmaus, D. Eastmond, X. Xin, M. Smith, Formaldehyde exposure and leukemia: a new meta-analysis and potencial mechanisms, Mut. Res. 681 (2009) 150–168.

[7] L. Zhang, X. Tang, N. Rothman, Occupational exposure to formaldehyde, hematoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells, Cancer Epidemiol. Biomarkers Prevention 19 (2010) 80–88.

[8] R. Baan, Y. Grosse, K. Straif, B. Secretan, F. El Ghissassi, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, C. Freeman, L. Galichet, V. Cogliano, On behalf of the WHO International Agency for Research on Cancer Monograph Working Group, A review of human carcinogens. Part F. Chemical agents and related occupations, The Lancet Oncol. 10 (2009) 1143–1144.

[9] G. Speit, H.-P. Gelbke, D. Pallapies, P. Morfeld, Occupational exposure to formaldehyde, hematotoxicity and leukemia-specific chromosome changes in cultured myeloid progenitor cells—letter, Cancer Epidemiol. Biomarkers Prev. 19 (7) (2010) 1882–1884.

[10] X. Ye, W. Yan, H. Xie, M. Zhao, C. Ying, Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters, Mut. Res. 588 (2005) 22–27.

[11] G. Speit, P. Schütz, J. Högel, O. Schmid, Characterization of the genotoxic potencial of formaldehyde in V79 cells, Mutagenesis 22 (6) (2007) 387–394.

[12] M. Fenech, Cytokinesis-block micronucleus cytome assay, Nat. Protoc. 5 (2007) 1084–1104.

[13] R. Mateuca, N. Lombaert, P. Aka, I. Decordier, M. Kirsch-Volders, Chromosomal changes: induction, detection methods and applicability in human biomonitoring, Biochimie 88 (2006) 1515–1531.

[14] I. Serrano-García, R. Montero-Montoya, Micronuclei and chromatid buds are results of related genotoxic events, Environ. Mol. Mutagen. 38 (2001) 38–45.

[15] M. Fenech, Chromosomal biomarkers of genomic instability relevant to cancer, Drug Discov. Today 22 (2002) 1128–1137.

[16] G. Iarmarcovai, S. Bonassi, A. Botta, R.A. Baan, T. Orsière, Genetic polymorphisms and micronucleus formation: a review of the literature, Mut. Res. 658 (2008) 215–233.

[17] M. Fenech, N. Holland, W. Chang, E. Zeiger, S. Bonassi, The Human MicroNucleus Project—an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans, Mut. Res. 428 (1999) 271–283.

[18] K. Umegaki, M. Fenech, Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils, Mutagenesis 15 (3) (2000) 261–269.

[19] M. Fenech, Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death, Mut. Res. 600 (2006) 58–66.

[20] M. Fenech, The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis, Mutagenesis 20 (2005) 225–269.

[21] P. Thomas, K. Umegaki, M. Fenech, Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay, Mutagenesis 18 (2003) 187–194.

[22] M. Fenech, J. Crott, Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes—evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay, Mut. Res. 504 (2002) 131–136.

[23] NIOSH – Manual of analytical methods, 4th ed., DHHS (NIOSH) Publication 94-113, 1994 August.

[24] J.D. McGlothlin, et al., Occupational exposure assessment and control using video exposure monitoring in the pharmaceutical industry, in: International Scientific Conference (IOHA 2005), 6, 19–23 September 2005, Pilanesberg National Park North West Province, South Africa. Pilanesberg, IOHA–International Occupational Hygiene. MVS-Mine Ventilation of South Africa, 2005.

[25] S. Viegas, C. Ladeira, C. Nunes, J. Malta-Vacas, M. Gomes, M. Brito, P. Mendonça, J. Prista, Genotoxic effects in occupational exposure to formaldehyde: a study in anatomy and pathology laboratories and formaldehyde-resins production, J. Occup. Med. Toxicol. 5 (25) (2010) 1–8.

[26] P. Tolbert, C. Shy, J. Allen, Micronuclei and other nuclear anomalies in buccal smears: a field test in snuff users, Am. J. Epidemiol. 8 (1991) 840–850.

[27] N. Goyer, C. Beaudry, D. Bégin, M. Bouchard, S. Buissonet, G. Carrier, O. Gely, M. Gérin, L. Lavoué, P. Lefebvre, N. Noisel, G. Perraut, B. Roberge, Impacts d'un abaissement de la values d'exposition admissible au formaldéhyde: industries de fabrication de formaldéhde et de résines à base de formaldéhyde, Institut de Recherche Robert-Sauvé en Santé et Securité du Travail, Móntreal, 2004.

[28] T. Orsière, I. Sari-Minodier, G. Iarmarcovai, A. Botta, Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes, Mut. Res. 605 (2006) 30–41.

[29] J.-L. He, H.-Y. Jin, Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay, Biomed. Environm. Sci. 11 (1998) 87–92.

[30] X. Ye, W. Yan, M. Zhao, C. Ying, Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters, Mut. Res. 588 (2005) 22–27.

[31] A. Suruda, P. Schulte, M. Boeniger, R. Hayes, G. Livingston, K. Steenland, P. Stewart, R. Herrick, D. Douthit, M. Fingerhut, Cytogenetic effects of formaldehyde exposure in students of mortuary science, Cancer Epidemiol. Biomarkers Prev. 2 (1993) 453–460.

[32] G. Speit, O. Schmid, M. Fröhler-Keller, I. Lang, G. Triebig, Assessment of local genotoxic effects of formaldehyde in humans measured by de micronucleus test with exfoliated buccal mucosa cell, Mut. Res. 627 (2007) 129–131.

[33] G. Speit, O. Schmid, Local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated epithelial cells, Mut. Res. 613 (2006) 1–9.

[34] S. Burgaz, O. Erdem, G. Çakmak, A. Karakaya, Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde, Biomarkers 2 (2002) 151–161.

[35] S. Bonassi, M. Fenech, C. Lando, Y.-P. Lin, M. Ceppi, W. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M. Bigatti, C. Bolognesi, C. Jia, M. Di Giorgio, L. Ferguson, A. Fucic, O. Lima, P. Hrelia, A. Krishnaja, T.-K. Lee, L. Migliore, L. Mikhalevich, E. Mirkova, P. Mosesso, W.-U. Müller, Y. Odagiri, M. Scarfi, E. Szabova, I. Vorobtsova, A. Vral, A. Zijno, Human MicroNucleus Project: international database comparison for results with cytokinesis-block micronucleus assay in human lymphocytes. I. Effect of laboratory protocol, scoring criteria, and host factor son the frequency of micronuclei, Environ. Mol. Mutagen. 37 (2001) 31–45.

[36] A. Wojda, E. Zietkiewicz, M. Witt, Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects, Mutagen. Adv. Access (2007) 1–6.

[37] N. Holland, C. Bolognesi, M. Kirsch-Volders, S. Bonassi, E. Zeiger, S. Knasmueller, M. Fenech, The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps, Mutat. Res. Rev. Mutat. Res. (2008), doi:10.1016/j.mrrev.2008.03.007.

[38] J. Catalán, K. Autio, E. Kuosma, H. Norppa, Age-dependent inclusion of sex chromosomes in lymphocyte micronuclei in man, Am. J. Hum. Genet. 63 (1998) 1464–1472.

[39] J. Catalán, G. Falck, H. Norppa, The X chromosome frequently lags behind in female lymphocyte anaphase, Am. J. Hum. Genet. 66 (2000) 687–691.

[40] J. Catalán, J. Surrallés, G. Falck, K. Autio, H. Norppa, Segregation of sex chromosomes in human lymphocytes, Mutagenesis 3 (2000) 251–255.

[41] E. Zietkiewicz, A. Wojda, M. Witt, Cytogenetic perspective of ageing and longevity in men and women, J. Appl. Genet. 50 (2009) 261–273.

[42] C. Bolognesi, C. Lando, A. Forni, E. Landini, R. Scarpato, L. Migliori, S. Bonassi, Chromosomal damage and ageing: effect of micronuclei frequency in peripheral blood lymphocytes, Age Ageing 28 (1999) 393–397.

[43] M. Kirsch-Volders, R. Mateuca, M. Roelants, A. Tremp, E. Zeiger, S. Bonassi, N. Holland, W. Chang, P. Aka, M. DeBoeck, L. Goddenis, V. Haufroid, H. Ishikawa, B. Laffon, R. Marcos, L. Migliore, H. Norppa, J. Teixeira, A. Zijno, M. Fenech, The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo, Cancer Epidemiol. Biomarkers Prev. 15 (5) (2006) 1038–1042.

[44] J.M. Battershill, K. Burnett, S. Bull, Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies, Mutagenesis 6 (2008) 237–423.

[45] S. Bonassi, M. Neri, C. Lando, M. Ceppi, Y. Lin, W. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, M. Fenech, Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project, Mut. Res. 543 (2003) 155–166.

[46] R. El-Zein, M. Schabath, C. Etzel, M. Lopez, J. Franklin, M. Spitz, Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk, Cancer Res. 66 (2006) 6449–6456.

[47] R. El-Zein, M. Fenech, M. Lopez, M. Spitz, C. Etzel, Cytokinesis-blocked cytome assay biomarkes identify lung cancer cases amongst smokers, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 1111–1119.

[48] A. Ramirez, P. Saldanha, Micronucleus investigation of alcoholic patients with oral carcinoma, Genet. Mol. Res. 1 (2002) 246–260.

[49] H. Stich, M. Rosin, Quantitating the synergistic effect of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells, Int. J. Cancer 31 (1983) 305–308.

7

Genotoxicity Biomarkers: Application in Histopathology Laboratories

Carina Ladeira[1,2], Susana Viegas[1,2], Elisabete Carolino[1], Manuel Carmo Gomes[3] and Miguel Brito[1]
[1]*Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa,*
[2]*Centro de Investigação e Estudos em Saúde Pública, Universidade Nova de Lisboa,*
[3]*Faculdade de Ciências, Universidade de Lisboa,*
Portugal

1. Introduction

Most cancers results from man-made and natural environmental exposures (such as tobacco smoke; chemical pollutants in air, water, food, drugs; radon; and infectious agents) acting in concert with both genetic and acquired characteristics. It has been estimated that without these environmental factors, cancer incidence would be dramatically reduced, by as much as 80%-90% (Perera, 1996). The modulation of environmental factors by host susceptibility was rarely evaluated. However, within the past few years, the interaction between environmental factors and host susceptibility factors has become a very active area of research (Perera, 2000). Molecular biology as a tool for use in epidemiological studies has significant potential in strengthening the identification of cancers associated with environmental exposures related to lifestyle, occupation, or ambient pollution. In molecular epidemiology, laboratory methods are employed to document the molecular basis and preclinical effects of environmental carcinogenesis (Portier & Bell, 1998).

Molecular epidemiology has become a major field of research and considerable progress has been made in validation and application of biomarkers and its greatest contribution has been the insights provided into interindividual variation in human cancer risk and the complex interactions between environmental factors and host susceptibility factors, both inherited and acquired, in the multistage process of carcinogenesis (Perera, 2000).

The possibility to use a biomarker to substitute classical endpoints, such as disease incidence or mortality is the most promising feature and one that is most likely to affect public health. The use of events that are on the direct pathways from the initiation to the occurrence of disease to surrogate the disease incidence is a very appealing approach, which is currently investigated in different fields (Bonassi & Au, 2002).

Biological monitoring of workers has three main aims: the primary is individual or collective exposure assessment, the second is health protection and the ultimate objective is occupational health risk assessment. It consists of standardized protocols aiming to the periodic detection of early, preferably reversible, biological signs which are indicative, if compared with adequate reference values, of an actual or potential condition of exposure,

effect or susceptibility possibly resulting in health damage or disease. These signs are referred to as biomarkers (Manno et al., 2010).

There has been dramatic progress in the application of biomarkers to human studies of cancer causation. Progress has been made in the development and validation of biomarkers that are directly relevant to the carcinogenic process and that can be used in large-scale epidemiologic studies (Manno et al., 2010).

There are many important aspects to consider when a biomonitoring study is designed. For instance, there is needed a detail information on genotoxin exposure, e.g. type of toxin, duration of exposure, commencing date of exposure relative to sampling date of buccal cells, in order to achieve a meaningful interpretation of data. It will also helps to identify key variables affecting the observed frequency of biomarkers, like age, gender, vitamin B status, genotype and smoking status (Thomas et al., 2009).

Based on the impact on genotoxicity biomarkers in peripheral blood lymphocytes on the design of biomonitoring studies, Battershill et al. (2008) study have considered a strong/sufficient correlation between micronucleus (MN) frequency and increasing age. The effect is more pronounced in females than in males, with the increase more marked after 30 years of age. There are studies that also demonstrated a strong correlation between age and MN frequency and suggested that chromosome loss is a determining factor in this increase.

In what concern to gender, is also documented a gender difference in the background incidence of MN in peripheral blood lymphocytes (PBL), with the frequency being consistently higher in females. A study that assessed MN, chromosomal aberrations and sister chromatid exchange showed highly significant elevations in MN in lymphocytes of women (29% when adjusted for age and smoking) whereas chromosomal aberrations and sister chromatid exchange remained unchanged. This may reflect aneuploidy detected in MN assays (Battershill et al., 2008).

In respect to smoking, although the link between smoking and cancer is strong and exposure to genotoxic carcinogens present in tobacco smoke has been convincingly demonstrated, interestingly the same convincing association is less apparent when assessing biomonitoring studies of genotoxicity. HUMN project study about tobacco smoke, the majority of the laboratories showed no significant differences between smokers and non-smokers and the pooled analysis, interestingly, indicated an overall decrease for all smokers compared to controls (Battershill et al., 2008).

It was verified a weak/insufficient evidence for association with genotoxicity end points and alcohol consumption. Alcohol consumption has been causally associated with cancer at a number of sites (e.g. head and neck cancer). Alcoholic beverages have not been reported to induce mutagenic effects in rodents. The evidence regarding an effect of drinking alcoholic beverages on increased MN or substitute for chromosomal aberrations formation in PBL is inconclusive (Battershill et al., 2008).

2. Biomarkers – General definitions

Biomarkers have been defined by the National Academy of Sciences (USA) as an alteration in cellular or biochemical components, processes, structure or functions that is measurable

in a biological system or sample. The traditional, generally accepted classification of biomarkers into three main categories - biomarkers of exposure, effect, and susceptibility; depending on their toxicological significance (Manno et al., 2010).

A biomarker can potentially be any substance, structure or process that could be monitored in tissues or fluids and that predicts or influences health, or assesses the incidence or biological behaviour of a disease. Identification of biomarkers that are on causal pathway, have a high probability of reflecting health or the progression to clinical disease, and have the ability to account for all or most of the variation in a physiological state or the preponderance of cases of the specified clinical outcome, have largely remained elusive (Davis et al., 2007).

A biomarker of exposure is a chemical or its metabolite or the product of an interaction between a chemical and some target molecule or macromolecule that is measured in a compartment or a fluid of an organism (Manno et al., 2010).

A biomarker of effect is a measurable biochemical, structural, functional, behavioural or any other kind of alteration in an organism that, according to its magnitude, can be associated with an established or potential health impairment or disease. A sub-class of biomarkers of effect is represented by biomarkers of early disease (Manno et al., 2010).

A biomarker of susceptibility may be defined as an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a chemical (Manno et al., 2010).

Although the different types of biomarkers are considered for classification purposes, as separate and alternative, in fact it is not always possible to attribute them to a single category. The allocation of a biomarker to one type or the other sometimes depends on its toxicological significance and the specific context in which the test is being used (Manno et al., 2010).

2.1 Genotoxicity biomarkers

As a subtype of biomarkers of effect there are biomarkers of genotoxicity, generally used to measure specific occupational and environmental exposures or to predict the risk of disease or to monitor the effectiveness of exposure control procedures in subjects to genotoxic chemicals (Manno et al., 2010).

Cytogenetic biomarkers are the most frequently used endpoints in human biomonitoring studies and are used extensively to assess the impact of environmental, occupational and medical factors on genomic stability (Barrett et al., 1997; Battershill et al., 2008) and lymphocytes are used as a surrogate for the actual target tissues of genotoxic carcinogens (Barrett et al., 1997). The evaluation of MN in PBL is the most commonly used technique, although cells such as buccal epithelium are also utilized (Battershill et al., 2008).

MN assay is one of the most sensitive markers for detecting DNA damage, and has been used to investigate genotoxicity of a variety of chemicals. MN testing with interphase cells is more suited as a cytogenetic marker because it is not limited to metaphases, and has the advantage of allowing rapid screening of a larger numbers of cells than in studies with sister chromatid exchanges or chromosomic aberrations (Ishikawa et al., 2003).

MN analysis, therefore, appears to be a good tool for investigating the effects of clastogens and aneuploidogens in occupational and environmental exposure in human epidemiological studies (Ishikawa et al., 2003) and are described as a promising approach with regard to assessing health risks (Battershill et al., 2008).

2.1.1 Cytokinesis-Block micronucleus assay

The scope and the application of cytokinesis-block MN assay (CBMN) in biomonitoring has also been expanded in recent years so that in addition to scoring MN in binucleate cells, there are proposals to evaluate MN in mononucleate cells (to provide a more comprehensive assessment of DNA damage), nucleoplasmic bridges (indicative of DNA misrepair, chromosome rearrangement or telomere endfusions) and nuclear buds (a measure of gene amplification or acentric fragments). Fenech (2007), has proposed that CBMN assay can be used to measure chromosomal instability, mitotic dysfunction and cell death (necrosis and apoptosis) and has suggested the term CBMN assay. Identification of the contents of MN (e.g. presence and absence of centromeres) is now considered important in the evaluation of MN in biomonitoring studies, providing insight into mechanisms underpinning the positive results reported, i.e. to differentiate between clastogens and aneugenic responses (Battershill, et al., 2008).

The CBMN assay is a comprehensive system for measuring DNA damage; cytostasis and cytotoxicity-DNA damage events are scored specifically in once-divided binucleated cells and include: micronucleus (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUDs). Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios (Fenech, 2002a, 2006, 2007).

MN originate from chromosome fragments or whole chromosomes that lag behind anaphase during nuclear division. The CBMN assay is the preferred method for measuring MN in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided binucleated cells, which are the cells that can express MN. In the CBMN assay, once-divided cells are recognized by their binucleated appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis.

The CBMN assay allows measuring chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis. Also measure NPB, a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair, and to measure NBUDs, a biomarker of gene amplification.

Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells (Fenech, 2002b, 2007).

NPB occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. There are various mechanisms that could lead to NPB formation following DNA misrepair of strand breaks in DNA. Typically, a dicentric chromosome and an acentric chromosome fragment are formed that result in the formation of an NPB and an MN, respectively. Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosomes and concatenated ring chromosomes which could also result in the

formation of NPB. An alternative mechanism for dicentric chromosome and NPB formation is telomere end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion. The importance of scoring NPB should not be underestimated because it provides direct evidence of genome damage resulting for misrepaired DNA breaks or telomere end fusions, which is otherwise not possible to deduce by scoring MN only (Fenech, 2007 ; Thomas et al., 2003).

NBUD are biomarkers of elimination of amplified DNA and/or DNA repair complexes. The nuclear budding process has been observed in cultures grown under strong selective conditions that induce gene amplification as well as under moderate folic acid deficiency. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes), which localized to distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The process of nuclear budding occurs during S phase and the NBUD are characterized by having the same morphology as an MN with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process. The duration of the nuclear budding process and the extrusion of the resulting MN from the cell remain largely unknown (Fenech, 2007; Serrano-García & Montero-Montoya, 2001; Utani et al., 2007).

Most chemical agents and different types of radiation have multiple effects at the molecular, cellular and chromosomal level, which may occur simultaneously and to varying extents depending on the dose. Interpretation of genotoxic events in the absence of data on effects in nuclear division rate and necrosis or apoptosis can be confounding because observed increases in genome damage may be due to indirect factors such as inhibition of apoptosis or defective/permissive cell-cycle checkpoints leading to shorter cell-cycle times and higher rates of chromosome malsegregation. Furthermore, determining nuclear division index (NDI) and proportion of cells undergoing necrosis and apoptosis provides important information on cytostatic and cytotoxic properties of the agent being examined that is relevant to the toxicity assessment. In human lymphocytes, the NDI also provides a measure of mitogen response, which is a useful biomarker of immune response in nutrition studies and may also be related to genotoxic exposure. The cytome approach in the CBMN cytome assay is important because it allows genotoxic (MN, NPB and NBUD in binucleated cells), cytotoxic (proportion of necrotic and apoptotic cells) and cytostatic (proportion and ratios of mono-, bi- and multinucleated cells, NDI) events to be captured within one assay (Fenech, 2005, 2007; Umegaki & Fenech, 2000).

In conclusion, the CBMN method has evolved into an efficient "cytome" assay of DNA damage and misrepair, chromosomal instability, mitotic abnormalities, cell death and cytostasis, enabling direct and/or indirect measurement of various aspects of cellular and nuclear dysfunction such as: unrepaired chromosome breaks fragments and asymmetrical chromosome rearrangement (MN or NPB accompanied by MN originating from acentric chromosomal fragments); telomere end fusions (NPB with telomere signals in the middle of the bridge and possibly without accompanying MN); malsegregation of chromosomes due to spindle or kinetochore defects or cell-cycle checkpoint malfunction (MN containing whole chromosomes or asymmetrical distribution of chromosome-specific centromere signals in the nuclei of BN cells); nuclear elimination of amplified DNA and/or DNA repair

complexes (NBUD); chromosomal instability phenotype and breakage-fusion-bridge cycles (simultaneous expression of MN, NPB and NBUD); altered mitotic activity and/or cytostasis (NDI) and cell death by necrosis or apoptosis (ratios of necrotic and apoptotic cells) (Fenech, 2007).

2.1.2 Micronucleus in exfoliated buccal cells

Regeneration is dependent on the number and division rate of the proliferating (basal) cells, their genomic stability and their propensity for cell death. These events can be studied in the buccal mucosa (BM), which is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to study subjects. This method is increasingly used in molecular epidemiology studies for investigating the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage, chromosome malsegregation and cell death (Thomas et al., 2009).

The assay has been successfully to study DNA damage as measured by MN or by the use of fluorescent probes to detect in BM is an indication of the regenerative capacity of this tissue. The BM provides a barrier to potential carcinogens that can be metabolized to generate potential reactive products. As up to 90% of all cancers appear to be epithelial in origin, the BM could be used to monitor early genotoxic events as a result of potential carcinogens entering the body through ingestion or inhalation. Exfoliated buccal cells have been used non-invasively to successfully show the genotoxic effects of lifestyle factors such as tobacco smoking, chewing of betel nuts and/or quids, medical treatments, such as radiotherapy as well as occupational exposure, exposure to potentially mutagenic and/or carcinogenic chemicals, and for studies of chemoprevention of cancer.

In this assay cells derived from the BM are harvested from the inside of a patient's mouth using a small-headed toothbrush. The cells are washed to remove the debris and bacteria, and a single-cell suspension is prepared and applied to a clean slide using a cytocentrifuge. The cells are stained with Feulgen and Light Green stain allowing both bright field and permanent fluorescent analysis that can be undertaken microscopically (Thomas et al., 2009).

The Buccal Mucosa Cytome (BMCyt) assay has been used to measure biomarkers of DNA damage (MN and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells). The protocol can also make use of molecular probes for DNA adduct, aneuploidy and chromosome break measures within the nuclei of buccal cells. Furthermore, chromosome-specific centromeric probes have been used to measure aneuploidy by determining the frequency of nuclei with abnormal chromosome number. Tandem probes have been successfully applied to measure chromosome breaks in specific important regions of the genome (Thomas et al., 2009).

The methodology and concepts described in this protocol may be applied to other types of exfoliated cells such as those of the bladder, nose and cervix but the morphological characteristics, sampling and scoring methods are neither properly described nor standardized for cells from these tissues (Thomas et al., 2009).

The time of sampling is also an important variable to consider. As the buccal cells turn over every 7-21 days, it is theoretically possible to observe the genotoxic effects of an acute exposure approximately 7-21 days later.

Ideally, repeat sampling, at least once every 7 days after acute exposure, should be performed for 28 days or more so that the kinetics and extent of biomarker induction can be thoroughly investigated. In the case of chronic exposure due to habitual diet or alcohol consumption or smoking it is recommend that multiple samples are taken at least once every 3 months to take into account seasonal variation (Thomas et al., 2009).

The uniformity of sampling is one of the many aspects to consider; therefore a circular expanding motion is used with toothbrush sampling to enhance sampling over a greater area and to avoid continual erosion in a single region of the BM. This is performed on the inside of both cheeks using a different brush for sampling left and right areas of the mouth to maximize cell sampling and to eliminate any unknown biases that may be caused by sampling one cheek only. It is important to note that repeated vigorous brushing of the same area can lead to increased collection of cells from the less differentiated basal layer. About transportation, in some investigations buccal cells may have to be collected from a distant site which may cause sample deterioration. About cell fixation, there are many possible alternatives of fixatives such as methanol: glacial acetic acid (3:1), 80% methanol or ethanol: glacial acetic (3:1). The staining technique recommend is Feulgen because is a DNA-specific stain and because permanent slides can be obtained that can be viewed under both transmitted and/or fluorescent light conditions. There are many false-positive results in MN frequency as a result of using Romanowsky-type stains such as Giemsa, May-Grunwald Giemsa and/or Leishmann's which leads to inaccurate assessment of DNA damage. Romanowsky stains have been shown to increase the number of false positives as they positively stain keratin bodies that are often mistaken for MN and are therefore not appropriate for this type of analysis. For these reasons, it is advisable to avoid Romanowsky stains in favour of DNA-specific fluorescent-based stains such as propidium iodide, DAPI, Feulgen, Hoechst 33258 or Acridine Orange (Thomas et al., 2009).

The criterion of scoring is originally based in the described by Tolbert et al. that are intended for classifying buccal cells into categories that distinguish between "normal" cells and cells that are considered "abnormal" on the basis of cytological and nuclear features, which are indicative of DNA damage, cytokinetic failure or cell death. Therefore, some definitions of the cytological findings are (Thomas et al., 2009):

Normal "differentiated" cells have a uniformly stained nucleus, which is oval or round in shape. They are distinguished from basal cells by their larger size and by their smaller nucleus-to-cytoplasm ratio. No other DNA-containing structures apart from the nucleus are observed in these cells. These cells are considered to be terminally differentiated relative to basal cells, as no mitotic cells are observed in this population.

Cells with MN are characterized by the presence of both a main nucleus and one more smaller nuclear structures called MN. The MN are round or oval in shape and their diameter should range between 1/3 and 1/16 of the main nucleus. MN has the same staining intensity and texture as the main nucleus. Most cells with MN will ontain only one MN but it is possible to find cells with two or more MN. Baseline frequencies for micronucleated cells in the BM are usually within the 0.5-2.5 MN/1000 cells range. Cells with multiple MN are rare in healthy subjects but become more common in individuals exposed to radiation or other genotoxic events.

Cells with nuclear buds contain nuclei with an apparent sharp constriction at one end of the nucleus suggestive of a budding process, i.e. elimination of nuclear material by budding.

The NBUD and the nucleus are usually in very close proximity and appear to be attached to each other. The NBUD has the same morphology and staining properties as the nucleus; however, its diameter may range from a half to a quarter of that of the main nucleus. The mechanism leading to NBUD formation is not known but it may be related to the elimination of amplified DNA or DNA repair (Thomas et al., 2009).

The scoring method should include coded slides by a person not involved in the study in order to be a blind study. The best magnification to the observation is 1000X. An automated procedure of scoring, by image cytometry have to be developed and validated. The authors suggested first determine the frequency of all the various cell types in a minimum of 1000 cells, following this step, the frequency of DNA damage biomarkers (MN and NBUD) is scored in a minimum of 2000 differentiated cells (Thomas et al., 2009).

At the end the results with the BMCyt are dependent on the level of exposure and potency of genotoxic or cytotoxic agents, genetic background and the age and gender of the donor cells being tested (Thomas et al., 2009).

Is important to define the role of BMCyt in human biomonitoring as a new tool, less invasive in comparison with the CBMN assay, and with many potentialities in molecular epidemiology (Thomas et al., 2009).

Genotoxicity biomonitoring endpoints such as micronucleus, chromosome aberrations and 8-OHdG and DNA repair measured by comet assay are the most commonly used biomarkers in studies evaluating environmental or occupational risks associated with exposure to potential genotoxins. A review made by Knudsen and Hansen (2007) about the application of biomarkers of intermediate end points in environmental and occupational health concluded that MN in lymphocytes provided a promising approach with regard to assessing health risks but concluded that the use of chromosome aberrations in future studies was likely to be limited by the laborious and sensitive procedure of the test and lack of trained cytogeneticists. Methodologies like comet assay in lymphocytes, urine and tissues are increasingly being used as markers of oxidative DNA damage (Battershill et al., 2008).

Studies investigating correlations between endpoints used in genotoxicity biomonitoring studies have yielded inconsistent results, where we can find studies that correlate cytogenetic and comet and studies there do not achieve a correlation between micronucleus, chromosome aberrations and comet. The relative sensitivities of the different endpoints discussed, together with the importance of other factors which influence the persistence of the biomarkers such as DNA repair, may plausibly impact on background levels in the studies considered and would need to be considered before the relationship regarding increases in genotoxicity endpoints with exposure to environmental chemicals or endogenous factors is explored (Battershill et al., 2008).

2.2 Application of genotoxicity biomarkers in an occupational setting – Histopathology laboratories

A biomonitoring study was conducted in 7 histopathology laboratories in Portugal in order to assess the genotoxicity effects in occupational exposure to formaldehyde (FA).

FA is a reactive, flammable and colourless gas with a strong and very characteristic pungent odour that, when combined with air, can lead to explosive mixtures. FA occurs as an

endogenous metabolic product of N-, O- and S-demethylation reactions in most living systems. It is used mainly in the production of resins and their applications, such as adhesives and binders in wood product, pulp and paper, synthetic vitreous fibre industries, production of plastics, coatings, textile finishing and also as an intermediate in the synthesis of other industrial chemical compounds. Common non-occupational sources of exposure to FA include vehicle emissions, particle boards and similar building materials, carpets, paints and varnishes, food and cooking, tobacco smoke and its use as a disinfectant (Conaway et al., 1996; Franks, 2005; IARC, 2006; Pala et al., 2008; Viegas & Prista, 2007).

Commercially, FA is manufactured as an aqueous solution called formalin, usually containing 37 to 40% by weight of dissolved FA (Zhang et al., 2009), which is commonly used in histopathology laboratories as a cytological fixative to preserve the integrity of cellular architecture for diagnosis.

Exogenous FA can be absorbed following inhalation, dermal or oral exposure, being the level of absorption dependent on the route of exposure. The International Agency for Research on Cancer (IARC) reclassified FA as a human carcinogen (group 1) in June 2004 based on *"sufficient epidemiological evidence that FA causes nasopharyngeal cancer in humans"* (IARC, 2006; Zhang et al., 2009). In their review, IARC also concluded that there was *"strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to FA"* (Zhang et al., 2009, 2010). However, some studies have also led to mixed results and inconclusive evidence (Franks, 2005; Speit et al., 2010).

The inhalation of vapours can produce irritation to eyes, nose and the upper respiratory system. Whilst occupational exposure to high FA concentrations may result in respiratory irritation and asthmatic reactions, it may also aggravate a pre-existing asthma condition. Skin reactions, following exposure to FA are very common, because the chemical is both irritating and allergenic (Pala et al., 2008). FA induces genotoxic and cytotoxic effects in bacteria and mammals cells (Ye et al., 2005) and its genotoxicity and carcinogenicity has been proved in experimental and epidemiological studies that used proliferating cultured mammalian cell lines and human lymphocytes (Pala et al., 2008; Speit et al., 2007) by DNA-protein cross-links, chromosome aberrations, sister exchange chromatides, and MN (Zhang et al., 2009).

The goal of this study was to compare the frequency of genotoxicity biomarkers, provided by CBMN assay in peripheral lymphocytes and MN test in buccal cells between workers of histopathology laboratories exposed to FA and individuals non-exposed to FA and other environmental factors, namely tobacco and alcohol consumption.

The study population consisted of 56 workers occupationally exposed to FA from 7 hospital histopathology laboratories located in Portugal (Lisbon and Tagus Valley region), and 85 administrative staff without occupational exposure to FA. The characteristics of both groups are described in Table 1.

Ethical approval for this study was obtained from the institutional Ethical Board and Director of the participating hospitals, and all subjects gave informed consent to participate in this study. Every person filled a questionnaire aimed at identifying exclusion criteria like history of cancer, radio or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the past six months, intake of vitamins or other supplements like folic acid as well

as information related to working practices (such as years of employment and the use of protective measures). In this study, none of the participants were excluded.

	Control group	Exposed group
Number of subjects	85	56
Gender		
Females	54 (64%)	37 (66%)
Masculine	31 (36%)	19 (34%)
Age (mean ±standard deviation, in years)	32.42±8.1	39.45±11.5
Range	20-53	20-61
Years of employment (mean ± sandard deviation, in years)	-	14.5
Range		1-33
Tobacco consumption		
Non-smokers	60 (70,6%)	45 (80,4%)
Smokers	25(29, 4%)	11 (19,6%)
Alcohol consumption		
Non-drinkers	19 (22,4%)	19 (33,9%)
Drinkers	66 (77,6%)	37 (66,1%)

Table 1. Characteristics of the studied sample.

2.2.1 Environmental monitoring of FA exposure

Exposure assessment was based on two techniques of air monitoring conducted simultaneously. First, environmental samples were obtained by air sampling with low flow pumps for 6 to 8 hours, during a typical working day. FA levels were measured by Gas Chromatography analysis and time-weighted average (TWA_{8h}) was estimated according to the National Institute of Occupational Safety and Health method NIOSH 2541 (NIOSH, 1994).

The second method was aimed at measuring ceiling values of FA using Photo Ionization Detection (PID) equipment (11.7 eV lamps) with simultaneous video recording. Instantaneous values for FA concentration were obtained on a per second basis. This method allows establishing a relation between workers activities and FA concentration values, as well to reveal the main exposure sources (McGlothlin et al., 2005; Viegas et al., 2010).

Measurements and sampling were performed in a macroscopic room, provided with fume hoods, always near workers breath.

2.2.2 Biological monitoring

Evaluation of genotoxic effects was performed by applying the CBMN assay in peripheral blood lymphocytes and exfoliated cells from the buccal mucosa.

Whole blood and exfoliated cells from the buccal mucosa were collected between 10 a.m. and 12 p.m., from every subject and were processed for testing. All samples were coded and analyzed under blind conditions. The criteria for scoring the nuclear abnormalities in lymphocytes and MN in the buccal cells were the ones described by, respectively, Fenech et al. (1999) and Tolbert et al. (1991).

Heparinized blood samples were obtained by venipuncture from all subjects and freshly collected blood was directly used for the micronucleus test. Lymphocytes were isolated using Ficoll-Paque gradient and placed in RPMI 1640 culture medium with L-glutamine and red phenol added with 10% inactivated fetal calf serum, 50 ug/ml streptomycin + 50U/mL penicillin, and 10 ug/mL phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37ºC in a humidified 5% CO_2 incubator for 44h, and cytochalasin-b 6 ug/mL was added to the cultures in order to prevent cytokinesis. After 28h incubation, cells were spun onto microscope slides using a cytocentrifuge. Smears were air-dried and double stained with May-Grünwald-Giemsa and mounted with Entellan®. One thousand cells were scored from each individual by two independent observers in a total of two slides. Each observer visualized 500 cells per individual. Cells from the buccal mucosa were sampled by endobrushing. Exfoliated cells were smeared onto the slides and fixed with Mercofix®. The standard protocol used was Feulgen staining technique without counterstain. Two thousand cells were scored from each individual by two independent observers in a total of two slides. Each observer visualized 1000 cells per individual. Only cells containing intact nuclei that were neither clumped nor overlapped were included in the analysis.

