Biomarkers of environmental exposure: genetic and epigenetic approaches

Chiara Scoccianti
Imperial College London
Epidemiology and Public Health Department
Thesis submitted for PhD degree

October 2011

Contents

List of abbreviations

ADC	Adenocarcinoma
ATP	Adenosine triphosphate
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CI	Confidence Interval
CpG	Cytosine-phosphate-guanine
DF	Disease free
dHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxyribonucleotidephosphates
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor gene
EPIC	European Prospective Investigation into Cancer and Nutrition
EUELC	European early lung cancer project
FDA	Food and drug administration
FFQ	Food frequency questionnaire
HDAC	Histone deacetylase
HR	Hazard ratio
IARC	International agency for research on cancer
IQR	Interquartile Range
ITC	Isothiocyanate
KRAS	Kirsten ras gene
LC	Large cell carcinoma
LINE-1	Long interspersed nuclear element 1
ME-PCR	Mutant enrichment PCR
MLH1	MutL homolog 1
MTHFR	Methylenetetrahydrofolate reductase
NF-kB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance

Nrf2	Nuclear factor E2-related factor 2
NSCLC	Non-small-cell lung cancer
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PD	Progressive disease
RASSF1A	Ras association domain-containing protein 1 isoform A
RFLP	Restriction fragment length polymorphism
SAH	S-adenosylhomocysteine
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SD	Standard Deviation
SFN-Lys	Sulforaphane lysine
SNP	Single nucleotide polymorphism
TEAA	Triethylammonium acetate
TKI	Tyrosine Kinase Inhibitor
TNM	International classification of malignant tumours • T: size of the tumour and presence of invasion to nearby tissue • N: presence of regional lymph nodes • M: presence of distant metastasis
TP53	Tumour protein 53 gene

Figures

Tables

List of publications arising from this thesis

Scoccianti C, Ricceri F, Ferrari P, Cuenin C, Sacerdote C, Polidoro S, Jenab M, Hainaut P, Vineis P, Herceg Z. **Methylation patterns in sentinel genes in peripheral blood cells of heavy smokers: Influence of cruciferous vegetables in an intervention study.** Epigenetics 2011; 1: 6(9).

Scoccianti C, Vesin A, Martel G, Olivier M, Brambilla E, Timsit JF, the EUELC Collaborators, Brambilla C, Field JK, Hainaut P. **Prevalence and prognostic value of TP53, KRAS and EGFR mutations in the EUELC cohort.** European Respiratory Journal. Peer-reviewed.

I have performed all laboratory analyses, participated in managing collaboration and produced data interpretation. Prof. Vineis, Dr. Hainaut, Dr. Herceg and Prof. Field have designed the studies presented in this Thesis. I acknowledge strong contribution in producing statistical data by Dr. Ricceri and Mr. Vesin. I express my gratitude to Miss Martel and Mr Cuenin who taught me the techniques used in this Thesis. I wish to sincerely thank my supervisors Prof. Paolo Vineis and Dr. Pierre Hainaut for all the support and guidance received during my studies at Imperial College and at the International Agency for Research on Cancer.
I dedicate this work to the endless love and enthusiasm of my family and children.

Abstract

Exposure assessment in cancer epidemiological studies relies on measurable intermediate molecular biomarkers with high sensitivity and specificity in order to prevent common problems due to misclassification of exposure. Studies on the early stages of carcinogenesis have helped to identify molecular changes that are detectable in pre-cancerous lesions and that are thought to occur as the result of specific exposures such as tobacco smoking. More recently, *in vitro* evidence started to support the potential cancer-protective role of various micro-nutrients acting through epigenetic and genetic mechanisms. Somatic mutations in "master" cancer genes and modifications of epigenetic patterns in the promoter region of specific genes involved in cell cycle, apoptosis or DNA repair may prove good candidates of carcinogenic and dietary exposure even if the evidence that these changes may be present and detectable in "normal" tissue are still scarce (due in part to the practical and ethical difficulty to conduct experimental prospective studies in healthy individuals).

In this thesis, I have developed two projects exploring the application of *TP53, KRAS, EGFR* mutations and of DNA methylation changes as biomarkers of exposure to tobacco smoking, in experimental and observational study designs. Somatic mutations were analysed by dHPLC, ME-PCR, RFLP and sequencing and DNA-methylation analysis was performed by pyrosequencing. Moreover, somatic mutations were analysed in a prospective context of lung cancer recurrence; also the capacity of dietary polyphenols and isothiocyanates to modify methylation patterns in smokers was assessed in an intervention trial.

The results show that somatic mutations are good markers of different forms of tobacco-related lung cancers but have limited short-term prognostic value, with the exception of *KRAS* mutations in adenocarcinoma. Methylation data suggested that a specific short-term dietary intervention may stabilize global epigenetic (LINE1 DNA methylation) patterns in peripheral white blood cells.

Chapter I: Introduction

Biomarkers in the molecular epidemiology of cancer

The most important incentive for physicians and research scientists in the field of cancer research is to detect cancer at an early stage, before it spreads and becomes incurable. One of the most effective ways to achieve this goal is to identify environmental and lifestyle factors that increase or reduce cancer risk, as these factors will be the milestones on which prevention strategies can be built.
The field of molecular epidemiology integrates molecular biology techniques into epidemiologic studies, with the aim of providing new insights into the distribution, causes and mechanisms of diseases across human populations. The term was first popularized in the context of infectious diseases, and was applied to cancer research in the early 1980s, thus giving birth to the field of *molecular cancer epidemiology*. Molecular cancer epidemiology aims to incorporate molecular biomarkers into epidemiology in order to reveal mechanisms and pathways that occur between initial exposure and the development of a characterized disease (Perera and Weinstein 1982). The discovery of biomarkers that reflect exposure to a carcinogen and/or its effect is of key interest, since cancer takes many years to develop (a latency of 10–40 years between first exposure and clinical diagnosis is commonly observed), thus offering a long temporal window for preventive intervention. The biological interactions between different types of carcinogens, e.g. initiators or promoters, as well as their interactions over time have been well characterised *in vitro* and in animal models and there is now the need to translate these findings into the clinic.

Biomarkers are commonly defined as biological measures of cellular, biochemical or molecular alteration in a biological sample (such as human tissue, cell or fluid), with the ability to predict the risk of human disease (Shulte and Perera 1993; Rothman et al. 1995). A classic example of biomarkers study is that from

MacMahon and colleagues on the geographical correlation between urinary estrogens concentration and breast cancer (MacMahon et al. 1982). This study provided support for the hypothesis that estrogens are important in breast cancer aetiology. Another essential contribution to the field of molecular cancer epidemiology was to uncover the link between aflatoxin and initiation/progression of hepatocellular carcinoma, in particular in the presence of hepatitis B virus infection (Wild et al. 1993). This important discovery allowed the establishment of prevention strategies for aflatoxin exposure in low resource countries where the toxin was ingested daily through the diet. The validation of urinary adducts as biomarker of exposure to aflatoxin (IARC 2002) was made possible by several considerations: (i) the strong potency of aflatoxin as human carcinogen, (ii) the availability of a relatively specific and sensitive biomarker highly correlated with biologically effective dose and (iii) the availability in several populations of individuals with very different exposure levels and patterns.

Biomarkers for clinical use

In many instances, a single biomarker lacks the sensitivity and/or specificity to support unambiguous detection or monitoring of a cancer disease. Exceptions include α-fetoprotein (AFP) levels that can be used for diagnosis, staging and risk assessment of testicular teratoma (Diamandis et al. 2002). This serum marker is also used for the detection and diagnosis of liver cancer, although high specificity is only achieved at high levels of plasma AFP that are detected in only a fraction of cancer patients (Luo et al. 2010).