2.2.3 Statistical analysis

The deviation of variables from the normal distribution was evaluated by the Shapiro-Wilk goodness-of-fit test. The association between each of the genotoxicity biomarkers and occupational exposure to FA was evaluated by binary logistic regression. The biomarkers were dichotomized (absent/present) and considered the dependent variable in regression models where exposure was an independent variable. Odds ratios were computed to evaluate the risk of biomarkers presence and their significance was assessed. The non-parametric Kuskal-Wallis and Mann-Whitney U-tests, were also used to evaluate interactions involving confounding factors. All statistical analysis was performed using the SPSS package for windows, version 15.0.

2.2.4 Results

FA exposure levels

Results of FA exposure values were determined using the two methods described – the NIOSH 2541 method for average concentrations (TWA_{8h}) and the PID method for ceiling concentrations. For the first exposure metric, FA mean level of the 56 individuals studied was 0.16 ppm (0.04 – 0.51 ppm), a value that lies below the OSHA reference value of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (0.18 – 2.93 ppm), a value well above the reference of the American Conference of Governmental Industrial Hygienists (ACGIH) for ceiling concentrations (0.3 ppm). As for the different tasks developed in histopathology laboratories, the highest FA concentration was identified during macroscopic specimens' exam. This task involves a careful observation and grossing of the specimen preserved in FA, therefore has direct and prolonged contact with its vapors (Table 2).

Tasks	Ceiling values (ppm)
Macroscopic specimen's exam	2.93
Disposal of specimen and used solutions	0.95
Jar filling	2.51
Specimen wash	2.28
Biopsy exam	1.91

Table 2. FA ceiling values (ppm) by tasks in the macroscopy room.

Genotoxicity biomarkers

For all genotoxicity biomarkers under study, workers exposed to FA had significantly higher mean values than the controls (Table 3).

In peripheral blood lymphocytes, significant differences (Mann-Whitney test, $p<0.001$) were observed between subjects exposed and non-exposed to FA, namely in mean MN (respectively, 3.96±0.525 *vs* 0.81±0.172), NPB (3.04±0.523 *vs* 0.18±0.056), and NBUD (0.98±0.273 *vs* 0.07±0.028). In buccal mucosa cells, the MN mean was also significantly higher ($p=0.002$) in exposed subjects (0.96±0.277) than in controls (0.16±0.058).

	Mean, MN lymphocytes ±S.E. (range)	Mean, NPB±S.E. (range)	Mean, NBUD±S.E. (range)	Mean, MN buccal cells ±S.E. (range)
Controls	0.81±0.172 (0-7)	0.18±0.056 (0-3)	0.07±0.028 (0-1)	0.16±0.058 (0-2)
Exposed	3.96±0.525 (0-14)	3.04±0.523 (0-15)	0.98±0.273 (0-13)	0.96±0.277 (0-9)
p-value	<0.001	<0.001	<0.001	0.002

Table 3. Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± mean standard error, range and p-value of Mann-Whitney test)

Discriminating by occupation, technologists group mean of MN in lymphocytes was 3.76±0.647; in NBP was 2.62±0.629; in NBUD was 1.09±0.401 and in MN in BM was 1.18±0.406. In pathologists, the means were 5.00±1.243; 3.75±1.467; 0.33±0.188 and in MN in BM was 0.58±0.434, respectively.

The odds ratios indicate an increased risk for the presence of biomarkers in those exposed to FA, compared to non-exposed (Table 4) and they were all significant ($p<0.001$).

	OR	CI 95%	p-value
MN lymphocytes	9.665	3.81-24.52	<0.001
NPB	11.97	4.59-31.20	<0.001
NBUD	9.631	3.12-29.70	<0.001
MN buccal cells	3.990	1.38-11.58	0.011

Table 4. Results of binary logistic regression concerning the association between FA and genotoxicity biomarkers, as evaluated by the odds ratio (OR).

Regarding the impact of the duration of exposure to FA, the mean values of MN in lymphocytes and in buccal cells tended to increase with years of exposure (Table 5) but the association was not statistically significant.

Group	Years of exposure	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB± S.E. (range)	Mean NBUD± S.E. (range)	Mean MN buccal cells ± S.E. (range)
Exposed	> 5	8	2.75±0.940 (0-8)	5.13±1.381 (0-10)	1.38±0.498 (0-3)	0.63±0.625 (0-5)
	6-10	19	3.05±0.775 (0-12)	2.42±0.668 (0-9)	1.53±0.731 (0-13)	0.63±0.326 (0-6)
	11 - 20	12	5.50±1.317 (0-14)	3.33±1.443 (0-14)	0.33±0.188 (0-2)	0.83±0.458 (0-5)
	>21	15	5.00±1.151 (0-13)	2.33±1.036 (0-15)	0.73±0.248 (0-2)	1.20±0.8 (0-9)

Table 5. Descriptive statistics according to years of exposure to formaldehyde of MN in lymphocytes and buccal cells, NPB, and NBUD means in the two groups (mean ± mean standard error, range)

Age and gender are considered the most important demographic variables affecting the MN index. However, Table 6 shows that the mean of all the genotoxicity biomarkers did not differ between men and women within the exposed and the controls (p> 0.05).

Groups	Gender	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB ± S.E. (range)	Mean NBUD ± S.E. (range)	Mean MN buccal cells± S.E. (range)
Exposed	Females	37	4.43±0.676 (0-14)	3.03±0.699 (0-15)	1.34±0.418 (0-13)	1.14±0.353 (0-8)
	Males	19	3.47±0.883 (0-13)	2.95±0.818 (0-14)	0.42±0.158 (0-2)	0.74±0.495 (0-9)
Controls	Females	54	0.87±0.229 (0-7)	0.22±0.078 (0-3)	0.11±0.043 (0-1)	0.11±0.057 (0-2)
	Males	31	0.71±0.255 (0-6)	0.10±0.071 (0-2)	0.00	0.26±0.122 (0-2)

Table 6. Descriptive statistics by gender of MN in lymphocytes and buccal cells, NPB, and NBUD means in the two groups (mean ± mean standard error, range)

In order to examine the effect of age, exposed and non-exposed individuals were stratified by age groups: 20-30, 31-40, and ≥ 41 years old (Table 7). There was no consistent trend regarding the variation of biomarkers with age, the only exception being the MN in lymphocytes in the exposed group (Kruskal-Wallis, p= 0.006), where the higher means where found in the older group. According to Mann-Whitney test, there is a statistical significant result between the elder and the older group (20–30 and > 41 years old, p= 0.02), however the comparison between 20-30 and 31–40 groups (p= 0.262) and 30–40 and > 41 groups (p= 0.065) did not reach statistical significance.

Groups	Age	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB± S.E. (range)	Mean NBUD± S.E. (range)	Mean MN buccal cells ± S.E. (range)
Exposed	20-30	18	2.19±0.526 (0-8)	3.56±0.926 (0-10)	1.63±0.816 (0-13)	0.75±0.470 (0-6)
	31-40	11	3.00±0.775 (0-8)	1.20±0.467 (0-4)	0.50±0.224 (880-2)	0.40±0.221 (0-2)
	>41	27	5.54±0.876 (0-15)	3.00±0.879 (0-15)	0.69±0.234 (0-5)	1.46±0.503 (0-9)
Controls	20-30	36	0.47±0.157 (0-3)	0.14±0.071 (0-2)	0.08±0.047 (0-1)	0.19±0.96 (0-2)
	31-40	35	1.14±0.326 (0-7)	0.20±0.099 (0-3)	0.06±0.040 (0-1)	0.14±0.83 (0-2)
	>41	14	0.86±0.501 (0-6)	0.21±0.155 (0-2)	0.07±0.71 (0-1)	0.14±0.143 (0-2)

Table 7. Age effects on descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± mean standard error, range).

The interaction between age and gender in determining the frequencies of genotoxicity biomarkers was investigated and found to be significant only for MN in lymphocytes in exposed subjects (Kruskal-Wallis, $p=0.04$). In general the MN tended to be more frequent in the > 41 years old category in both genders; however women had the higher means.

Regarding smoking habits, a non-parametric analysis rejected the null hypothesis that biomarkers are the same for the four categories (control smokers and non-smokers, exposed smokers and non-smokers) (Kruskall-Wallis, $p<0.001$). However, the analysis of the interactions between FA exposure and tobacco smoke between exposed and controls (Mann-Whitney test) showed that FA exposure, rather than tobacco, has a preponderant effect upon the determination of biomarker frequencies. In the control group, non-smokers had slightly higher MN means in buccal cells in comparison with smokers; although the result did not reach statistical significance (Mann-Whitney, $p> 0.05$).

As for alcohol consumption, because uptake reported in enquires may differ considerably from real consumption, all consumers were gathered into a single entity, in contrast with non-consumers. Nevertheless, no one acknowledged having "heavy drink habits" in the questionnaires.

Overall, biomarkers in controls exhibited higher mean frequencies among alcohol consumers than among non-consumers. Among those exposed, however, mean frequencies were slightly lower among drinkers, suggesting that exposure was the major predominant factor in determining the high biomarker frequencies of those who are exposed. Differences between drinkers and non-drinkers were not statistically significant, to the exception of MN in lymphocytes in controls (Mann-Whitney, $p=0.011$), where drinkers have higher means. The interaction between alcohol consumption and smoking habits was statistically significant (Kruskal-Wallis, $p=0.043$), as subjects that do not smoke and do not drink tend to have lower frequencies of MN in buccal cells than those who drink and smoke, with a gradient of frequencies in between.

2.2.5 Discussion

Long exposures to FA, as those to which some workers are subjected for occupational reasons, are suspected to be associated with genotoxic effects that can be evaluated by biomarkers (Conaway et al., 1996; IARC, 2006; Viegas & Prista, 2007; Zhang et al., 2009). In this study the results suggest that workers in histopathology laboratories are exposed to FA levels that exceed recommended exposure limits. Macroscopic specimens' exam, in particular, is the task that involves higher exposure, because it requires a greater proximity to anatomical species impregnated with FA, as supported by the studies of Goyer et al. (2004) and Orsière et al. (2006).

A statistically significant association was found between FA exposure and biomarkers of genotoxicity, namely MN in lymphocytes, NPB, NBUD and MN in buccal cells. Chromosome damage and effects upon lymphocytes arise because FA escapes from sites of direct contact, such as the mouth, originating nuclear alterations in the lymphocytes of those exposed (He & Jin, 1998; IARC, 2006; Orsière et al. 2006; Ye et al., 2005). Our results thus corroborate previous reports (Ye et al., 2005) that lymphocytes can be damaged by long term FA exposure. Moreover, the changes in peripheral lymphocytes indicate that the cytogenetic effects triggered by FA can reach tissues faraway from the site of initial contact (Suruda et al., 1993). Long term exposures to high concentrations of FA indeed appear to have a potential for DNA damage; these effects were well demonstrated in experimental studies with animals, local genotoxic effects following FA exposure, namely DNA-protein cross links and chromosome damage (IARC, 2006).

In humans, FA exposure is associated with an increase in the frequency of MN in buccal epithelium cells (Burgaz et al., 2002; Speit et al., 2006, 2007b), as corroborated by the results presented here.

Suruda el al. (1993) claim that although changes in oral and nasal epithelial cells and peripheral blood cells do not indicate a direct mechanism leading to carcinogenesis, they present evidence that DNA alteration took place. It thus appears reasonable to conclude that FA is a cancer risk factor for those who are occupationally exposed in histopathology laboratories (IARC, 2006).

MN and NPB measured in lymphocytes had higher means in pathologists compared with technologists. This result can be explained by the exposure to higher concentrations of pathologists that perform macroscopic exam. Also this chemical mode of action is more related with the concentration than with time of exposure expressed by TWA results.

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking and alcohol consumption, upon the association between disease and exposure (Bonassi et al., 2001; Fenech at al., 1999). Concerning gender, studies realized by Fenech et al. (1999) and Wojda et al. (2007) reported that biomarker frequencies were greater in females than in males by a factor of 1.2 to 1.6 depending on the age group. With the exception of MN in the buccal cells of controls, the results presented here point to females having higher frequencies than males in all genotoxicity biomarkers, although the differences usually lacked statistical significance. Such trend is concordant with previous studies that reported higher MN frequency in lymphocytes in females and a slightly higher MN frequency in buccal cells in males (Holland et al., 2008) and that can be explained by preferential aneugenic events involving

the X-chromosome. A possible explanation is the micronucleation of the X chromosome, which has been shown to occur in lymphocytes in females, both *in vitro* and *in vivo*, and that can be accounted for by the presence of two X chromosomes. This finding might explain the preferential micronucleation of the inactive X (Catalán et al., 1998, 2000a, 2000b).

Aging in humans appears to be associated with genomic instability. Cytogenetically, ageing is associated with a number of gross cellular changes, including altered size and morphology, genomic instability and changes in expression and proliferation (Bolognesi et al., 1999; Zietkiewicz et al., 2009). It has been shown that a higher MN frequency is directly associated with decreased efficiency of DNA repair and increased genome instability (Kirsch-Volders et al., 2006; Orsière et al., 2006). The data has shown a significant increase of MN in lymphocytes in the exposed group. This can be explained in light of genomic instability, understood as an increased amount of mutations and/or chromosomal aberrations that cytogenetically translate into a greater frequency of changes in chromosome number and/or structure and in the formation of micronuclei (Zietkiewicz et al., 2009). The involvement of micronucleation in age-related chromosome loss has been supported by several studies showing that the rate of MN formation increases with age, especially in women (Catalán et al., 1998). This study provides evidence that age and gender interact to determine the frequency of MN in the lymphocytes of exposed subjects. The higher incidence of MN in both genders is more manifest in older age groups and the effect of gender becomes more pronounced as age increases. Several reports link this observation to an elevated loss of X chromosomes (Battershill et al., 2008).

Tobacco smoke has been epidemiologically associated to a higher risk of cancer development, especially in the oral cavity, larynx, and lungs, as these are places of direct contact with the carcinogenic tobacco's compounds. In this study, smoking habits did not influence the frequency of the genotoxicity biomarkers; moreover, the frequencies of MN in buccal cells were unexpectedly higher in exposed non-smokers than in exposed smokers, though the difference was not statistically significant. In most reports, the results about the effect of tobacco upon the frequency of MN in human lymphocytes were negative as in many instances smokers had lower MN frequencies than non-smokers (Bonassi et al., 2003). In the current study, the analysis of the interaction between FA exposure and smoking habits indicates that exposure is preponderant in determining the frequency of biomarkers. Nevertheless, the effect of smoking upon biomarkers remains controversial. Some studies reported an increased frequency of MN in lymphocytes, NPB, and NBUD as a consequence of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Still in this study no associations were observed between tobacco and nuclear abnormalities (El-Zein et al., 2006, 2008).

As for alcohol consumption, it did not appear to influence the frequency of genotoxicity biomarkers in study, to the exception of MN in lymphocytes in controls (Mann-Whitney, $p=0.011$), with drinkers having higher means. Alcohol is definitely a recognized genotoxic agent, being cited as able to potentiate the development of carcinogenic lesions (Ramirez & Saldanha, 2002). In our study, drinkers in the control group had higher mean frequencies of all biomarkers than non-drinkers, but the differences were only significant for MN in lymphocytes. Stich and Rosin (1983) study of alcoholic individuals, reported absence of significant differences concerning MN frequencies in buccal cells. That is important to corroborate our result, because of the lack of "heavy drinkers" in our study. The same study

concluded that neither alcohol nor smoking, alone, increase MN frequency in buccal cells, but a combination of both resulted in a significant elevation in micronucleated cells in the buccal mucosa. However, the synergism between alcohol consumption and tobacco has not been observed to act upon all biomarkers and, in several studies of lifestyle factors, it was difficult to differentiate the effect of alcohol from that of smoking (Holland et al., 2008).

The CBMN assay is a simple, practical, low cost screening technique that can be used for clinical prevention and management of workers subjected to occupational carcinogenic risks, namely exposure to a genotoxic agent such as FA. The results obtained in this study provide unequivocal evidence of association between occupational exposure to formaldehyde in histopathology laboratory workers and the presence of nuclear changes.

Given these results, preventive actions must prioritize safety conditions for those who perform macroscopic exams. In general, exposure reduction to FA in this occupational setting may be achieved by the use of adequate local exhaust ventilation and by keeping biological specimen containers closed during the macroscopic exam.

3. Conclusion

Another important application of biological monitoring, besides exposure assessment, is the use of biomarkers, at either individual or group level, for the correct interpretation of doubtful clinical tests. These are usually performed as part of occupational health surveillance program when exposure assessment data are unavailable or are deemed unreliable. Health surveillance is the periodical assessment of the workers' health status by clinical, biochemical, imaging or instrumental testing to detect any clinically relevant, occupation-dependent change of the single worker's health. Biomarkers are usually more specific and sensitive than most clinical tests and may be more effective, therefore, for assessing a causal relationship between health impairment and chemical exposure when a change is first detected in exposed workers (Manno et al., 2010).

Experience in biological monitoring gained in the occupational setting has often been applied to assess (the effects of) human exposure to chemicals in the general environment. The use of biological fluids/tissues for the assessment of human exposure, effect or susceptibility to chemicals in the workplace represents, together with the underlying data (e.g. personal exposure and biological monitoring measurements, media-specific residue measurements, product use and time-activity information), a critical component of the occupational risk assessment process, a rapidly advancing science (Manno et al., 2010).

Au et al. (1998), advise to put more emphasis on monitoring populations which are known to be exposed to hazardous environmental contaminant and on providing reliable health risk evaluation. The information can also be used to support regulations on protection of the environment.

4. Acknowledgment

This work was supported by Autoridade Portuguesa para as Condições de Trabalho (ACT – Portuguese Authority for Work Conditions). http://www.act.gov.pt/. Project reference: 075MNA/06.

5. References

Au, W.; Cajas-Salazar, N. & Salama, S. (1998). Factors contributing to discrepancies in population monitoring studies. *Mutation Research*, 400, pp. 467 – 478.

Barrett, J.; Vainio, H.; Peakall, D. & Goldstein, B. (1997). 12th Meeting of the scientific group on methodologies for the safety evaluation of chemicals: susceptibility to environmental hazards. *Environmental Health Perspectives*, 104, Suppl 4, pp. 699 – 737.

Battershill, J.; Burnett, K. & Bull, S. (2008). Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. *Mutagenesis*, 6, pp. 423-437.

Bolognesi, C., Lando, C.; Forni, A.; Landini, E.; Scarpato, R.; Migliori, L.& Bonassi, S. (1999). Chromosomal damage and ageing: effect of micronuclei frequency in peripheral blood lymphocytes. *Age and Ageing*, 28, pp. 393-397.

Bonassi, S.; Fenech, M., Lando, C., Lin, Y-P., Ceppi, M.; Chang, W.; Holland, N.; Kirsch-Volders, M.; Zeiger, E., Baan, S.; Barale, R., Bigatti, M., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L., Fucic, A., Lima, O., Hrelia, P., Krishnaja, A., Lee, T-K., Migliore, L., Mikhalevich, L.; Mirkova, E.; Mosesso, P., Müller, W.-U., Odagiri, Y., Scarfi, M.; Szabova, E.; Vorobtsova, I., Vral, A. & Zijno, A. (2001). Human MicroNucleus Project: International database comparison for results with cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factor son the frequency of micronuclei. *Environmental and Molecular Mutagenesis*, 37, 31-45.

Bonassi, S.; Au, W. (2002). Biomarkers in molecular epidemiology studies for health risk prediction. *Mutation Research*, 511, pp. 73 – 86.

Bonassi, S.; Neri, M.; Lando, C.; Ceppi, M.; Lin, Y.; Chang, W.; Holland, N., Kirsch-Volders, M.; Zeiger, E. & Fenech, M. (2003). Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. *Mutation Research*, 543, pp. 155-166.

Burgaz, S.; Erdem, O.; Çakmak, G. & Karakaya, A. (2002). Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. *Biomarker*, 2, pp. 151-161.

Catalán, J.; Autio, K.; Kuosma, E. & Norppa, H. (1998). Age-dependent inclusion of sex chromosomes in lymphocyte micronuclei in man. *Am. J. Hum. Genet.*, 63, pp.1464-1472.

Catalán, J.; Falck, G. & Norppa, H. (2000a). The X chromosome frequently lags behind in female lymphocyte anaphase. *American Journal of Human Genetics*, 66, pp. 687-691.

Catalán, J.; Surrallés, J.; Falck, G., Autio, K. & Norppa, H. (2000b). Segregation of sex chromosomes in human lymphocytes. *Mutagenesis*, 3, 251-255.

Conaway, C.; Whysner, J.; Verna, L. & Williams, G. (1996). Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. *Pharmacol. Ther.* 71, pp. 29 – 55.

Davis, C.; & Milner, J. (2007). Biomarkers for diet and cancer prevention research: potentials and challenges. *Acta Pharmacol Sin*, Vol. 9, 28, pp. 1262-1273.

El-Zein, R.; Schabath, M.; Etzel, C.; Lopez, M.; Franklin, J. & Spitz M. (2006) . Cytokinesis-Blocked Micronucleus Assay as a Novel Biomarker for Lung Cancer Risk. *Cancer Reearch*, 66, pp. 6449 – 6456.

El-Zein, R.; Fenech, M.; Lopez, M., Spitz, M. & Etzel, C. (2008). Cytokinesis-Blocked Cytome Assay Biomarkes Identify Lung Cancer Cases amongst Smokers. *Cancer Epidemiol. Biomarkers Prev* 17, pp. 1111 – 1119.

Fenech, M.; Holland, N.; Chang, W.; Zeiger, E. & Bonassi, S. (1999). The Human MicroNucleus Project – An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutation Research*, 428, pp. 271 – 283.

Fenech, M. & Crott, J. (2002a). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes – evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research*, 504, pp. 131-136.

Fenech, M. (2002b). Chromosomal biomarkers of genomic instability relevant to cancer. *Drug Discovery Today*, 22, pp. 1128-1137.

Fenech, M. (2005). The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. *Mutagenesis*, 20, pp. 225 – 269.

Fenech, M. (2006). Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research*, 600, pp.58-66.

Fenech, M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols*, 5, pp. 1084-1104.

Franks, S. (2005). A mathematical model for the absorption and metabolism of formaldehyde vapour by humans. *Toxicology and Applied Pharmacology*, 206, pp. 309 – 320.

Goyer, N.; Beaudry, C. ; Bégin, D. ; Bouchard, M. ; Buissonet, S. ; Carrier, G. ; Gely, O. ; Gérin, M. ; Lavoué, L., Lefebvre, P. ; Noisel, N. ; Perraut, G. & Roberge, B. (2004). Impacts d'un abaissement de la values d'exposition admissible au formaldéhyde : industries de fabrication de formaldéhde et de résines à base de formaldéhyde. Móntreal : Institut de Recherche Robert-Sauvé en Santé et Securité du Travail 2004.

He, J-L. & Jin, H.-Y. (1998). Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. *Biomed. Environm. Sci.*, 11, pp. 87-92.

Holland, N.; Bolognesi, C.; Kirsch-Volders, M.; Bonassi, S.; Zeiger, E., Knasmueller, S. & Fenech, M. (2008). The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps, *Mutation Research*: doi: 10.1016/j.mrrev.2008.03.007.

IARC. (2006). *IARC Monographs on the Evaluation of Carcinogenic Risks to Human, volume 88: Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxy-2-propanol.* World Health Organization, Lyon.

Ishikawa, H.; Tian, Y. & Yamauchi, T. (2003). Influence of gender, age and lifestyle factor son micronuclei frequency in healthy japanese populations. *Journal of Occupational Health*, 45, pp. 170-181.

Kirsch-Volders, M.; Mateuca, R.; Roelants, M.; Tremp, A., Zeiger, E.; Bonassi, S.; Holland, N.; Chang, W.; Aka, P.; DeBoeck, M.; Goddenis, L.; Haufroid, V.; Ishikawa, H.; Laffon, B.; Marcos, R.; Migliore, L., Norppa, H.; Teixeira, J.; Zijno, A. & Fenech, M. (2006). The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in Human Lymphocytes in vivo. *Cancer Epidemiol Biomarkers Prev*, Vol. 5, 15, pp. 1038-1042.

Knudsen, L. & Hansen, A. (2007). Biomarkers of intermediate endpoints in environmental and occupational health. *International Journal of Hygiene and Environmental Health*, 210, pp. 461 – 470.

Manno, M.; Viau, C.; Cocker, J.; Colosio, C.; Lowry, L.; Mutti, A.; Nordberg, M. & Wang, S. (2010). Biomonitoring for occupational health risk assessment (BOHRA). *Toxicology Letters*, 192, pp. 3 – 16.

McGlothlin, J. & col. (2005). Occupational exposure assessment and control using video exposure monitoring in the pharmaceutical industry. In INTERNATIONAL SCIENTIFIC CONFERENCE (IOHA 2005), 6, 19-23 September 2005, Pilanesberg National Park North West Province, South Africa. Pilanesberg: IOHA – International Occupational Hygiene. MVS – Mine Ventilation of South Africa.

NIOSH – Manual of analytical methods. 4th ed. DHHS (NIOSH) Publication 94-113. August, 1994.

Orsière, T.; Sari-Minodier, I.; Iarmarcovai, G. & Botta, A. (2006). Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. *Mutation Research*, 605, pp.30 – 41.

Pala, M.; Ugolini, D.; Ceppi, M.; Rizzo, F.; Maiorana, L.; Bolognesi, C.; Schilirò, T.; Gilli, G.; Bigatti, P.; Bono, R. & Vecchio, D. (2008). Occupational exposure to formaldehyde and biological monitoring of Research Institute workers. *Cancer Detection and Prevention*, 32, pp. 121 – 126.

Perera, F. (1996). Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. *Journal of the National Cancer Institute*, 8, pp. 496 – 509.

Perera, F. & Weinstein, I. (2000). Molecular epidemiology: recent advances and future directions. *Carcinogenesis*, 21, pp. 517 – 524.

Portier, C. & Bell, D. (1998). Genetic susceptibility: significance in risk assessment. *Toxicology Letters* 102 – 103, pp. 185 -189.

Ramirez, A. & Saldanha, P. (2002). Micronucleus investigation of alcoholic patients with oral carcinoma. *Genetics and Molecular Research*, 1, pp. 246 – 260.

Serrano-García, L. & Montero-Montoya, R. (2001). Micronuclei and Chromatid Buds are result of related genotoxic events. *Environmental and Molecular Mutagenesis*, 38, pp. 38 – 45.

Speit, G. & Schmid, O. (2006). Local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated epithelial cells. *Mutation Research*, 613, pp. 1-9.

Speit, G.; Schütz, P.; Högel, J. & Schmid, O. (2007a). Characterization of the genotoxic potencial of formaldehyde in V79 cells. *Mutagenesis, Vol.* 6, 22, pp. 387 – 394.

Speit, G.; Schmid, O.; Fröhler-Keller, M.; Lang, I. & Triebig, G. (2007b). Assessment of local genotoxic effects of formaldehyde in humans measured by de micronucleus test with exfoliated buccal mucosa cell. *Mutation Research*, 627, pp. 129-131.

Speit, G.; Gelbke H.-P.; Pallapies, D. & Morfeld, P. (2010). Occupational Exposure to formaldehyde, hematotoxicity and leukemia-specific chromosome changes in cultured myeloid progenitor cells – Letter, *Cancer, Epidemiology, Biomarkers & Prevention*, 7, 19, pp. 1882 – 1884.

Stich, H. & Rosin, M. (1983). Quantitating the synergistic effect of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells. *Int. J. Cancer* 31, pp. 305 – 308.

Suruda, A.; Schulte, P.; Boeniger, M.; Hayes, R.; Livingston, G.; Steenland, K., Stewart, P.; Herrick, R.; Douthit, D. & Fingerhut, M. (1993). Cytogenetic effects of formaldehyde exposure in students of mortuary science. *Cancer Epidemiology, Biomarkers & Prevention*, 2, 453 – 460.

Thomas, P.; Holland, N.; Bolognesi, C.; Kirsch-Volders, M.; Bonassi, S.; Zeiger, E.; Knasmueller, S. & Fenech, M. (2009). Buccal micronucleus cytome assay. *Nature Protocols*, 6, pp. 825-837.

Thomas, P.; Umegaki, K. & Fenech, M. (2003). Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. *Mutagenesis*, 18, pp. 187– 194.

Tolbert, P.; Shy, C. & Allen, J. (1991). Micronuclei and other nuclear anomalies in buccal smears: a field test in snuff users. *American Journal of Epidemiology*, 8, pp. 840 – 850.

Umegaki, K.; & Fenech, M. (2000). Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis, Vol.* 3, *15*, pp. 261-269.

Utani, K.; Kawamoto, J. & Shimizu, N. (2007). Micronuclei bearing extrachromosomal chromatin are transcriptionally competent and may perturb the cancer cell phenotype. *Mol. Cancer Res*, 5, pp. 695 – 704.

Viegas, S. & Prista, J. (2007). Cancro Nasofaríngeo e Exposição a Formaldeído: avaliação da história profissional em 63 casos registados, *Soc. Portuguesa de Medicina do Trabalho, 6*, pp. 13 – 22.

Viegas, S.; Ladeira, C.; Nunes, C.; Malta-Vacas, J., Gomes, M., Brito, M.; Mendonça, P. & Prista, J. (2010). Genotoxic effects in occupational exposure to formaldehyde: a study in anatomy and pathology laboratories and formaldehyde- resins production. Journal of Occupational Medicine and Toxicology 5, 25, pp. 1 – 8.

Wojda, A.; Zietkiewicz, E. & Witt, M. (2007). Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects. Mutagenesis Advance Access, pp. 1 – 6.

Ye, X.; Yan, W.; Xie, H.; Zhao, M. & Ying, C. (2005). Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutation Research, *588*, pp. 22-27.

Zhang, L.; Steinmaus, C.; Eastmond, D.; Xin, X. & Smith, M. (2009). Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. *Mutation Research*, 681, pp. 150 - 168.

Zhang, L.; Tang, X. & Rothman, N. (2010). Occupational exposure to formaldehyde, hematoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. *Cancer Epidemiology, Biomarkers & Prevention*, 19, pp. 80 - 88.

Zietkiewicz, E.; Wojda, A.; Witt, M. (2009). Cytogenetic perspective of ageing and longevity in men and women. *Journal of Applied Genetics*, 50, pp. 261-273.

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18 -27 . ISSN: 1646-9704

Exposição ocupacional a formaldeído: avaliação da exposição e efeitos genotóxicos

Carina Ladeira[1-2*], Susana Viegas[1-2], Elisabete Carolino[1], Mário Gomes[1], João Prista[2-3], Manuel Carmo Gomes[4], Miguel Brito[1]

1. Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa
2. Centro de Investigação e Estudos em Saúde Pública (CIESP), Escola Nacional de Saúde Pública, Universidade Nova de Lisboa
3. Escola Nacional de Saúde Pública, Universidade Nova de Lisboa
4. Faculdade de Ciências, Universidade de Lisboa
* Autor correspondente: carina.ladeira@estesl.ipl.pt

RESUMO: O formaldeído (FA) foi classificado, em 2004, pela *International Agency for Cancer Research* como agente cancerígeno. Este agente químico ocupa a 25ª posição em toda a produção química dos Estados Unidos da América, com mais de 5 milhões de toneladas produzidas por ano. Devido à sua importância económica e uso diversificado, muitos indivíduos estão expostos profissionalmente a FA. Com o estudo desenvolvido pretendeu-se avaliar a exposição a FA em dois contextos ocupacionais distintos – na produção de FA e resinas e em laboratórios de anatomia patológica (AP) e relacionar com eventuais efeitos para a saúde, comparando a frequência de micronúcleos (MN) em linfócitos do sangue periférico e em células esfoliadas da mucosa bucal dos trabalhadores expostos a FA com indivíduos não expostos (controlos). Como amostra foram estudados 80 trabalhadores ocupacionalmente expostos a FA: 30 trabalhadores da fábrica de produção de FA e resinas e 50 trabalhadores de 10 laboratórios de AP. Foi constituído um grupo controlo de 85 indivíduos com atividades profissionais que não envolviam a exposição a formaldeído ou qualquer outro agente químico com propriedades genotóxicas. Aplicaram-se duas metodologias distintas de avaliação ambiental do FA com o objetivo de conhecer a exposição profissional. Compararam-se os resultados obtidos com os valores limite para a exposição média ponderada (TLV-TWA=0,75 ppm) e para a concentração máxima (VLE-CM=0,3 ppm). A totalidade dos laboratórios apresentou resultados superiores ao valor de referência existente para a concentração máxima. Nenhum dos resultados obtidos para a exposição média ponderada foi superior ao valor de referência. O exame macroscópico obteve os valores das concentrações máximas mais elevadas em 90% dos laboratórios. Os valores de MN foram mais elevados nos indivíduos expostos a FA comparativamente com os controlos. No caso dos MN nos linfócitos, a média foi de 3,96 nos expostos e de 0,81 nos não expostos. Os MN nas células esfoliadas da boca apresentaram uma média de 0,96 nos expostos e de 0,16 nos controlos. Os resultados obtidos nesta acção de biomonitorização podem revelar-se particularmente úteis para as organizações responsáveis em definir os níveis aceitáveis para a exposição humana a FA.

Palavras-chave: genotoxicidade, micronúcleos, exposição ocupacional, formaldeído

Occupational exposure to formaldehyde: exposure and genotoxic effects assessment

ABSTRACT: Since 2004, formaldehyde (FA) has been classified by the International Agency for Cancer Research as a carcinogen. The FA ranks 25th in the overall United States chemical production, with more than 5 million tons produced each year. Due to its economic importance and varied use, many individuals are exposed to FA at their occupational settings. This study aimed to assess the exposure to FA in two occupational settings – FA production factory and pathology anatomy (PA) laboratories – and relate it to possible health effects by comparing frequency of micronuclei (MN) in peripheral blood

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18-27 . ISSN: 1646-9704

lymphocytes and exfoliated cells from the oral mucosa of workers exposed to FA with individuals not exposed to this agent (controls). This study was performed in 80 workers occupationally exposed to FA: 30 workers of the FA factory and 50 workers in 10 PA laboratories. The control group comprised 85 subjects without exposure. We have applied two different methodologies for environmental monitoring of FA. The results were compared with the reference to the exposure weighted average (TLV-TWA = 0.75 ppm) and ceiling concentration (VLE-MC = 0.3 ppm). All laboratories had results higher than the reference value to CM (1.41 ppm). None of the results obtained for the TWA exposure (0.16 ppm) were higher than the reference value. Macroscopic examination obtained the highest values of CM in 90% of laboratories. MN values were higher in individuals exposed to FA as compared to controls. As for MN in lymphocytes, the average was 3.96 in exposed compared with 0.81 in the unexposed. The MN in exfoliated cells of the buccal mucosa had an average of 0.96 in exposed, compared with 0.16 in controls. The results of this biomonitoring can be particularly useful to organizations responsible for defining acceptable levels for human exposure to FA.

Keywords: genotoxicity, micronucleus, occupational exposure, formaldehyde

Introdução

Estudos epidemiológicos que utilizam a biologia molecular – epidemiologia molecular possuem um potencial significativo na identificação de patologias oncológicas associadas com a exposição ambiental relacionada com estilos de vida, ocupação ou poluição[1]. Os biomarcadores de genotoxicidade são utilizados para avaliar os efeitos derivados de exposição, ambiental ou ocupacional, sendo classificados como preditivos do risco de doença[2].

O ensaio dos micronúcleos (MN) é um dos biomarcadores mais sensíveis na deteção do dano no DNA e tem sido utilizado na investigação de efeitos genotóxicos numa variedade de agentes químicos[3]. A avaliação de MN em linfócitos do sangue periférico é a técnica mais comummente utilizada. No entanto, a utilização de células esfoliadas da mucosa bucal também é frequente[4].