Cancer biomarkers may also be useful to distinguish patients with respect to clinical outcome ahead of a drug treatment. This capacity characterizes the predictive value of a biomarker. The introduction into clinical practice of screening breast cancer for oestrogen receptor positive status has represented a major achievement, since those patients (which represent around 70% of breast cancer patients) have a favourable prognosis, and more importantly, may better benefit

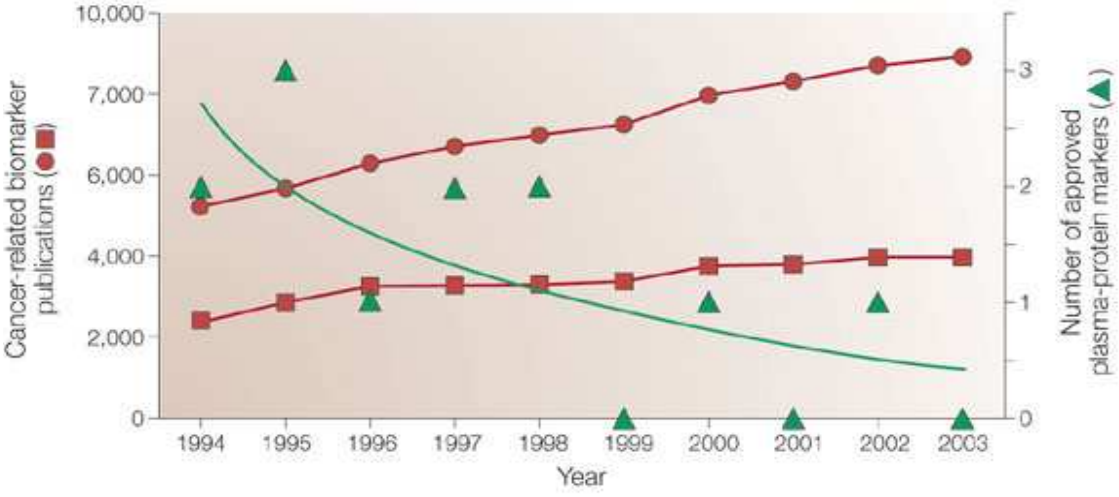
from endocrine treatment (McGuire et al., 1997; Harris et al. 2007). Nowadays, virtually all clinical trials have introduced oestrogen and progesterone receptors testing to distinguish between groups of breast cancer patients with different responses to therapy and outcomes. Furthermore, rapid developments in the identification of biomarkers distinguishing between different molecular phenotypes of breast cancer provide valuable tools for assigning patients to specific treatment protocols.

One of the most spectacular developments in using molecular cancer biomarkers in clinical practice is the identification of specific oncogene mutations that generate constitutively activated enzymes, which can be blocked by specific pharmaceutical drugs. After the seminal example of Imatinib (Gleevec), which blocks the activated c-KIT tyrosine kinase oncogene in some forms of leukaemia and in gastro-intestinal stromal tumours, the “mutation biomarker/small drug” paradigm is now applied with success to the treatment of lung adenocarcinomas, in which activating mutations in the *EGFR* gene encoding the epidermal growth factor receptor are common (in particular in never-smoking women). Patients with this mutation have been shown to have excellent clinical response to treatment by tyrosine kinase inhibitors Gefitinib (Iressa) or Erlotinib (Tarceva). In the past 5 years, a number of other “-inibs” (enzyme inhibitors) have been phased into clinical trials to target specific molecular end-points in many different types of cancers.

There exist many forms of tumour markers, such as hormones, enzymes, receptors, genetic mutations, amplifications or translocation, to evaluate “normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Working Group 2001). The main difficulty in using biomarkers is to set up the conditions for their objective measure and evaluation. In order to measure them both easily and reliably, assays must provide high analytical and diagnostic sensitivity and specificity (Kulasingam and Diamandis 2008).

This promise is offered by recent analytical technologies (e.g. gene-expression profiling or protein arrays), which have significantly increased the number of candidate DNA, RNA and protein biomarkers. Despite the advances in molecular biology, there is still a considerable gap in translating these bench results into bedside applications. Paraphrasing Taylor Coleridge, “there is water everywhere but still little to drink”; in fact, even if the emerging biomarkers proposed are countless, very few of them have been taken through the extensive validation pipe-line required for their routine usage in clinical cancer care or in prevention. Few biomarkers have been clinically approved by the US Food and Drug Administration (FDA) and even less have been integrated into the clinical practice. Figure 1 compares Medline publications under the keyword “cancer biomarker” with the number of FDA-approved plasma protein markers per year from 1994 up to 2003 (Ludwig and Weinstein 2005). While the number of publications on biomarkers (and hence the number of candidate biomarkers) has steadily increased, the number of biomarkers that went through full validation has decreased. This comparison shows a gap between biomarker research and clinical application. The main reason for this gap is the extensive workload and costs associated with validation, which can sometimes discourage investment at the level needed to fully evaluate a biomarker. Yet, in laboratory practice, researchers are continuously confronted with the common drawback of lack of appropriate biomarker validation. Lack of validation often appears when trying to reproduce data from a published study; this could be either due to a lack of robust validation in the original study, or to poor sensitivity and specificity of the biomarkers identified, or, in the worst case, to over-optimistic presentation and interpretation of initial data. As a result, it is often extremely difficult to validate data and to repeat a study. Robust biomarkers and a low inter-operator and inter-institution variability are eventually attained by setting, whenever possible, large collaborative studies, since they enable the creation of laboratory standards and the screening of a larger panel of samples (thus also increasing statistical power).

Figure 1: Medline publications (with "biomarker" as heading, red squares; as text word, red circles) and number of FDA-approved markers per year (green triangles)



From Ludwig et al. 2005

Biomarker families

There are many different ways of classifying biomarkers. In molecular epidemiology, the most accepted way is to distinguish between three broad families of biomarkers (i.e. biomarkers of exposure, of effect and of susceptibility), according to their contribution to the suspected chain of causality linking environmental exposure to a disease end-point.

For IARC to classify an agent as *carcinogenic to humans* both exposures *in vivo* (assessed on the basis of animal and human studies) and biological mechanistic evidences are combined (IARC 2006). In fact, if the same biological response occurs across species, there is higher probability that we are observing an appropriate biomarker. Common examples of carcinogenic exposure that are validated with reliable biomarkers are dietary toxins (e.g. aflatoxin B1), chemical and physical carcinogens (e.g. UV), tobacco smoking, and alcohol beverages.