Nos estudos epidemiológicos em humanos, a análise de MN é uma boa ferramenta na investigação dos efeitos de agentes clastogéneos e aneuploidogéneos decorrentes da exposição ocupacional e ambiental[3] e está descrita como uma abordagem promissora na avaliação de risco em saúde[4].

A avaliação da exposição inclui cinco etapas fundamentais: recolha dos dados, identificação do perigo, formação de grupos de exposição, seleção dos referenciais de exposição adequados e, ainda, seleção de métodos de avaliação da exposição[5–8].

Recentemente o estudo da exposição às concentrações máximas tem vindo a ser considerado de particular interesse, uma vez que ocorre uma exposição elevada a nível dos tecidos e órgãos alvo, alterando potencialmente o metabolismo, sobrecarregando os mecanismos de proteção e reparação e amplificando as respostas tecidulares[9-10]. Devido a estes aspetos, níveis elevados de exposição num curto período de tempo podem estar implicados na etiologia de doenças crónicas ocupacionais que tradicionalmente estão associadas a exposições cumulativas por períodos de longa duração[10-11].

De acordo com o *Report on Carcinogens*, o formaldeído (FA) ocupa a 25ª posição em toda a produção química dos Estados Unidos da América com mais de 5 milhões de toneladas produzidas por ano[12]. Devido à sua importância económica e uso diversificado, muitos indivíduos estão expostos ao FA a nível ambiental e/ou ocupacional[13].

O FA é utilizado na produção de resinas com ureia, fenol e melamina, mobília e outros produtos de madeira e possui uma aplicação importante como desinfetante e preservante, razão pela qual pode ocorrer exposição ocupacional relevante nos laboratórios de Anatomia Patológica (AP) e em morgues[14–16].

Recentemente, a *International Agency for Research on Cancer* (IARC) reafirmou a classificação do FA no grupo 1, baseada na suficiente associação à incidência de cancro nasofaríngeo em humanos. A IARC concluiu que existe também suficiente evidência de associação do FA com o desenvolvimento de leucemia, nomeadamente leucemia mielóide[14,17-18].

Os efeitos para a saúde da exposição a FA parecem estar maioritariamente relacionados com a intensidade de períodos de exposição a concentrações elevadas do que com exposições longas a concentrações consideradas aceitáveis. Estes factos suportam a ideia de que a estratégia baseada na determinação das concentrações máximas possivelmente será a melhor para avaliar a exposição e obter dados para a avaliação do risco[14,18].

O objetivo principal desta investigação foi avaliar a exposição a FA em dois contextos ocupacionais – fábrica de produção de FA e resinas e laboratórios de AP e relacionar com eventuais efeitos para a saúde.

Materiais e métodos

O estudo foi realizado em Portugal, considerou uma amostra de 80 trabalhadores ocupacionalmente expostos a FA: 30 trabalhadores da fábrica de produção de FA e resinas e 50 trabalhadores de 10 laboratórios de AP. Foi constituído um grupo controlo de 85 indivíduos com atividades profissionais

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18 -27 . ISSN: 1646-9704

que não envolviam a exposição a formaldeído ou qualquer outro agente químico com propriedades genotóxicas.

Todos os participantes do estudo preencheram um termo de consentimento informado e um questionário acerca das condições de saúde, história médica, toma de medicamentos e/ou suplementos, estilos de vida, bem como informação acerca de práticas laborais, como a utilização de equipamento de proteção individual.

1. Avaliação da exposição

A avaliação ambiental foi realizada no período de Setembro de 2007 a Março de 2008. Em ambos os contextos ocupacionais foram identificados diferentes grupos de exposição. Nos laboratórios de AP foram definidos três: patologistas, técnicos de AP e auxiliares de ação médica. Na fábrica foram também identificados três grupos distintos: produção de resinas, impregnação e controlo de qualidade. Estas definições foram baseadas essencialmente na similaridade das atividades desenvolvidas, sendo esta informação obtida pela observação direta das atividades.

A quantificação da exposição a FA teve por base a aplicação de dois métodos de avaliação ambiental distintos: o Método 1 implicou o recurso a um equipamento de medição das concentrações de formaldeído por leitura direta; e o Método 2 que consistiu na aplicação do Método NIOSH 2541[19].

2. Métodos

Método 1

Este método implicou a utilização de um equipamento de leitura direta que efetua a medição das concentrações de FA por *Photo Ionization Detection* (PID), registando essa concentração no ar, ao segundo, sendo designado por *First Check*, da *ION Science* (www.ionscience.com). Foi utilizada uma lâmpada de 11,7 eV, indicada para estudar ambientes contaminados com formaldeído[20].

A necessidade de selecionar um equipamento de medição que realizasse o registo das concentrações de FA em cada segundo deveu-se ao facto de se pretenderem identificar as rápidas alterações na concentração do FA e associá-las com as atividades desenvolvidas no momento da medição, as quais foram alvo de filmagem simultânea.

O equipamento foi colocado ao nível do aparelho respiratório dos profissionais, durante a execução das atividades estudadas, visto ser esta a via de penetração preferencial do agente químico no organismo[14].

Antes do início das medições analisou-se a situação de trabalho com o objetivo de decompor a atividade em acontecimentos distintos e sucessivos, permitindo a observação de detalhes, a identificação de quando e onde medir as concentrações de FA e, ainda, a definição de grupos de exposição.

Os valores mais elevados de concentração obtidos em cada atividade estudada por este método foram comparados com o valor limite de exposição para a concentração máxima (VLE-CM=0,3 ppm), estabelecido na Norma Portuguesa NP 1796:2007[21].

Método 2

O Método 2 consiste na aplicação do método NIOSH 2541[19].

Este método implica recorrer a amostragem ativa através da utilização de amostradores individuais (bombas de amostragem de baixo caudal) e de material de retenção colocado próximo do aparelho respiratório dos trabalhadores.

No caso do FA e por se tratar de um agente químico muito reativo, o material de retenção tem que ter na sua composição dinitrofenilhidrazina para que esta reaja com o FA e torne a sua adsorção possível. Posteriormente, o material de retenção é processado e analisado por cromatografia gasosa[19,22].

O caudal das bombas de amostragem foi verificado antes e após cada utilização. Foram assegurados caudais de recolha inferiores a 0,10 litros/minuto e foi recolhido, por cada bomba de amostragem, um volume total de ar inferior a 36 litros[19].

As amostras foram posteriormente sujeitas a um processamento analítico específico, por cromatografia gasosa, conforme descrito no método NIOSH 2541.

Os resultados obtidos foram comparados com o valor limite disponível para a concentração média ponderada (TLV – TWA= 0,75 ppm), estabelecido pela *Occupational Safety and Health Administration* (OSHA), por não existir um referencial português.

3. Biomonitorização humana

De forma a avaliar os efeitos da exposição ocupacional foi realizado o estudo de biomarcadores de efeito. Os biomarcadores de efeito aplicados foram, especificamente, biomarcadores genotóxicos, através do estudo de micronúcleos (MN) em duas matrizes biológicas distintas: linfócitos de sangue periférico e células esfoliadas da mucosa bucal.

Para a quantificação de MN em linfócitos do sangue periférico foi utilizado o protocolo validado do ensaio de MN por bloqueio da citocinese, desenvolvido por Fenech[23], que utiliza citocalasina-B para bloquear a citocinese para que os linfócitos possuam uma aparência binucleada.

Os linfócitos foram isolados por gradiente utilizando o Ficoll-Paque e colocados, posteriormente, em meio de cultura RPMI 1640 com L-glutamina, fenol vermelho, soro fetal de bovino inativo a 10%, 50 µg/ml estreptomicina + 50U/ml de penicilina e 10 µg/ml de fitohemaglutinina. Foram realizadas culturas em duplicado para cada amostra e incubadas numa estufa a 37° C, humificada com 5% de CO_2 por 44h, sendo posteriormente adicionadas 6 µg/ml citocalasina-B, de forma a inibir a citocinese. Após 28h de incubação, as células foram projetadas, através de citocentrifugação, em lâminas de vidro. As lâminas foram secas ao ar, coradas com a técnica de May-Grünwald Giemsa e montadas com entellan®. As frequências de células binucleadas com MN foram determinadas em 1.000 linfócitos no conjunto das duas lâminas por sujeito da amostra.

Para o teste de MN para as células esfoliadas da mucosa bucal obteve-se a amostra através da raspagem do interior

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18-27 . ISSN: 1646-9704

das duas bochechas com escova *endobrush* e a realização posterior de esfregaço diretamente em duas lâminas. As amostras foram imediatamente fixadas com Mercofix®, um fixador com capacidades de preservação à base de metanol. As lâminas foram coradas com a técnica de Feulgen sem contraste[24], específica para DNA. As lâminas foram secas ao ar e montadas com entellan®. Foram avaliadas no total das duas lâminas 2.000 células que possuíssem o núcleo intacto e que não existisse sobreposição entre citoplasmas e núcleos. O critério de avaliação de anormalidades nucleares em linfócitos e de MN em células esfoliadas da mucosa bucal encontra-se descrito, respectivamente, em Fenech, et al.[25] e Tolbert, et al.[26].

Resultados

1. Avaliação da exposição

Os valores da exposição a FA foram determinados utilizando os métodos anteriormente descritos: PID para obtenção dos valores das concentrações máximas e o NIOSH 2541 para as concentrações médias ponderadas (TWA_{8h}) (*cf.* Tabelas 1 e 2).

Todos os valores obtidos se situam abaixo do valor limite para as concentrações médias ponderadas (TLV-TWA), estabelecido pela OSHA (0,75 ppm). Em alguns laboratórios obtiveram-se valores muito baixos, não tendo sido possível determiná-los por serem inferiores ao limite de deteção do método aplicado[19].

Em oposição, para as concentrações máximas, todos os resultados obtidos para cada grupo de exposição dos 2 contextos ocupacionais excederam o valor limite (0,3 ppm). Nos laboratórios foram obtidos valores entre 0,18 ppm e 5,02 ppm, com um valor médio de 2,52 ppm. Na fábrica, os valores registados situaram-se entre 0,0 ppm e 1,02 ppm. Igualmente, as 3 atividades estudadas na fábrica obtiveram resultados superiores ao valor limite.

Na produção de resinas, o valor de concentração mais elevado foi obtido durante a colheita de amostras no reator das resinas. Neste caso e na operação da máquina de impregnação não existem disponíveis dispositivos de exaustão localizada. Apenas na tarefa do grupo de exposição "controlo de qualidade" existia uma pequena *hotte* que não é usualmente utilizada aquando da análise da qualidade das resinas.

No caso dos laboratórios, todos eles apresentaram, pelo menos, uma tarefa com resultados superiores ao valor limite para as concentrações máximas (0,3 ppm) (*cf.* Figura 1). Considerando as 83 tarefas estudadas nos laboratórios (*cf.* Tabela 1), 93% dos resultados foram superiores ao valor limite.

O maior valor de exposição foi observado durante a tarefa "exame macroscópico" de material biológico fixado em FA. Esta tarefa é desenvolvida numa bancada de macroscopia com exaustão local e, em todos os laboratórios estudados, se verificou o seu normal funcionamento.

A tarefa "registo de dados" apresentou igualmente valores elevados de concentração de FA, sendo importante referir que esta etapa ocorre durante o exame macroscópico (*cf.* Tabela 3).

Através da avaliação de 69 exames macroscópicos, tarefa mais frequentemente desenvolvida nestes laboratórios, constatou-se que cerca de 93% dos valores de concentração de FA foram superiores, mais uma vez, ao valor limite.

Neste contexto ocupacional, o valor de concentração máxima mais elevado foi identificado no grupo de exposição "patologistas" e o valor médio mais elevado foi obtido no grupo de exposição "técnicos de AP" (*cf.* Tabela 4).

É importante considerar que não foi observada a utilização de equipamento de proteção individual (máscara de proteção respiratória) nos dois contextos ocupacionais considerados.

2. Biomonitorização humana

A Tabela 5 evidencia que a média de MN nos trabalhadores expostos ocupacionalmente a FA é maior comparativamente ao grupo controlo, quer em linfócitos de sangue periférico ($p < 0,001$) quer em células esfoliadas da mucosa bucal ($p<0,001$).

Quando analisado cada contexto ocupacional em separado, verificam-se diferenças estatisticamente significativas

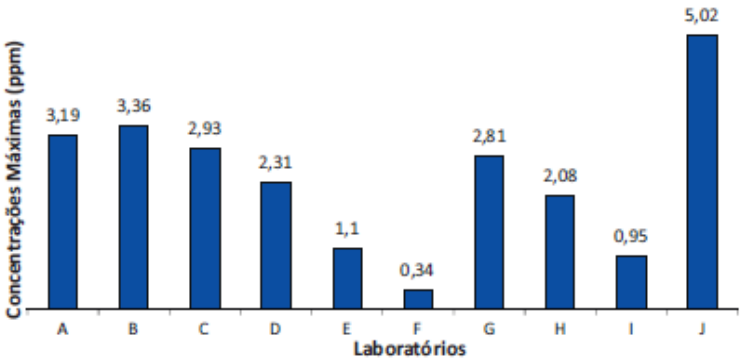


Figura 1: Valor de concentração máxima obtida nos laboratórios de anatomia patológica.

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18 -27 . ISSN: 1646-9704

Tabela 1: Resultados da exposição a FA nos laboratórios de anatomia patológica estudados (MP – Média Ponderada, CM – Concentração Máxima)

Laboratórios	Grupos de exposição	FA (MP) n=29 (ppm)	FA CM) n=83 (ppm)	Intervalo dos valores de CM (ppm)	Média dos valores de CM (ppm)
A	Auxiliar de Ação Médica	0,27	2,51	1,05 – 2,51	1,78
	Patologistas	<LOD	3,19	0,34 – 3,19	1,04
	Técnicos de AP	0,16	NM	--------	
B	Auxiliar de Ação Médica	0,15	0,62	0,62	------
	Patologistas	0,24	2,71	1,49 – 3,36	2,23
	Técnicos de AP	0,16	3,36	1,91 – 3,36	2,31
C	Auxiliar de Ação Médica	0,12	0,53	0,53	------
	Patologistas	0,47	2,93	1,53 – 2,93	2,18
	Técnicos de AP	0,51	2,28	2,22 – 2,28	2,25
D	Auxiliar de Ação Médica	< LOD	NM	------	
	Patologistas	0,07	2,31	2,09 – 2,31	2,21
	Técnicos de AP	0,11	0,85	0,85	------
E	Auxiliar de Ação Médica	< LOD	NM	------	------
	Patologistas	0,06	1,10	0,95 – 1,10	1,03
	Técnicos de AP	0,07	0,85	0,85	------
F	Auxiliar de Ação Médica	0,09	NM	------	------
	Patologistas	0,23	0,34	0,22 – 0,34	0,28
	Técnicos de AP	0,12	0,28	0,28	------
G	Auxiliar de Ação Médica	0,16	0,71	0,64 – 1,71	0,67
	Patologistas	0,05	2,81	0,18 – 2,81	0,56
	Técnicos de AP	0,04	1,26	1,26	------
H	Auxiliar de Ação Médica	0,25	0,68	0,68	------
	Patologistas	0,11	2,08	1,21 – 2,08	1,65
	Técnicos de AP	0,25	0,68	0,68	------
I	Auxiliar de Ação Médica	0,05	0,95	0,95	------
	Patologistas	< LOD	0,47	0,21 – 0,47	0,34
	Técnicos de AP	0,06	NM	------	------
J	Auxiliar de Ação Médica	NM	NM	------	------
	Patologistas	0,13	5,02	1,15 – 5,02	3,24
	Técnicos de AP	0,08	4,32	4,32	------

* Valores mais elevados para cada grupo de exposição
< LOD – Abaixo do limite de detecção
NM – Não Mensurável

Tabela 2: Resultados da exposição a FA na fábrica (MP – Média Ponderada, CM – Concentração Máxima)

Grupos de exposição	FA (MP) n=3 (ppm)	FA (CM) n=3 (ppm)	Intervalo dos valores de CM (ppm)	Média dos valores de CM (ppm)
Produção de resinas	NM	Recolha de amostra do reator 1,02	0,01 – 1,02	0,15
Impregnação	< LOD	Operação na máquina de impregnação 1,04	0,00 – 1,04	0,21
Controlo de qualidade	< LOD	Análise de uma amostra de resina 0,52	0,01 – 0,52	0,08

< LOD – Abaixo do limite de deteção
NM – Não Mensurável

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18-27 . ISSN: 1646-9704

Tabela 3: Exposição a FA nas atividades desenvolvidas nos laboratórios (CM – Concentração Máxima)

Tarefas	Número	Valores de CM (ppm)	Trabalhadores expostos
Exame macroscópico	69	5,02	Patologista
Descarte do material biológico e de FA usado	5	0,95	Técnicos de AP e Auxiliares de Ação Médica
Mudança de reagentes	2	2,51	Auxiliares de Ação Médica
Registo de dados	3	4,32	Técnicos de AP
Lavagem de material biológico	2	2,28	Técnicos de AP
Biópsias	2	1,91	Técnicos de AP

Tabela 4: Resultados de CM para cada grupo de exposição (CM – Concentração Máxima)

Grupos de exposição	Atividades estudadas	Intervalo (ppm)	Média (ppm)	Desvio-padrão (ppm)
Auxiliar de Ação Médica	9	0,28 – 2,51	0,86	0,58
Patologista	65	0,21 – 5,02	1,42	1,07
Técnicos de AP	14	0,68 – 4,32	2,04	0,95

* Algumas atividades envolvem exposição simultânea dos dois grupos

Tabela 5: Média de MN no grupo exposto – fábrica e laboratórios de anatomia patológica e no grupo controlo

	Controlos	Expostos		
		Fábrica	Laboratório de Anatomia Patológica	Total
MN linfócitos Média ± Desvio-padrão	1,17±1,95	1,76±2,07	3,70±3,86	2,97±3,42
MN células epiteliais da boca Média ± Desvio-padrão	0,13±0,48	1,27±1,55	0,64±1,74	0,88±1,69

entre as médias de MN em linfócitos (p <0,001) e células esfoliadas da mucosa bucal (p <0,005) entre o grupo de expostos pertencentes ao laboratório e o grupo de controlo. No que concerne ao grupo da fábrica, foram detetadas diferenças significativas nas frequências de MN apenas nas células esfoliadas da mucosa bucal (p <0,001).

Finalmente, a comparação das frequências de MN entre os dois grupos de exposição (fábrica e laboratórios) permitiu verificar que a frequência de MN em linfócitos foi significativamente maior nos laboratórios (p <0,005); no entanto, no que diz respeito às células esfoliadas da mucosa bucal não se registaram diferenças significativas (p=0,108).

Discussão

A avaliação da exposição nos dois contextos ocupacionais estudados permitiu observar exposição a concentrações elevadas de FA durante curtos períodos de tempo, confirmando estudos previamente publicados[15,27-28]. A importância deste resultado reside no reforço da evidência de que os efeitos para a saúde decorrentes da exposição a FA parecerem estar relacionados essencialmente com a exposição a concentrações elevadas durante curtos períodos de tempo[14,29]. Exposições com estas características são de especial interesse, uma vez que podem produzir doses de exposição elevadas nos órgãos e tecidos-alvo, alterando potencialmente o seu metabolismo, sobrecarregando mecanismos de reparação e proteção e amplificando as respostas tecidulares[9-10]. Pyatt, et al.[29] sugerem mesmo que a maioria dos estudos epidemiológicos anteriores estariam seriamente limitados por falta de dados acerca da exposição a concentrações máximas. Nesses estudos, com efeito, os efeitos para a saúde estavam associados exclusivamente a dados da exposição média ponderada (TWA_{8h})[29]. Até 2004, apenas dois estudos associaram os efeitos para a saúde com resultados da exposição das concentrações máximas, obtendo valores de risco relativo superiores em comparação com os estudos anteriores[16,30-31].

Recentemente, um estudo desenvolvido em embalsamadores reportou que a taxa de mortalidade por leucemia aumentava significativamente, não apenas com os anos de atividade, mas também com a exposição a concentrações elevadas durante curtos períodos de tempo[18,32].

Os resultados nos laboratórios demonstraram que o "exame macroscópico" é a tarefa que envolve maior exposição a FA. Este facto deve-se, provavelmente, à elevada precisão e boa visualização necessária para a consecução

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18 -27 . ISSN: 1646-9704

deste exame, levando a que os patologistas necessitem de grande proximidade ao material biológico fixado com FA promovendo, assim, uma maior exposição[15,28]. Apesar de ser o grupo de exposição "patologista" quem normalmente realiza esta tarefa, o grupo "técnicos de AP" obteve simultaneamente a exposição mais elevada às TWA_{8h} e a maior média dos valores de concentração máxima. Este resultado pode ser justificado pelo facto de este grupo estar envolvido num maior número de atividades que implicam a manipulação de FA durante o turno de trabalho.

No caso da fábrica, a tarefa "recolher amostra do reator" resultou numa exposição elevada, devido provavelmente à proximidade ao reator e à sua abertura. É importante referir que este tipo de informação – determinantes da exposição, fontes de emissão e trabalhadores expostos – apenas foi possível obter através da filmagem realizada em simultâneo com a medição das concentrações (método 1, *cf.* Figura 2), a qual possibilita o relacionamento direto da exposição com a atividade do trabalhador[33-35]. Adicionalmente, as medições em tempo real possibilitam a avaliação das medidas de controlo da exposição e a sua eficácia[36].

Em concordância com outros estudos[37-39], foi possível concluir que o estudo da exposição a concentrações médias ponderadas fornece escassa informação e de menor utilidade na identificação das atividades que devem ser alvo prioritário de medidas de eliminação e/ou controlo da exposição.

Exposições de longa duração a FA, como as que caracterizam habitualmente os contextos ocupacionais, são suspeitas de estarem associadas a efeitos genotóxicos, passíveis de mensuração por biomarcadores[14,40-41]. Neste estudo, os resultados demonstraram que os trabalhadores dos laboratórios de AP e da fábrica estão expostos a valores de FA que excedem os valores de referência e foi igualmente observada uma associação estatisticamente significativa entre a exposição a FA e os biomarcadores de genotoxicidade utilizados.

O dano cromossómico nos linfócitos pode ser justificado pelo facto de o FA ultrapassar o local de contacto direto, como a cavidade bucal, originando alterações nucleares nos linfócitos[16,28,42-43]. Os resultados apresentados nesta investigação corroboram estudos anteriores[42] que reportam que os linfócitos podem ser comprometidos por exposições de longa duração, indicando que os efeitos citogenéticos provocados pelo FA se devem ao alcance de tecidos para além do local inicial de contacto[43].

As exposições de longa duração a elevadas concentrações de FA possuem um elevado potencial de dano generalizado do DNA. Estudos experimentais em animais demonstram efeitos genotóxicos locais após exposição a FA, originando ligações cruzadas DNA-proteína, aberrações cromossómicas estruturais e células aberrantes[4].

Neste estudo verificou-se que a frequência de MN em linfócitos de sangue periférico foi significantemente maior no grupo dos laboratórios em comparação com o grupo da fábrica, possivelmente devido ao maior número de anos de atividade e, consequentemente, de exposição, no primeiro grupo.

Em humanos, a exposição a FA está associada ao aumento da frequência de MN nas células do epitélio bucal[45-47], como corroborado nos resultados apresentados nesta investigação.

Suruda, et al.[44] reportam que, apesar de a presença de alterações nas células epiteliais bucais, nasais e sangue não indicar um mecanismo direto conducente a carcinogénese, indica pelo menos ocorrência de alterações no DNA. Parece bastante razoável concluir que o FA é um fator de risco para os trabalhadores expostos nos dois contextos ocupacionais estudados[14].

Considerações finais

Os resultados obtidos neste estudo confirmam a evidência de associação entre a exposição ocupacional a FA em trabalhadores nos laboratórios de anatomia patológica e a presença de alterações nucleares. No que concerne ao grupo da fábrica, diferenças significativas nas frequências de MN foram detetadas apenas nas células esfoliadas da mucosa bucal, o que se poderá dever, entre outros fatores,

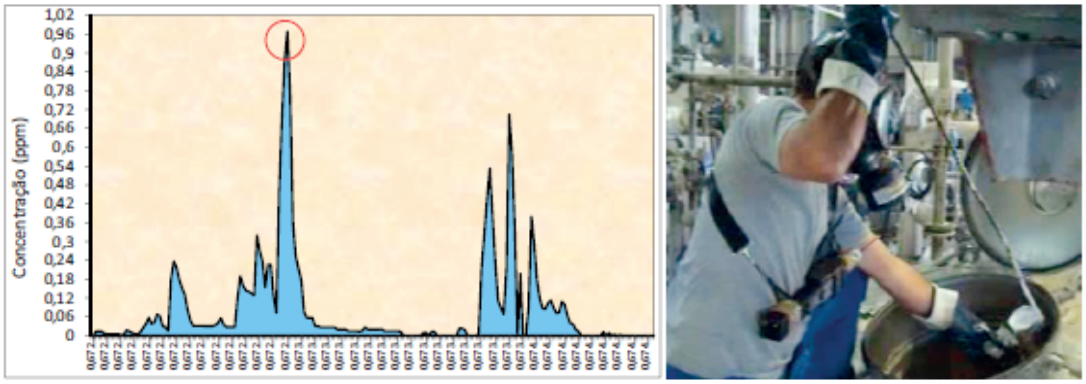


Figura 2: Informação disponibilizada através de PID e recolha de imagem em simultâneo.

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18-27 . ISSN: 1646-9704

Tabela 6: Média de MN nos grupos de exposição dos laboratórios de anatomia patológica

	Laboratórios de anatomia patológica		
	Patologista	Técnico de AP	Auxiliar de Ação Médica
MN linfócitos Média ± Desvio-padrão	5,00±1,24	3,76±0,647	4,13±1,55
MN células epiteliais da boca Média ± Desvio-padrão	0,58±0,434	1,18±0,406	0,88±0,611

Tabela 7: Média de MN nos grupos de exposição da fábrica

	Fábrica		
	Produção de resinas	Impregnação	Controlo de qualidade
MN linfócitos Média ± Desvio-padrão	1,85±2,48	1,16±1,04	4,5±0,7
MN células epiteliais da boca Média ± Desvio-padrão	0,66±0,94	1,75±1,79	3,5±0,5

ao facto de terem ainda pouco tempo de exposição a FA por se tratar de uma unidade de produção recente.

Nos laboratórios de AP, a redução da exposição a FA pode ser conseguida com a utilização adequada dos dispositivos de exaustão localizada e colocação dos recipientes que contêm o material biológico em áreas com ventilação adequada. Na fábrica, as medidas de prevenção devem considerar a automatização de alguns processos, como a recolha de amostras do reator e, adicionalmente, a promoção do uso do dispositivo de ventilação localizada existente no laboratório de controlo de qualidade.

Os métodos de avaliação da exposição aqui aplicados permitem concluir que um método que faculte dados acerca das concentrações máximas de formaldeído presentes no ambiente de trabalho será mais adequado por disponibilizar informações mais detalhadas e pertinentes para a ação preventiva.

O ensaio dos MN por bloqueio da citocinese é uma técnica simples, prática, pouco dispendiosa e relativamente não invasiva que pode ser utilizada para a vigilância da saúde de trabalhadores em contextos ocupacionais que envolvam risco carcinogénico, nomeadamente a exposição a agentes genotóxicos, como o FA.

Referências Bibliográficas

1. Portier CJ, Bell DA. Genetic susceptibility: significance in risk assessment. Toxicol Lett. 1998;102-103:185-9.
2. Manno M, Viau C, Cocker J, Colosio C, Lowry L, Mutti A, et al. Biomonitoring for occupational health risk assessment (BOHRA). Toxicol Lett. 2010;192(1):3-16.
3. Ishikawa H, Tian Y, Yamauchi T. Influence of gender, age and lifestyle factor son micronuclei frequency in healthy Japanese populations. J Occup Health. 2003;45(3):179-81.
4. Battershill JM, Burnett K, Bull S. Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. Mutagenesis. 2008;23(6):423-37.
5. Herber RF, Duffus JH, Christensen JM, Olsen E, Mark MV. Risk assessment for occupational exposure to chemicals: a review of current methodology. Pure Appl Chem. 2001;73(6):993-1031.
6. U. S. Environmental Protection Agency. Guidelines for exposure assessment. Washington, DC: US EPA; 1992. ISBN 989-8076-02-1.
7. Stewart P, Stenzel M. Exposure assessment in the occupational setting. Appl Occup Environ Hyg. 2000;15(5):435-44.
8. Ritter L, Solomon K, Sibley P, Hall K, Keen P, Mattu G, et al. Sources, pathways, and relative risks of contaminants in surface water and groundwater: a perspective prepared for the Walkerton inquiry. J Toxicol Environ Health A. 2002;65(1):1-142.
9. Smith T. Studying peak exposure: toxicology and exposure statistics. In: Marklund S, editor. Exposure assessment in epidemiology and practice. Stockholm: National Institute for Working Life; 2001. p. 207-9.
10. Preller L, Burstyn I, De Pater N, Kromhout H. Characteristics of peaks of inhalation exposure to organic solvents. Ann Occup Hyg. 2004;48(7):643-52.
11. Checkoway H, Rice CH. Time-weighted averages, peaks, and other indices of exposure in occupational epidemiology. Am J Ind Med. 1992; 21(1):25-33.
12. National Toxicology Program. Formaldehyde (Gas) – CAS no. 50-00-0: substance profile, report on carcinogens [Internet]. 11th ed. Washington, DC: National Institutes of Health; 2005. Available from: http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s089form.pdf
13. Nazaroff WW. Indoor air chemistry: cleaning agents, ozone and toxic air contaminants. Berkeley, CA: California Air Resources Board, California Environmental Protection Agency; 2006.
14. International Agency for Research on Cancer. Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol. Lyon: IARC; 2006. ISBN 92-832-1288-6.

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18 -27 . ISSN: 1646-9704

15. Goyer N, Beaudry C, Bégin D, Bouchard M, Buissonnet S, Carrier G, et al. Impacts d'un abaissement de la valeur d'exposition admissible au formaldéhyde: industries de fabrication de formaldéhyde et de résines à base de formaldéhyde. Montréal: Institut de Recherche Robert-Sauvé en Santé et en Sécurité du Travail; 2004.
16. Zhang L, Steinmaus C, Eastmond DA, Xin XK, Smith MT. Formaldehyde exposure and leukaemia: a new meta-analysis and potential mechanisms. Mutat Res. 2009;681(2-3):150-68.
17. Baan R, Grosse Y, Straif K, Secretan B, El Ghissassi F, Bouvard V, et al. A review of human carcinogens – Part F: chemical agents and related occupations. Lancet Oncol. 2009;10(12):1143-4.
18. Hauptmann M, Stewart PA, Lubin JH, Freeman LE, Hornung RW, Herrick RF, et al. Mortality from lymphohematopoietic malignancies and brain cancer among embalmers exposed to formaldehyde. J Natl Cancer Inst. 2009;101(24):1696-708.
19. NIOSH. Applications manual for the revised NIOSH lifting equation. Cincinnati, OH: National Institute for Occupational Safety and Health; 1994.
20. Langhorst ML. Photoionization detector sensitivity of organic compounds. J Cromatogr Sci. 1981;19(2): 98-103.
21. Instituto Português da Qualidade. NP 1796:2007 – Segurança e saúde do trabalho: valores limite de exposição profissional a agentes químicos existentes no ar dos locais de trabalho. Caparica: IPQ; 2007.
22. Brown RH. The sampling of gases and vapours: principles and methods. In Gardiner K, Harrington JM, editors. Occupational hygiene. 3rd ed. Massachussetts: Blackwell Publishing; 2005. p. 208-21.
23. Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2:1084-104.
24. Nersesyan A, Kundi M, Atefie K, Schulte-Hermann R, Knasmüller S. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. Cancer Epidemiol Biomarkers Prev. 2006;15(10):1835-40.
25. Fenech M, Holland N, Chang W, Zeiger E, Bonassi S. The HUman MicroNucleus Project: An international collaborative study on the use of micronucleus technique for measuring DNA damage in humans. Mutat Res. 1999;428(1-2):271-83.
26. Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear abnormalities in buccal smears: a field test in snuff users. Am J Epidemiol. 1991;134(8):840-50.
27. Shaham J, Bomstein Y, Gurvich R, Rashkovsky M, Kaufman Z. DNA–protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde. Occup Environ Med. 2003;60(6):403-9.
28. Orsière T, Sari-Minodier I, Iarmarcovai G, Botta A. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. Mutat Res. 2006;605(1-2):30-41.
29. Pyatt D, Natelson E, Golden R. Is inhalation exposure to formaldehyde a biological plausible cause of lymphohematopoietic malignancies? Regul Toxicol Pharmacol. 2008;51(1):119-33.
30. Hauptmann M, Lubin JH, Stewart PA, Hayes RB, Blair A. Mortality from solid cancers among workers in formaldehyde industries. Am J Epidemiol. 2004;159(12):1117-30.
31. Pinkerton LE, Hein MJ, Stayner LT. Mortality among a cohort of garment workers exposed to formaldehyde: an update. Occup Environ Med. 2004;61(3):193-200.
32. Dreyfuss JH. Occupational formaldehyde exposure linked to increased risk of myeloid leukemia and death. CA Cancer J Clin. 2010;60(3):135-6.
33. Ryan TJ, Burroughs GE, Taylor K, Kovein, RJ. Video exposure assessments demonstrate excessive laboratory formaldehyde exposures. Appl Occup Environ Hyg. 2003;18(6):450-7.
34. Rosén G, Andersson IM, Walsh PT, Clark RD, Säämänen A, Heinonen K, et al. A review of video exposure monitoring as an occupational hygiene tool. Ann Occup Hyg. 2005;49(3):201-17.
35. Mcglothlin JD. Occupational exposure assessment and control using video exposure monitoring in the pharmaceutical industry. In International Scientific Conference (IOHA 2005), 6, 19-23 September 2005, Pilanesberg National Park North West Province, South Africa. Pilanesberg: IOHA – International Occupational Hygiene Association; SAIOH – Southern African Institute for Occupational Hygiene; MVS – Mine Ventilation Society of South Africa; 2005.
36. Yokel RA, MacPhail RC. Engineered nanomaterials: exposures, hazards, and risk prevention. J Occup Med Toxicol. 2011;6:7.
37. Susi P, Schneider S. Database needs for a task-based exposure assessment model for construction. Appl Occup Environ Hyg. 1995;10(4):394-9.
38. Kromhout H. Design of measurement strategies for workplace exposures. Occup Environ Med. 2002;59(5):349-54.
39. Meijster T, Tielemans E, Schinkel J, Heederik D. Evaluation of peak exposures in the Dutch flour processing industry: implications for intervention strategies. Ann Occup Hyg. 2008;52(7):587-96.
40. Viegas S, Prista J. Cancro nasofaríngeo e exposição a formaldeído: avaliação da história profissional em 63 casos registados. Soc Port Med Trabalho. 2007;(6):13-22.
41. Conaway CC, Whysner J, Verna LK, Williams GM. Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. Pharmacol Ther. 1996;71(1-2):29-55.
42. Ye X, Yan W, Xie H, Zhao M, Ying C. Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutat Res. 2005;588(1):22-7.
43. He JL, Jin LF, Jin HY. Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. Biomed Environ Sci. 1998;11(1):87-92.
44. Suruda A, Schulte P, Boeniger M, Hayes RB, Livingston GK, Steenland K, et al. Cytogenetic effects of formaldehyde

SAÚDE & TECNOLOGIA . MAIO | 2012 | #7 | P. 18-27 . ISSN: 1646-9704

exposure in students of mortuary science. Cancer Epidemiol Biomarkers Prev. 1993:2(5):453-60.
45. Speit G, Schmid O, Fröhler-Keller M, Lang I, Triebig G. Assessment of local genotoxic effects of formaldehyde in humans measured by de micronucleus test with exfoliated buccal mucosa cell. Mutat Res. 2007;627(2):129-35.
46. Speit G, Schmid O. Local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated epithelial cells. Mutat Res. 2006;613(1):1-9.
47. Burgaz S, Erdem O, Çakmak G, Erdem N, Karakaya A, Karakaya AE. Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. Biomarkers. 2002;7(2):151-61.