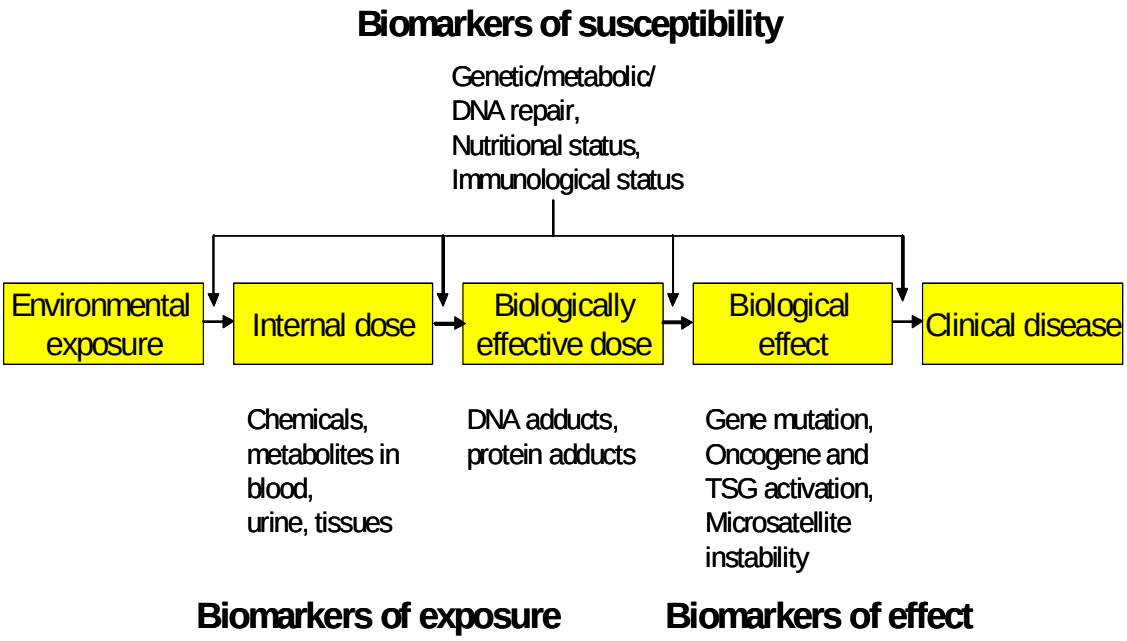
The carcinogenesis from exposure to cancer is a complicated picture where multiple molecular and cellular events take place over a long period of time, influencing each other and ultimately transforming a normal cell into a malignant, neoplastic one. During this process, the cellular interaction with chemical or physical carcinogens commonly leads to “initiation”, i.e. the acquisition of diverse genetic and epigenetic alterations which somehow “prime” a target cell to become cancerous. Further to initiation, a phase of “promotion” is required for the clonal expansion of initiated cells; promotion is often defined as a reversible and/or preventable event. Promotion then leads to irreversible “progression”, which characterizes the evolution from benign to fully malignant, invasive lesions. This general model, although largely questioned by many recent developments in molecular cancer biology, still provides an elegant framework for identifying biomarkers associated with different stages of carcinogenesis.

At least theoretically, at each step of the process it would be possible to define a biomarker; either to assess exposure to potential environmental hazards, to gain insight into disease mechanisms by describing early changes, to express epiphenomena of preclinical disease or to understand acquired or inherited susceptibility (Perera and Weinstein 2000; Vineis and Perera 2007). During initiation, promotion or progression, different molecules or events may accumulate in tissues or in biological fluids. Their effect may simply reflect exposure or ongoing physiopathological changes without providing direct evidence of detriment to survival and good health, or may prove to be associated to the future or current, sub-clinical development of a disease. In the latter case, biomarkers may provide a pre-clinical application and link a genotype to a phenotype.

Biomarkers are commonly measured in easily accessible surrogate tissues, e.g. urine or blood samples, and are broadly divided into three classes or “families” (Figure 2). The classification is temporal and assumes that the carcinogenic process is a continuum where one step leads to the following one. In reality

sometimes the classification overlaps, e.g., DNA adducts that are used as biomarkers of exposure may also imply a biological effect since failure to repair DNA adducts may lead to mutations in genes that “drive” carcinogenesis.

Figure 2: **Examples of biomarkers of exposure, of effect and of susceptibility**



In general, *biomarkers of exposure* are those biomarkers that can inform on the nature of the process by which environmental factors have influenced or caused carcinogenesis. In many instances, biomarkers of exposures are those that can be detected in the early stages of the process. These biomarkers are preferably specific to a particular chemical of exposure. Examples are biomarkers of internal dose and of biologically effective dose of the exposure compound.

Biomarkers of effect reflect the biological effect that follows the initial exposure. Examples are biomarkers of early biological effect, such as alterations in liver enzyme levels and activity in subjects at high risk of chronic liver disease and liver cancer. They also include anatomo-pathological markers of precursor diseases such as metaplastic, hyperplastic or other atypical tissue lesions.

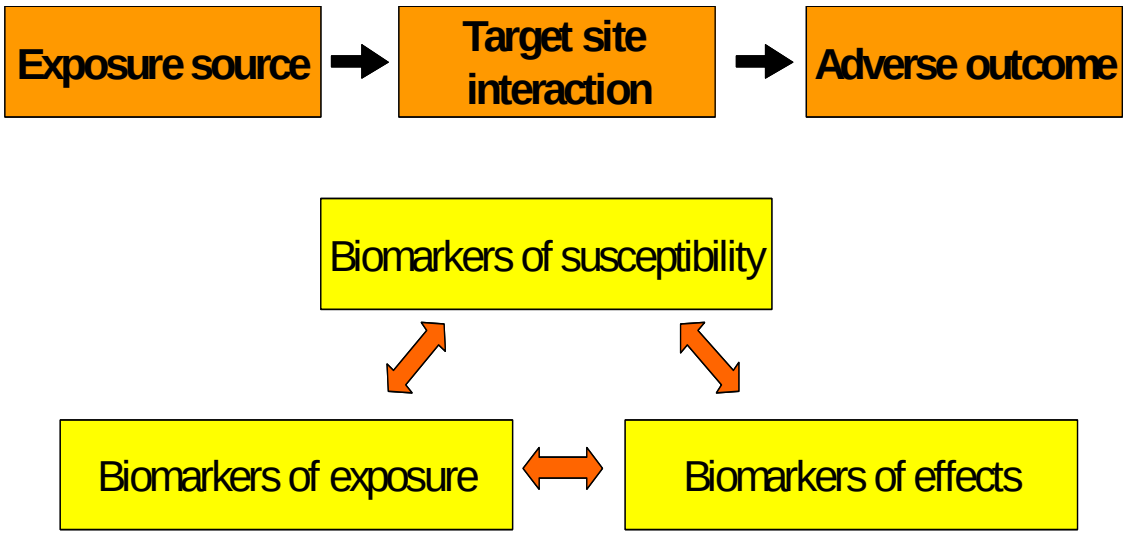
Biomarkers of effect may be non-specific to the carcinogenic agent but, compared to biomarkers of exposure, they may better reflect complex exposures and cumulative exposures over time.

Biomarkers of susceptibility indicate the often constitutive ability of an individual to respond to a given exposure. Biomarkers of susceptibility may include gene loci associated to risk for a particular type of cancer such as lung cancer (Brennan et al. 2011), as well as polymorphisms of specific genes associated with the metabolism of a compound that eventually alter the risk of cancer (Boccia et al. 2009). Inherited genetic differences in metabolism are generally small at the individual level, due to the sequential involvement of many different enzymes in any metabolic pathway and to their redundancy. However, they may become very significant at population level, and may result in different effects for the same exposure across entire groups or populations. Many single nucleotide polymorphisms (SNPs) may alter the expression or activity of a gene product and may modulate the body response to a toxicant. Examples of biomarkers of susceptibility are enzymes responsible for the metabolism of xenobiotics and for DNA repair.

As in the case of biomarkers of exposure, the use of biomarkers of effect to measure a disease outcome (typically cancer) in an epidemiological study may increase the specificity and the sensitivity in defining the outcome. For example, microarray-based techniques, used to measure the expression of a large number of genes, have led to the discovery that breast cancers may show profoundly different patterns of genetic expression, allowing their classification into up to six sub-groups not easily distinguished on the basis of histopathological features. These sub-classifications are now proving to be extremely helpful when assigning patients to specific treatment regimens.
The proposed concept of “biomarkers families” suggests that if a member of a family (i.e. category of biomarkers) is established as measurement of risk, then

other members too, as well as “relatives”, could be candidates as reliable biomarkers, as suggested in Figure 3.

Figure 3: Theoretical correlation among the main biomarkers families



Biomarkers of exposure, of effect and of susceptibility are all *intermediate biomarkers* whose aim is to describe the endo-phenotype that develops during the pathogenesis of environment-related diseases.