Artigo recebido em 18.10.2011 e aprovado em 31.01.2012.

Environmental and Molecular Mutagenesis 54:213–221 (2013)

Research Article

The Influence of Genetic Polymorphisms in *XRCC3* and *ADH5* GENES on the Frequency of Genotoxicity Biomarkers in Workers Exposed to Formaldehyde

Carina Ladeira,[1,2*] Susana Viegas,[1,2] Elisabete Carolino,[1] Manuel C. Gomes,[3] and Miguel Brito[1]

[1] *Escola Superior de Tecnologia da Saúde de Lisboa – Instituto Politécnico de Lisboa, Portugal*

[2] *Centro de Investigação e Estudos em Saúde Pública – Universidade Nova de Lisboa, Portugal*

[3] *Faculty of Sciences – University of Lisbon, Portugal*

The International Agency for Research on Cancer classified formaldehyde as carcinogenic to humans because there is ''sufficient epidemiological evidence that it causes nasopharyngeal cancer in humans''. Genes involved in DNA repair and maintenance of genome integrity are critically involved in protecting against mutations that lead to cancer and/or inherited genetic disease. Association studies have recently provided evidence for a link between DNA repair polymorphisms and micronucleus (MN) induction. We used the cytokinesis-block micronucleus (CBMN assay) in peripheral lymphocytes and MN test in buccal cells to investigate the effects of *XRCC3* Thr241Met, *ADH5* Val309Ile, and Asp353Glu polymorphisms on the frequency of genotoxicity biomarkers in individuals occupationally exposed to formaldehyde ($n = 54$) and unexposed workers ($n = 82$). *XRCC3* participates in DNA double-strand break/recombination repair, while *ADH5* is an important component of cellular metabolism for the elimination of formaldehyde. Exposed workers had significantly higher frequencies ($P < 0.01$) than controls for all genotoxicity biomarkers evaluated in this study. Moreover, there were significant associations between *XRCC3* genotypes and nuclear buds, namely *XRCC3* Met/Met (OR = 3.975, CI 1.053–14.998, $P = 0.042$) and *XRCC3* Thr/Met (OR = 5.632, CI 1.673–18.961, $P = 0.005$) in comparison with *XRCC3* Thr/Thr. *ADH5* polymorphisms did not show significant effects. This study highlights the importance of integrating genotoxicity biomarkers and genetic polymorphisms in human biomonitoring studies. Environ. Mol. Mutagen. 54:213–221, 2013. © 2013 Wiley Periodicals, Inc.

Key words: genetic susceptibility; genotoxicity biomarkers; occupational exposure

INTRODUCTION

In June 2004, the International Agency for Research on Cancer (IARC) classified formaldehyde as carcinogenic to humans (Group 1) because there is ''sufficient epidemiological evidence that formaldehyde causes nasopharyngeal cancer in humans'' and also concluded that there was ''strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to formaldehyde'' [IARC, 2006; Zhang et al., 2009].

Epidemiological studies have provided strong evidence for a causal relationship between exposure to formaldehyde and cancer in humans. Causality is indicated by consistent findings of increased risks of nasopharyngeal cancer, sinonasal cancer, and lymphohematopoietic cancer, specifically myeloid leukemia [Zhang et al., 2010a], among individuals with higher measures of exposure to formaldehyde (exposure level or duration), which cannot be explained by chance, bias, or confounding alone [National Toxicology Program, 2011]. However, some studies led to mixed results and inconclusive evidence [Franks, 2005] prompting a re-evaluation of former studies that had suggested a causal association between form-

*Correspondence to: Carina Ladeira, Av. D. João II, lote 4.69.01, 1990-096 Lisboa. E-mail: carina.ladeira@estesl.ipl.pt

Received 13 July 2012; provisionally accepted 8 November 2012; and in final form 14 November 2012

DOI 10.1002/em.21755

Published online 28 January 2013 in Wiley Online Library (wileyonlinelibrary.com).

Environmental and Molecular Mutagenesis. DOI 10.1002/em

aldehyde exposure and mortality from leukemia, myeloid leukemia and nasopharyngeal cancer [Marsh and Youk, 2004; Marsh et al., 2010; Rhomberg et al., 2011].

In spite of some controversy, studies in humans have demonstrated that inhaled formaldehyde can cause genotoxicity in lymphocytes, including DNA-protein cross-links, DNA strand breaks, micronucleus formation, and chromosomal aberrations [National Toxicology Program, 2011]. The cytokinesis-block micronucleus (CBMN) assay is frequently used in biomonitoring studies and can be considered as a ''cytome'' assay covering chromosomal changes, such as micronuclei (MN), nucleoplasmic bridges, and nuclear buds. The detection of MN is extensively used in molecular epidemiology as a biomarker of chromosomal damage, genome instability, and increased cancer risk. The occurrence of MN represents an integrated response to chromosome-instability and altered cellular viabilities caused by genetic defects and/or exogenous exposures to genotoxic agents [Hedberg, 2001]. MN contain either acentric chromosomal fragments formed by unrepaired double-strand breaks, or lagged chromosomes that have failed to segregate into a daughter macronucleus during mitosis [Fenech et al., 1999; Fenech, 2002; Mateuca et al., 2006; Iarmarcovai et al., 2006, 2008]. Nucleoplasmic bridges (NBP) are biomarkers of dicentric chromosomes resulting in telomere end-fusions or DNA misrepair [Fenech et al., 2002; Thomas et al, 2003; Fenech, 2005, 2006]. These events occur when centromeres of dicentric chromosomes are pulled to opposite poles of the spindle during anaphase. Nuclear buds (NBUD) are characterized by the same morphology as MN, except that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmatic material depending on the stage of the nuclear budding process. They are considered as biomarkers of the elimination of amplified DNA and/or DNA repair complexes [Tolbert et al., 1991; Fenech et al., 2002; Thomas et al., 2003; Fenech, 2006]. Our previous studies reported evidence that long-term exposures to formaldehyde and high peak formaldehyde concentrations are associated with an increase in the frequency of MN in lymphocytes and exfoliated buccal mucosa cells in workers at anatomy and pathology laboratories and at formaldehyde-resins production factories [Viegas et al., 2010] and with an increase in MN, NPB and NBUD in workers at histopathology laboratories [Ladeira et al., 2011].

Recently, association studies have linked genotypes, which account for interindividual differences in the response to genotoxic exposure, to the occurrence of MN as a measure of genetic damage due to environmental exposures [Dhillon et al., 2011]. Polymorphisms in various genes involved in DNA repair, activation/deactivation of carcinogens/chemicals/drugs/alcohol, folate metabolism pathway, and micronutrient transport have all been shown to affect MN formation [Dhillon et al., 2011]. Genes involved in DNA repair and maintenance of genome integrity are critically involved in protecting against DNA lesions that lead to cancer and/or inherited genetic disease [Matullo et al., 2001]. Single-nucleotide polymorphisms (SNPs) in these genes are recognized as potential cancer susceptibility factors [Figueiredo et al., 2004]. Molecular epidemiology studies have shown that the inheritance of certain genetic variants at one or more loci results in a reduced DNA repair capacity and an increase in the individual risk of cancer [Winsey et al., 2000; Matullo et al., 2001].

The X-ray repair cross-complementing gene 3 (*XRCC3*) participates in DNA double-strand break/recombination repair and is a member of an emerging family of Rad-51-related proteins that participate in the homologous recombination (HR) pathway to maintain chromosome stability, repair DNA damage, and correct chromosome segregation in mammalian cells [Bolognesi et al., 1999; Catalán et al., 2000; Matullo et al., 2001; Bonassi et al., 2003; El-Zein et al., 2006; Kirsch-Volders et al., 2006; Iarmarcovai et al., 2006; Battershill et al., 2008; Mateuca et al., 2008]. This pathway is of great importance in preventing chromosomal fragmentation, translocations, and deletions, which can lead to carcinogenesis [Winsey et al., 2000]. The Rad 51 paralogue XRCC3 promotes the HR repair of double strand breaks induced either directly or indirectly following replication of closely spaced single strand breaks [Mateuca et al., 2008]. XRCC3 is required for the assembly and stabilization of Rad51 [Winsey et al., 2000; Shen et al., 2002]. In addition to repairing double strand-breaks, XRCC3 also plays a role in the repair of more global DNA damage arising from carcinogen treatment [Araujo et al., 2002].

XRCC3 is located on chromosome 14 (14q32.3) and its most studied polymorphism is a transition between cytosine and thymine in exon 7 (*XRCC3*-18067C> T) at codon 241 that results in the substitution of a threonine by a methionine [Bonassi et al., 2003; Wang et al, 2003; Battershill et al., 2008; El-Zein et al., 2008]. The XRCC3 Thr241Met variation does not reside in the adenosine triphosphate-binding domain, the only functional domain identified in the protein [Manuguerra et al., 2006]; however, conversion from a hydroxyl amino acid to one with a sulfhydryl group represents a substantial change in protein functional characteristics [Winsey et al., 2000]. This polymorphism has been proposed as an allele of low penetrance associated with breast and lung cancer, acute myeloid leukemia, risk of upper aerodigestive tract cancer [Stich and Rosin, 1983; Ramirez and Saldanha, 2002; El-Zein et al., 2008] and risk for melanoma skin cancer and bladder carcinoma [Wang et al., 2003]. Mammalian alcohol dehydrogenases (ADH; EC 1.1.1.1.) are zinc-containing dimeric enzymes that catalyze the reversible oxidation of a wide variety of alcohols, using NAD^+ as the preferred coenzyme. They form a gene family divided into at least five distinct classes with about 60% amino acids in common as identified in interclass comparisons [Hur et al., 1992]. Alcohol dehydrogenase 5 (ADH5), originally known as formaldehyde dehydrogenase (FDH), differs in cat-

Environmental and Molecular Mutagenesis. DOI 10.1002/em

alytic profile from all other alcohol dehydrogenases because it appears to have no ethanol oxidation activity and its best known substrate is S-nitrosoglutathione [Wu et al., 2007]. ADH5 is composed of nine exons and eight introns [Hur et al., 1992] and is located on chromosome 4 (4q23) [Just et al., 2001] and has been detected in all human tissues and at all stages of development. This is the only ADH identified thus far that is capable of oxidizing formaldehyde in a glutathione dependent reaction [Kaiser et al., 1991; Engeland et al., 1993; Lee et al., 2003]. ADH5 is an important component of cellular metabolism for the elimination of formaldehyde serving as the prime guardian against formaldehyde [Hedberg, 2001] and offering enzymatic defence against both formaldehyde and nitrosative stress in human oral tissue and in epithelial cell lines. Although formaldehyde is rapidly metabolized, it is an electrophile that reacts with a variety of endogenous molecules, including glutathione, proteins, nuclei acids, and folic acid [National Toxicology Program, 2011].

Two ADH5 polymorphisms are known: *ADH5* Val309Ile, a transition of a cytosine to a thiamine in codon 309 that consists in the substitution of a valine by an isoleucine; and *ADH5* Asp353Glu, a transversion of an adenine to a cytosine in codon 353 that results in the substitution of an aspargine by a glutamine. To our knowledge, no association has been found between ADH5 polymorphisms and disease [Wang et al., 2010].

The CBMN assay was extensively used over the past decade in molecular epidemiology studies [El-Zein et al., 2006; Fenech, 2006; Battershill et al., 2008]. It was based upon this technique that Dhillon et al. [2011] suggested that the genotype might influence the frequency of MN in lymphocytes and that NPB and NUBD measurements should be investigated with regard to the impact of genotype on these biomarkers. In previous reports, we have provided evidence for an association between exposure to formaldehyde and genotoxicity biomarkers [Viegas et al., 2010; Ladeira et al., 2011]. In this study we focus upon the association between genotoxicity biomarkers and genetic polymorphisms in key genes involved in DNA repair and formaldehyde metabolism. The goal of this study is to compare individuals occupationally exposed to formaldehyde and matched controls with regard to the effects of *XRCC3* Thr241Met, *ADH5* Val309Ile and Asp353Glu polymorphisms on the frequency of genotoxicity biomarkers detected by the CBMN assay in peripheral lymphocytes and the MN test in buccal cells.

MATERIALS AND METHODS

Subjects

This study was conducted with a group of 54 workers occupationally exposed to formaldehyde at six histopathology hospital laboratories in Portugal (Lisbon and Tagus Valley region), and a group of 82 administrative staff members with no known exposure to formaldehyde.

Ethical approval was obtained from the Institutional Ethical Board and Service Director of the hospitals, and all subjects gave informed consent to participate. Each person answered a questionnaire aimed at identifying exclusion criteria such as a history of cancer, radio or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the past six months, intake of vitamins or supplements like folic acid, as well as information related to working practices, such as years of employment and the use of protective measures.

Environmental Monitoring of Formaldehyde Exposure

Exposure assessment was based on two techniques of air monitoring conducted simultaneously [Viegas et al., 2010]. First, environmental samples were obtained by air sampling with low flow pumps for 6–8 hr during a typical working day. Formaldehyde levels were measured by gas chromatography analysis and time-weighted average (TWA_8) was estimated according to the National Institute of Occupational Safety and Health method - NIOSH 2541 [NIOSH, 1994].

The second method was aimed at measuring ceiling values of formaldehyde using Photo Ionization Detection (PID) equipment (11.7 eV lamps) with simultaneous video recording [McGlothlin, 2005]. Instantaneous values of FA concentration were obtained on a per second basis. A relationship could thus be established between worker activities and ceiling values and the main sources of exposure could be identified.

Genotoxic Effects Evaluation

Evaluation of genotoxic effects was conducted by applying the CBMN assay in peripheral blood lymphocytes and the MN test in exfoliated cells from the buccal mucosa. Whole blood and exfoliated cells (buccal mucosa cells) were collected from each subject between 10 A.M. and 12 P.M. and were processed for testing. All samples were coded and analyzed under blind conditions. The criteria for scoring the nuclear abnormalities in lymphocytes and MN in the buccal cells were the ones described by Fenech et al. [1999] and Tolbert et al. [1991], respectively.

Cytokinesis-Block Micronucleus Assay

Heparinized blood samples were obtained by venipuncture from all subjects and freshly collected peripheral blood was used for the CBMN assay. Lymphocytes were isolated using a Ficoll-Paque gradient and placed in RPMI 1640 culture medium with L-glutamine and phenol red added with 10% inactivated fetal calf serum, 50 μg/ml streptomycin + 50U/mL penicillin, and 10 μg/mL of phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37°C in a humidified 5% CO_2 incubator for 44 h, and 6 μg/mL cytochalasin B was added to the cultures to prevent cytokinesis. After 28 h incubation, cells were spun onto microscope slides using a cytocentrifuge. Smears were air-dried and double stained with May-Grünwald-Giemsa and mounted with Entellan®. One thousand cells were scored from each individual by two independent observers on two slides. Each observer visualized 500 cells/individual.

Buccal Mucosa Cells

Cells from the buccal mucosa were collected with an endobrush swab. Exfoliated cells were smeared onto slides and fixed with Mercofix®. The Feulgen staining technique without counterstain was used. Two thousand cells were scored from each individual by two independent observers on two slides. Each observer visualized 1,000 cells/individual. Only cells that were neither clumped nor overlapped and contained intact nuclei were included in the analysis.

Polymorphisms Analysis

Whole blood samples were collected and stored at −20°C until total white blood cell DNA was extracted using the standard protocol of phenol-chloroform. The *XRCC3* Thr241Met (rs861539), ADH5 Val309Ile (rs28730628), and *ADH5* Asp353Glu (rs16996593) polymorphisms were

Environmental and Molecular Mutagenesis. DOI 10.1002/em

TABLE I. Comparison of the Two Samples

	Control group	Exposed group	*P*-value
Number of subjects	82	54	
Sex			
Female	53 (64.6%)	35 (64.8%)	0.983
Male	29 (35.4%)	19 (35.2%)	
Age			
(mean ±standard deviation, in years)	32.79±8.03	39.80±11.56	<0.001
Range	20-53	20-61	
Tobacco consumption			
Non-smokers	57 (69.5%)	43 (79.6%)	0.191
Smokers	23 (30.5%)	11 (20.4%)	
Alcohol consumption			
Non-drinkers	18 (22%)	18 (33.3%)	0.141
Drinkers	64 (78%)	36 (66.7%)	

Descriptive statistics and *P*-value of Qui-square test for sex, tobacco and alcohol consumption and *t*-test of independent samples for age.

TABLE II. Frequency of Genotypes and Alleles in the Study Samples (P-Value of Fisher-Exact Test)

Genes	Genotypes	All	Exposed	Controls	*P*-value
XRCC3	Met/Met	33 (24.3%)	13 (24.1%)	20 (24.4%)	0.669
Met 241Thr	Met/Thr	49 (36.0%)	22 (40.7%)	27 (32.9%)	
	Thr/Thr	54 (39.7%)	19 (35.2%)	35 (42.7%)	
	Met	115 (0.423)	48 (0.44)	67 (0.409)	0.621
	Thr	157 (0.577)	60 (0.56)	97 (0.591)	
ADH5	Val/Val	50 (36.8%)	21 (38.9%)	29 (35.4%)	0.719
Val309Ile	Val/Ile	86 (63.2%)	33 (61.1%)	53 (64.6%)	
	Val Ile	186 (0.684)	75 (0,694)	111 (0,677)	0.795
		86 (0.316)	33 (0,306)	53 (0,323)	
ADH5	Asp/Asp	59 (43.4%)	24 (44.4%)	35 (42.7%)	0.863
Asp353Glu	Asp/Glu	77 (56.6%)	30 (55.6%)	47 (57.3%)	
	Asp	195 (0.717)	78 (0,722)	117 (0,713)	
	Glu	77 (0.283)	30 (0,278)	47 (0,287)	0.892

determined using the TaqMan SNP genotyping assay with Real Time PCR (Applied Biosystems).

To perform the genotype analysis of *XRCC3* and *ADH5* polymorphisms the target fragments were amplified in 20 µl reaction mixture containing 10 µl *TaqMan Universal PCR Master Mix*, 1 µl primers, 5 µl Miliq water, and 4 µl DNA. Real Time PCR, *iCycler iQ® Multicolor Real-Time PCR Detection System* (BIO-RAD), was then conducted as follows: 2 min of the initial step at 50°C, 30 sec and 10 min at 95°C, 50 cycles of 15 sec and 1 min at 92°C and 60°C, respectively, and a final temperature stay at 4°C. All inconclusive samples were reanalyzed.

Statistical Analysis

Variables were compared with the Normal distribution using the Shapiro-Wilk test. Rejection of the null hypothesis of underlying normality led us to proceed with nonparametric procedures to compare means and check associations. The association between each of the genotoxicity biomarkers, occupational exposure to formaldehyde, and *XRCC3* and *ADH5* polymorphisms was evaluated by binary logistic regressions. The biomarkers were dichotomized (absent/present) and considered the dependent variable, taking absence as the reference. Occupational exposure, genetic polymorphisms of XRCC3, and ADH5 were considered independent variables.

Each genetic polymorphism has three possible genotypes. Two dummy variables were considered for each polymorphism and the last category was taken as the reference, namely, the Thr/Thr genotype for *XRCC3* Thr241Met; the Val/Ile genotype for *ADH5* Val309Ile; and the Asp/Glu genotype for *ADH5* Asp353Glu.

The nonparametric Mann-Whitney and Kruskal-Wallis tests were also used to compare the groups. Statistical analyses were performed using the SPSS package for Windows, version 19.0. The analysis of genotype and allele frequency and Fisher-exact test was made with the GenPop program.

RESULTS

Population characteristics such as sex, age, tobacco habits, and alcohol consumption for the control and exposed groups are shown in Table I. The frequencies of genotypes and alleles of the studied polymorphisms in the two study populations are shown in Table II. No significant differences were observed in genotype and allele frequencies for the three polymorphisms analysed (Fisher's exact test, $P > 0.05$).

Formaldehyde Exposure

The mean level of formaldehyde exposure of the 54 exposed individuals was 0.16 ppm (min–max: 0.04–0.51 ppm), a value below the Occupational Safety and Health Administration (OSHA) reference of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (min–max: 0.18–2.93 ppm), a value well above the 0.3 ppm reference of the American Conference of Governmental Industrial Hygienists (ACGIH) for ceiling concentrations. The highest formaldehyde concentration was observed during macroscopic examination of biological samples by the exposed workers.

The effect of formaldehyde exposure on the frequencies of genotoxicity biomarkers is shown in Table III. Significant increases ($P < 0.001$ for lymphocytes and $P = 0.006$ for buccal mucosa cells) were found in the exposed workers relative to controls for all the genotoxicity biomarkers examined.

XRCC3 Polymorphisms

Results of binary logistic regression provided evidence for a statistically significant association between *XRCC3* polymorphisms and NBUD. Specifically, *XRCC3* Met/Met (OR = 3.975, $CI_{95\%}$ 1.053–14.998, $P = 0.042$) and *XRCC3* Thr/Met (OR = 5.632, $CI_{95\%}$ 1.673–18.961, $P = 0.005$) are risk factors for NBUD in comparison with *XRCC3* Thr/Thr. As shown in Table IV, lower means of NBUD were found in carriers of Thr/Thr polymorphism for both in exposed and controls. All the other biomarkers showed higher means in exposed workers, however, no increase was statistically significant.

Environmental and Molecular Mutagenesis. DOI 10.1002/em

TABLE III. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Two Samples (Mean ± Mean Standard Error, Range, and P-Value of Mann-Whitney Test)

	Mean, MN lymphocytes ± S.E. (range)	Mean, NPB±S.E. (range)	Mean, NBUD±S.E. (range)	Mean, MN buccal cells ±S.E. (range)
Controls	0.83 ± 0.18 (0–7)	0.18 ± 0.06 (0–3)	0.07 ± 0.03 (0–1)	0.17 ± 0.06 (0–2)
Exposed	4.00 ± 0.52 (0–14)	3.1 ± 0.54 (0–13)	0.79 ± 0.3 (0–5)	1.0 ± 0.267 (0–9)
P-value	<0.001	<0.001	<0.001	0.006

TABLE IV. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by XRCC3 Met241Thr Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	XRCC3	*N*	Mean MN lymphocytes ± S.E.	Mean NPB± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
	Met/Met	13	2.92 ± 0.93 (0–12)	2.00 ± 1.14 (0–15)	0.38 ± 0.18 (0–2)	1.00 ± 0.71 (0–9)
Exposed	Thr/Met	22	5.05 ± 0.98 (0–14)	3.91 ± 0.84 (0–13)	1.50 ± 0.33 (0–2)	1.05 ± 0.38 (0–5)
	Thr/Thr	19	3.53 ± 0.80(0–12)	2.95 ± 0.90(0–13)	0.21 ± 0.12(0–2)	0.95 ± 0.51(0–8)
	P–value		0.372	0.156	0.002	0.733
	Met/Met	20	1.15 ± 0.46 (0–7)	0.25 ± 0.12 (0–2)	0.2 ± 0.09 (0–1)	0.25 ± 0.14 (0–2)
Controls	Thr/Met	27	0.70 ± 0.30 (0–6)	0.15 ± 0.12 (0–3)	0.04 ± 0.04 (0–1)	0.11 ± 0.82 (0–2)
	Thr/Thr	35	0.74 ± 0.23 (0–6)	0.14 ± 0.07 (0–2)	0.03 ± 0.29 (0–1)	0.17 ± 0.10 (0–2)
	p-value		0.621	0.450	0.045	0.664

TABLE V. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by ADH5 Val309Ile Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	ADH5	*N*	Mean MN lymphocytes ± S.E.	Mean NPB± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
Exposed	Val/Val	21	2.57 ± 0.65 (0–11)	3.19 ± 0.89 (0–14)	0.62 ± 0.28 (0–5)	0.95 ± 0.41 (0–6)
	Val/Ile	33	4.91 ± 0.75 (0–14)	3.06 ± 0.69 (0–15)	0.88 ± 0.21 (0–5)	1.03 ± 0.39 (0–9)
Controls	*P*–value		0.024	0.957	0.274	0.713
	Val/Val	29	0.97 ± 0.28 (0–6)	0.17 ± 0.07 (0–1)	0.00 ± 0.00 (0)	0.14 ± 0.10 (0–2)
	Val/Ile	53	0.75 ± 0.23 (0–7)	0.17 ± 0.08 (0–3)	0.11 ± 0.04 (0–1)	0.19 ± 0.08 (0–2)
	P-value		0.176	0.370	0.061	0.546

TABLE VI. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by ADH5 Asp353Glu Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	ADH5	*N*	Mean MN lymphocytes ± S.E.	Mean NPB ± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
Exposed	Asp/Asp	24	4.08 ± 0.91 (0–14)	4.21 ± 0.96 (0–15)	0.71 ± 0.23 (0–3)	0.92 ± 0.37 (0–6)
	Asp/Glu	30	3.93 ± 0.67 (0–12)	2.23 ± 0.57 (0–14)	0.83 ± 0.25 (0–5)	1.07 ± 0.43 (0–9)
	P-value		0.700	0.217	0.740	0.983
Controls	Asp/Asp	35	0.86 ± 0.23 (0–6)	0.29 ± 0.12 (0–3)	0.06 ± 0.04 (0–1)	0.29 ± 0.12 (0–2)
	Asp/Glu	47	0.81 ± 0.26 (0–7)	0.09 ± 0.04 (0–1)	0.09 ± 0.04 (0–1)	0.09 ± 0.05 (0–2)
	P-value		0.211	0.204	0.633	0.202

ADH5 Polymorphisms

The descriptive statistics concerning the relationship between genotoxicity biomarkers and the two *ADH5* polymorphisms studied is shown in Tables V and VI. In this study, we did not find any individuals homozygous for the variant allele of the two *ADH5* polymorphisms investigated. Results of binary logistic regression did not show statistically significant associations between *ADH5* polymorphisms and the genotoxicity biomarkers studied. However, a borderline significant association ($P = 0.06$) was found with NBUD, as the Asp/Asp genotype had lower means than the Asp/Glu genotype. As shown in Table V, there was a statistically significant difference between Val/Val and Val/Ile genotypes for the *ADH5* Val309Ile polymorphism in the exposed group (Kruskal-Wallis, $P =$

Environmental and Molecular Mutagenesis. DOI 10.1002/em

0.024) with carriers of the heterozygote genotype having higher mean values than the homozygotes.

DISCUSSION

Exposure to formaldehyde in occupational settings is often prolonged enough to lead to the accumulation of DNA damage and increase in mutation risk [Mateuca et al., 2006]. Previous studies have suggested that genetic polymorphisms in specific genes affect chromosome damage levels associated with environmental exposures to genotoxic agents [Umegaki et al., 2000]. Genetic polymorphisms are potentially important in MN formation, depending on level of exposure, biological matrix studied and ethnicity of the studied population [Umegaki et al., 2000]. Chromosomal instability and impaired cell viability have been correlated with XRCC3 mutations and several other genes known or thought to be involved in HR [Bolognesi et al., 1999; Brenneman et al., 2000]. Previous studies have revealed a requirement for the HR pathway in processing DNA damage induced by formaldehyde [Zhang et al., 2010b].

In this study, we report a statistically significant association between XRCC3 Thr241Met polymorphism and NBUD. The carriers of the *XRCC3* Met/Met and Thr/Met genotypes had higher NBUD frequencies than their Thr/Thr genotype counterparts. Gene amplification plays a crucial role on the malignant transformation of human cells as it mediates the activation of oncogenes or the acquisition of drug resistance [Utani et al., 2007]. Excess DNA may be expelled from the nucleus by the formation of NBUD and subsequent micronucleation [Lindberg et al., 2007]. Studies have described in vivo budding of nuclear material in cell lines where changes in chromosomal numbers were occurring, and the spontaneous formation of NBUD structures was seen as a possible mechanism for the loss of chromosomes and for the generation of MN [Fenech et al. 2011]. Therefore, NBUD should also be considered genotoxic biomarkers with an origin comparable with that of MN [Serrano-García and Montero-Montoya, 2001].

Previous studies have shown that carriers of the XRCC3 heterozygous genotype had increased levels of chromatid breaks and sister-chromatid exchanges in smokers and increased DNA adducts in lymphocytes [Fenech et al., 1999] suggesting that this polymorphism is associated with low DNA repair capacity and may increase the risk of many types of cancer [Benhamou et al., 2004; Han et al., 2006; Batershill et al., 2008]. Studies from Yoshihara et al. [2004] and Lindh et al. [2006] suggested that XRCC3 Thr241Met variants contribute to the induction of MN arising from chromosome loss. Carriers of the Met/Met alleles would present higher MN frequencies than their wild-type Thr/Thr allele counterparts [Mateuca et al., 2008]. A significant increase of MN frequency in the Thr/Met genotype of XRCC3 was reported in workers exposed to oil from the Prestige accident, indicating that this polymorphism must be taken into account in chronic exposure scenarios [Pérez-Cadahía et al., 2008]. Shen et al. [2002] suggested that the Met/Met genotype may contribute to a subset of squamous cell carcinoma of the head and neck and Figueiredo et al. [2004] found that both carriers of Met/Met and Thr/Met genotypes have an increased risk for breast cancer. The Met/Met genotype may cause genetic instability and lead to an increased susceptibility to various cancers due to the inability of genotype carriers to complement the centrosome amplification defect and to a decrease of apoptotic rates [Lindt et al. 2006], factors that may prevent aberrant cells from entering apoptosis. However, other studies did not find evidence for the influence of XRCC3 genotype in the MN basal frequency [Iarmarcovai et al., 2006].

The functional differences between the XRCC3 alleles are not entirely understood. The amino acid substitution of a threonine by a methionine has the potential to affect protein structure and integrity [Dhillon et al., 2011]. Variants leading to diminished XRCC3 function may be predicted to confer an increased risk of cancer due to accumulated levels of DNA damage. As many genes are involved in the repair of DNA damage, there is also the possibility that these polymorphisms might be in linkage disequilibrium with other causative factors [Figueiredo et al., 2004].

Our study did not provide conclusive evidence that some *ADH5* polymorphisms may influence the carrier's capacity to protect against DNA damage. A borderline association ($P = 0.06$) was found between the frequency of NBUD and the homozygous Asp/Asp genotype, as compared to the Asp/Glu heterozygous genotype. These individuals may be more prone to nuclear alterations following a possible alteration in formaldehyde metabolism and adduct formation. Another interesting result was the statistically significant difference in carriers of the Val/Ile genotype in comparison with Val/Val genotype of the ADH5 Val309Ile polymorphism in MN in lymphocytes in the exposed group. The carriers of the heterozygous genotype showed higher means of MN in lymphocytes in the exposed group but not in the control group suggesting that the carriers of Val/Ile genotype metabolize poorly formaldehyde and present more DNA damage. Our results are in agreement with the findings of Just et al. [2011], who investigated three different polymorphisms in the transcribed regions of *ADH5* for inter-individual differences against the genotoxicity of formaldehyde in the German population and found no biologically relevant variants. The biological significance of ADH5 polymorphisms in relation to disease remains uncertain.

A better understanding of MN induction driven by genetic polymorphism affecting DNA repair and/or genome stability, in particular XRCC3 Thr241Met, requires larger scale studies and the assessment of other relevant polymorphism interacting with individual DNA repair capacity [Mateuca et al., 2008]. The association between

SNPs in relevant genes and the frequency of MN in lymphocytes is a valuable tool for this purpose, as the latter is one of the best validated DNA damage biomarker known to be sensitive to a wide range of endogenous, environmental, and lifestyle factors that can harm the genome [Dhillon et al., 2011]. Some genetic polymorphisms of xenobiotic-metabolizing enzymes have been observed to influence the level of genotoxic damage in humans. This may facilitate the identification of risk groups and increase the sensitivity of biomarkers in biomonitoring [Norppa, 2001]. However, studies that report an association between genotypes and biomarkers, such as MN, have some limitations in design and analysis. Common limitations are group sample size, usually too small to evaluate rare polymorphisms, and the wide range of allele frequency variation for each genotype in different ethnic populations. The statistical analysis is often plagued with problems of lack of power (due to insufficient sample size) and confounding can seldom be precluded given the amount of potential factors involved that have not been measured [Chung et al., 2010; Hunter, 2005].

In conclusion, this study showed that occupational exposure to formaldehyde increased the frequencies of genotoxicity biomarkers. Our results showed a significant statistical association between XRCC3 Thr241Met polymorphism and NBUD. ADH5 polymorphisms did not show significant association with the genotoxicity biomarkers studied. Several association studies have recently addressed the link between DNA repair polymorphism and MN induction, but the evidence that DNA repair polymorphisms influence MN frequencies remains limited [Mateuca et al., 2008]. This study highlights the importance of applying biomarkers of effect, such as genotoxicity biomarkers, and individual susceptibility biomarkers, such as genetic polymorphisms, to human biomonitoring studies in occupational exposure settings.

AUTHOR CONTRIBUTIONS

C. Ladeira, M.C. Gomes, and M. Brito conceived the idea and designed the study. C. Ladeira performed de CBMN assay and MN test for the assessment of the genotoxicity biomarkers and Real Time PCR analysis for genetic polymorphisms analysis. S. Viegas performed the exposure assessment. E. Carolino performed the statistical data analysis. All authors have read and approved the final manuscript.

REFERENCES

Araujo F, Pierce A, Stark J, Jasin M. 2002. Variant XRCC3 implicated in cancer is functional in homology-directed repair of double-strand breaks. Oncogene 21:4176–4180.

Battershill JM, Burnett K, Bull S. 2008. Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: Impact on design of biomonitoring studies. Mutagenesis 6:423–237.

Benhamou S, Tuimala J, Bouchardy C, Dayer P, Sarasin A, Hirvonen A. 2004. DNA repair gene XRCC2 and XRCC3 polymorphisms and susceptibility to cancers of the upper aerodigestive tract. Int J Cancer 112:901–904.

Bolognesi C, Lando C, Forni A, Landini E, Scarpato R, Migliori L, Bonassi S. 1999. Chromosomal damage and ageing: Effect of micronuclei frequency in peripheral blood lymphocytes. Age Ageing 28:393–397.

Bonassi S, Neri M, Lando C, Ceppi M, Lin Y, Chang W, Holland N, Kirsch-Volders M, Zeiger E, Fenech M. 2003. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: Results from the Human MicroNucleus project. Mutat Res 543:155–166.

Brenneman M, Weiss A, Nickoloff J, Chen D. 2000. XRCC3 is required for efficient repair of chromosome breaks by homologous recombination. Mutat Res 459:89–97.

Catalán J, Surrallés J, Falck G, Autio K, Norppa H. 2000. Segregation of sex chromosomes in human lymphocytes. Mutagenesis 3:251–255.

Chung C, Magalhães W, Gonzalez-Bosquet J, Chanock S. 2010. Genome-wide association studies in cancer—Current and future directions. Carcinogenesis 31:111–120.

Dhillon V, Thomas P, Iarmarcovai G, Kirsch-Volders M, Bonassi S, Fenech M. 2011. Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. Mutagenesis 26:33–42.

El-Zein R, Schabath M, Etzel C, Lopez M, Franklin J, Spitz M. 2006. Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. Cancer Res 66:6449–6456.

El-Zein R, Fenech M, Lopez M, Spitz M, Etzel C. 2008. Cytokinesis-blocked cytome assay biomarkes identify lung cancer cases amongst smokers. Cancer Epidemiol Biomarkers Prev 17:1111–1119.

Engeland K, Höög J-O, Holmquist B, Estonius M, Jörnvall H. 1993. Mutation of Arg-115 of human class III alcohol dehydrogenase: A binding site required for formaldehyde dehydrogenase activity and fatty acid activation. Proc Natl Acad Sci USA 90:2491–2494.

Fenech M, Holland N, Chang W, Zeiger E, Bonassi S. 1999. The Human MicroNucleus Project—An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res 428:271–283.

Fenech M. 2002. Chromosomal biomarkers of genomic instability relevant to cancer. Drug Discov Today 22:1128–1137.

Fenech M, Crott J. 2002. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes—Evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutat Res 504:131–136.

Fenech M. 2005. The Genome Health Clinic and Genome Health Nutrigenomics concepts: Diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. Mutagenesis 20:225–269.

Fenech M. 2006. Cytokinesis-block micronucleus assay evolves into a ''cytome'' assay of chromosomal instability, mitotic dysfunction and cell death. Mutat Res 600:58–66.

Fenech M, Kirsch-Volders M, Natarajan A.T, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26:125–132.

Figueiredo J, Knight J, Briollais L, Andrulis I, Ozcelik H. 2004. Polymorphisms XRCC1-R399Q and XRCC3-T241M and the risk of breast cancer at the ontario site of the Brest Cancer Family Registry. Cancer Epidemiol Biomarkers Prevention 13:583–591.

Franks SJ. 2005. A mathematical model for the absorption and metabolism of formaldehyde vapour by humans. Toxicol Appl Pharmacol 206:309–320.

Environmental and Molecular Mutagenesis. DOI 10.1002/em

Han S, Zhang H, Wang Z, Xie Y, Tang R, Mao Y, Li Y. 2006. DNA repair gene XRCC3 polymorphisms and cancer risk: a meta-analysis of 48 case-control Studies. Eur J Hum Gene 14:1136–1144.

Hedberg, J. 2001. Function, Expression and Polymorphism of Human Alcohol Dehydrogenase 3/Glutathione-Dependent Formaldehyde Dehydrogenase. Stockholm: Karolinska Institute.

Hunter D. 2005. Gene-environment interactions in human diseases. Nat Rev Gene 6:287–298.

Hur MW, Edenberg H. 1992. Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase. Gene 121:305–311.

IARC, 2006. IARC Monographs on the Evaluation of Carcinogenic Risks to Human, volume 88: Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxy-2-propanol. Lyon: World Health Organization.

Iarmarcovai G, Sari-Minodier I, Orsière T, De Méo M, Gallice P, Bideau C, Iniesta D, Pompili J, Bergé-Lefranc JL, Botta A. 2006. A combined analysis of XRCC1, XRCC3, GSTM1 and GSTT1 polymorphisms and centromere content of micronuclei in welders. Mutagenesis 2:159–165.

Iarmarcovai G, Bonassi S, Botta A, Bann RA, Orsière T. 2008. Genetic polymorphisms and micronucleus formation: A review of the literature. Mutat Res 658:215–233.

Just W, Zeller J, Riegert C, Speit G. 2011. Genetic polymorphisms in the formaldehyde dehydrogenase gene and their biological significance. Toxicol Lett 207:121–127.

Kaiser R, Holmquist B, Vallee B, Jörnvall H. 1991. Human class III alcohol dehydrogenase/glutathione-dependent formaldehyde dehydrogenase. J Protein Chem 1:69–73.

Kirsch-Volders M, Mateuca R, Roelants M, Tremp A, Zeiger E, Bonassi S, Holland N, Chang W, Aka P, DeBoeck M, Goddenis L, Haufroid V, Ishikawa H, Laffon B, Marcos R, Migliore L, Norppa H, Teixeira J, Zijno A, Fenech M. 2006. The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. Cancer Epidemiol Biomarkers Prev 15:1038–1042.

Ladeira C, Viegas S, Carolino E, Gomes MC, Brito M. 2011. Genotoxicity biomarkers in occupational exposure to formaldehyde—The case of histopathology laboratories. Mutat Res 721:15–20.

Lee S-L, Wang M-F, Lee A-I, Yin S-J. 2003. The metabolic role of human ADH3 functioning as ethanol dehydrogenase. FEBS Lett 544:143–147.

Lindberg H, Wang X, Järventaus H, Falck G, Norppa H, Fenech M. 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat Res 617:33–45.

Lindh A, Rafii S, Schultz N, Cox A, Helleday T. 2006. Mitotic defects in XRCC3 variants T241M and D213N and their relation to cancer susceptibility. Hum Mol Gene 7:1217–1224.

Manuguerra M, Saletta F, Karagas M, Berwick M, Veglia F, Vineis P, Matillo G. 2006. XRCC3 and XPD/ERCC2 single nucleotide polymorphisms and the risk of cancer: A HuGE review. Am J Epidemiol 164:297–302.

Marsh G, Youk A. 2004. Reevaluation of mortality risks from leukemia in formaldehyde cohort study of the National Cancer Institute. Regulatory Toxicol Pharmacol 40:113–124.

Marsh G, Youk A, Morfeld P, Collins J, Symons J. 2010. Incomplete follow-up in the National Cancer Institutés formaldehyde worker study and the impact on subsequent reanalyses and causal evaluations. Regulatory Toxicol Pharmacol 58:233–236.

Mateuca R, Lombaert N, Aka NP, Decordier I, Kirsch-Volders M. 2006. Chromosomal changes: Induction, detection methods and applicability in human biomonitoring. Biochimie 88:1515–1531.

Mateuca R, Roelants M, Iarmarcovai G, Aka P, Godderis L, Tremp A, Bonassi S, Fenech M, Bergé-Lefranc, Kirsch-Volders M. 2008. *hOGG1*^{326}, *XRCC1*^{399}, *XRCC3*^{241} polymorphisms influence micronucleus frequencies in human lymphocytes *in vivo*. Mutagenesis 23:35–41.

Matullo G, Palli D, Peluso M, Guarrera S, Carturan S, Celentano E, Krogh V, Munnia A, Tumino R, Polidoro S, Piazza A, Vineis P. XRCC1, XRCC3, XPD gene polymorphisms, smoking and ^{32}P-DNA adducts in a sample of healthy subjects. Carcinogenesis 9:1437–1445.

McGlothlin JD. 2005. Occupational exposure assessment and control using video exposure monitoring in the pharmaceutical industry. In INTERNATIONAL SCIENTIFIC CONFERENCE (IOHA 2005), 6, 19-23 September 2005, Pilanesberg National Park North West Province, South Africa. Pilanesberg: IOHA – International Occupational Hygiene. MVS – Mine Ventilation of South Africa.

National Toxicology Program. 2011. Report on Carcinogens, Twelfth Edition. Department of Health and Human Services. 195–205.

NIOSH. 1994. Manual of Analytical Methods, 4th ed. DHHS (NIOSH) Publication 94–113.

Norppa H. 2001. Genetic polymorphism and chromosome damage. Int J Hyg Environ Health 204:31–38.

Pérez-Cadahía B, Laffon B, Valdiglesias V, Pásaro E, Méndez J. 2008. Cytogenetic effects induced by *Prestige* oil on human populations: The role of polymorphisms in genes involved in metabolism and DNA repair. Mutat Res 653:117–123.

Ramirez A, Saldanha P. 2002. Micronucleus investigation of alcoholic patients with oral carcinoma. Gene Mol Res 1:246–260.

Rhomberg L, Bailey L, Goodman J, Hamade A, Mayfield D. 2011. Is exposure to formaldehyde in air causally associated with leukemia? A hypothesis-based weight-of-evidence analysis. Crit Rev Toxicol 41:555–621.

Serrano-García L, Montero-Montoya R. 2001. Micronuclei and chromatid buds are the result of related genotoxic events. Environmental Mol Mutagen 38:38–45.

Shen H, Sturgis E, Dahlstrom K, Zheng Y, Spitz M, Wei Q. 2002. A variant of the DNA repair gene XRCC3 and risk of squamous cell carcinoma of the head and neck: A case-control analysis. Int J Cancer 99:869–872.

Stich H, Rosin M. 1983. Quantitating the synergistic effect of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells. Int J Cancer 31:305–308.

Thomas P, Umegaki K, Fenech M. 2003. Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. Mutagenesis 18:187–194.

Tolbert P, Shy C, Allen J. 1991. Micronuclei and other nuclear anomalies in buccal smears: A field test in snuff users. Am J Epidemiol 8:840–850.

Umegaki K, Fenech M. 2000. Cytokinesis-block micronucleus assay in WIL2-NS cells: A sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. Mutagenesis 15:261–269.

Utani K, Kawamoto J-k, Shimizu N. 2007. Micronuclei bearing acentric extrachromosomal chromatin are transcriptionally competent and may perturb the cancer cell phenotype. Mol Cancer Res 5:695–704.

Viegas S, Ladeira C, Nunes C, Malta-Vacas J, Gomes M, Mendonça P, Brito M, Prista J. 2010. Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. J Occupational Med Toxicol 5:1–8.

Wang Y, Liang D, Spitz M, Zhang K, Dong Q, Amos C, Wu X. 2003. XRCC3 genetic polymorphism, smoking, and lung carcinoma risk in minority populations. Cancer 98:1701–1706.

Wang LL, Yang AK, Li Y, Liu JP, Zhou SF. 2010. Phenotype prediction of deleterious nonsynonymous single nucleotide polymorphisms in human alcohol metabolism-related genes: A bioinformatics study. Alcohol 44:425–438.

Winsey S, Haldar N, Marsh H, Bunce M, Marshall S, Harris A, Wojnarowska F, Welsh K. 2000. A variant within the DNA repair gene XRCC3 is associated with the development of melanoma skin cancer. Cancer Res 60:5612–5616.

Environmental and Molecular Mutagenesis. DOI 10.1002/em

Wu H, Romieu I, Sienra-Monge JJ, Rio-Navarro B, Anderson D, Jenchura C, Li H, Ramirez-Aguilar M, Lara-Sanchez I, London S. 2007. Genetic variation in S-nitrosogluthatione reductase (GSNOR) and childhood asthma. J Allergy Clin Immunol 2:322–328.

Yoshihara T, Ishida M, Kinomura A, Katsura M, Tsuruga T, Tashiro S, Asahara T, Miyagawa K. 2004 XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. Embo J 23:670–680.

Zhang L, Steinmaus C, Eastmond D, Xin X, Smith M. 2009. Formaldehyde exposure and leukemia: A new meta-analysis and potential mechanisms. Mutat Res 681:150–168.

Zhang L, Tang X, Rothman N, Vermeulen R, Ji Z, Shen M, Qiu C, Guo W, Liu S, Reiss B, Freeman L, Ge Y, Hubbard A, Hua M, Blair A, Galvan N, Ruan X, Alter B, Xin K, Li S, Moore L, Kim S, Xie Y, Hayes R, Azuma M, Hauptmann M, Xiong J, Stewart P, Li L, Rappaport S, Huang H, Fraumeni J, Smith Jr., Lan Q. 2010a. Occupational exposure to formaldehyde, hematoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. Cancer Epidemiol Biomarkers Prev 19:80–88.

Zhang L, Freeman L, Nakamura J, Hecht S, Vandenberg J, Smith M, Sonawane B. 2010b. Formaldehyde and leukemia: Epidemiology, potential mechanisms and implications for risk assessment. Environ Mol Mutagen 51:181–191.

Accepted by—
L. Zhang

Journal of Toxicology and Environmental Health, Part A, 77:879–887, 2014

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2014.910158

Taylor & Francis
Taylor & Francis Group

ASSESSMENT OF GENOTOXIC EFFECTS IN NURSES HANDLING CYTOSTATIC DRUGS

C. Ladeira[1,2], S. Viegas[1,2], M. Pádua[1], M. Gomes[1,3], E. Carolino[1], M. C. Gomes[4], M. Brito[1]

[1]Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa, Lisbon, Portugal
[2]CMDT, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal
[3]Chemistry Department, REQUIMTE/CQFB, and SINTOR–UNINOVA, Faculty of Sciences and Technology, Universidade de Lisboa, Lisbon, Portugal
[4]Faculty of Sciences, University of Lisbon, Lisbon, Portugal

Several antineoplastic drugs have been classified as carcinogens by the International Agency for Research on Cancer (IARC) on the basis of epidemiological findings, animal carcinogenicity data, and outcomes of in vitro genotoxicity studies. 5-Fluorouracil (5-FU), which is easily absorbed through the skin, is the most frequently used antineoplastic agent in Portuguese hospitals and therefore may be used as an indicator of surface contamination. The aims of the present investigation were to (1) examine surface contamination by 5-FU and (2) assess the genotoxic risk using cytokinesis-block micronucleus assay in nurses from two Portuguese hospitals. The study consisted of 2 groups: 27 nurses occupationally exposed to cytostatic agents (cases) and 111 unexposed individuals (controls). Peripheral blood lymphocytes (PBL) were collected in order to measure micronuclei (MN) in both groups. Hospital B showed a higher numerical level of contamination but not significantly different from Hospital A. However; Hospital A presented the highest value of contamination and also a higher proportion of contaminated samples. The mean frequency of MN was significantly higher in exposed workers compared with controls. No significant differences were found among MN levels between the two hospitals. The analysis of confounding factors showed that age is a significant variable in MN frequency occurrence. Data suggest that there is a potential genotoxic damage related to occupational exposure to cytostatic drugs in oncology nurses.

Antineoplastic dugs are a heterogeneous group of chemicals that are widely used in the treatment of cancer and some nonneoplastic diseases. These agents share an ability to inhibit tumor growth by disrupting cell division and killing actively growing cells. These drugs were shown to be mutagenic, carcinogenic, and teratogenic (Fucic et al., 1998; Sessink and Bos, 1999; Bouraoui et al., 2011; Gulten et al., 2011; Buschini et al., 2013).

Oncology nurses and other health care staff in oncology units are at risk of exposure to antineoplastic agents. This exposure is primarily due to environmental contamination that might result from inhalation and percutaneous absorption or, less commonly, ingestion of antineoplastic agents at subtherapeutic concentrations. Protective equipment such as gloves, masks, gowns, caps, and protective eyewear and preparation of drugs in biological safety cabinets are normally available in the workplaces and hinder exposure (Gulen et al., 2011). Specifically, in nursing, exposure to antineoplastic agents may occur during preparation, administration, or disposal of equipment, or when human excreta are handled. Contamination of work surfaces and also permeation of gloves by some antineoplastic

Address correspondence to Dr. C. Ladeira, Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa, Av. D. João II, lote 4.69.01, 1990-096 Lisboa, Parque das Nações, Lisbon, Portugal. E-mail: carina.ladeira@estesl.ipl.pt

drugs were previously reported in several studies (Laffon et al., 2005; Kopjar et al., 2009; Gulen et al., 2011). Further, vaporization of spilled antineoplastic drugs may represent an additional route of exposure to health care workers through inhalation. However, contact with contaminated surfaces seems to be the predominant route of exposure due to dermal absorption (Sessink and Bos, 1999; Fransman et al., 2004). Therefore, monitoring of surfaces contamination is a common way to assess occupational exposure, with the most common method used being wipe sampling (Hedmer et al., 2004, 2008). Exposure in a hospital setting is normally due to the use of several antineoplastic drugs simultaneously. Nevertheless, the effects of such mixtures on cell and human health are not predictable and are unique due to differences in practices between hospital oncology departments, in the number of patients, in protective devices available, and in the experience and safety procedures of medical staff (Kopjar et al., 2009).

Although various methods of monitoring biological effects have been established, none indicates an apparent correlation between exposure levels and development of cancer. Therefore, an internal dosimeter needs to be considered in the detection of genotoxic and potential carcinogenic risks (Bouraoui et al., 2011). Cytogenetic assays may be used to achieve this goal. The conceptual basis for application is that DNA damage is the initial event in the pathogenesis of disease. Thus, cytogenetic surveillance might serve as an indicator enabling early detection of exposure to genotoxic agents (Rekhadevi et al., 2007; Bouraoui et al., 2011). Micronuclei (MN) in peripheral blood lymphocytes (PBL) are extensively used as a biomarker of genomic instability due to exposure to genotoxic agents and might be used to detect early biological effects in human biomonitoring programs (Bouraoui et al., 2011).

The cytokinesis-block micronucleus assay (CBMN) has been used to assess cellular DNA damage as an eventual effect of occupational exposed to antineoplastic drugs in nurses (Cavallo et al., 2005, 2007; Bouraoui et al., 2011). The CBMN assay has the ability to detect both clastogenic (chromosome breakage) and aneugenic (chromosome loss) effects, enabling one to measure genomic damage within PBL that may have occurred while circulating within the body in the quiescent phase. Further, recently Villarini et al. (2012) reported that MN frequency in surrogate cells such as PBL is a reliable predictor for cancer risk in human populations.

It is known that exposures to even small concentrations of certain drugs may be hazardous for workers who handle them or work near them (National Institute for Occupational Safety and Health [NIOSH], 2004; Constantinidis et al., 2011). 5-Fluorouracil (5-FU) has been used since the early 1950s as the main antineoplastic agent in treatment of gastrointestinal tumors and has been used frequently in combination with other agents for the treatment of a variety of solid tumors (International Agency for Research on Cancer [IARC], 1981). Data on chromosomal aberrations (CA) produced by 5-FU suggest that the drug has clastogenic potential; however, no apparent evaluation has be conducted on the carcinogenic risk of 5-FU in humans (IARC, 1981). In addition, 5-FU is one of the most frequently antineoplastic agents employed and is readily absorbed through the skin. Considering these two factors, 5-FU was considered as an indicator of surfaces contamination and exposure as reported in other studies (Larson et al., 2003; Castiglia et al., 2008; Schierl et al., 2009; Hedmer and Wohlfart, 2012; Kopp et al., 2013). The aims of the present investigation were to (1) determine surface contamination by 5-FU and (2) assess genotoxic risk using CBMN assay in nurses from two Portuguese hospitals.

MATERIAL AND METHODS

Subjects of Study

This study was performed in accordance with standards of ethics and received the

necessary approvals. The population under study was composed of 27 occupationally exposed nurses and 111 nonexposed subjects. The control group was constituted by workers in an academic institution without contact with cytostatic drugs. All participants were informed regarding the aim and experimental details of the study and provided informed consent. Each participant completed a standardized questionnaire that covered a detailed medical, family, and dietary history, including variables known to influence cytogenetic endpoints, such as exposure to potential mutagens, smoking, alcohol consumption, oncological therapeutics, and lifestyle activities.

Exposure Assessment

Surfaces contamination by 5-FU was investigated in two hospitals by wipe sampling in areas where antineoplastic drugs were administered, as recommended by Hedmer et al. (2004, 2008). 5-Fluorouracil was considered a suitable indicator for occupational exposure to antineoplastic drugs because the drug is frequently used in preparations and in high amounts in both hospitals (Castiglia et al., 2008). Sensitive analytical methods were previously established for this drug. In both hospitals, sampling was developed in two different days. Regarding antineoplastic drug administration, the days were indicated by workers and services as normal working days. Before wiping, gauzes were moistened with ethyl acetate. Sampling was performed by consecutive wiping to cover an area of 10 × 10 cm. Selected sample areas were preparation tables, drugs administration devices, chairs for drug administration, worktops, treatment registration tables, and protection devices such as gloves and masks. All wipe samples were extracted according to Schmaus et al. (2002). Analysis of the samples was blinded and performed by high-performance liquid chromatography with diode array detection (HPLC-DAD) with a level of quantification (LOQ) 10 ng/cm^2 as described by Schmaus et al. (2002).

Genotoxicity Assessment

Assessment of genotoxic effects was conducted by applying the CBMN assay in PBL. Heparinized blood samples were obtained by venipuncture from each subject between 10 a.m. and 12 p.m. All samples were coded and analyzed under blind conditions. The CBMN assay was performed as described by Ladeira et al. (2011, 2013). The criterion for scoring MN in PBL was described by Fenech et al. (1999).

Statistical Analysis

Statistical analysis was performed using the SPSS software program package for Windows (version 21.0). Variables were compared with the normal distribution using the Shapiro–Wilk test ($p > .05$). Rejection of the null hypothesis of underlying normality led us to proceed with nonparametric procedures to compare groups and check associations. The association between genotoxicity biomarkers, occupational exposure to cytostatics, age, gender, tobacco habits, and alcohol consumption was evaluated by multiple linear regression analysis. The biomarkers were dichotomized (absent/present) and considered dependent variables, taking absence as the reference. Occupational exposure, age, gender, and lifestyle factors were considered independent variables. The nonparametric Mann–Whitney test was used to compare groups.

RESULTS

Population characteristics including gender distribution, age, tobacco habits, and alcohol consumption for the control and exposed groups are shown in Table 1. In total, 133 surfaces samples were taken in both hospitals and 26 (19.55%) showed measurable values of 5-FU (Table 2). Hospital A showed a higher percentage of contaminated samples (50%) than Hospital B (8.57%), but no difference was found between contamination levels. A higher value was obtained in Hospital A

TABLE 1. Characteristics of the Sample

	Control	Exposed
Number of subjects	111	27
Gender		
Female	54 (48.6%)	5 (18.5%)
Male	57 (51.4%)	22 (81.5%)
Age		
(mean ± SE, yr)	34.25 ± 0.88	34.89 ± 1.47
Range	20-61	25-55
Years of employment		
(mean ± SD, yr)	n.a.	6.01
Range		0.17-30
Tobacco consumption		
Nonsmokers	75 (67.6%)	24 (92.9%)
Smokers	36 (32.4%)	3 (7.1%)
Alcohol consumption		
Nondrinkers	45 (40.5%)	19 (70.4%)
Drinkers	66 (59.5%)	8 (29.6%)

Note. n.a., Nonapplicable.

TABLE 2. 5-Fluorouracil in Both Hospital Samples

	Hospital A ($n = 28$)	Hospital B ($n = 105$)
Contaminated (> LOQ)	14 (50%)	9 (8.57%)
Mean (ng/cm^2) ± SD	16.42 ± 3.41	19.94 ± 3.39
Range	10.95–60.51	10.66–41.25
p Value	0.208	

Note. Limit of detection (LOD) = 3 ng/cm^2. Limit of quantification (LOQ) = 10 ng/cm^2.

(60.51 ng/cm^2) in a worktop that supports the drug administration process.

The frequencies of MN in control an in exposed population are presented in Table 3. In PBL, significant increases were observed in exposed subjects compared to nonexposed subjects. Comparing the exposed workers separately by hospital, no significant differences were detected, although MN mean in Hospital A was numerically higher than in Hospital B, as shown in Table 4. Using multiple linear regression analyses, only exposure to cytostatics and age were found to significantly influence MN frequency in PBL. Gender, tobacco, and alcohol consumption did not markedly affect results. Searching for the influence of the years of exposure to cytostatics, no significant correlation was detected between years of exposure and presence of MN.

TABLE 3. MN in lymphocytes

	Mean, MN lymphocytes ± SE (range)
Controls	2.09 ± 0.312 (0–15)
Exposed	10.11 ± 2.053 (1–58)
p Value	<.001

Note. Data are given as mean in the studied population: mean ± mean standard error (range); *p* value is of Mann–Whitney test.

TABLE 4. MN in lymphocytes between hospitals

	Mean, MN lymphocytes ±SE (range)
Hospital A workers	9.91 ± 1.93 (2–20)
Hospital B workers	7.07 ± 0.75 (1–11)
p Value	.323

Note. Data are given as mean in the studied population: mean ± mean standard error (range); *p* value is of Mann–Whitney test.

DISCUSSION

Health care workers handling antineoplastic drugs usually implement collective and individual protective measures. However, contamination of the work environment is still possible, and safety measures employed may not be sufficient to prevent exposure. In addition, workers may not utilize all the safety measures required for handling such substances.

Surface monitoring of antineoplastic drug contamination may be used as a surrogate for dermal exposure, and therefore as a reliable indicator of occupational exposure to these drugs. The existence of contamination by antineoplastic drugs on different surfaces in the workplace implies a potential increased risk for health care workers to become dermally exposed (Hedmer and Wohlfart, 2012). Our results showed contamination by 5-FU, indicating possible exposure to other antineoplastic drugs. Although Hospital B handles high quantities of this drug and other antineoplastic drugs, Hospital A showed a higher number of contaminated samples. These differences may be attributed to less safety procedures in Hospital A due to the fact that this is a more recent oncology unit.

The nature of overall damage induced after in vivo exposure is quite complex, taking into

account different modes of action, where various classes of antineoplastic drugs induce different mutations and cytogenetic aberrations. Further, synergistic and additive effects also need to be anticipated. Since the state of the art in cancer chemotherapy is constantly changing, the duration of daily exposure and the spectrum and quantities of antineoplastic drugs handled by an individual usually are difficult to anticipate retrospectively. Considering that the efficient treatment of most malignancies involves the use of polychemotherapy, nurses are in most cases simultaneously exposed to complex mixtures of antineoplastic agents with different potential to damage DNA as well as to produce certain types of damage that might be more susceptible to detection (Kopjar et al., 2009).

With respect to genotoxicity, a significant increase of MN frequency in nurses handling antineoplastic drugs was noted, similar to others studies (Fucic et al., 1998; Deng et al., 2005; Cavallo et al., 2007; Cornetta et al., 2008; Bouraoui et al., 2011; El-Ebiary et al., 2011). The results of cytogenetic studies are often ambiguous. A study performed in Austria (Pilger et al., 2000) and a study in Sweden (Thiringer et al., 1991) reported no significant differences in MN frequency between hospital pharmacy personnel and unexposed controls. Maluf and Erdtmann (2000), Hessel et al. (2001), Cavallo et al. (2005), and Laffon et al. (2005) also demonstrated no significant change in MN frequency in workers exposed to antineoplastic drugs. These contradictory observations might be attributed to differences in the antineoplastic drugs handled, or to protective measures available and used effectively. Further, based on genetic make-up, human response to genotoxic xenobiotics may vary due to the presence of individual differences in DNA damage-repairing capacity (Rekhadevi et al., 2007; El-Ebiary et al., 2011).

Multiple linear regression analysis failed to demonstrate that years of exposure to cytostatic agents influenced the frequency of MN. Our data were corroborated by studies of Thiringer et al. (1991), Hessel et al. (2001), Bouraoui et al. (2011), El-Ebiary et al. (2011), and Villarini et al. (2012) that also did not find an association between years of exposure to antineoplastic drugs and MN frequency. However, there are controversial results with respect to exposure duration. Studies by Kevekordes et al. (1998), Kasuba et al. (1999), Laffon et al. (2005), Cavallo et al. (2007), Rekhadevi et al. (2007), and Kopjar et al. (2009) noted a correlation between years of exposure and rise of MN rate. In addition, Laffon et al. (2005) reported this effect after 10 yr of cumulative exposure.

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking, and alcohol consumption, upon the association between disease (effect) and exposure (Fenech et al., 1999; Bonassi et al., 2001).

Cytogenetically, aging is associated with a number of gross cellular changes, including altered size and morphology, genomic instability, and changes in expression and proliferation (Bolognesi et al., 1999; Zietkiewicz et al., 2009). Kirsch-Volders et al. (2006) and Orsière et al. (2006) showed that a higher MN frequency was directly associated with decreased efficiency of DNA repair and increased genome instability. Data demonstrated a significant increase of MN frequency in PBL in exposed workers, suggesting that MN frequencies tended to rise with age as previously reported by Hessel et al. (2001) and Kopjar et al. (2009).

Tobacco smoke has been epidemiologically associated with a higher risk of cancer development. In the present study, smoking habits did not markedly affect the frequency of MN in either the exposed or control subjects. Concerning gender, studies by Fenech et al. (1999) and Ladeira et al. (2011) noted that biomarker frequencies in lymphocytes were greater in females compared to males. In our study, gender was not markedly associated with an increase in frequency of MN in both groups. Results regarding smoking habits and gender were corroborated by Villarini et al. (2012).

As many newly developed antineoplastic drugs are designed to attack specific intracellular targets, their harmful effects might easily "escape" detection by majority of standard

endpoints. To accurately evaluate cytogenetic outcomes of such exposure, the CBMN assay needs to be used (Kopjar et al., 2009). The CBMN value in assessment of genotoxic damage among occupationally exposed personnel was also affirmed in this study. Data demonstrated a significant increase of MN in PBL in the exposed group. This might be attributed to genomic instability, as evidenced by an enhanced amount of mutations and/or chromosomal aberrations that cytogenetically translate into a greater frequency of changes in chromosome and formation of MN (Zietkiewicz et al., 2009).

CONCLUSIONS

Health risk is influenced by exposure duration and potency and toxicity of the hazardous material. To provide workers with the greatest protection, employers need to implement necessary administrative and engineering controls and ensure that workers apply safety procedures for handling hazardous drugs and use proper protective equipment (NIOSH, 2004). The present study demonstrated that there is surface contamination in the workplace, and the cytogenetic endpoint studied (CBMN) was indicative of signs of exposure. Since genotoxicity may be due to combined effects of all or some of the antineoplastic drugs, it is not possible to attribute damage to any particular agent. Results of this study as well as previous investigations performed on subjects occupationally exposed to antineoplastic drugs using different genotoxicity endpoints suggest that mixtures of antineoplastic drugs in chronic occupational exposure may act as clastogens on DNA of somatic cells (Rekhadevi et al., 2007).

Our data suggest that safety measures adopted by oncology nurses of both hospitals were not sufficient to avoid contamination/exposure and prevent adverse health effects related to the antineoplastic drug handled. Further, our findings address the need for regular biomonitoring of personnel occupationally exposed to antineoplastic drugs, which indicated an enhanced health risk assessment in oncology nurses (El-Ebiary et al., 2011).

FUNDING

This study was supported by ACT-Autoridade para as Condicoes de Trablho. The assistance of the Lisbon School of Health Technology is greatly appreciated.

REFERENCES

Bonassi, S., Fenech, M., Lando, C., Lin, Y.-P., Ceppi, M., Chang, W., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L., Fucic, A., Lima, O., Hrelia, P., Krishnaja, A., Lee, T.-K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Müller, W.-U., Odagiri, Y., Scarfi, M., Szabova, E., Vorobtsova, I., Vral, A., and Zijno, A. 2001. Human MicroNucleus Project: International database comparison for results with cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ. Mol. Mutagen*. 37: 31–45.

Bolognesi, C., Lando, C., Forni, A., Landini, E., Scarpato, R., Migliori, L., and Bonassi, S. 1999. Chromosomal damage and ageing: Effect of micronuclei frequency in peripheral blood lymphocytes. *Age Ageing* 28: 393–397.

Bouraoui, S., Brahem, A., Tabka, F., Mrizek, N., Saad, A., and Elghezal, H. 2011. Assessment of chromosomal aberrations, micronuclei and proliferation rate index in peripheral lymphocytes from Tunisian nurses handling cytotoxic drugs. *Environ. Toxicol. Pharmacol*. 31: 250–257.

Buschini, A., Villarini, M., Feretti, D., Mussi, F., Dominici, L., Zerbini, I., Moretti, M., Ceretti, Bonfiglioli, R., Carrieri, M., Gelatti, U., Rossi, C., Monarca, S., and Poli, P. 2013. Multicentre study for the evaluation of mutagenic/carcinogenic risk in nurses

exposed to antineoplastic drugs: Assessment of DNA damage. *Occup. Environ. Med.* doi:10.1136/oemed-2013-101475.

Castiglia, L., Miraglia, N., Pieri, M., Simonelli, A., Basilicata, P., Genovese, G., Guadagni, R., Acampora, A., Sannolo, N., and Scafarto, M. V. 2008. Evaluation of occupational exposure to antiblastic drugs in an Italian hospital oncological department. *J. Occup. Health* 50: 48–56.

Cavallo, D., Ursini, C., Rondinone, B., and Iavicoli, S. 2009. Evaluation of a suitable DNA damage biomarker for human biomonitoring of exposed workers. *Environ. Mol. Mutagen*. 50: 781–790.

Cavallo, D., Ursini, C. L., Omodeo-Salè, E., and Iavicoli, S. 2007. Micronucleus induction and FISH analysis in buccal cells and lymphocytes of nurses administering antineoplastic drugs. *Mutat. Res*. 628: 11–18.

Cavallo, D., Ursini, C., Perniconi, B., Francesco, A., Giglio, M., Rubino, F., Marinaccio, A. and Iavicoli, S. 2005. Evaluation of genotoxic effects induced by exposure to antineoplastic drugs in lymphocytes and exfoliated buccal cells of oncology nurses and pharmacy employees. *Mutat. Res*. 587: 45–51.

Constantinidis, T. C., Vagka, E., Dallidou, P., Basta, P., Drakopoulos, V., Kakolyris, S., and Chatzaki, E. 2011. Occupational health and safety of personnel handling chemotherapeutic agents in Greek hospitals. *Eur. J. Cancer Care* 20: 123–131.

Cornetta, T., Padua, L., Testa, A., Ievoli, E., Festa, F., Tranfo, G., Baccelliere, L., and Cozzi, R. 2008. Molecular biomonitoring of population of nurses handling antineoplastic drugs. *Mutat. Res*. 638: 75–82.

Deng, H., Zhang, M., He, J., Wu, W., Jin, L., Zheng, W., Lou, J., and Wang, B. 2005. Investigating genetic damage in workers occupationally exposed to methotrexate using three genetic endpoints. *Mutagenesis* 20: 351–357.

El-Ebiary, A., Abuelfadl, A. A., and Sarhan, N. 2011. Evaluation of genotoxicity induced by exposure to antineoplastic drugs in lymphocytes of oncology nurses and pharmacists. *J. Appl. Toxicol*. 33: 196–201.

Fenech, M., Holland, N., Chang, W., Zeiger, E., and Bonassi, S. 1999. The Human MicroNucleus Project—An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat. Res*. 428: 271–283.

Fransman, W., Vermeulen, R., and Kromhout, H. 2004. Occupational dermal exposure to cyclophosphamide in Dutch hospitals: A pilot study. *Ann. Occup. Hyg*. 48: 237–244.

Fucic, A., Jazbec, A., Mijic, A., Seso-Simic, D., and Tomek, R. 1998. Cytogenetic consequences after occupational exposure to antineoplastic drugs. *Mutat. Res*. 416: 59–66.

Gulten, T., Evke, E., Ercan, I., Evrensel, T., Kurt, E., and Manavoglu, O. 2011. Lack of genotoxicity in medical oncology nurses handling antineoplastic drugs: Effect of work environment and protective equipment. *Work* 39: 485–489.

Hedmer, M., Tinnerberg, H., Axmon, A., and Jönsson, B. A. G. 2008. Environmental and biological monitoring of antineoplastic drugs in four workplaces in a Swedish hospital. *Int. Arch. Occup. Environ. Health* 81: 899–911.

Hedmer, M., Jönssona, B., and Nygren, O. 2004. Development and validation of methods for environmental monitoring of cyclophosphamide in workplaces. *J. Environ. Monit*. 6: 979–984.

Hedmer, M., and Wohlfart, G. 2012. Hygienic guidance values for wipe sampling of antineoplastic drugs in Swedish hospitals. *J. Environ. Monit*. 14: 1968–1975.

Hessel, H., Radon, K., Pethran, A., Maisch, B., Gröbmair, S., Sautter, I., and Fruhmann, G. 2001. The genotoxic risk of hospital, pharmacy and medical personnel occupationally exposed to cytostatic drugs—Evaluation by the micronucleus assay. *Mutat. Res*. 497: 101–109.

IARC. 1981. Some antineoplastic and immunosuppressive agents. *IARC Monogr. Eval. Carcinogen. Risks Hum*. Vol 26.

Jackson, M., Stack, H. F., and Waters, M. D. 1996. Genetic activity profiles of anticancer drugs. *Mutat. Res*. 355: 171–208.

Kasuba, V., Rozgaj, R., and Garaj-Vrhovac, V. 1999. Analysis of sister chromatid exchange and micronuclei in peripheral blood lymphocytes of nurses handling cytostatics drugs. *J. Appl. Toxicol*. 19: 401–404.