In particular, *markers of internal dose* measure the amount of compound (or its metabolites) that the individual receives during exposure (Wild et al. 1990). Such markers may give additional information on the compound itself, such as revealing metabolites of other sources of exposure (including endogenous exposure), or genetic polymorphisms for metabolic enzymes. The product of interaction of a compound (or a class of compounds, such as polycyclic aromatic hydrocarbons) with its site of toxicological action, e.g. DNA adducts or protein adducts, is a marker of the compound’s *biologically effective dose* (sometimes called *tissue dose*) (Denissenko et al. 1996; Jarabek et al. 2009). These markers include types of DNA damage that directly reflect exposure to genotoxic carcinogens. In the carcinogenesis process they immediately precede the development of *biomarkers of altered structure/function* such as somatic

mutations, gene-promoter methylation and modifications of chromatin structure (Downs 2007).

Biomarkers of effect may be extremely useful in understanding pathways and mechanisms of carcinogenicity in relation to exposure. A common example is the analysis of *TP53* mutations in lung cancer in relation to smoking status (Hainaut and Pfeifer 2001) since the pattern of mutations in lung cancers of non-smokers is very different from that in smokers. The link between biological and epidemiological findings was provided by studies on carcinogens present in tobacco, and specifically on polycyclic aromatic hydrocarbons such as benzo(a)pyrene, which induces G to T transversions at characteristic hotspots of the tumour suppressor *TP53*.

In other cases, biomarkers of effect still encounter many challenges to provide additional evidence for risk of an etiologically defined cancer. A highly promising field is DNA methylation profiling in cancer cases. Recently methylation of the *CDKN2A* promoter has been associated with tobacco smoking (Vaissière et al. 2009) and recurrence of early lung cancer stages (Brock et al. 2008).

Challenges in applying biomarkers to epidemiological studies: pre-analytical variations in large cohorts

Analytical variations observed during biomarker validation can be due to both biological inter- and intra-individual variations and to laboratory variation. The degree of variability relates to the biomarker accuracy in measuring the relevant exposure. Not only can the biomarker levels vary due to genetic and disease states, but there are also important issues concerning sample collection, storage conditions and quality controls of laboratory methods, statistics and reporting of data. The reproducibility among laboratories reflects the measure of accuracy, i.e. the measurement error of the biomarker which stay in between the biomarker's true value (or gold standard) and the measured biomarker.

Pre-analytical variation usually reflects accuracy in sample collection, storage conditions and quality controls; analytical variation refers to laboratory methods, statistics and reporting of data. These variations can be minimized by relying on *standard operating procedures* during all phases of the analysis. Techniques have to be reproducible, both intra and inter-laboratories, measurements have to prove sensitive, accurate and precise, and references have to be available to make results comparable over a period of time.
Well validated laboratory techniques are fundamental when studying the causality of a certain biomarkers expression upon environmental exposure, especially when the environmental exposure triggers very subtle changes in the biomarker expression (as in the case of gene promoter methylation upon dietary exposure). The validity of a laboratory assay is reflected in its reliability, i.e. how often the same results are obtained from multiple retesting, ideally in different laboratory contexts and using different instrument platforms, and it is correlated to the measurement error and the stability of the biological sample.

Measurement error, to which laboratory analyses are prone, is also called *laboratory drift* and can be due to a batch effect, to a storage effect or to repeated freeze-thaw cycles (Rundle et al. 2005).
Batches are created whenever it is unfeasible to process all of the samples together. *Batch effects* can create random noise or bias and are due to technological issues (e.g. the number of wells in a PCR plate), to logistic issues (e.g. transport and shipping limitations) or more simply to different availability of laboratory staff. This effect may lead to a misclassification of the exposure (Schulte and Perera 1993). In the ideal case of an even distribution of the measurement error among cases and controls (non-differential error) it may lead to an underestimation of the biomarker's association with the disease; in case the bias is unevenly distributed (differential error) it would be important to have the same proportion of cases and controls in each batch, and in general all measurements should be compared with a standard. In the present report, all

measurements have been conducted at least in duplicate with appropriate controls in each batch and following validated laboratory procedures.

Storage effect may arise whenever samples are not analysed immediately after collection; in fact, the level of some biomarkers can be easily influenced by both storage conditions (Table 1) and duration. Since it may take many years for a large multicenter study to assemble all samples and since biomarker levels can decline over time, samples must be stored in a consistent manner that does not vary by recruitment site and time, thus minimizing the storage effect on biomarker levels.

A variant of the storage effect arises when the volume of sample used for a particular test is smaller than the stored aliquots. Consequently, the remaining portion of the aliquot is stored again and thus may undergo several *freeze-thaw cycles.* Freeze-thaw cycles may alter chemical as well other properties of a biological sample through several physical and chemical mechanisms (Brey et al. 1994); they can degrade DNA (Lahiri and Schnabel 1993) and the situation is even more delicate for proteins, RNAs or metabolites. During freezing, the higher concentration of solutes in the liquid phase increases ionic strength, as well as it changes pH, and it may cause protein precipitation and denaturation (Van den Berg and Rose 1959). Thus, since biomarker levels could be influenced by the sequence in which the hypotheses are tested, the freeze-thaw cycles necessitate careful planning.

Table 1: Examples of storage recommendations for biomarkers

Method of collection	Bio-specimens	Examples of biomarkers/biomaterials	Recommendation and condition for collection and storage	References/ web sites
invasive	serum	micronutrients (vitamins and minerals)	Fasting for a maximum of 12 hours	Chiplonkar, 2004
		sex hormones	In females recording of day 1 of the ongoing and next menstrual cycle	Verkasalo, 2001
			Follow-up: age at menopause	Kaaks, 2005
		lipids	Fasting for a maximum of 12 hours	Appel, 2005
		uric acid	Serum separation within the 32 hours after venipuncture	Bovanton, 2002
		glucose	Stabilized by sodium fluoride, separation within 3 hours after venipuncture	Zhang, 1998
		metabonomic	Fasted subjects, clotting for 20-35 min on ice, avoid repeated freezing and thawing	Teahan, 2006
	plasma	vitamin C	Stabilisation with metaphosphoric acid, EDTA, perchloric acid or DTT Short term storage at -70°C (1 week) and long term storage at -196°C	Jenab, 2005
		DNA	Do not use heparin as an anti-coagulant (inhibition of PCR); separate plasma within 2 hours after venipuncture	Jen, 2000
	tissue	protein	Freezing within 30 min after resection	Jackson, 2005
		RNA	RNAlater or snap freezing in liquid nitrogen	Winnepenninckx, 2006 Mutter, 2004
non-invasive	urine	erythropoietin	Immediate addition of complete protease inhibitor cocktail	Khan, 2005 Beullens, 2006
		fluoride, magnesium, calcium	24h urine collection	Zohouri, 2006 Rylander, 2004 Anatol, 2005
	buccal cells	DNA	Collection by cytobrush gives the best DNA yield and purity	Mulot, 2005
	cervical cells	RNA	Snap freezing in liquid nitrogen	Wang, 2006
	hair	DNA, drugs and metals	Sequencial washing with detergent, water and organic solvent	McNevin, 2005 Lachenmeier, 2006 Pereira, 2004
	nail	metals	Hand washed with distilled water and medicated soap devoid of metal contamination, nails cut with clean stainless steel scissors, nails washed sequentially with non-ionic detergent, acetone and water	Mehra, 2005
	white blood cells	RNA	Process blood quickly to avoid changes in gene expression	http://www.affymetrix.com/support/technical/technotes/blood_technote.pdf#search=%22blood%20separation%20RNA%22
		antibody	Process blood on the day of collection	Weiblen, 1984

From Caboux et al. 2008

Challenges in applying biomarkers to epidemiological studies: biomarker validation

During the past few decades, great efforts have been invested in the identification of biomarkers of carcinogen exposure and early effects, and the development of analytical methods for their detection and quantification. The sensitivity of these assays may enable the measurement of the concentrations of metabolites, or adducts with macromolecules, of many environmentally relevant carcinogens at very low levels of exposure, or the detection and quantification of early genetic effects (ECNIS 2006). Biomarkers must always undergo the critical process of validation to ascertain their biological relevance to both exposure and disease in order to assess the "accuracy, precision, and effectiveness of results" (ECNIS 2007). The validity of an exposure biomarker might be compared with that of other exposure assessment methods, such as questionnaires and environmental monitoring. The main criteria to be met remain the relevance of the biomarker to the exposure of interest, its specificity (e.g. chemicals often share common metabolites) and the characteristics of the assay, including sensitivity, source of variability and effect modifiers.
Validation is required for any new method to ensure that it is capable of giving reproducible and reliable results, when it is used by different operators employing the same equipment in the same or different laboratories. The type of validation programme required depends entirely on the particular method and its proposed applications.