Kevekordes, S., Gebel, T. W., Hellwig, M., Dames, W., and Dunkelberg, H. 1998. Human effect monitoring in cases of occupational exposure to antineoplastic drugs: A method comparison. *Occup. Environ. Med*. 55: 145–149.

Kiffmeyer, T., and Hadtstein, C. 2007. Handling of chemotherapeutic drugs in the OR: Hazards and safety considerations. *Cancer Treat. Res*. 134: 275–290.

Kirsch-Volders, M., Mateuca, R., Roelants, M., Tremp, A., Zeiger, E., Bonassi, S., Holland, N., Chang, W., Aka, P., DeBoeck, M., Goddenis, L., Haufroid, V., Ishikawa, H., Laffon, B., Marcos, R., Migliore, L., Norppa, H., Teixeira, J., Zijno, A., and Fenech, M. 2006. The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. *Cancer Epidemiol. Biomarkers Prev*. 15: 1038–1042.

Kopjar, N., Garaj-Vrhovac, V., Kasuba, V., Rozgaj, R., Ramic, S., Pavlica, V., and Zeljezic, D. 2009. Assessment of genotoxic risks in Croatian health care workers occupationally exposed to cytotoxic drugs: a multibiomarker approach. *Int. J. Hyg. Environ. Health* 212: 414–431.

Kopjar, N., and Garaj-Vrhovac, V. 2001. Application of the alkaline comet assay in human biomonitoring for genotoxicity: A study on Croatian medical personnel handling antineoplastic drugs. *Mutagenesis* 16: 71–78.

Kopp, B., Crauste-Manciet, S., Guibert, A., Mourier, W., Guerrauly-Moro, M.-N., Ferrari, S., Jomier, J.-Y., Brossard, D., and Schierl, R. 2013. Environmental and biological monitoring of platinum-containing drugs in two hospital pharmacies using positive air pressure isolators. *Ann. Occup. Hyg.*, 57: 374–383.

Kusnetz, E., and Condon, M. 2003. Acute effects from occupational exposure to antineoplastic drugs in a para-professional health care worker. *Am. J. Ind. Med*. 44: 107–109.

Ladeira, C., Viegas, S., Carolino, E., Gomes, M. C., and Brito, M. 2013. The influence of genetic polymorphisms in XRCC3 and ADH5 genes on the frequency of genotoxicity biomarkers in workers exposed to formaldehyde. *Environ. Mol. Mutagen*. 54: 213–221.

Ladeira, C., Viegas, S., Carolino, E., Prista, J., Gomes, M. C., and Brito, M. 2011. Genotoxicity biomarkers in occupational exposure to formaldehyde—The case of histopathology laboratories. *Mutat. Res*. 721: 15–20.

Laffon, B., Teixeira, J.P., Loureiro, J., Torres, J., Pásaro, E., Méndez, J. and Mayan, O. 2005. Genotoxic effects in a population of nurses handling antineoplastic drugs, and relationship with genetic polymorphisms in DNA repair enzymes. *Am. J. Ind. Med*. 28: 128–136.

Larson, R., Khazaeli, M., and Dillon, H. 2003. A new monitoring method using solid sorbent media for evaluation of airborne cyclophosphamide and other antineoplastic agents. *Appl. Occup. Environ. Hyg*. 18: 120–131.

Mader, R., Kokalj, A., Kratochvil, E., Pilger, A., and Rüdiger, H. W. 2008. Longitudinal biomonitoring of nurses handling antineoplastic drugs. *J. Clin. Nurs*. 18: 263–269.

Mahboob, M., Rahman, M. F., Rekhavedi, P.V., Sailaja, N., Balasubramanyam, A., Prabhakar, P. V., Singh, S. P., Reddy, U. A., Rao, S., and Grover, P. 2012. Monitoring of oxidative stress in nurses occupationally exposed to antineoplastic drugs. *Toxicol. Int*. 19: 20–24.

Maluf, S., and Erdtmann, B. 2000. Follow-up study of the genetic damage in lymphocytes of pharmacists and nurses handling antineoplastic drugs evaluated by cytokinesis-block micronuclei analysis and single cell gel electrophoresis. *Mutat. Res*. 471: 21–27.

Moretti, M., Bonfiglioli, R., Feretti, D., Pavanello, S., Mussi, F., Grollino, M., Villarini, M., Barbieri, A., Ceretti, E., Carrieri, M., Buschini, A., Appolloni, M., Dominici,

L., Sabatini, L., Gelatti, U., Bartolucci, G., Poli, P., Stronati, L., Mastrangelo, G., and Monarca, S. 2011. A study protocol for the evaluation of occupational mutagenic/carcinogenic risks in subjects exposed to antineoplastic drugs: A multicentric project. *BMC Public Health* 11: 195.

NIOSH Alert. 2004. Preventing occupational exposures to antineoplastic and other hazardous drugs in health care settings. Department of Health and Human Services, Centres for Disease Control and Prevention. *Natl. Inst. Occup. Safety Health* 165: 1–50.

Orsière, T., Sari-Minodier, I., Iarmarcovai, G., and Botta, A. 2006. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. *Mutat. Res*. 605: 30–41.

Pilger, A., Kohler, I., Stettner, H., Mader, R.M., Rizovski, B., Terkola, R., Diem, E., Franz-Hainzl, E., Konnaris, C., Valic, E., and Rudiger, H.W. 2000. Long-term monitoring of sister chromatid exchanges and micronucleus frequencies in pharmacy personnel occupationally exposed to cytostatic drugs. *Int. Arch. Occup. Environ. Health* 73: 442–448

Rekhadevi, P. V., Sailaja, N., Chandrasekhar, M., Mahboob, M., Rahman, M. F., and Grover, P. 2007. Genotoxicity assessment in oncology nurses handling anti-neoplastic drugs. *Mutagenesis* 22: 395–401.

Schierl, R., Böhlandt, A., and Nowak, D. 2009. Guidance values for surface monitoring of antineoplastic drugs in German pharmacies. *Ann. Occup. Hyg*. 53: 703–711.

Schmaus, G., Schierl, R., and Funck, S. 2002. Monitoring surface contamination by antineoplastic drugs using gas chromatography-mass spectrometry and voltammetry. *Am. J. Health Syst. Pharm*. 59: 956–961.

Saski, M., Dakeishi, M., Hoshi, S., Ishii, N., and Murata, K. 2008. Assessment of DNA damage in japanese nurses handling antineoplastic drugs by the comet assay. *J. Occup. Health* 50: 7–12.

Sessink, P., and Bos, P. 1999. Drugs Hazardous to healthcare workers—Evaluation of methods for monitoring occupational exposure to cytostatic drugs. *Drug Safety* 20: 347–359.

Sessink, P., Kroese, E. D., van Kranen, H. J., and Bos, R. P. 1995. Cancer risk assessment for health care workers occupationally exposed to cyclophosphamide. *Int. Arch. Occup. Environ. Health* 67: 317–323.

Thiringer, G., Granung, G., Holmén, A., Högstedt, B., Järvholm, B., Jönsson, D., Persson, L., Wahlström, J., and Westin, J. 1991. Comparison of methods for the biomonitoring of nurses handling antitumor drugs. *Scand. J. Work Environ. Health* 17: 133–138.

Villarini, M., Dominici, L., Fatigoni, C., Muzi, G., Monarca, S., and Moretti, M. 2012. Biological effect monitoring in peripheral blood lymphocytes from subjects occupationally exposed to antineoplastic drugs: Assessment of micronuclei frequency. *J. Occup. Health* 54: 405–415.

Villarini, M., Dominici, L., Piccinini, R., Fatigoni, C., Ambrogi, M., Curti, G., Morucci, P., Muzi, G., Monarca, S., and Moretti, M. 2011. Assessment of primary, oxidative and excision repaired DNA damage in hospital personnel handling antineoplastic drugs. *Mutagenesis* 26: 359–369.

Ündeger, Ü., Basaran, N., Kars, A., and Güç, D. 1999. Assessment of DNA damage in nurses handling antineoplastic drugs by the alkaline COMET assay. *Mutat. Res*. 439: 277–285.

Ursini, C., Cavallo, D., Colombi, A., Giglio, M., Marinaccio, A., and Iavicoli, S. 2006. Evaluation of early DNA damage in healthcare workers handling antineoplastic drugs. *Int. Arch. Environ. Health* 80: 134–140.

Yoshida, J., Kosaka, H., Tomioka, K., and Kumagai, S. 2006. Genotoxic risks to nurses from contamination of the work environment with antineoplastics drugs in Japan. *J. Occup. Health* 48: 517–522.

Zietkiewicz, E., Wojda, A., and Witt, M. 2009. Cytogenetic perspective of ageing and longevity in men and women. *J. Appl. Genet*. 50: 261–273.

Chapter 3 - Human nutrition, DNA damage and cancer: a review

C. Ladeira[1,2], M.C. Gomes[3] and M. Brito[1]
[1]Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico De Lisboa, Av. D. João II, Lote 4.01.69, 1900-096 Lisboa, Portugal; carina.ladeira@estesl.ipl.pt
[2]Center for Malaria & Tropical Diseases (CMDT), Centro de Investigação e Estudos em Saúde Pública, Escola Nacional de Saúde Pública, Universidade Nova De Lisboa, Avenida Padre Cruz, 1600-560 Lisboa, Portugal
[3]Faculty of Sciences, University of Lisbon, Campo Grande, DBV, Edifício C2, 1749-016 Lisboa, Portugal

Abstract

Dietary habits are recognized to be an important factor influencing cancer risk and tumour behaviour. Diet can influence cancer development in several ways, as direct action of carcinogens in food can damage DNA, and some diet components (macro or micronutrients) can block or induce enzymes involved in activation or deactivation of carcinogenic substances. Moreover, inadequate intake of some molecules involved in DNA synthesis, repair or methylation can influence mutation rate or changes in the gene expression mechanism. From a mechanistic view of carcinogenesis, food mutagens are classified as genotoxic and non-genotoxic. Genotoxic agents cause DNA damage resulting in gene point mutations, deletions and insertions, recombination, rearrangements and amplifications, as well as chromosomal aberrations. Non-genotoxic agents are less distinctively defined in terms of their modes of action, but they are presumed to indirectly affect cell proliferation as tumours promoters, with or without accompanying chronic cell damage. Other mechanism that diet can influence DNA mutation, and consequently cancer risk, is energy balance and growth rates, since nutrition will influence hormone levels and growth factors that will influence the rate of cell division, cell cycling and consequently influence time for DNA repair and/or replication of DNA lesions. Nutritional genomics studies the functional interaction of food and its components, macro and micronutrients, with genome at molecular, cellular, and systemic level. One of the goals is to identify biomarkers that will provide better guidance on the relation between nutrition and health. Also relevant are the implications of genetic polymorphisms and their role in the interaction between diet, environmental factors, lifestyles, and cancer risk. The recognition of the importance of adequate dietary levels of micronutrients in maintaining genomic stability is very significant because the latter is also affected by inadequate nutrient intake, such as lack of vitamins A, D, E, folate, selenium and others.

Keywords: mutagens, carcinogenesis, genotoxic, antioxidants, diet

N.B. Tomlekova, M.I. Kozgar and M.R. Wani (eds.) Mutagenesis: exploring novel genes and pathways
DOI 10.3920/978-90-8686-787-5_3, © Wageningen Academic Publishers 2014

3.1 Introduction

Mutagens have been identified not only in foods but also in food contaminants. These include toxins produced by fungal pathogens or pesticide/herbicide residues used to produce food in large supply. Dietary deficiencies may also turn out to be mutagenic (Ferguson, 2010). Diet-related mutagenesis plays an etiologic role in chronic diseases, including cardiovascular disease and cancer (Ferguson, 2010; Ferguson and Philpott, 2008).

Three major factors involved in human carcinogenesis are cigarette smoking, infection and inflammation, and nutrition and dietary carcinogens (Sugimura, 2000). From a mechanistic view of carcinogenesis, food mutagens are classified as genotoxic and non-genotoxic agents (Sutandyo, 2010). Genotoxic agents cause genetic alterations related to carcinogenesis and constituents inducing tumour promotion-associated phenomena. Genotoxic agents are clearly defined as causing DNA damage resulting in gene point mutations, deletions and insertions, recombinations, rearrangements and amplifications, as well as chromosomal aberrations (Sugimura, 2000). Non-genotoxic agents are less distinctively defined in terms of their modes of action, but they are presumed to indirectly affect cell proliferation as tumours promoters, with or without accompanying chronic cell damage. These agents are generally macrocomponents, like high fat (Sugimura, 2000; Sutandyo, 2010) or high caloric diets. Many dietary mutagens are DNA reactive, leading to distinct spectra of base-pair substitution mutations and structural chromosome changes (Ferguson, 2010).

The early recognition of the importance of adequate dietary levels of micronutrients in maintaining genomic stability has become more significant since the latter is also affected by inadequate nutrient intake, such as lack of folate and selenium (Ferguson, 2010; Ferguson and Philpott, 2008). It has become increasingly apparent that the long term implications of exposure to dietary mutagens are significantly dependent on the genetic background (Ferguson, 2010).

3.2 Mutagenesis

There is strong evidence that mutations play a causal role in carcinogenesis. It appears that tumour initiation occurs through mutation of somatic cells, whereas later stages may involve other processes, including rapid cell proliferation, gene amplification, and chromosomal rearrangements (Ferguson and Philpott, 2008).

In this respect some important concepts in mutagenesis need to be clarified, namely: mutation, mutagens and antimutagens. Mutation refers to a heritable change in nucleotide sequence or number occurring due to alteration in the sequence of a gene product. The DNA lesions occur spontaneously or may be induced by several physical, chemical or biological agents and can cause permanent alteration in DNA structure, and consequently mutation, which have been implicated in the etiopathology of cancer and other degenerative diseases (Bhattacharya, 2011).

3. Human nutrition, DNA damage and cancer

Mutagens are substances which can induce mutations. Many diverse environmental, industrial, dietary and natural chemicals are capable of inducing mutation and genotoxic effects. The mutagenic effects of genotoxic chemicals are additive, cumulative and sometimes irreversible (Bhattacharya, 2011).

An antimutagen can prevent the transformation of a mutagenic compound into mutagen, inactivate the mutagen, prevent the reaction between mutagen and DNA, or induce the DNA repair mechanisms. The antimutagens can be classified as: desmutagens and bio-antimutagens. Desmutagens are substances, which inactivate the mutagens partially or fully by enzymatic or chemical interaction before the mutagen attacks the genes (apparent antimutagens). Bio-antimutagens suppress the process of DNA lesion after genes are damaged by mutagens. They act on repair and replication processes of the mutagen damaged DNA resulting in a decline in mutation frequency (true antimutagens) (Bhattacharya, 2011).

It is important to understand the significance of dietary sources of human mutation, where and whether this extrapolates to carcinogens (Ferguson, 2010). Reports on the existence of a persuasive correlation between mutagenicity and carcinogenicity triggered the use of this correlation and related mutagenicity tests as a tool for discovering potential environmental mutagens as well as a predictive test for carcinogenesis (Ferguson and Philpott, 2008).

Much of the early work on mutagenicity had considered dietary mutagens from external sources (Brandt and Watson, 2003; Kizil *et al.*, 2011; Srám and Binková, 2000), however Ames (2010), suggested that natural chemicals, present in human diet as complex mixtures, may be a more important source. Examples of components of a natural diet that are DNA-reactive mutagens are: ptaquiloside, pyrrolizidine alkaloids and mutagens formed through cooking or processing, such as heterocyclic amines (HA), polycyclic aromatic hydrocarbons (PAH) and N-nitroso compounds (Ferguson and Philpott, 2008; Sugimura, 2000; Sutandyo, 2010) or through fungal contamination, mycotoxins – aflatoxin B_1 and ochratoxin A (Marin *et al.*, 2013; Sirot *et al.*, 2013; Wang and Groopman, 1999). These are examples of chemical agents that have carcinogenic effects, namely, aflatoxin B_1 is related with hepatocellular carcinoma and HA and PAH induce genotoxic damage or cancer in the gastrointestinal tract of animals receiving these compounds orally (Strickland and Goopman, 1995).

Accumulating literature also suggests endogenously produced oxygen radicals are another important source of mutagenesis. Ames (2010), pointed out that the human diet contains a great variety of natural mutagens and carcinogens, many of which may act through the generation of oxygen radicals. It is also emphasized, the importance of natural antimutagens and anticarcinogens that could be an important part of the body's defence mechanism against these agents (Ferguson, 2010; Ferguson and Philpott, 2008).

It is important to consider the role of inflammation, namely persistent or chronic, for the increasing in mutation rate (Collins and Ferguson, 2004; Ferguson, 2010). Much of these effects are associated with reactive oxygen and nitrogen species produced during the inflammatory

process. Among the affected, the two most important molecules for mutagenesis are lipids and nucleic acids, producing, in the latter, DNA strand breaks, oxidized bases, abasic sites, DNA-DNA intrastrand adducts, and DNA-protein cross-links. The nucleic acid products may also interact with DNA. Thus, dietary influences that enhance chronic inflammation will themselves enhance the rate of mutation (Ferguson, 2010).

Mutagenesis is not the only pathway that links dietary exposures and cancers. There are growing evidences that epigenetic factors including changes in DNA methylation patterns are causing cancer and can be modified by dietary components (Collins and Ferguson, 2004; Sutandyo, 2010).

3.3 Nutritional research and dietary mutagens

For those mutagens which produce a distinctive mutational signature, molecular epidemiology has proved invaluable in tracking their human implications (Ferguson, 2010). Figure 3.1 explains the steps to consider between biomonitoring for specific dietary mutagens exposures and cancer development. The role of molecular markers in the assessment of individual exposure to carcinogenic agents provides specific examples where mutation patterns have been linked to

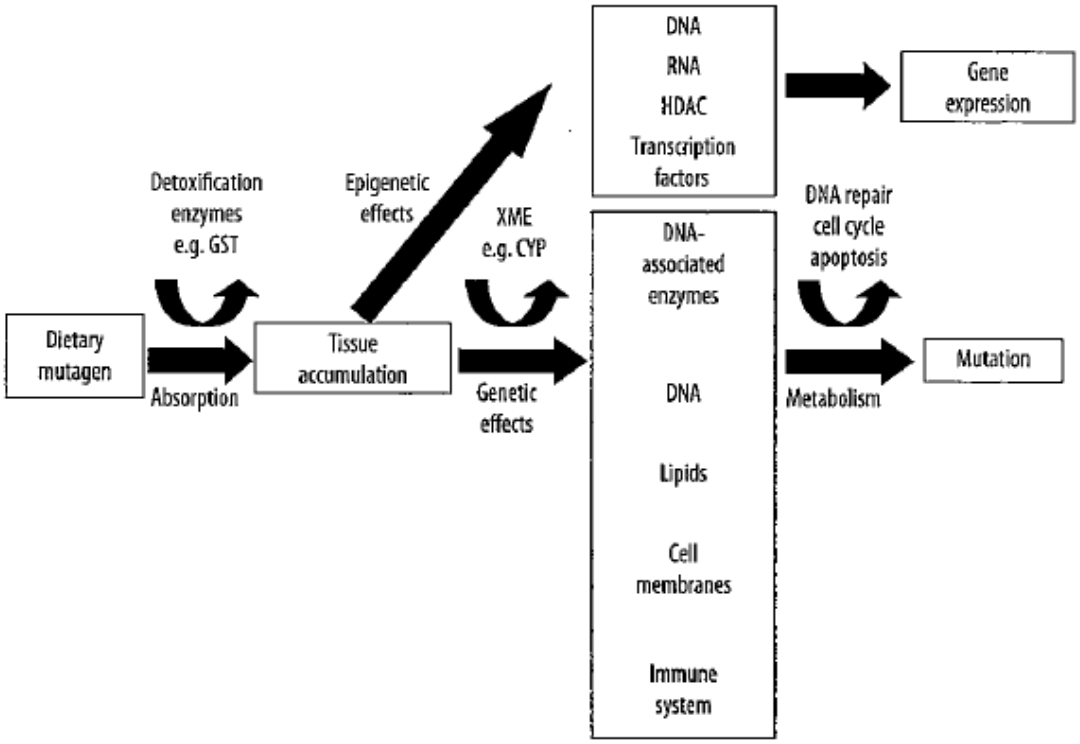


Figure 3.1 Steps to consider in biomonitoring for dietary mutagens. Adapted from Ferguson, 2010. GST = gluthatione-S-transferase; XME = xenobiotic metabolizing enzyme; CYP = cytochrome P450; HDAC = histone deacetylase.

specific carcinogenic exposures. Also relevant are the implications of genetic polymorphisms and their role in the interaction between diet, environmental factors, lifestyles, and cancer risk (Zaridze, 2008).

Dietary habits are recognized to be an important modifiable environmental factor influencing cancer risk and tumour behaviour. Although some studies have estimated that about 30-40% of all cancers are related to dietary habits, the actual percentage is highly dependent on the foods consumed and the specific type of cancer (Davis and Milner, 2007; Strickland and Groopman, 1995; Sutandyo, 2010).

Nutrition science has evolved into a multidisciplinary field that applies molecular biology and integrates individual health with the epidemiologic investigation of population health (Go *et al.*, 2003). Nutritional genomics studies the functional interaction of food and its components, macro and micronutrients, with the genome at the molecular, cellular, and systemic level (Ordovas and Corella, 2004). In nutritional genomics, two terms are used: nutrigenomics and nutrigenetics. Nutritional genomics, defined as the interaction between nutrition and an individual's genome or the response of an individual to different diets, will likely provide important clues about responders and non-responders (Davis and Milner, 2004).

Nutritional genomics provides the means to develop molecular biomarkers of early, pivotal changes between health maintenance and disease progression (Elliot and Ong, 2002), applying systems biology to build models that will integrate information about intake, gene polymorphisms, gene expression, phenotypes, diseases, effect biomarkers and susceptibility biomarkers (Ordovas and Corella, 2004).

A nutritional biomarker can be any biological specimen that indicates the nutritional status with respect to intake or metabolism of dietary constituents. It can be a biochemical, functional or clinical index of status of an essential nutrient or other dietary constituent. Nutritional biomarkers can have three categories depending on their use: (1) a means of validation of dietary instruments; (2) surrogate indicators of dietary intake; or (3) integrated measures of nutritional status for a nutrient (Potischman and Freudenheim, 2003).

Nutrigenetics refers to the impact of genetic variability between individuals in their response to a specific dietary pattern, functional food or supplement for a specific health outcome (Bull and Fenech, 2008). It examines the effect of genetic variation on the interaction between diet and disease. The specific fields of genome-health nutrigenomics and genome-health nutrigenetics are proposed on the premise that a more useful approach to the prevention of diseases caused by genome damage is to take into consideration. Inappropriate nutrient supply can cause sizeable levels of mutation or alter expression of genes required for genome maintenance. Genetic polymorphisms may alter the activity of genes that affect the bioavailability of micronutrients and/or the affinity for micronutrient cofactors in key enzymes involved in DNA metabolism or repair (Bull and Fenech, 2008).

Nutrigenetics attempts to identify and characterize gene variants associated or responsible for differential responses to nutrients. The goal of nutrigenetics is to generate recommendations regarding the risks and benefits of specific diets or dietary components to the individual as a personalized or individualized nutrition (Ordovas and Corella, 2004).

Genetic polymorphisms may be partially responsible for variations in individual response to bioactive food components (Davis and Milner, 2004). Several genetic polymorphisms have been identified like folate metabolism (Carmona *et al.*, 2008; Guerreiro *et al.* 2007), iron homoeostasis, bone health, lipid metabolism, immune function and others (Elliot and Ong, 2002), that can have significant association with nutrients in health/disease outcomes. Some common polymorphisms in genes involved in nutrient metabolism, metabolic activation and/or detoxification could establish the magnitude – whether there is a positive or negative response to a food component (Davis and Milner, 2004). An example is the case of folate metabolism, there are common polymorphisms in genes that control folate metabolism that have been linked to conditions such as neural tube defects, Down's syndrome, homocystineamia and cancer (Elliott and Ong, 2002). If the mechanisms by which these polymorphisms disturb folate mechanism and alter disease risk can be elucidated, it should be possible to develop dietary or therapeutic strategies for 'at risk' individuals to redress the balance. Polymorphisms have also been identified in genes involved in lipid metabolism that are important in determining an individual's plasma low density lipoprotein cholesterol concentration, a marker of cardiovascular disease risk. It is important to consider the logistics and costs of routine genetic screening for many genes, the provision of appropriate counselling, and public attitudes and ethical issues associated with such screening in relation to, say, life insurance and family planning (Elliott and Ong, 2002).

Nutrigenomics focuses on the effect of nutrients on the genome, proteome, metabolome, epigenome and transcriptome. Because it is a complex area of knowledge there are many different definitions regarding this concept (Ordovas and Corella, 2004). The term nutrigenomics emerged from the mapping of the human genome and provides researchers with the tools for using systems biology into exploitation of the relationship between nutrition and health (Go *et al.*, 2003).

An integrated framework that simultaneously examines genetics and associated polymorphisms with diet-related diseases (nutrigenetics), nutrient induced changes in DNA methylation and chromatin alterations (nutritional epigenomics), nutrient induced changes in gene expression (nutritional transcriptomics), and altered formation and/or bioactivation proteins (proteomics) will allow for greater understanding of the interrelationships between diet and cancer risk and tumour behaviour (Davis and Milner, 2004). Since the response to a bioactive food component may be subtle, careful attention will need to be given to characterizing how the quantity and timing of exposure influence small molecular weight cellular constituents (metabolomics). Managing this enormous amount of information will necessitate new and expanded approaches of bioinformatics (Davis and Milner, 2004).

Nutrigenomics will promote and increase understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phases of diet-

related disease and the extent to which individual sensitizing genotypes contribute to such diseases. Eventually, nutrigenomics will lead to evidence-based dietary intervention strategies for restoring health and fitness for preventing diet-related disease (Afman and Müller, 2006).

In short, nutrigenomics is the study of molecular relationships between nutritional stimuli and the response of the genes by application of high-throughput functional genomic technologies in nutrition research. Applied wisely, it will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phase of a diet-related disease, and to what extend individual sensitizing genotypes contribute to such disease (Ordovas and Corella, 2004). Such techniques can facilitate the definition of optimal nutrition at the level of populations, particular groups, and individuals. This in turn should promote the development of new food derived treatments and functionally enhanced foods to improve health (Elliott and Ong, 2002).

3.4 Diet and DNA damage

Dietary patterns involve complex interactions of food and nutrients summarizing the total diet or key aspects of the diet for a population under study. In that sense it is important to focus in each nutrient, but also in the whole diet itself. For instance, several studies have highlighted the protective effect of the so call Mediterranean diet (high consumption of vegetables, legumes, fruits, nuts and minimally processed cereals, and mono-unsaturated lipids, moderately high consumption of fish, low consumption of dairy and meat products and regular but moderate intake of alcohol) in cancer prevention (Couto *et al.*, 2011, 2013).

Of major importance are also epidemiological studies on the role of environmental exposure to carcinogens in diet and specific cancers whose incidence is known to vary considerably among countries (Strickland and Groopman, 1995). The link between diet and cancer is revealed by the large variation in incidence and by the observed changes in incidence in those communities who migrated to a different geographic area and culture (Anand *et al.*, 2008). The substantial increases in the risk of cancers are observed in populations migrating from low- to high-risk areas, suggesting that international differences in cancer incidence can be attributed primarily to environmental or lifestyle factors rather than genetic factors (Anand *et al.*, 2008; Strickland and Groopman, 1995).

Diet can influence cancer development in several ways, namely direct action of carcinogens in food that can damage DNA, diet components (macro or micronutrients) that can block or induce enzymes involved in activation or deactivation of carcinogenic substances (Willett and Giovannucci, 2006). Moreover, inadequate intake of some molecules involved in DNA synthesis, repair or methylation can influence mutation rate or changes in gene expression. Other mechanism whereby diet can influence DNA mutation, and consequently cancer risk, are energy balance and growth rates, since nutrition will influence hormone levels and growth factors that will influence the rate of cell division, cell cycling and consequently influence time for DNA repair

and/or replication of DNA lesions (Willett and Giovannucci, 2006). High levels of insulin like growth factor are associated with some cancers, namely colon cancer (Pollak, 2000).

Several studies support the idea that diet can influence the risk of cancer; however information concerning the precise dietary factor that determines human cancer is an ongoing debate (Ames, 2001; Anand *et al.*, 2008; Couto *et al.*, 2011; Key *et al.*, 2004). A lot of epidemiological studies, involving food frequency questionnaires, have been developed providing important information concerning diet and cancer, however, diet is a complex composite of various nutrients (macro and micronutrients) and non-nutritive food constituents that makes the search for specific factors almost limitless.

The definition of nutrient is variable and continues to evolve. A nutrient is classically defined as a constituent of food necessary for normal physiological function and essential nutrients are those required for optimal health. The postgenomic era classifies nutrient as a 'fully characterized (physical, chemical, physiological) constituent of a diet, natural or designed, that serves as a significant energy yielding substrate or a precursor for the synthesis of macromolecules or of other components needed for normal cell differentiation, growth, renewal, repair, defence and/or maintenance or a required signalling molecule, cofactor or determinant of normal molecular structure/function and/or a promoter of cell and organ integrity' (Go *et al.*, 2003).

3.4.1 Energy balance

Calorie restriction (undernutrition without malnutrition) prevents a variety of cancers in experimental animal model. The influence of calorie restriction on carcinogenesis is effective in several species, for a variety of tumour types, and for both spontaneous tumours and chemically induced cancers (Hursting *et al.*, 2003). In rodents, experiments with caloric restriction showed suppression in the carcinogenic action of diethylnitrosamine (Lagoupolos and Stalder, 1987) and also inhibition of radiation induced cancers (Gross and Dreyfuss, 1990).

The possible mechanisms associating calorie restriction to cancer prevention evolve regulation of cellular proliferation and apoptosis (decrease in DNA replication), reduction in metabolic rate, in oxidative damage and in inflammation mediators (reduction in reactive oxygen species and consequent reduction in DNA damage) (Masoro, 2005). Inversely, the association between obesity (a positive energy balance) and cancer can be partially explained by alterations in hormone levels and metabolism that could influence cell proliferation, differentiation and apoptosis (Willett and Giovannucci, 2006).

Moreover, it is well established that excess calorie intake, resulting in fat deposits, is a risk factor for cancer. Digestion, absorption, metabolism and excretion of excess nutrients require oxidative metabolism and produce more active oxygen species which cause DNA damage (Hwang and Bowen, 2007; Sugimura, 2000; Sutandyo, 2010).

3.4.2 Macronutrients

A nutrient can be defined as a chemical whose absence from the diet results in a specific change in health. Because the body needs large quantities of fat, protein and carbohydrates, these are called macronutrients. Moreover, only these ones are source of energy, which makes it difficult to access their influence in cancer development independently from energy intake. Several case-control studies have demonstrated a higher risk of colorectal cancer with increased total energy intake. Thus, excess intake of any of the important energy-supplying macronutrients in the diet (e.g. protein, fat and carbohydrate) could contribute to a higher risk of colorectal cancer (Sun *et al.*, 2012). Kimura *et al.* (2007), who investigated the associations of different types of meat, fish and individual types of fatty acids with colorectal cancer, showed no clear association between red meat or associated fat intake and colorectal cancer and thus did not provided support for the hypothesis that high consumption of red meat increases the risk of colorectal cancer. However, the amount and the relative proportions of different fats in diet should be taken into account. For instance several works have not found an association between breast cancer risk and fat intake (Holmes *et al.*, 1999). Epidemiological studies suggest that a high omega-3 to omega-6 ratio may be the optimal strategy to decrease breast cancer risk (Lorgeril and Salen, 2012), since omega-3 fatty acids do have anticancer properties. Nevertheless, other epidemiological investigations have suggested that a positive correlation exists between fat intake and incidences of breast, colon and prostate cancers (Sugimura, 2000).

Epidemiological studies have suggested that people who consume diet high in omega-3 fatty acids may experience a lower prevalence of cancer, and many small trials have attempted to assess the effects of omega-3 fatty acid in the diet, either as omega-3 fatty acid-rich foods or as dietary supplements. A systematic review made by MacLean *et al.* (2006) regarding the effects of omega-3 fatty acids on cancer risk, which compiled a large body of literature spanning numerous cohorts from many countries and with different demographic characteristics did not provide evidence of a significant association between omega-3 fatty acids and cancer incidence. The authors concluded that dietary supplementation with omega-3 fatty acids is unlikely to prevent cancer (MacLean *et al.*, 2006).

As for fat, carbohydrate ingestion and cancer development should be analysed taking into account the total amount of carbohydrates and the type of glycids ingested. It is well known that diets rich in refined carbohydrates exacerbate many of the metabolic effects of obesity, namely hypertriglyceridemia, hyperglycaemia and hyperinsulinemia. Many studies associate increased cancer risk with diets rich in starches like sugar and sucrose (Willett and Giovannucci, 2006). In contrast, a diet rich in dietary fibre has been associated with low risk of cancer, since fibre can have a potential to dilute carcinogens, to speed up bulk transition, reducing time for carcinogens absorption, and also serves as a substrate to generate short-chain fatty-acids used by colonic epithelial cells (Kumar *et al.*, 2012). In this sense, a diet with a low proportion of carbohydrates might increase the risk of cancer development (Lagergren *et al.*, 2013).

Concerning protein intake, there is no epidemiological evidence of a clear association between high protein ingestion and risk of cancer. However, if we consider that a high amino acid intake could lead to an enhance energy intake with consequent increase in growth rate a relationship might emerge. The positive association between protein-rich red meat consumption and cancer can have other explanations besides protein intake. For instance there are several N-nitroso mutagenic compounds formed during meat preparation, another possible explanation is that high meat consumption is associated with high proportions of omega-6 fatty acids that are associated with cancer risk as mentioned, also the types of meat consumed appears to influence cancer risk (Yang *et al.*, 2012).

Humans are exposed to N-nitroso compounds in diet from a variety of cured meats and fish products. The occurrence of N-nitroso compounds in food is due to the use of sodium nitrite to colour, flavour and preserve meat and fish (Lijinsky, 1999). N-nitrosamines can be formed *in vivo* during simultaneous ingestion of nitrite or nitrogen oxides and a nitrosatable substrate such as a secondary amine. Dietary N-nitrosamines have been linked to oesophageal and other gastrointestinal cancers (Goldman and Shields, 2003). Regarding mutagens formed through cooking or processing, other than N-nitroso compounds, which are formed endogenously from nitrates in the body reaction with amines, heterocyclic amines (HA) and polycyclic aromatic hydrocarbons (PAH) are cases studies related with high temperature cooking (Collins and Ferguson, 2004; Ferguson, 2002; Ferguson and Philpott, 2008; Goldman and Shields, 2003; Strickland and Goopman, 1995; Sutandyo, 2010). The HA are produced during high-temperature cooking of meat by pyrolysis of proteins, amino acids or creatine and can be present in normal human diet in substantial concentrations depending on cooking habits (Goldman and Shields, 2003; Sugimura, 2000; Sutandyo, 2010). The PAH, namely benzo(a)pyrene (BaP) and related PAH are known products of incomplete combustion processes, although they may be formed directly in well-cooked (broiled, barbecued, or smoked) meats and fish; they also occur commonly as environmental contaminants on food plants, such as cereals and vegetables; and tobacco smoking (Ferguson and Philpott, 2008; Goldman and Shields, 2003; Strickland and Goopman, 1995; Sutandyo, 2010).

3.4.3 Micronutrients

Micronutrients are a set of approximately 40 substances, including vitamins, essential minerals and other compounds required in small amounts for normal metabolism, that are essential for human health (Ames, 1998; Lal and Ames, 2011). Micronutrients are capable of acting via a number of mechanisms to block DNA damage, mutation, and carcinogenesis by oxygen radicals, PAHs and other chemical carcinogens (Collins and Ferguson, 2004; Perera, 1996). Mutations have been related to the deficit, rather than the excess, of micronutrients (Ferguson and Philpott, 2008).