Technical validity may be defined as the lack of systematic error in measuring the biomarker in comparison to a standard. The degree of reproducibility is tested on results obtained by analysing the same sample under a variety of normal test conditions such as different analysts, laboratories, instruments, reagents and different days (for dHPLC also matter assay temperatures and small variations in mobile phase). Systematic errors may result from the methodology, the

instrument or the operator, and can affect both the accuracy and the precision of the measurement.

The components of analytical validity are mainly sensitivity, specificity and test reliability and they should apply to all kinds of biomarkers, including intermediate biomarkers of exposure and early response. Sensitivity and specificity evaluate how well the test detects the marker when it is present and when it is absent, respectively. *Sensitivity* has two meanings: i) the proportion of true positive results that the test will report as positive (i.e. absence of false negatives) and ii) the ability to detect a small proportion of positive material in a large amount of normal tissue (e.g. tumour DNA in a background of wild-type DNA). It is the first meaning that we shall usually refer to when describing the performance of a mutation detection assay, and of course it is desirable that the sensitivity is as close to 100% as possible, although it is not easy to establish this other than by empirical studies. *Specificity* is the absence of false positive results; only true positives are scored in a 100% specific assay. In mutation detection, this can be made more demanding by asking to report only pathogenic mutations and not normal sequence variants. When detecting somatic mutations by chromatography or DNA methylation levels by pyrosequencing for example, it is important to define a limit of detection. This is the lowest concentration in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions and once the background noise of the technique has been reduced as much as possible. In particular, for somatic mutations that are detected in a background of wild-type DNA, but in general for any screening assay, the minimum percentage of biomarker should be inspected in comparison to internal controls (both positives and negatives).

When a technique yields high sensitivity, we may have stronger confidence in interpreting the correspondence of the measurement with a conceptual entity. Example of a sensitive technique is immunohistochemistry for detecting the stress-induced nuclear accumulation of p53 protein. Under stress conditions wild-type p53 protein accumulates in the nucleus to block DNA synthesis and hence

cell division (Martinez et al. 1991); but it has a half-life of less than 20 minutes and does not normally accumulate at levels that are high enough to be detectable by immunohistochemical methods. In contrast, the mutated *TP53* gene codes for a protein that has a considerably prolonged half-life and that can be detected by immunohistology. Accumulation of proliferating-cell nuclear p53 detected by immunohistology is a sensitive method for assessing p53 abundance and status in cancerous samples and is simple to perform (Melhem et al. 1995). However, interpreting the results may sometimes be tricky since the absence of a detectable protein may occur when *TP53* gene contains a nonsense or frameshift mutation. Therefore, when this technique is followed by a validated one for *TP53* mutation detection (e.g. dHPLC and/or bidirectional sequencing), we obtain a robust laboratory method for screening somatic mutations in lung cancer.

Biomarker validation requires the choice of the appropriate *target sample* for measurement. Biomarkers can be measured in exhaled air, blood, urine and in tissue samples. Often the actual target organ or cell is not readily available for measurements and biomarkers of exposure are thus often surrogate measures of doses or effects at the target. The ideal biomarker has been described as chemical-specific, detectable at low levels, inexpensive to analyse and quantitatively related to prior exposures (Kulasingam and Diamandis 2008). The ideal biomarker should also be available using non-invasive techniques, meaning that biological materials should be easily accessible in sufficient amounts under routine conditions and without unacceptable discomfort or health risk for the patient. For these reasons blood and urine are most commonly used as source of biomarkers, since cells in blood may provide surrogate endpoints for the effects in internal organs. Hair, teeth, nails and exfoliated buccal cells have also been used for biomonitoring, but knowledge of these media requires further improvement and validation (Esteban and Castano 2009). The choice of target material may also influence the exposure time that a marker will reflect. Levels of chemicals in blood usually reflect a short time period of exposure (a few hours or days) whereas adduct levels in urine may reflect a much longer time of exposure.

After data collection, one can evaluate the ability of the marker to describe exposure and its specificity and selectivity. The relationship of the biomarker to the observed effects may be then investigated by evaluating a dose-response pattern. Possible shortcomings arising at this stage could be a lack of pharmacokinetic models describing a certain compound's metabolism or a substantial endogenous production of the studied biomarker. For example, in the case of formaldehyde, the normal endogenous metabolism in humans is higher that the recorded occupational exposure limits, suggesting the need to look for alternative biological sources of the biomarker. In other words, it is important in this case both to establish practical thresholds for the exogenous compound and to improve the sensitivity of the assays.

Large scale biomarker analysis using "-omics" technologies: state of validation

In recent years, the field of biomarkers has considerably expanded and gained in complexity through the emergence of technologies collectively identified as "omics", allowing the simultaneous analysis of multiple markers in a single specimen. The use of the suffix "-omics" entails extensive coverage of a particular type of molecule and analysis of the whole set of this particular molecule in a given specimen target. Thus, genomics, proteomics and metabonomics encompass the analysis of, respectively, the whole genome, proteome and metabonome. From a methodological viewpoint, -omics techniques often consist in the multiplexing of the same techniques as those used for detection of a single biomarker, within a miniaturized matrix (micro-array). Thus, all the problems and difficulties in biomarker discovery, assessment and validation are the same for -omics as for single-biomarker approaches. There are however two major differences. First, with "omics", a new type of biomarker can be defined, arising from the identification of a pattern of changes

simultaneously affecting a wide range of molecules (thus defining a “signature”). Second, the analysis of data from “omics” and, significantly, the identification of “signatures” critically depend upon heavy computing capacity (bioinformatics). Therefore, with “omics”, the problem of biomarker validation is compounded by adding to logistic and laboratory issues, a whole range of issues including bioinformatic methods, biostatistics and availability of extensive databases serving as resources for the correct identification of biomarkers.

-Omics technologies offer means for characterizing exposures to several important classes of environmental and life-style factors with a multi-targets approach. The integration of complementary –omics technologies open the path towards a more complete systems biology model which highlights novel responses to exposure within particular biological pathways. The concept is particularly appealing when studying intermediate biomarkers and when investigating the carcinogenic fingerprints of environmental exposure ahead of disease onset.

Typical -omics fields are genomics, proteomics, metabolomics, as well as transcriptomics and epigenomics. Genomics based biomarkers are found through DNA chip-arrays, quantitative real time PCR, reverse transcriptase polymerase chain reaction, DNA sequencing, fluorescent in situ hybridization etc. Gene expression profiling of two to several thousand genes may provide diagnostic, prognostic, or predictive information about tumours. Genomic microarrays represent a powerful technology for gene-expression studies, arrays are high resolution “lenses” which allow the analysis of a massive amount of data per experiment, comprehensive of thousands of individual genes. Results from high-density arrays have for instance enabled to classify breast cancer types into prognostic categories based on the expression of certain genes (Weigelt et al. 2005). Unfortunately, despite these encouraging results, the use of gene-arrays is still not recommended for widespread clinical use (Diamandis et al. 2006) since validation studies often do not report high reliability of the original data.