Epidemiological studies performed by the American Institute for Cancer Research/World Cancer Research Fund have also shown that individuals who consume large amounts of fruits and vegetables rich in micronutrients with antioxidant properties (such as vitamin C, vitamin E, carotenoids and flavonoids) show a lower incidence of lung, stomach, oesophagus, breast, colon,

liver, pancreas, endometrium, oral cavity and pharynx cancer (Prado *et al.*, 2010; Sutandyo, 2010), presumably because many micronutrients are required as cofactors in DNA maintenance reactions, including DNA synthesis, DNA repair, DNA methylation and apoptosis (Ferguson, 2002).

Micronutrient deficiency or excess can have modifying effects on genomic integrity that may involve nutrient-nutrient or nutrient-gene interactions and may depend on an individual's genetic constitution (Fenech *et al.*, 2005; Thomas *et al.*, 2011). Therefore, determining the intake levels of micronutrients required to maintain genome stability is an essential step in the definition of optimal diets for the prevention of cancer and other diseases caused by genome damage (Fenech *et al.*, 2005).

Complex gene-environment and nutrient-nutrient interactions are also risk determinants for most disease states. Thus, the individual's genes, environmental exposures and physiological state must all be considered when determining disease risk.

3.5 Antioxidants and chemoprevention

In a biological system, an antioxidant can be defined as any substance which, when present at low concentration in relation to oxidizable substrates, would significantly inhibit or delay oxidative processes, while often being oxidized itself (Wanasundara and Shahidi, 2005; Kumar, 2011). The oxidazable substrate may be any molecule that is found in foods or biological materials, including carbohydrates, DNA, lipids and proteins (Wanasundara and Shahidi, 2005).

Antioxidants delay autoxidation by inhibiting formation of free radicals or by interrupting the propagation of free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation; (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides; (3) quenching O_2^- preventing formation of peroxides; (4) breaking the autoxidative chain reaction; and/or (5) reducing localized O_2 concentration (Brewer, 2011).

Antioxidants defend against both enzymatic and non-enzymatic reactions protecting the body against oxidative damage. Cellular DNA may be protected against oxidation by antioxidants, and oxidised DNA lesions are removed by several repair systems such as base excision repair and nucleotide excision repair that have overlapping specificity and may interact or function as back-up systems (Guarnieri *et al.*, 2008). Antioxidants may be molecules that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized (Lu *et al.*, 2010).

Non-enzymatic antioxidants are frequently added to the food to prevent lipid oxidation. Several lipid antioxidants can exert pro-oxidant effect towards other molecule under certain

circumstances thus antioxidants for food and therapeutic use must be characterized carefully (Kumar, 2011).

Antioxidants can be classified as primary or natural and secondary or synthetic. Natural antioxidants comprise enzymes, such as superoxide dismutase, catalase and glutathione peroxidase; they also include low molecular weight antioxidants, such as lipid and water soluble antioxidants (Hamid *et al.*, 2010; Kumar, 2011; Lu *et al*, 2010).

The antioxidant enzymes are complemented by small-molecule antioxidants, some of which are derived exclusively from diet and are vitamins. These small-molecule antioxidants are extra and intra-cellular, and include ascorbic acid (vitamin C), glutathione, and tocopherols (vitamin E mostly). The mechanisms by which these antioxidants act at the molecular and cellular level include roles in gene expression and regulation, apoptosis, and signal transduction, being thus involved in fundamental metabolic and homeostatic processes (Frei, 1999; Hamid *et al.*, 2010).

Hamid *et al.* (2010) classifies the natural antioxidants in three categories: mineral antioxidants, vitamins and phytochemicals. Mineral antioxidants are co-factor of antioxidant enzymes. Their absence will definitely affect metabolism of many macromolecules such as carbohydrates, and examples include selenium, copper, iron, zinc and manganese. Antioxidant vitamins are needed for most body metabolic functions; they include vitamin B, C and E. Finally, phytochemicals are phenolic compounds that are neither vitamins nor minerals that have become thought of as important in modulating oxidative stress (Cooke *et al.*, 2002; Hamid *et al.*, 2010). These compounds are also termed polyphenols and are grouped under flavones (e.g. chrysin; fruit skin), flavanols (e.g. catechin, epicatechin, epigallocatechin; green and black teas, wine), flavonones (e.g. nariongin and taxifolin; citrus fruits), flavonols (e.g. quercetin; many fruits and vegetables), methylated flavones and O-glycoside derivates of the flavonoids which are the most abundant form of these compounds (Cooke *et al.*, 2002; Hamid *et al.*, 2010).

Phytochemicals may alter multiple molecular targets within a specific biological process, and may exhibit additive or synergistic effects as well as antagonistic interactions. Several studies suggested that the regular consumption of fruits, vegetables, and spices have health benefits including risk reduction of developing cancer; these food items are known to contain phytochemicals such as polyphenols, terpenes, and alkaloids, commonly present in low levels in plants. Flavonoids, for instance, have been reported to possess potential for the prevention of several cancers specially those of the gastrointestinal tract, like oral cavity and colon cancer (Ramos *et al.*, 2011).

Synthetic antioxidants are the most effective and are synthetic chemicals. They are phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions. Examples approved by the US Food and Drug Administration (FDA), are butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT) tertiary butylated hydroxyl quinine (TVHQ), ethoxyquin, galattes and others (Brewer, 2011; Hamid *et al.*, 2010; Kumar, 2011; Wanasundara and Shahidi, 2005). In what concerns food, antioxidants are defined, according to the FDA, as substances used to preserve food by retarding deterioration, rancidity or discoloration due to

oxidation (Kumar, 2011; Wanasundara and Shahidi, 2005). Antioxidants may occur as natural constituents of foods, may intentionally be added, or may be formed during processing. The main purpose of using an antioxidant as a food additive is to maintain food quality and extend its shelf-life rather than improving food quality (Wanasundara and Shahidi, 2005). A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables, tea, and others (Kumar, 2011; Tanaka *et al.*, 2001; Weisburger, 1999).

Chemoprevention is the process of using natural or synthetic compounds to block, reverse, or prevent the development of cancers through the action on multiple cellular mechanisms. Generally, these cellular mechanisms can be grouped in two: (1) anti-mutagenesis, that includes the inhibition of the uptake, formation/activation of carcinogens, their detoxification, the blockage of carcinogen-DNA binding, and the enhancement of fidelity of DNA repair; (2) anti-proliferation/anti-progression, that includes modification of signal transduction pathways, inhibition of oncogene activity, and promotion of the cellular modulation of hormone/growth factor activity (Ramos *et al.*, 2011).

Potential chemopreventive agents are to be found both among nutrients and non-nutrients in diet (Tanaka *et al.*, 2001). Dietary components with potential cancer chemopreventive activity include vitamins, fibre, and minerals. If chemopreventive agents, as supplements, are to be suitable for the large-scale prevention of cancer in the general population, they should have high acceptance, low cost, oral consumability, high efficacy, no or low toxicity, and a known mechanism of action (Bartsch and Gerhäuser, 2009). Promising chemopreventive agents currently investigated in preclinical and clinical studies include naturally occurring anti-inflammatory agents, antiestrogens, micronutrients, phytochemicals, and some synthetic analogues (Banakar *et al.*, 2004; Bartsch and Gerhäuser, 2009).

3.6 Vitamins

The role of vitamins in cancer chemoprevention has been increasingly under scrutiny. Antioxidants (vitamins A, D, E) are known to be reducing agents and these molecules are capable of slowing or preventing the oxidation of other molecules (Awodele *et al.*, 2010).

Vitamins A, D and E belong to the family of fat-soluble vitamins. Their intakes have been associated with reduced risk of several chronic diseases, particularly some cancers and heart diseases. In contrast to water-soluble vitamins, fat-soluble vitamins are stored in the liver and fatty tissues and are only slowly excreted from the body. Thus, they may have deleterious or toxic consequences if consumed at very high levels (Jenab *et al.*, 2009). They are at the end of oxidative chain reactions, removing free radicals and preventing the oxidation of unsaturated fats; and are clearly documented anti-genotoxic and antimutagenic potential antioxidants (Awodele *et al.*, 2010).

3.6.1 Vitamin A and cancer

Retinol (vitamin A) and its metabolites (retinoids) are important micronutrients that regulate many biological processes such as cellular growth and differentiation. The classical mechanism of action by retinoids is through activation of retinoic acid receptors and retinoid X receptors (Fritz *et al.*, 2011; Pasquali *et al.*, 2013). In experimental models, retinoids suppress the transforming effects of carcinogens, inhibit growth of premalignant cells, enhance differentiation of malignant cells and induce apoptosis (Stich *et al.*, 1984; Kristal, 2004).

The term vitamin A (all-*trans* retinol) is often used as a general term for all compounds that exhibit the biological activity of retinol, while the term retinoid refers to both naturally occurring and synthetic compounds bearing a structural resemblance to all-*trans* retinol. Biologically important oxidation products of retinol are retinal and retinoic acids that occur in several isomeric forms such as 11-cis retinal, 9-cis, or all-trans-retinoic acid, but the important biologically active form of vitamin A is retinoic acid (Polidori and Stahl, 2009).

The oxidant activity of retinol and its derivatives is moderate; however, the compound plays a major role in cellular signalling, for example, as a ligand of a family of nuclear receptors involved in the regulation of gene expression. *In vitro* cell culture studies, studies on animal's models, and different types of human studies; all support the idea that carotenoids and vitamin A play a role in the prevention of cancer (Polidori and Stahl, 2009), namely because of the antigenotoxic and anticarcinogenic effects of both (De Flora *et al.*, 1999). Genotoxic effects include a variety of end-points which can be evaluated both *in vitro* and in animal models, such as DNA damage, point mutation of differential specificity, numerical and structural chromosomal alterations and impairment of DNA repair mechanisms (De Flora *et al.*, 1999).

Vitamin A and its derivatives are essential to processes such as vision and cell differentiation, particularly during embryological development, as well as in carcinogenesis, glycoprotein synthesis, epithelial cell integrity, immune cell maintenance and human growth hormone production (Fritz *et al.*, 2011; Jenab *et al.*, 2009; Kristal, 2004; Polidori and Stahl, 2009). Deficiency of this vitamin is associated with night blindness, loss of vision, xeropthalmia, growth retardation, foetal reabsorption, and immunodeficiency (Fritz *et al.*, 2011; Kristal, 2004; Polidori and Stahl, 2009). Almost all epithelial tissues contain receptors for retinoic acid, and a deficiency of vitamin A has consistently been implicated as an important causal factor of cancers in human beings (Zhang *et al.*, 2012). Experiments in animals have also shown that vitamin A deficiency predisposes to the development of squamous intraepithelial lesions (Zhang *et al.*, 2012).

The body obtains vitamin A from two sources: preformed vitamin A (retinol and retinal in the form of retinyl esters), and provitamin A carotenoids (beta-carotene, alpha-carotene, beta-cryptoxanthin) (Fritz *et al.*, 2011; Jenab *et al.*, 2009). Retinol itself is rarely found in foods. Preformed vitamin A is found in cod liver oil, butter, eggs, animal products and fortified grains. Provitamin A carotenoids are found in highly pigmented vegetables such as carrots, squash, tams,

and green leafy vegetables. Once in the body, retinol is ultimately converted into retinoic acid and its isoforms, collectively known as retinoids (Fritz *et al.*, 2011).

In recent years many studies have described a protective role of vitamin A in several diseases related to lung development (e.g. asthma, chronic obstructive pulmonary disease, and parenchymal lung diseases). These studies exploit the ability of vitamin A as a scavenger of toxic metabolites widely known as free radicals (Pasquali *et al.*, 2013).

The World Health Organization (WHO) recommends that adults ingest an average of 5,000 IU/day of vitamin A on their diet. The physiological range of retinol in cells varies between 0.2 and 5 μl. Vitamin A has been recommended in a wide range of doses for treatment of some conditions, mainly in the field of dermatologic disturbances and oncology (infants, children and young adults during leukaemia treatment). Retinoids were claimed to exert important antioxidant functions in biological systems, and this belief stimulated the use of retinoids as antioxidants and nutritional supplements in the prevention and treatment of diverse diseases (Pasquali *et al.*, 2013). Preneoplastic and neoplastic diseases successfully treated with retinoids include oral leukoplasia, cervical dysplasia and xeroderma pigmentosum (premalignant), and acute promyelocitic leukemia. Modest but encouraging results have been found in the treatment of other cancer types including: head and neck cancer, oesophageal, cutaneous T-cell lymphoma, neuroblastoma and mesothelioma (Fritz *et al.*, 2011; Kristal, 2004). However, some studies observed that vitamin A may induce toxic effects to different cell types. Retinol and its derivatives may exert pro-oxidant effects which may cause oxidative damage, cell cycle disruption, and transformation and/or cell death (Pasquali *et al.*, 2013).

Cell culture as well as other *in vitro* assays confirmed that retinoids also presented cytotoxic and/or pro-oxidant effects, causing oxidative damage to biomolecules. The explanation could be the increased lipid peroxidation by retinol, as well as the protein carbonylation, and decreased protein thiol content. Moreover, the activities of antioxidant enzymes, such as catalase and superoxide dismutase were also modulated by retinol. It is known that retinol auto-oxidation *in vitro* increases $O_2^{-\cdot}$ (Pasquali *et al.*, 2013).

The study of Pasquali *et al.* (2013) demonstrate that retinol causes an increase reactive oxygen and nitrogen species (ROS/RNS) production in human lung cancer A549 cells, which leads to NF-kB activation and decreased the receptor for advanced glycation end-products expression, this has recently been considered a key event in lung cancer development and progression; retinol, on the other hand, was previously considered an antioxidant, anticancer agent, but it has been observed to induce deleterious and pro-neoplastic effects. Also the review made by De Flora *et al.* (1999), concluded that the impact of supplementation with vitamin A could vary significantly, ranging from showing benefit to producing small but significant increases in lung cancer incidence amongst high risk individuals such as tobacco smokers and asbestos-exposed workers. This highlights the importance of just proceeds to supplementation under nutritional or medical specialist advice.

Beta-carotene and retinol efficacy trial (CARET) results showed that participants receiving the combination of β-carotene and vitamin A had no chemopreventive benefit and had excess lung cancer incidence and mortality (Omenn *et al.*, 1996). Also the Cheng and Neuhouser (2012) study which investigated the association between vitamin A intake and serum hydroxyvitamin D, showed a limited statistical evidence of the beneficial association of vitamin D with reduced lung cancer mortality, nevertheless this association may be diminished among those who are supplement users with excess circulating vitamin A or vitamin A/β-carotene. The study of Fritz *et al.* (2011) suggested that there is no evidence for an association between treatment and prevention of lung cancer and vitamin A and related retinoids. This study also enlightened important factors regarding daily supplementation of vitamin A, such as the increase of several parameters of oxidative stress in rat lungs. Furthermore, data from clinical trials also indicate an increase in incidence of lung cancer and colorectal cancer in smokers and asbestos-exposed men that receive oral supplementation with vitamin A and/or beta-carotene, the same being true for cardiovascular disease incidence (Pasquali *et al.*, 2013). Results obtained from Klerk *et al.* (1998) investigation suggested that retinol supplementation in subjects exposed to crocidolite (blue asbestos) may reduce the incidence of mesothelioma, however there was a small increase in risk of mesothelioma for those on β-carotene, similar to what was found in the CARET study (Omenn *et al.*, 1996). The study by Miyazaki *et al.* (2012), demonstrated a clear positive association between dietary vitamin A intake and the incidence of gastric cancer in the general Japanese population. However this data should be critically interpreted because such association was enhanced by the positivity for *Helicobacter pylori*, a well-established powerful risk factor for gastric cancer. Although this study also suggested that dietary vitamin A was significantly associated with the incident of gastric cancer, such association was not observed for dietary retinol or carotenoids alone, suggesting that the combination of both may act as a risk factor for gastric cancer.

Miyazaki *et al.* (2012) justified their results by the autoxidation of retinoids, generating free radicals, which play a role in DNA damage, coupled with a higher dietary vitamin A intake promoting mucosal damage in the stomach. It is well known that infection with *H. pylori* also induces DNA damage in gastric mucosal cells, through oxidative stress, acting together in synergy. A study by Park *et al.* (2012) showed that dietary supplementation with vitamin A inhibits colon cancer metastasis to the liver, the major storage site for vitamin A and the target organ for colon cancer metastasis, in a mouse model. Taken together, these data suggest that dietary vitamin A supplementation may prove useful for reducing the number of metastatic tumours that develop, and thus the overall amount of cancerous tissue per liver in patients prone to colorectal cancer metastasis.

Deregulation of retinoid metabolism has been found in several cancers, including the prostate cancer and prostate cancer tissue is known to have a lower concentration of retinoic acid than a normal prostate. Studies of multivitamin supplements, mostly using retinyl palmitate as a source of vitamin A, have failed to find an association with prostate cancer risk (Patterson *et al.*, 1999). Finally, prospective studies based on serum retinol have revealed increased, decreased and no prostate cancer risk associated with higher retinol concentrations. Since serum retinol is homeostatically controlled, it is difficult to interpret these associations as a reflection of dietary

retinol intake (Kristal, 2004). Neither dietary nor supplemented vitamin A intake is related to prostate cancer risk, and there is no evidence that they are useful as chemopreventive agents. Currently available synthetic retinoids will also not be useful as prostate cancer chemopreventive agents due to their high toxicity (Kristal, 2004).

The results from the meta-analysis performed by Zhang *et al.* (2012) indicated that vitamin A intake is inversely associated with risk of cervical cancer; however there was no significant association between blood retinol level and cervical cancer risk.

Administration of topic vitamin A revealed regression and even remission of leukoplakia, a white lesion of the mucosa that does not represent a histological condition but due to the possibility of malignant transformation, these lesions must be assessed and managed closely (Epstein and Gorsky, 1999). It was observed that in hipervitaminosis A, the levels of plasma and tissue retinol do not correlate with the increased intake, suggesting that retinol is converted to several other metabolites when increasing doses are ingested (Pasquali *et al.*, 2013). These data reinforce the importance of keeping retinol status within the normal physiologic range and the importance of carefully observing the outcome of vitamin supplementations in epidemiologic and experimental studies.

3.6.2 Vitamin D, vitamin D receptor and cancer

Biological and epidemiological data suggest that vitamin D levels may influence cancer development. Vitamin D is not a true micronutrient for most mammals, since it is primarily synthesized in skin cells in the presence of adequate sunlight providing UVB. Vitamin D deficiency, and insufficiency, has become a well-recognized problem worldwide (Polidori and Stahl, 2009). Besides its 'classical' role in mediating calcium and phosphate homeostasis, 1,25-dihydroxyvitamin D3 has 'nonclassical' roles that include antiproliferative, antiangiogenic, and prodifferentiating effects in a wide range of tumour cells (Bao *et al.*, 2010; Polidori and Stahl, 2009; Uitterlinden *et al.*, 2004), it can also activate apoptotic pathways and inhibit cell migration, supporting claims of its potential role in cancer prevention and cure (Deeb *et al.*, 2007; Raimondi *et al.*, 2009). These effects are mediated through perturbation of several important cellular signalling pathways.

There are many terms and synonyms used in the description of various compounds referred to broadly as *vitamin D*. In general, the letter D without a numeral modifier is used when a distinction between D_2 and D_3 forms is not necessary. Therefore, the common term vitamin D may be inclusive of all forms of vitamin D, including ingestible forms or serum levels. Vitamin D_2 is ergosterol, vitamin D_3 is calcitriol, 25(OH)D or 25-hydroxyvitamin D are synonymous of 25-(OH) vitamin D (calcidiol) and $1,25(OH)_2D3$ is 1,25(OH)2D3 or $1,25(OH)_2D_3$ (Hines *et al.*, 2010).

$1,25(OH)_2D_3$ is the biologically active form of vitamin D, and it exerts its effects mainly through binding to nuclear vitamin D receptor (VDR) and further binding to specific DNA sequences, namely vitamin D response elements. Through this genomic pathway, $1,25(OH)_2D_3$ exerts

transcriptional activation and repression of targeted genes by binding to the *VDR* (Cui and Rohan, 2006; Polidori and Stahl, 2009). The *VDR* (OMIM 601769) is a crucial mediator for the cellular effects of vitamin D and additionally it interacts with other cell-signalling pathways that influence cancer development (Orlow *et al.*, 2012; Raimondi *et al.*, 2009). It is an intracellular hormone receptor that specifically binds the biologically active form of vitamin D, $1,25(OH)_2D_3$, and interacts with specific nucleotide sequences of targeted genes to produce a variety of biologic effects. The *VDR* gene is located on chromosome 12q12-q14 and several single-nucleotide polymorphisms have been identified that may influence cancer risk (Kalláy *et al.*, 2002; Maruyama *et al.*, 2006; Raimondi *et al.*, 2009; Uitterlinden *et al.*, 2004).

The involvement of *VDR* in multiple pathways and points of convergence within these pathways indicates the potential importance of *VDR* in the aetiology of cancer (Raimondi *et al.*, 2009). Binding of *VDRs* by $1,25(OH)_2D_3$ leads to increased differentiation and apoptosis as well as reduced proliferation, invasiveness, angiogenesis and metastasis (Bao *et al.*, 2010).

A study by Maruyama *et al.* (2006), found that the expression of human *VDR* gene is directly up-regulated by the p53, which confirmed an association between p53 and *VDR* and helps to elucidate one aspect of the biological significance of elevated *VDR* expression.

Numerous studies *in vitro* and *in vivo* have shown proapoptotic and anticancer effects upon biding of $1,25(OH)_2D_3$ to the *VDR* for many types of cancer, namely in cells derived from tumours of the breast, prostate, pancreas, colon, bladder, cervix, thyroid, pituitary, skin (squamous cell carcinoma, basal cell carcinoma and melanoma), glioma, neuroblastoma, leukemia and lymphoma cells (Maruyama *et al.*, 2006; Raimondi *et al.*, 2009). Also *VDR* polymorphisms have been implicated in several immune and inflammatory disorders, including mycobacterial and human immunodeficiency virus susceptibility, diabetes, psoriasis, and Crohn's disease, although the precise mechanisms of action of these diverse disease-related effects remain speculative, such as asthma and atopic risk (Raby *et al.*, 2004).

Normal respiratory epithelial cells have high levels of *VDR*, however, in lung cancer tissues, these components of the vitamin D pathway are suppressed, leading to a decrease in $1,25(OH)_2D_3$, deterring vitamin D's anti-proliferative function. These contrasts between normal lung and malignant cells suggest that vitamin D may be important for maintenance of normal and anti-proliferative functions in the lung (Cheng and Neuhouser, 2012).

The study of Cheng and Neuhouser (2012) concluded that serum 25(OH)D concentrations were inversely associated with lung cancer mortality in non-smokers. The mechanism by which vitamin D reduces lung cancer risk and progression may involve modulating the immune function of lung epithelial cells and inhibiting tumour proliferation, angiogenesis, and metastasis. The study of Bao *et al.* (2010) higher 25(OH)D score was associated with a significantly lower risk of pancreatic cancer.

There are biological reasons to suspect that the active form of vitamin D, may be related to ovarian cancer incidence and mortality. The study by Cook *et al.* (2010) demonstrated absence of a consistent or strong evidence to support the claim made in numerous review articles that vitamin D exposures reduce the risk for ovarian cancer occurrence or mortality. However, Grant (2010) claims the existence of good evidence that solar UVB and vitamin D reduces the risk of ovarian cancer. Grant *et al.* (2013) studied the association of common variants in the *VDR* gene, such as *ApaI, FokI, TaqI* and *BsmI* and risk of ovarian cancer and concluded that such risk may be altered by *VDR* polymorphisms and race. Mostowska *et al.* (2013) indicate that the *VDR B* gene variant might be a moderate risk factor of ovarian cancer development in the Polish population. The study by Edvardsen *et al.* (2011) found no significant associations between vitamin D effective UV radiation dose, or vitamin D intake, or sun-seeking holidays, or use of solarium, or frequency of sunburn, and breast cancer risk. However, Yin *et al.* (2010) reported that serum 25(OH)D levels are inversely associated with breast cancer risk, although the statistical significance remained to be confirmed. $1,25(OH)_2D_3$ exerts anti-cancer effects and effects on calcium homeostasis by binding to *VDR*. The *BsmI* polymorphism present two forms denoted *B* and *b*, and has previously linked to breast cancer risk as the study of Lowe *et al.* (2005) found that women with the *bb* genotype had almost twice the risk of breast cancer compared with those to *BB* genotype. Also, in prostate cancer, the *BsmI VDR* genotype was not associated with prostate cancer risk overall, but when men with low levels of 25(OH)D were analysed separately there was an increased risk of the disease associated with the *bb* genotype (Lowe *et al.*, 2005; Ma *et al.*, 1998). However, the case-control study by Taylor *et al.* (1996) verified that the *VDR* genotype represents an important determinant of prostate cancer risk. Also Correa-Cerro *et al.* (1999) indicated a weak but general role of the *TaqI* polymorphisms in prostate cancer susceptibility. Medeiros *et al.* (2002) concluded that there is a role played by *VDR* polymorphisms in the susceptibility to prostate cancer being cancer susceptibility associated with an interaction between host prediction and exposure, and hypothesized that the contribution of *VDR* genotypes to prostate cancer susceptibility varies among populations and geographic localization.

Orlow *et al.* (2012) found that eight *VDR* single nucleotide polymorphisms, located in the promoter, coding 3' gene regions, were associated with melanoma and confer a modest but statistically significant change in risk of becoming a multiple primary melanoma. The risk increases or decreases, depending on the polymorphisms.

Prospective observational studies showed that higher plasma 25(OH)D levels are associated with a significant reduction in risk of colorectal cancer and, once colorectal cancer is diagnosed, it is associated with improved survival (Bischoff-Ferrari *et al.*, 2006; Ng *et al.*, 2009, Vieth *et al.*, 2007). Kim *et al.* (2001) found evidence that the variant allele *B* of the *BsmI VDR* polymorphism was inversely associated with colorectal adenoma risk. In addition, the data suggested that dietary vitamin D intake modifies the association between *BsmI VDR* genotype and colorectal adenoma risk. Ingles *et al.* (2001), suggested that *VDR FokI* polymorphism influences development of colorectal adenomas and that the effect may be modified by calcium and vitamin D status. However, in relation to *BsmI* polymorphism the authors found no significant association. Reports documented that serum levels of active vitamin D were significantly lower in renal cell carcinoma

of Japanese patients compared to controls. The study of Ikuyama *et al.* (2002) indicated that the *TaqI* polymorphism exhibited a significant association with the risk of developing this type of tumour and is a risk factor for clinical and pathological advanced disease in Japanese.

3.6.3 Vitamin E and cancer

Vitamin E was first described by Evans and Bishop as an essential nutrient for reproduction in rats (Polidori and Stahl, 2009) and is a general term including α-, β-, δ- and γ-forms of the tocopherol and tocotrienol chemical classes (Jenab *et al.*, 2009). Vitamin E has the ability to chemically act as a lipid based (lipoprotein and membranes) free radical chain breaking molecule and to exert its action by protecting the organism against the attack of those radicals. Vitamin E has been shown to influence cellular signalling, enzymatic activity and gene expression.

The claim that vitamin E has, like vitamin A and vitamin D derivatives, cell regulatory properties unrelated to its radical chain breaking potential, is supported by a number of experimental results (Zingg and Azzi, 2004). The most potent form of natural vitamin E, α-tocopherol, is taken up, transported and retained by the body much more efficiently than the other natural and synthetic derivatives. Since they all have equal radical chain breaking properties, it is to date still unexplained why nature specifically selected the α form of tocopherol, and it is an open question whether vitamin E deficiency syndromes could be completely prevented by supplying β-, γ- and δ-tocopherols or tocotrienols. On the one hand α-tocopherol has some specific characteristics, for instance the fully methylated chromanol-head group may be required for optimal interactions with enzymes and/or 'α-tocopherol receptors' (Zingg and Azzi, 2004). On the other hand, the β-, γ- and δ-tocopherols and the tocotrienols may have biological effects that interfere with normal cellular processes, so that they need to be specifically recognized, metabolized by the liver and later eliminated. A unique feature of δ-tocopherol is the location of the reactive -OH group between two methyl groups; after reacting with lipid peroxide the unpaired electron can delocalize over the fully substituted chromanol ring which is known to increase its stability and chemical reactivity (Zingg and Azzi, 2004).

In the alpha-tocopherol beta-carotene cancer prevention study, vitamin E and β-carotene failed to prevent upper aerodigestive tract cancers (Wright *et al.*, 2007). Vitamin E was recognized as possible blocking and suppressing agent for oesophageal cancer on account of its antioxidative function of scavenging electrophiles and inhibiting oxidative DNA damage (Yang *et al.*, 2012). Yang *et al.* (2012) demonstrated that vitamin E and selenium supplementation was time selective in the chemopreventive of N-Nitrosomethylbenzymanine-induced oesophageal carcinogenesis. An early-stage supplementation significantly prevented cancer development, whereas late-stage supplementation did not show a clear benefit. Data present from the animal model provide further experimental support to the hypothesis that the efficacy of cancer chemoprevention by nutrients may time selective during the multistage of carcinogenesis. Tomasetti *et al.* (2004) observed that vitamin E analogue efficiently kills malignant mesothelioma cells and sensitises them to immunologic inducer of apoptosis tumour necrosis factor-related apoptosis-inducing ligand, showing therefore anticancer activity.

Lotan *et al.* (2012) found no preventative effect of selenium or vitamin E, alone or in combination on bladder cancer in men. The SELECT research group had previously reported no reduction in cancer risk following the long-term supplementation with vitamin E or selenium, reporting a non-significant trend for increased prostate cancer risk with vitamin E supplementation (Lippman *et al.*, 2008). Gaziano *et al.* (2009) showed in a long-term trail of male physicians that neither vitamin E nor vitamin C supplementation reduced the risk of prostate cancer or even total cancer, namely colorectal, or other common cancers. Klein *et al.* (2011), reported that men who used vitamin E supplements were at 17% increased risk for cancer as compared to men taking placebo, with the increased risk for developing cancer being seen as soon as 3 years after enrolling in the trial. Beilby *et al.* (2010) also reported null associations between prostate cancer and serum folate, lycopene, β-carotene, retinol and vitamin E. Rodriguez *et al.* (2004) showed lack of support for a strong role of vitamin E in prostate cancer prevention, although a modest protective effect among smokers could not be ruled out.

The Heart Outcomes Prevention Evaluation (HOPE) trial and the HOPE-ongoing outcomes (HOPE-TOO) studies, which administered a daily dose of natural source vitamin E (400IU) and a matching placebo, concluded that in patients with vascular disease or diabetes mellitus, long-term vitamin E supplementation not only does not prevent cancer or major cardiovascular events, but also may increase the risk for heart failure, therefore the investigators recommended that vitamin E supplements should not be used in patients with these diseases (Lonn *et al.*, 2005). Miller III *et al.* (2005) also studied the effect of a high-dosage of vitamin E supplementation and concluded that a high-dosage (≥400 IU/d) vitamin E supplements may increase all-cause mortality and should therefore be avoided.

3.6.4 Other micronutrient by-substances and cancer

Results from some clinical trials raise concern that certain micronutrients could promote growth of pre-existing tumours or precancerous lesions. For example, supplementation with folic acid could possible promote colorectal cancer. An animal study also suggested that folate supplementation at early stage (prior to the existence of preneoplastic lesions) could inhibit colorectal cancer formations; however, supplementation at a later stage could promote carcinogenesis (Cole *et al.*, 2007; Fenech and Ferguson, 2001; Kim, 2004).

Folate is necessary for DNA synthesis, repair and methylation. A low folate concentration has been implicated as a potential promoter of carcinogenesis, for example, in colorectal cancer, lung, breast, pancreatic, gastric, oesophageal and prostate malignancies (Ames, 1998, 2001; Ames and Wakimoto, 2002; Beilby *et al.*, 2010; Ferguson and Philpott, 2008; Sutandyo, 2010). Folate plays a key role in a number of processes related to DNA integrity, such as DNA synthesis and methylation. *In vitro* studies have shown that folic-acid deficiency causes a dose-dependent increase in uracil incorporation into human lymphocyte DNA. Folate administration reduces DNA uracil incorporation and the occurrence of chromosome breaks in human cells (Ames and Wakimoto, 2002; Cooke *et al.*, 2002). Carmona *et al.* (2008) concluded that 5' and 3' UTR thymidylate synthase polymorphisms modulate the risk of colorectal cancer independently of the

intake of methyl group donors. Also, Guerreiro *et al.* (2008) concluded that the risk of colorectal cancer associated with the C677T polymorphism in 5,10-methylenetetrahydrofolate reductase in Portuguese patients depends on the intake of methyl-donor nutrients.

The role of carotenoids as biological antioxidants has been the focus of numerous investigations. The most prominent dietary ones include β-carotene, lycopene, lutein, β-cryptoxanthin, and, α-carotene. Several studies point to carotenoid-rich green leafy vegetables being able to lower cancer risks, and β-carotene in particular is considered to be an important protective ingredient. Beta-carotene has antioxidant properties and may inhibit carcinogenesis by several mechanisms, for example, prevention of DNA damage induced by free radicals, interference with the metabolic activation of carcinogens, or prevention of the binding of carcinogens to DNA (Cooke *et al.*, 2002; Fenech and Ferguson, 2001; Ramos *et al.*, 2011). Beta-carotene given as a supplement, however, increased lung cancer incidence and mortality rates in smokers or was without effect in healthy subjects (Hwang and Bowen, 2007; Ramos *et al.*, 2011).

Lycopene, a predominant carotenoid in tomato, watermelon, guava, papaya, apricots, pink grapefruit, blood oranges and other foods, is a potent antioxidant in chemical reactions. The mechanism of action of lycopene is known to have a protective role in carcinogenesis, especially in prostate cancer (Hamid *et al.*, 2010; Sutandyo, 2010).

Epidemiologic studies have provided evidence that high consumption of tomatoes effectively lowers the risk of ROS-mediated diseases such as cardiovascular diseases and cancer by improving the antioxidant carotenoid reported to be more stable and potent singlet oxygen quenching agent compared to other carotenoids. In addition to its antioxidants properties, lycopene shows an array of biological effects including cardio-protective, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities. The cancer activities of lycopene have been demonstrated both *in vitro* and *in vivo* tumour models (Hamid *et al.*, 2010).

Epidemiological evidence indicates that intake of foods that are naturally rich in vitamin C is associated with reduced risk for cardiovascular, neurodegenerative disease and various cancers but the extent to which vitamin C contributes to this effect remains unclear (Fenech and Ferguson, 2001).

Selenium is a mineral, not an antioxidant nutrient. However, it is an important component of antioxidant enzymes, namely selenoproteins such as glutathione peroxidises, thioredoxin reductases, and selenoprotein P, which contain molecular selenium in the form of selenocysteines within their active centre. They are involved in the defence of reactive oxygen species, which otherwise may cause DNA damage alterations of protein function (Ames, 2001; Hwang and Bowen, 2007). Plant foods like rice and wheat are the major dietary sources of selenium in most countries. Selenium supplementation has moved from the realm of correcting nutritional deficiencies to one of pharmacological intervention, especially in the clinical domain of cancer chemoprevention and in the control of heart failure (Hamid *et al.*, 2010). Dietary selenium significantly inhibits the induction of skin, liver, colon, and mammary tumours in experimental

animals by a number of different carcinogens, as well as the induction of mammary tumours by viruses (Ames, 1983).