The expression of proteins is often studied by proteomics. This type of “omics” encompasses highly sophisticated pipe-line for the purification of a large variety of proteins over several orders of magnitude of abundance, their fractionation into small peptide units and the complete characterization of the mass and amino-acid sequence of these peptides by mass spectrometry. The multiple individual mass fragments are then automatically compared against databases to identify and “reconstruct” the proteins from which they derive. Data from such proteomic approaches can be used to discover new protein markers that can be further assessed and validated using simpler, routine technologies such as immunodetection (e.g. enzyme-linked immunosorbent assays). Alternatively, complex peptidic patterns can be used to generate specific “signatures”, although in this case it is often extremely difficult to ensure the reproducibility of analyses across laboratories and instrument platforms (Chan et al. 2006).

The past few years have seen the advent of metabonomics (or metabolomics). This methodology aims at providing an extensive identification of the set of metabolites present in a given sample. It employs two complementary technologies, mass spectrometry-based and 1H Nuclear Magnetic Resonance, to process a variety of biological specimens. Analysis of metabolic fingerprints leads to a list of metabolites that can be interpreted for mechanisms of toxicity as well as for eventual biomarkers of exposure.

One of the most daunting challenges risen by the “-omics” is to summarize and to purge the huge amount of data from spurious results. Bioinformatics has the possibility to model the heterogeneity of pathways and to reveal shared biological patterns (Abu-Asab et al. 2011) within the data. In the case of cancer, and after clearing inconsistencies caused by logistical and technical problems, most of the heterogeneity is due to the fact that clonal, driver and most likely irreversible aberrations on one side and non-expanded, passenger or reversible aberrations on the other side, are both potentially taking place during the carcinogenesis

process (Loeb et al. 2008). In cancer, clonal and mostly irreversible alterations are hypothesized to drive the carcinogenic process by the selective pressure given by a proliferative advantage. These alterations could act as potential clinical biomarkers since they are the most common among individuals with the same disease. Bioinformatics could map both “random” alterations occurring in a subset of individuals and “common” alterations, and create models where shared clonal alterations would be considered as the “baseline” reference. In this way it would be possible to biologically describe the identity of diseased against normal non-diseased specimens. Ideally, the resulting molecular pathway could be translated into the clinical setting for early detection, diagnosis, prognosis and treatment assessment.

As underlined in this introduction, the current approach of biomarker studies does not entirely fulfil its immediate, explicit objective of finding new markers for screening, detection or prognosis. Several methodological problems have been identified including the lack of reproducibility of analytic methods or of data analysis among different laboratories. Moreover, there is a lack of harmonization on protocols for sample collection, processing and storage. In the case of Genome-Wide Association Studies replication of findings is built into the study, this is not common in molecular epidemiology studies. The ‘Strengthening the Reporting of Observational Studies in Epidemiology’ (STROBE) initiative was established aiming at providing guidance on how to report observational research in order to improve the quality of reporting observational studies and studies investigating associations between exposures and health outcomes (Gallo et al. 2011). These guidelines and recommendations have been recently complemented by the BRISQ recommendations (Biospecimen Reporting for Improved Study Quality, (Moore et al. 2011) which specifically address data collection and annotation for specimen biobanks.

Although seen as «translational research», cancer biomarker research is actually a new approach for understanding the mechanisms of carcinogenesis and the

power and flexibility of -omics technologies address molecular carcinogenesis on a wider scale. Biomarkers could be successful in identifying similarities among patient subgroup, by focusing on specific pathways to molecularly define a subset of tumours categorized at diagnosis. A big achievement would be represented by discovering biomarkers that provide the best stratification of clinical outcome, in order to reliably target patients who are most likely to benefit from a particular agent. Consequently, biomarkers should demonstrate evidence-based clinical validity and utility in prospective, well-designed clinical studies across multiple institutions, with well-established standards for laboratory analyses and assessment of exposures. Once the validation of a personalized medicine based on the discovered biomarkers occurs, it remains to evaluate if the commercial incentives to develop these complex assays are in place for a broader clinical use. In fact, the financial aspect of the overall process should also be carefully considered, since the biomarker must be identified, an assay to measure it reliably in clinical samples (ideally in a non-invasive manner) must be developed and the usefulness of the biomarker to make a clinical distinction must be demonstrated.

In conclusion, the concept of “personalized medicine” must be addressed with caution since implementing biomarkers requires clinical trials and robust, reproducible evidence through systematic technical and epidemiological validation studies.

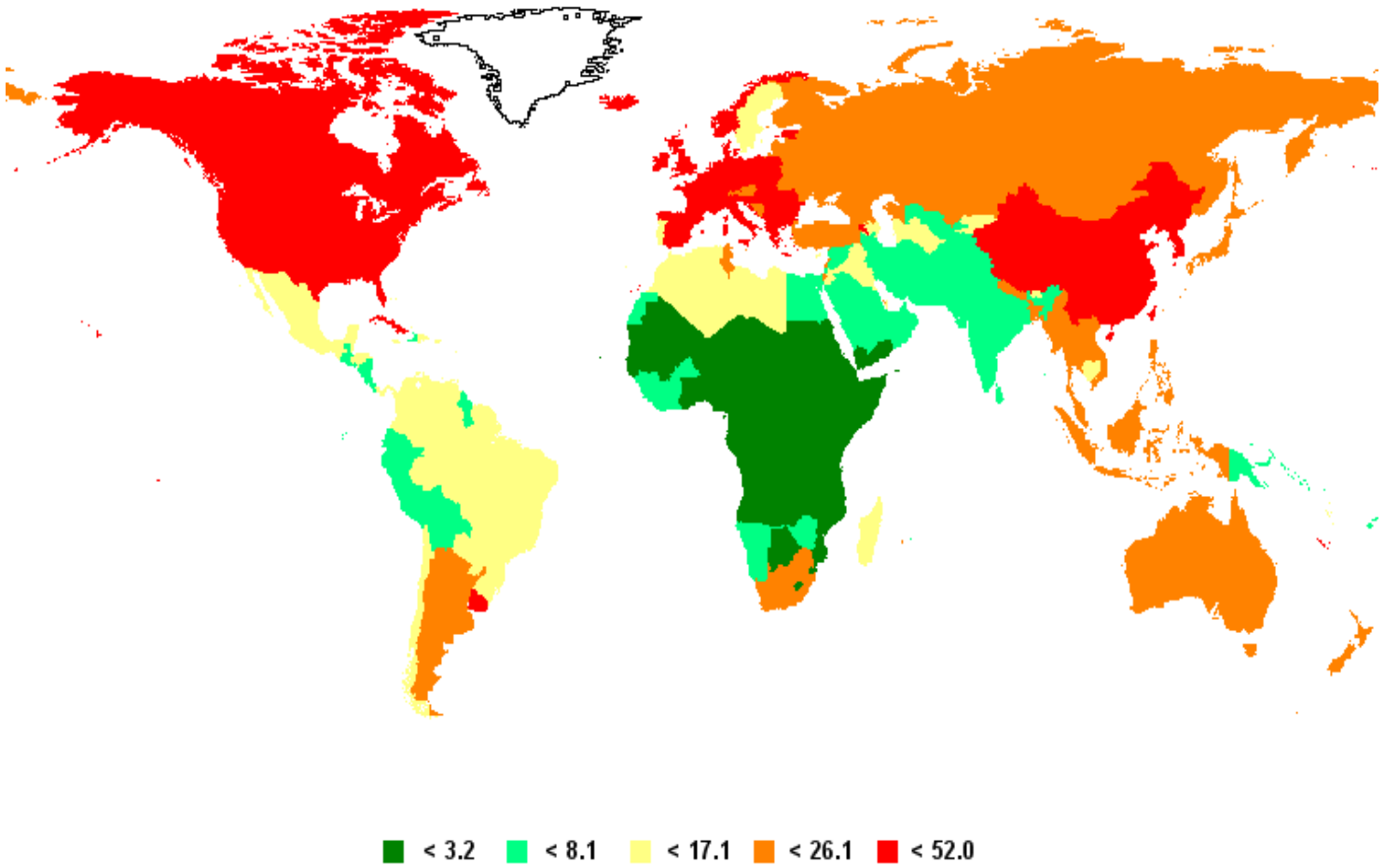
Lung cancer: a paradigm to discover and validate biomarkers associated with environmental exposures

Lung cancer is the most common cancer in the world today (12.7% of all new cancers, 18.2% of cancer deaths with a ratio of mortality to incidence of 0.86). There were an estimated 1.61 million new cases and 1.38 million deaths in 2008. In Figure 4, from Globocan 2008, the age-standardized prevalence rates for men and women are combined to generate total prevalence. It has to be taken into account that there are important gender differences in incidence, where lung cancer is the most common cancer in men worldwide (1.1 million cases, 16.5% of all cancers) and the fourth most frequent cancer in women (516000 cases, 8.5% of all cancers).