Zinc deficiency causes a variety of health effects, namely associated with developmental defects and increased DNA damage rates (Ames, 1998, 2001; Fenech and Ferguson, 2001). Zinc is found in all body tissues, is one of the most abundant intracellular elements and is a component of more than a thousand DNA-binding proteins that contain zinc fingers, as well as copper-zinc superoxide dismutase, the oestrogen receptor and synaptic transmission proteins. Losing the function of zinc-containing DNA-repair enzymes compromises the ability of the cell to repair DNA damage, thus promoting tumorigenesis (Ames, 2006; Ames and Wakimoto, 2002). Functioning of p53, a zinc protein which is mutated in half of human tumours is disrupted on loss of zinc (Ames, 2001). Copper is a co-factor for a number of critical enzymes, including cytochrome c oxidase, Cu/Zn superoxide dismutase, seruloplasmin, tyrosinase, lysyl oxidase, domaine-monooxygenase and peptidyl glycine α-amidating monooxygenase (Fenech and Ferguson, 2001).

The epidemiological data on iron and cancer are mainly limited to studies of iron excess. Excessive iron has long been known to catalyse oxidation *in vitro*. Increased risk of human cancer is associated with excess of iron. The increased risk of hepatic carcinoma in individuals with cirrhosis caused by haemochromatosis indicates a link between iron overload and cancer. Several studies have reported associations between increased levels of iron and colorectal cancer. But iron deficiency, as well as iron excess, leads to oxidative DNA damage (Ames, 2001; Ames and Wakimoto, 2002), namely being associated with diminished immune function and neuromuscular abnormalities (Ames, 2006).

Niacin, or nicotinic acid, is one of the few vitamins that have an intimate role in DNA synthesis, DNA repair and cell death (Ames, 1998; Fenech and Ferguson, 2001; Hageman *et al.*, 1998). Niacin is required as substrate for poly (ADP-ribose) polymerase which is involved in cleavage and re-joining of DNA and telomere length maintenance. The consequence of its deficiency is increased DNA oxidation, DNA breaks, and an elevated chromosome damage rate (Fenech and Ferguson, 2001; Hageman *et al.*, 1998). According to the Expert Group on Vitamins and Minerals (2003), there are no genotoxicity and carcinogenicity data available for nicotinic acid.

3.7 Conclusions and future perspectives

Diet itself is a complex mixture, and the impact of diet on mutagenesis seems to be even more complex. The mutagenic effects of individual diets and dietary components have been studied in wide range of systems and technologies are now becoming available to enable more a comprehensive understanding of the interactions between different dietary components. It will be important to consider the implications of mutagenesis to both population groups and to the individual, therefore when the genotype is taken into account the complexity increases.

Although it is not possible to entirely avoid mutagenic food components or certain dietar regimes, rational developments of antimutagens as chemopreventive agents, coupled wit technologies appropriate to nutrigenomics, lead to an optimistic outlook for the future drawin of personalized nutrition plans, aimed at protecting against diet-related mutagenesis (Ferguso and Philpott, 2008).

Knowledge on nutrition and carcinogenic factors can also contribute to large-scale suppressio of carcinogenesis by spreading the word and the practice of anti-carcinogenesis action. Diet ver much depends on locality, history, race and religion but, at the same time, the food industry ca and should be an effective partner in cancer prevention (Sugimura, 2000).

In the past, DNA interaction has been a central dogma of the field, but it is becoming increasingl clear that other cellular targets and whole systems, such as the immune system, are potentia targets for dietary mutagens (Ferguson, 2010).

Molecular biomonitoring provides an opportunity to address certain issues of exposure, susceptibility, and risk in diet-associated human carcinogenesis (Strickland and Goopman, 1995). Molecular epidemiologic approaches, coupled with bioinformatics, will provide important evidence for the role of specific dietary mutagens in certain human cancers. Optimizing nutritional approaches towards the reduction of mutagenesis will require the innovative application of many of the newer technologies which are becoming available. It is essential not to underestimate their future importance (Ferguson, 2010). Nutrition and food carcinogens continue to be a most challenging subject for research towards cancer control (Sugimura, 2000).

Prevention of DNA damage and/or enhanced DNA repair activity by dietary agents constitutes an important strategy to prevent mutations and consequently inhibit the carcinogenic process (Ramos *et al.*, 2011). With more research, we will develop a better understanding of the mechanisms by which specific micronutrients regulate normal cell function, and how their deficiencies can alter normal metabolism. 'Tuning-up' human metabolism, which varies with genetic constitution and changes with age, could prove to be a simple and inexpensive way to minimize DNA damage, prevent cancer, improve health and prolong a healthy lifespan (Ames and Wakimoto, 2002).

References

Afman, L. and Müller, M., 2006. Nutrigenomics: from molecular nutrition to prevention of disease. Journal of the American Dietetic Association 106: 569-576.

Ames B., 2010. Prevention of mutation, cancer, and other age-associated diseases by optimizing micronutrient intake. Journal of Nucleic Acids 22: 1-11.

Ames, B. and Wakimoto, P., 2002. Are vitamin and mineral deficiencies a major cancer risk? Nature 2: 694-704.

Ames, B., 1983. Dietary carcinogens and anticarcinogens – oxygen radicals and degenerative diseases. Science 221: 1256-1264.

Ames, B., 1998. Micronutrients prevent cancer and delay aging. Toxicology Letters 102/103: 5-18.

Ames, B., 2001. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. Mutation Research 475: 7-20.

Ames, B., 2006. Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage. Proceedings of the National Academy of Sciences of the USA 103: 17589-17594.

Anand, P., Kunnumakara, A., Sundaram, C., Harikumar, K., Tharakan, S., Lai, O., Sung, B. and Aggarwal, B., 2008. Cancer is a preventable disease that requires major lifestyle changes. Pharmaceutical Research 25: 2097-2116.

Awodele, O., Olayemi, S., Alimba, C., Egbejiogu, C. and Akintonwa, A., 2010. Protective effect of vitamin C and or vitamin E on micronuclei induction by rifampicin in mice. Tanzania Journal Health Research 12(2): 1-7.

Banakar, M., Paramasivan, S., Chattopahyay, M., Datta, S., Chakraborty, P., Chatterjee, M., Kannan, K. and Thygarajan, E., 2004. 1α,25-dihydroxyvitamin D_3 prevents DNA damage and restores antioxidant enzymes in rat hepatocarcinogenesis induced by diethylnitrosamine and promoted by Phenobarbital. World Journal Gastroenterology 10: 1268-1275.

Bao, Y., Ng, K., Wolpin, B.B., Michaud, D.S., Giovannucci, E. and Fuchs, C.S., 2010. Predicted vitamin D status and pancreatic cancer risk in two prospective cohort studies. British Journal of Cancer 102: 1422-1427.

Bartsch, H. and Gerhäuser, C., 2009. Molecular mechanisms of cancer induction and chemoprevention. In: Knasmüller, S., DeMarini, D., Johnson, I. and Gerhäuser, C. (eds.) Chemoprevention of cancer and DNA damage by dietary factors. Wiley-Blackwell, Weinheim, Germany, pp. 3-19.

Beilby, J., Ambrosini, G.L., Rossi, E., Klerk, N.K. and Musk, A.W., 2010. Serum levels of folate, lycopene, β-carotene, retinol and vitamin E and prostate cancer. European Journal of Clinical Nutrition 64: 1235-1238.

Bhattacharya, S., 2011. Natural antimutagens: a review. Research Journal of Medicinal Plant 5: 116-126.

Bischoff-Ferrari, H., Giovanucci, E., Willett, W., Dietrich, T. and Dawson-Hughes, B., 2006. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. American Journal Clinical Nutrition 84: 18-28.

Brandt, H. and Watson, W., 2003. Monitoring human occupational and environmental exposures to polycyclic aromatic compounds. The Annals of Occupational Hygiene 47: 349-378.

Brewer, M.S., 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Comprehensive Reviews in Food Science and Food Safety 10: 221-247.

Bull, C. and Fenech, M., 2008. Genome-health nutrigenomics and nutrigenetics: nutritional requirements or 'nutriomes' for chromosomal stability and telomere maintenance at the individual level. Proceedings of the Nutrition Society 67: 146-156.

Carmona, B., Guerreiro, C., Cravo, M., Nobre-Leitão, C. and Brito, M., 2008. 5' and 3' UTR thymidylate synthase polymorphisms modulate the risk of colorectal cancer independently of the intake of methyl group donors. Molecular Medicine Reports 1: 747-752.

Cheng, T. and Neuhouser, M., 2012. Serum 25-hydroxyvitamin D, vitamin A, and lung cancer mortality in the US population: a potential nutrient-nutrient interaction. Cancer Causes Control 23: 1557-1565.

Cole, B.F., Baton, J.A., Sandler, R.S., Haile, R.W., Ahnen, D.J., Bresalier, R.S., McKeown-Eyssen, G., Summers, R.W., Rothstein, R.I., Burke, C.A., Snover, D.C., Church, T.R., Allen, J., Robertson, D., Beck, G., Bond, J., Byers, T., Mandel, J., Mott, L., Pearson, L., Barry, E., Rees, J., Marcon, N., Saibil, F., Ueland, P. and Greenberg, E. for the Polyp Prevention Study Group, 2007. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. Journal of the American Medical Association 297: 2351-2359.

Collins, A. and Ferguson, L., 2004. Nutrition and carcinogens. Mutation Research 551: 1-8.

Cook, L., Neilson, H., Lorenzetti, D. and Lee, R., 2010. A systematic literature review of vitamin D and ovarian cancer. American Journal of Obstetrics Gynecology 203: 70.e1-70.e8.

Cooke, M.S., Evand, M.D., Mistry, N. and Lunec, J., 2002. Role of dietary antioxidants in the prevention of *in vivo* oxidative DNA damage. Nutrition Research Reviews 15: 19-41.

Correa-Cerro, L., Berthon, P., Häussler, J., Bochum, S., Drelon, E., Mangin, P., Fournier, G., Paiss, T., Cussenot, O. and Vogel, W., 1999. Vitamin D receptor polymorphisms as markers in prostate cancer. Human Genetics 105: 281-287.

Couto, E., Boffetta, P., Lagiou, P., Ferrari, P., Buckland, G., Overvad, K., Dahm, C.C., TjØnneland, A., Olsen, A., Clavel-Chapelon, F., Boutron-Ruault, M.C., Cottet, V., Trichopoulos, D., Naska, A., Benetou, V., Kaaks, R., Rohrmann, S., Boeing, H., Von Ruesten, A., Panico, S., Pala, V., Vineis, P., Palli, D., Tumino, R., May, A., Peeters, P.H., Bueno-de-Mesquita, H.B., Büchner, F.L., Lund, E., Skeie, G., Engeset, D., Gonzalez, C.A., Navarro, C., Rodríguez, L., Sánchez, M.J., Amiano, P., Barricarte, A., Hallmans, G., Johansson, I., Manjer, J., Wirfärt, E., Allen, N.E., Crowe, F., Khaw, K.T., Wareham, N., Moskal, A., Slimani, N., Jenab, M., Romaguera, D., Mouw, T., Norat, T., Riboli, E. and Trichopoulou, A., 2011. Mediterranean dietary pattern and cancer risk in the EPIC cohort. British Journal of Cancer 104: 1493-1499.

Couto, E., Sandin, S., Löf, M., Ursin, G. and Adami, H.O., 2013. Mediterranean dietary pattern and risk of breast cancer. PLoS ONE 8: e55374.

Cui, Y. and Rohan, T., 2006. Vitamin D, calcium, and breast cancer risk: a review. Cancer Epidemiology, Biomarkers and Prevention 15: 1427-1437.

Davis, C. and Milner, J., 2004. Frontiers in nutrigenomics, proteomics, metabolomics and cancer prevention. Mutation Research 551: 51-64.

Davis, C. and Milner, J., 2007. Biomarkers for diet and cancer prevention research: potentials and challenges. Acta Pharmacologica Sinica 28: 1262-1273.

De Flora, S., Bagnasco, M. and Vainio, H., 1999. Modulation of genotoxic and related effects by carotenoids and vitamin A in experimental models: mechanistic issues. Mutagenesis 14: 153-172.

Deeb, K., Trump, D. and Johnson, C., 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nature Reviews 7: 684-700.

Edvardsen, K., VeierØod, M., Brustad, M., Braaten, T., Engelsen, O. and Lund, E., 2011. Vitamin D-effective solar UV radiation, dietary vitamin D and breast cancer risk. International Journal of Cancer 128: 1425-1433.

Elliot, R. and Ong, T.J., 2002. Nutritional genomics. British Medical Journal 324: 1438-1442.

Epstein, J. and Gorsky, M., 1999. Topical application of vitamin A to oral leukoplakia. Cancer 86: 921-927.

Fenech, M. and Ferguson, L., 2001. Vitamins/minerals and genomic stability in humans. Mutation Research 475: 1-6.

Fenech, M., Baghurst, P., Luderer, W., Turner, J., Record, S., Ceppi, M. and Bonassi, S., 2005. Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, β-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability – results from a dietary intake and micronucleus index survey in South Australia. Carcinogenesis 26: 991-999.

Ferguson, L. and Philpott, M., 2008. Nutrition and mutagenesis. Annual Review of Nutrition 28: 313-329.

Ferguson, L., 2002. Natural and human-made mutagens and carcinogens in the human diet. Toxicology 181-182: 79-82.

Ferguson, L., 2010. Dietary influences on mutagenesis – where is this field going? Environmental and Molecular Mutagenesis 51: 909-918.

Frei, B., 1999. Molecular and biological mechanisms of antioxidant action. Journal of the Federation of American Societies for Experimental Biology 13: 963-964.

Fritz, H., Kennedy, D., Fergusson, D., Fernandes, R., Doucette, S., Cooley, K., Seely, A., Sagar, S., Wong, R. and Seely, D., 2011. Vitamin A and retinoid derivatives for lung cancer: a systematic review and meta-analysis. PLoS ONE 6: e21107.

Gaziano, J., Glynn, R., Christen, W., Kurth, T., Belanger, C., MacFadyen, J., Bubes, V., Manson, J., Sesso, H. and Buring, J., 2009. Vitamins E and C in the prevention of prostate cancer and total cancer in men – the physicians' health study II randomized controlled trial. Journal of the American Medical Association 301: 52-62.

Go, V., Butrum, R. and Wong, D., 2003. Diet, nutrition, and cancer prevention: the postgenomic era. International Research Conference on Food, Nutrition, and Cancer 133: 3830S-3836S.

Goldman, R. and Shields, P., 2003. Food mutagens. Journal of Nutrition 133: 965S-973S.

Grant, D., Hoyo, C., Akushevich, L., Iversen, E., Whitaker, R., Marks, J., Berchuck, A. and Schildkraut, J., 2013. Vitamin D receptor (VDR) polymorphisms and risk of ovarian cancer in Caucasian and African American women. Gynecologic Oncology 129: 173-178.

Grant, W., 2010. Letters to the editors: Good evidence exists that solar ultraviolet-B and vitamin D reduce the risk of ovarian cancer. American Journal of Obstetrics and Gynecology 203: e10.

Gross, L. and Dreyfuss, Y., 1990. Prevention of spontaneous and radiation-induced tumors in rats by reduction of food intake. Proceedings of the National Academy of Sciences of the USA 87: 6795-6797.

Guarnieri, S., Loft, S., Riso, P., Porrini, M., Risom, L., Poulsen, H., Dragsted, L. and Moller, P., 2008. DNA repair phenotype and dietary antioxidant supplementation. British Journal of Nutrition 99: 1018-1024.

Guerreiro, C., Carmona, B., Gonçalves, S., Carolino, E., Fidalgo, P., Brito, M., Leitão, C. and Cravo, M., 2008. Risk of colorectal cancer associated with the C677T polymorphism in 5,10-methylenetetrahydrofolate reductase in Portuguese patients depends on the intake of methyl-donor nutrients. American Journal of Clinical Nutrition 88: 1413-1418.

Guerreiro, C., Cravo, M., Brito, M., Vidal, P., Fidalgo, P. and Leitão, C., 2007. The D1822V APC polymorphism interacts with fat, calcium, and fiber intakes in modulating the risk of colorectal cancer in Portuguese persons. American Journal of Clinical Nutrition 85: 1592-1597.

Hageman, G., Stierum, R., Van Herwijnen, M., Van der Veer, M. and Kleinjans, J., 1998. Nicotinic acid supplementation: effects on niacin status, cytogenetic damage, and poly(ADP-ribosylation) in lymphocytes of smokers. Nutrition and Cancer 32: 113-120.

Hamid, A., Aiyelaagbe, O., Usman, L., Ameen, O. and Lawal, A., 2010. Antioxidants: its medicinal and pharmacological applications. African Journal of Pure and Applied Chemistry 4: 142-151.

Hines, S.L., Jorn, H.K., Thompson, K.M. and Larson, J.M., 2010. Breast cancer survivors and vitamin D: a review. Nutrition 26: 255-262.

Holmes, M.D., Hunter, D.J., Colditz, G.A., Stampfer, M.J., Hankinson, S.E., Speizer, F.E., Rosner, B. and Willett, W.C., 1999. Association of dietary intake of fat and fatty acids with risk of breast cancer. Journal of the American Medical Association 281: 914-920.

Hursting, S.D., Lavigne, J.A., Berrigan, D., Perkins, S.N. and Barrett, C., 2003. Calorie restriction, aging and cancer prevention: mechanisms of action and applicability to humans. Annual Reviews Medicine 54: 131-152.

Hwang, E.S. and Bowen, P., 2007. DNA damage, a biomarker of carcinogenesis: its measurement and modulation by diet and environment. Critical Reviews in Food Science and Nutrition 47: 27-50.

Ikuyama, T., Hamasaki, T., Inatomi, H., Katoh, T., Muratani, T. and Matsumoto, T., 2002. Association of vitamin D receptor gene polymorphism with renal cell carcinoma in Japanese. Endocrine Journal 49: 433-438.

Ingles, S.A., Wang, J., Coetzee, G.A., Lee, E.R., Frankl, H.D. and Haile, R.W., 2001. Vitamin D receptor polymorphism and risk of colorectal adenomas (United States). Cancer Causes Control 12: 607-614.

Jenab, M., Salvini, S., Van Gils, C.H., Shakya-Shrestha, S., Buijsse, B., Verhagen, H., Touvier, M., Biessy, C., Wallström, P., Bouckaert, K., Lund, E., Wassseth, M., Roswall, N., Joensen, A.M., Linseisen, J., Boeing, H., Vasilopoulou, E., Dilis, V., Sieri, S., Sacerdote, C., Ferrari, P., Manjer, J., Nilsson, S., Welch, A.A., Travis, R., Boutron-Ruault, M.C., Niravong, M., Bueno-de-Mesquita, H.B., Van der Schouw, Y.T., Tormo, M.J., Barricarte, A., Riboli, E., Bingham, S. and Slimani, N., 2009. Dietary intakes of retinol, β-carotene, vitamin D and vitamin E in the European Prospective Investigation into Cancer and Nutrition cohort. European Journal of Clinical Nutrition 63: S150-S178.

Kállay, E., Bareis, P., Bajna, E., Kriwanek, S., Bonner, E., Toyokuni, S. and Cross, H., 2002. Vitamin D receptor activity and prevention of colonic hyperproliferation and oxidative stress. Food and Chemical Toxicology 40: 1191-1196.

Key, T., Schatzkin, A., Willett, W., Allen, N., Spencer, E. and Travis, R., 2004. Diet, nutrition and the prevention of cancer. Public Health Nutrition 7(1A): 187-200.

Kim, H., Newcomb, A., Ulrich, C., Keener, C., Bigler, J., Farin, F., Bostick, R. and Potter, J., 2001. Vitamin D receptor polymorphism and the risk of colorectal adenomas: evidence of interaction with dietary vitamin D and calcium. Cancer Epidemiology, Biomarkers and Prevention 10: 869-874.

Kim, Y.I., 2004. Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. Environmental Molecular Mutagenesis 44: 10-25.

Kimura, Y., Kono, S., Toyomura, K., Nagano, J., Mizoue, T., Moore, M., Mibu, R., Tanaka, M., Kakeji, Y., Maehara, Y., Okamura, T., Ikejiri, K., Futami, K., Yasunami, Y., Maekawa, T., Takenaka, K., Ichimiya, H. and Imaizumi, N., 2007. Meat, fish and fat intake in relation to subsite-specific risk of colorectal cancer: the Fukuoka colorectal cancer study. Cancer Science 98: 590-597.

Kizil, M., Oz, F. and Besler, T., 2011. A review on the formation of carcinogenic/mutagenic heterocyclic aromatic amines. Journal of Food and Processing Technology 2(5): 1-5.

Klein, E.A., Thompson, Jr., I.M., Tangen, C.M., Crowley, J.J., Lucia, M.S., Goodman, P.J., Minasian, L.M., Ford, L.G., Parnes, H.L., Graziano, J.M., Karp, D.D., Lieber, M.M., Walther, P.J., Klotz, L., Parsons, J.K., Chin, J.L., Darke, A.K., Lippman, S.M., Goodman, G.E., Meyskens, Jr., F.L. and Baker, L.H., 2011. Vitamin E intake and prostate cancer risk. Journal of the American Medical Association 306: 1549-1556.

Klerk, N.H., Musk, A.W., Ambrosini, G.L., Eccles, J.L., Hansen, J., Olsen, J., Watts, V.L., Lund, H.G., Pang, S.G., Beilby, J. and Hobbs, M.S.T., 1998. Vitamin A and cancer prevention II: comparison of the effects of retinol and β-carotene. International Journal of Cancer 75: 362-367.

Kristal, A., 2004. Vitamin A, retinoids and carotenoids as chemopreventive agents for prostate cancer. Journal of Urology 171: S54-S58.

Kumar, S., 2011. Free radicals and antioxidants: human and food system. Advances in Applied Science Research 2(1): 129-135.

Kumar, V., Sinha, A.K., Makkar, H.P., De Boeck, G. and Becker, K., 2012. Dietary roles of non-starch polysaccharides in human nutrition: a review. Critical Reviews in Food Science and Nutrition 52: 899-935.

Lagergren, K., Lindam, A. and Lagergren, J., 2013. Dietary proportions of carbohydrates, fat, and protein and risk of oesophageal cancer by histological type. PLoS ONE 8: e54913.

Lagopoulos, L. and Stalder, R., 1987. The influence of food intake on the development of diethylnitrosamine-induced liver tumours in mice. Carcinogenesis 8: 33-37.

Lal, A. and Ames, B., 2011. Association of chromosome damage detected as micronuclei with haematological diseases and micronutrient status. Mutagenesis 26: 57-62.

Lijinsky, W., 1999. N-nitroso compounds in the diet. Mutation Research 443: 129-138.

Lippman, S.M., Klein, E.A., Goodman, P.J., Lucia, M.S., Thompson, I.M., Ford, L.G., Parnes, H.L., Minasian, L.M., Gaziano, J.M., Hartline, J.A., Parksons, J.K., Bearden III, J.D., Crawford, E.D., Goodman, G.E., Claudio, J., Winquist, E., Cook, E.D., Karp, D.D., Walther, P., Lieber, M.M., Kristal, A.R., Darke, A.K., Arnold, K.B., Ganz, P.A., Santella, R.M., Albanes, D., Taylor, P.R., Probdtfield, J.L., Jagpal, T.J., Crowley, J.J., Meyskens, F.L., Baker, Jr., L.H. and Coltman, Jr., C.A., 2008. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the selenium and vitamin E cancer prevention trial (SELECT). Journal of the American Medical Association 301: 39-51.

Lonn, E., Bosh, J., Yusuf, S., Sheridan, P., Pogue, J., Arnold, J., Ross, C., Arnold, A., Sleight, P., Probstfield, J., Dagenais, G., and HOPE and HOPE-TOO Trial Investigators, 2005. Effects of long-term vitamin E supplementation on cardiovascular events and cancer. Journal of the American Medical Association 293(11): 1338-1347.

Lorgeril, M. and Salen, P., 2012. New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. BMC Medicine 21: 10-50.

Lotan, Y., Goodman, P., Youssef, R., Svatek, R., Shariat, S.F., Tangen, C., Thompson, I.M. and Klein, E., 2012. Evaluation of vitamin E and Selenium supplementation for the prevention of bladder cancer in SWOG coordinated SELECT. Journal of Urology 187(4S): e515-e516.

Lowe, L.C., Guy, M., Mansi, J.L., Peckitt, C., Bliss, J., Wilson, R.G. and Colston, K.W., 2005. Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population. European Journal of Cancer 41: 1164-1169.

Lu, J-M., Lin, P., Yao, Q. and Chen, C., 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. Journal Cellular and Molecular Medicine 14: 840-860.

Ma, J., Stampfer, M., Gann, P., Hough, H., Giovannucci, E., Kelsey, K., Hennekens, C. and Hunter, D. 1998. Vitamin D receptor polymorphisms, circulating vitamin D metabolites, and risk of prostate cancer in United States physicians. Cancer Epidemiology, Biomarkers and Prevention 7: 385-390.

MacLean, C., Newberry, S., Mojica, W., Khanna, P., Issa, A., Suttorp, M., Lim, Y.W., Traina, S., Hilton, L., Garland, R. and Morton, S., 2006. Effects of omega-3 fatty acids on cancer risk – a systematic review. Journal of the American Medical Association 295: 403-416.

Marin, S., Ramos, A.J., Cano-Sancho, G. and Sanchis, V., 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology 60: 218-237.

Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., Suzuki, H., Akino, K., One-Toyota, M., Maruyama, Y., Tatsumi, H., Imai, K., Shinomura, Y. and Tokino, T., 2006. Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. Cancer Research 66: 4574-4583.

Masoro, E.J., 2005. Overview of caloric restriction and ageing. Mechanisms of Ageing and Development 126: 913-922.

Medeiros, R., Morais, A., Vasconcelos, A., Costa, S., Pinto, D., Oliveira, J. and Lopes, C., 2002. The role of vitamin D receptor gene polymorphisms in the susceptibility to prostate cancer of a southern European population. Journal of Human Genetics 47: 413-418.

Miller III, E., Pastor-Barriuso, R., Dalal, D., Riemersma, R., Appel, L. and Guallar, E., 2005. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Annals of Internal Medicine 142: 37-46.

Miyazaki, M., Doi, Y., Ikeda, F., Ninomiya, T., Hata, J., Uchida, K., Shirota, T., Matsumoto, T., Iida, M. and Kiyohara, Y., 2012. Dietary vitamin A intake and incidence of gastric cancer in a general Japanese population: the Hisayama study. Gastric Cancer 15: 162-169.

Mostowska, A., Sajdak, S., Pawlik, P., Lianeri, M. and Jagodzinski, P., 2013. Vitamin D receptor gene BsmI, FokI polymorphisms in relation to ovarian cancer risk in the Polish population. Genetic Testing and Molecular Biomarkers 17: 183-187.

Ng, K., Wolpin, B.M., Meyerhardt, J.A., Wu, K., Chan, A.T., Hollis, B.W., Giovannucci, E.L., Stampfer, M.J., Willet, W.C. and Fuchs, C.S., 2009. Prospective study of predictors of vitamin D status and survival in patients with colorectal cancer. British Journal of Cancer 101: 916-923.

Omenn, G., Goodman, G., Thornquist, M., Balmes, J., Cullen, M., Glass, A., Keogh, J., Meyskens Jr.F., Valanis, B., Williams, Jr., J., Barnhart, S., Cherniack, M., Brodkin, C. and Hmmar, S., 1996. Risk factors for lung cancer and for intervention effects in CARET, the Beta-carotene and retinol efficacy trial. Journal of National Cancer Institute 88: 1550-1559.

Ordovas, F. and Corella, D., 2004. Nutritional genomics. Annual Review of Genomics Human Genetics 5: 71-118.

Orlow, I., Roy, P., Reiner, A., Yoo, S., Patel, H., Paine, S., Armstrong, B., Kricker, A., Marrett, L., Millikan, R., Thomas, N., Gruber, S., Anton-Culver, H., Rosso, S., Gallagher, R., Dwyer, T., Kanetsky, P., Busam, K., From, L., Begg, C. and Berwick, M., 2012. Vitamin D receptor polymorphisms in patients with cutaneous melanoma. International Journal of Cancer 130: 405-418.

Park, E., Pinali, D., Lindley, K. and Lane, M., 2012. Hepatic vitamin A preloading reduces colorectal cancer metastatic multiplicity in a mouse xenograft model. Nutrition and Cancer 64: 732-740.

Pasquali, M., Gelain, D, Zeidán-Chuliá, F., Pires, A., Gasparotto, J., Terra, S. and Moreira, J., 2013. Vitamin A (retinol) downregulates the receptor for advanced glycation endproducts (RAGE) by oxidant-dependent activation of p38 MAPK and NF-kB in human lung cancer A549 cells. Cellular Signalling 25: 939-954.

Patterson, R.E., Kristal, A.R. and Neuhouser, M.L., 1999. Vitamin supplements and cancer risk: epidemiologic research and recommendations. In: Bendich, A. and Deckelbaum, R.J. (eds.) Preventive nutrition II. Humana Press, Totowa, New Jersey, NJ, USA, pp. 21-43.

Perera, F.P., 1996. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. Journal of the National Cancer Institute 8: 496-509.

Polidori, M. and Stahl, W., 2009. Carotenoids and vitamin A. In: Knasmüller, S., DeMarini, S., Johnson, I. and Gerhäuser, C. (eds.) Chemoprevention of cancer and DNA damage by dietary factors. Wiley-Blackwell, Weinheim, Germany, chapter 23.

Pollak. M., 2000. Insulin-like growth factor physiology and cancer risk. European Journal of Cancer 36: 1224-1228

Potischman, N. and Freudenheim, J., 2003. Biomarkers of nutritional exposure and nutritional status: an overview. Journal of Nutrition 133: 873S-874S.

Prado, R., Santos, B., Pinto, C., Assis, K., Salvadori, D. and Ladeira, M., 2010. Influence of diet on oxidative DNA damage, uracil misncorporation and DNA repair capability. Mutagenesis 5: 483-487.

Raby, B., Lazarus, R., Silverman, E., Lake, S., Lange, C., Wjst, M. and Weiss, S., 2004. Association of vitamin D receptor gene polymorphisms and childhood and adult asthma. American Journal of Respiratory and Critical Care Medicine 170: 1057-1065.

Raimondi, S., Johansson, H., Maisonneuve, P. and Gandini, S., 2009. Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk. Carcinogenesis 30: 1170-1180.

Ramos, A., Lima, C. and Pereira-Wilson, C., 2011. DNA damage protection and induction of repair by dietary phytochemicals and cancer prevention: what do we know? In: Chen, C. (ed.) Selected topics in DNA repair. InTech, Rijeka, Croatia, pp. 237-270.

Rodriguez, C., Jacobs, E., Mondul, A., Calle, E., McCullough, M. and Thun, M., 2004. Vitamin E supplements and risk of prostate cancer in U.S. men. Cancer Epidemiology, Biomarkers and Prevention 13: 378-382.

Sirot, V., Fremy, J.M. and Leblanc, J.C., 2013. Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food and Chemical Toxicology 52: 1-11.

Srám, R. and Binková, B., 2000. Molecular epidemiology studies on occupational and environmental exposure to mutagens and carcinogens. Environmental Health Perspectives 108: 57-70.

Stich, H., Rosin, M. and Vallejera, M., 1984. Reduction with vitamin A and beta-carotene administration of proportion of micronucleated buccal mucosal cells in Asian betel nut and tobacco chewers. Lancet 232: 1204-1206.

Strickland, P. and Groopman, J., 1995. Biomarkers for assessing environmental exposure to carcinogens in the diet. The American Journal of Clinical Nutrition 61: 710S-720S.

Sugimura, T., 2000. Nutrition and dietary carcinogens. Carcinogens 21: 387-395.

Sun, Z., Liu, L., Wang, P., Roebothan, B., Zhao, J., Dicks, E., Cotterchio, M., Buehler, S., Campbell, P., Mclaughlin, J. and Parfrey, P., 2012. Association of total energy intake and macronutrient consumption with colorectal cancer risk: results from a large population-based case-control study in Newfoundland and Labrador and Ontario, Canada. Nutritional Journal 11: 18.

Sutandyo, N., 2010. Nutritional carcinogenesis. Acta Medica Indonesian Journal Internal Medicine 42: 36-42.

Tanaka, T., Kohno, H. and Mori, H., 2001. Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. Asian Journal of Cancer Prevention 2: 165-177.

Taylor, J., Hirvonen, A., Watson, M., Pittman, G., Mohler, J. and Bell, D., 1996. Association of prostate cancer with vitamin D receptor gene polymorphism. Cancer Research 56: 4108-4110.

Thomas, P., Wu, J., Dhillon, V. and Fenech M., 2011. Effect of dietary intervention on human micronucleus frequency in lymphocytes and buccal cells. Mutagenesis 26: 69-76.

Tomasetti, M., Gellert, N., Procopio, A. and Neuzil, J. 2004. A vitamin E analogue supresses malignant mesothelioma in a preclinical model: a future drug against a fatal neoplastic disease? International Journal of Cancer 109: 641-642.

Uitterlinden, A., Fang, Y., Van Meurs, J., Pols, H. and Van Leeuwen, J., 2004. Genetics and biology of vitamin D receptor polymorphisms. Gene 338: 143-156.

Vieth, R., Bischoff-Ferrari, H., Boucher, B., Dawson-Hughes, B., Garland, C., Heaney, R., Holick, M., Hollis, B., Lamberg-Allardt, C., McGrath, J., Norman, A., Scragg, R., Whiting, S., Willett, W. and Zittermann, A., 2007. The urgent need to recommend an intake of vitamin D that is effective. American Journal of Clinical Nutrition 85: 649-650.

Wanasundara, P. and Shahidi, F., 2005. Antioxidants: science, technology, and applications. In: Shahidi, F. (ed.) Baliley's industrial oil and fat products, Sixth Edition, Vol. 1. Wiley Interscience, Hoboken, NJ, USA, pp. 431-489.

Wang, J.S. and Groopman, J.D., 1999. DNA damage by mycotoxins. *Mutat Res.* 424: 167-181.

Weisburger, J., 1999. Mechanisms of action of antioxidants as exemplified in vegetables, tomatoes and tea. Food and Chemical Toxicology 37: 943-948.

Willett, W. and Giovannucci, E., 2006. Epidemiology of diet and cancer risk. In: Shils, M., Shike, M., Ross, A., Caballer, B. and Cousins, R. (eds.) Modern nutrition in health and disease, 10th ed. Lippincott Williams and Wilkins, Philadelphia, PA, USA, pp. 1267-1269.

Wright, M.E., Virtamo, J., Hartman, A.M., Pietinen, P., Edwards, B.K., Taylor, P.R., Huttunen J.K. and Albanes, D., 2007. Effects of alpha-tocopherol and beta-carotene supplementation on upper aerodigestive tract cancers in a large, randomized controlled trial. Cancer 109: 891-898.

Yang, H., Jia, X., Chen, X., Yang, C.S. and Li, N., 2012. Time-selective chemoprevention of vitamin E and selenium on esophageal carcinogenesis in rats: the possible role of nuclear factor kappa B signalling pathway. International Journal of Cancer 131: 1517-1527.

Yang, W.S., Wong, M.Y., Vogtmann, E., Tang, R.Q., Xie, L., Yang, Y.S., Wu, Q.J., Zhang, W. and Xiang, Y.B., 2012. Meat consumption and risk of lung cancer: evidence from observational studies. Annals of Oncology 23: 3163-3170.

Yin, L., Grandi, N., Raum, E., Haug, U., Arndt, V. and Brenner, H., 2010. Meta-analysis: serum vitamin D and breast cancer risk. European Journal of Cancer 46: 2196-2205.

Zaridze, D., 2008. Molecular epidemiology of cancer. Biochemistry 73: 532-542.

Zhang, X., Dai, B., Zhang, B. and Wang, Z., 2012. Vitamin A and risk of cervical cancer: a meta-analysis. Gynaecologic Oncology 124: 366-373.

Zingg, J.M. and Azzi, A., 2004. Non-antioxidant activities of vitamin E. Current Medical Chemistry 11: 1113-1133.