In industrialized countries, the past century has witnessed a lung cancer epidemic due to tobacco smoking. Despite progress in smoking prevention in many developed countries, this tobacco-related lung cancer epidemic is spreading at an unabated rate in many emerging and low-resources countries. Given the demonstrated role of tobacco carcinogens as causal agents for lung cancer, this cancer represents a paradigm for research on biomarkers associated with lifestyle habits and environmental exposures. Tobacco smoke is a complex mixture that contains many carcinogens. Yet, despite its complexity and the wide diversity of the patterns of exposure to tobacco, this exposure is measurable, quantifiable and the risk associated with it has been well defined by numerous large-scale epidemiological studies.

Therefore, studies in smokers and in patients with lung cancer associated with smoking offer a perfect opportunity to discover, assess and validate biomarkers of this particular form of environmental exposure. In this Thesis, I have used this epidemiological context as focal point for developing different approaches on biomarkers of exposures.

Figure 4: Estimated age-standardised lung cancer incidence rate per 100,000 individuals by country



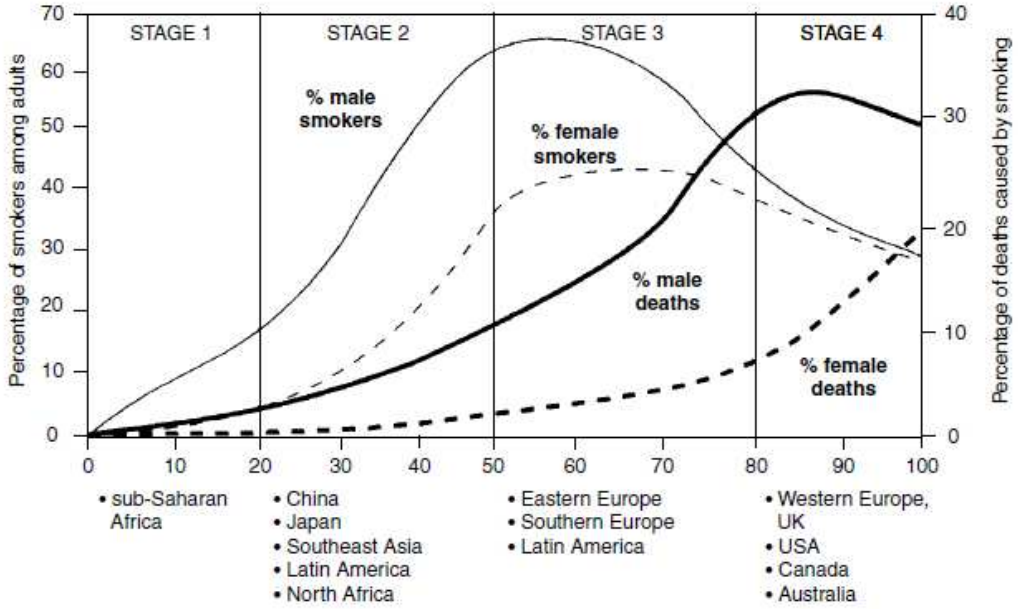
From Globocan (2008)

Epidemiology of lung cancer worldwide

The overwhelming majority of cancer cases are associated with environmental factors. Only a fraction of lung cancer cases (5% to 10%) are caused by genetic susceptibility and inheritance, although there is accumulating evidence that genetic susceptibility plays an important role in modulating how an exposed person responds to environmental lung carcinogens (Brennan et al. 2011). Lung cancer is extremely strongly associated with smoking in developed/industrialized countries and differences in geographical incidence are strongly linked to the evolution of smoking-habits, particularly among women and in developing countries. Industrialized countries in Northern and Western Europe, North America, and the Western Pacific region are generally at approaching this stage. Nearly 80% of the more than one billion smokers worldwide live in low- and medium-income countries, where the burden of tobacco-related illness and death is heaviest even if a proportion of lung cancer cases are attributable to causes other than smoking (Youlden et al. 2008). Incidence lung cancer rates are high but decreasing in Europe and Northern America, while low but increasing in Middle and Western Africa. If trends continue, eight million people a year will die from tobacco-related causes by 2030 and 80% of these deaths will occur in low- and middle-income countries (WHO 2011).

The conceptual framework that links the various stages of the tobacco epidemic into a continuum, rather than a series of isolated events is the WHO model of the four stages of the evolving epidemic (Figure 5). The power of this model, originally proposed by Lopez et al. (Lopez et la. 1994) is that it allows virtually every country to find itself in relation to the larger pandemic.

Figure 5: Four stages of the tobacco epidemic



From Lopez et al. 1994

Almost all lung cancers are carcinomas, with other histologies counting for much less than 1%. The disease is clinically divided into two subtypes: non-small-cell lung cancer (NSCLC), representing almost 80% of all lung cancers, and small cell lung cancer (SCLC) that comprise about 10-15% of cases (IARC 2007). These two types of lung cancer are two different diseases, each with its own recommended therapies.

NSCLC, which originates from bronchial or alveolar epithelial cells, is further subdivided into three histological subtypes: adenocarcinoma (ADC; derived from bronchioalveolar epithelial cells), squamous cell carcinoma (SCC; that arises from bronchial epithelial cells through a squamous metaplasia/dysplasia process) and large cell carcinoma (LC). SCLC, in contrast, originates from cells with neuroendocrine differentiation that are present within the normal lung mucosa. SCLC is composed of several different pathological entities distinguished by their proliferative potential as well as histological characteristics. Both NSCLC and

SCLC, are strongly associated with tobacco smoking, although the magnitude of this association differs between histological types.

In recent decades, the number of squamous cell carcinomas has decreased while an increase of adenocarcinomas has been recorded. SCC represents 44% of lung cancers in men and 25% in women worldwide except for certain Asian populations (Chinese, Japanese) and North American (USA, Canada) where ADC incidence exceed that of SCC in men. ADC represents 28% of lung cancer cases in men and 42% in women worldwide. ADC is the most frequent histology in women, particularly in Asian women, with the exception of Poland and England where SCC predominates. Classically, tobacco smoking was considered to be more strongly associated with SCC than with ADC but the incidence trends do not correlate with the smoking prevalence. Changes in the manufacture and composition of cigarettes (e.g. filters), and the corresponding changes in smoke composition along with nicotine-compensating smoking patterns are suggested to contribute to the observed epidemiologic profiles (Khuder 2001). Another hypothesis is that the changes in cigarette composition have reduced the yield of polycyclic aromatic hydrocarbons, inducers of SCC, while increasing the yields of carcinogenic tobacco-specific N-nitrosamines, inducers of ADC (Hoffmann et al. 1997). These factors, along with advances in histological classification and detection methods for tumours in the distal airways, have contributed to the emerging predominance of ADC in lung cancers.

Lung cancer is one of the most insidious and aggressive neoplasms since it usually causes clinical symptoms only at a stage when the tumour has already invaded the lung parenchyma at least locally. Many patients who report with symptoms – coughing, respiratory distress – already have advanced forms of cancer. Furthermore, the perception of symptoms is often delayed because it is blurred by the underlying background of chronic bronchitis that occurs in many lifelong smokers. Resection remains the basis of therapy. However, fewer than 20 to 30% of lung cancer patients have lesions that are sufficiently localized to allow local (lobular) tumour resection. The survival rate is 48% for completely

resected cases detected when the disease is still localized, but only 15% of lung cancers are diagnosed at this early stage. As a result, the five-year overall lung cancer survival rate is still very low at around 15% (Jemal et al. 2010) and decreases by increasing stage of cancer at diagnosis. Combined modality treatments including surgery, radiotherapy and chemotherapy, have greatly progressed in the past 30 years. Still, a critical issue remains the frequency of unnecessary treatments, thus indicating the need for biomarkers of early diagnosis and appropriate therapy.

Lung cancer risk associated with tobacco smoking is strongly related to smoking duration and declines with increasing duration of cessation (more rapidly for SCC than ADC). Nevertheless, the estimated cumulative risk of lung cancer death among former smokers remains high, ranging from approximately 6% in smokers who quit smoking at the age of 50, to 10% for smokers who quit at age 60 and started in early adulthood (around 18 years old), while that for lifetime smokers in the United Kingdom was estimated between 9% and 16% (Doll et al. 2004).
The well established risk factor of tobacco smoking makes it a good model for studying exposure to risk as well as to protective factors (e.g. dietary factors). Screening for early lung cancer is being evaluated in a number of randomized trials and it is possible that screening high-risk individuals might be of great importance to public health intervention. Incorporating biomarkers of exposure and early effect into studies will further clarify the effects of cumulative exposure, smoking intensity and duration in relation to lung cancer risk and to subgroup susceptibility.

Genetic and epigenetic modifications in tobacco-induced carcinogenesis of the lung

The causal role of tobacco smoking in lung cancer incidence has been recognized by public health and regulatory authorities since the mid-1960s and first evaluated by the IARC Monographs in 1986 (IARC 1986) as a guide to regulatory and public health agencies in their decision making. The strong dose–response relationship between tobacco smoking and lung cancer previously reported (Medical Research Council 1957) was again confirmed in both questionnaire-based and biomarker-based studies (IARC 2004a).

Polycyclic aromatic hydrocarbons (PAHs), are formed as complex mixtures during many combustion processes and are implicated in the causation of lung cancer. The biomarker of exposure to PAHs that has been used in many studies is the excretion of 1-hydroxypyrene in urine (Aquilina et al. 2010). Many PAHs have been shown to be carcinogenic in animals via a genotoxic mode of action. Benzo(a)pyrene is the best studied PAH and was recently classified as a human carcinogen by IARC (IARC 2010). The strong dose–response relationship between tobacco smoking and lung cancer (Medical Research Council 1957; IARC 1986, 2004a and 2010) is confirmed by both questionnaire-based and biomarker-based studies. However, not all smokers develop lung cancer, indicating an inter-individual variation in susceptibility to tobacco smoke. Accordingly, it is reasonable to assume that tobacco-related lung cancer is caused by the interplay between tobacco smoke and other factors, including environmental factors and individual (genetic or acquired) susceptibility. Unravelling the molecular basis of tobacco carcinogenesis continues to inspire epidemiological studies incorporating genetic, molecular markers and refined statistical modelling techniques.

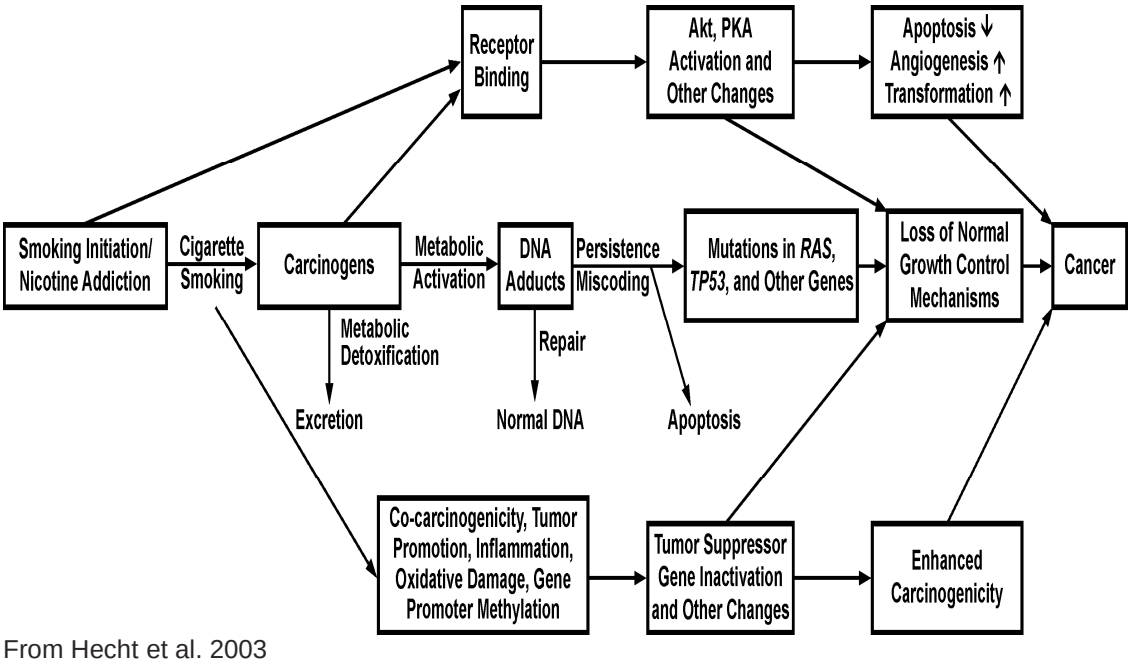
Molecular genetic studies have shown that lung cancer cells acquire a number of genetic lesions, including activation of dominant oncogenes and inactivation of

tumour suppressor genes or recessive oncogenes (Hanahan and Weinberg 2000 and 2011). Several acquired characteristics of tumours can be caused by specific point mutations. In smokers, the most common mutated genes are *TP53* and *KRAS*, the latter being primarily found in adenocarcinomas (ADC). In ADC in never-smokers, after the identification of *EGFR* mutations in 2005, a wide panel of oncogenes has been detected as recurrent target of mutations, including *ALK*, *PI3K*, *MET* or *BRAF* (Sharma et al. 2010). This specificity in mutational targets according to tobacco smoking status further supports the notion that tobacco smoking causes lung cancer by inducing mutations in specific genes that directly contribute to the cancer phenotype.

The role of *TP53* as the ‘‘guardian of the genome’’ is central in forcing genetically damaged cells into growth arrest, senescence or apoptosis. The p53 protein is a transcription factor that regulates the expression of a large panel of genes involved in multiple aspects of growth suppression and genetic stability. The protein functions can be lost during the course of tumour progression, either through inactivating mutations or via other mechanisms such as complex binding of p53 to specific viral or cellular proteins. By switching off p53 functions, these mutations facilitate the acquisition of the large number of genetic alterations required for developing a fully invasive/metastatic phenotype. The accumulation of such genetic changes is triggered by the numerous carcinogens as well as inflammatory agents contained in cigarette smoke.

The scheme below shows the carcinogenic process leading to lung cancer development (Figure 6).

Figure 6: Scheme linking tobacco-smoke carcinogens and lung cancer



From Hecht et al. 2003

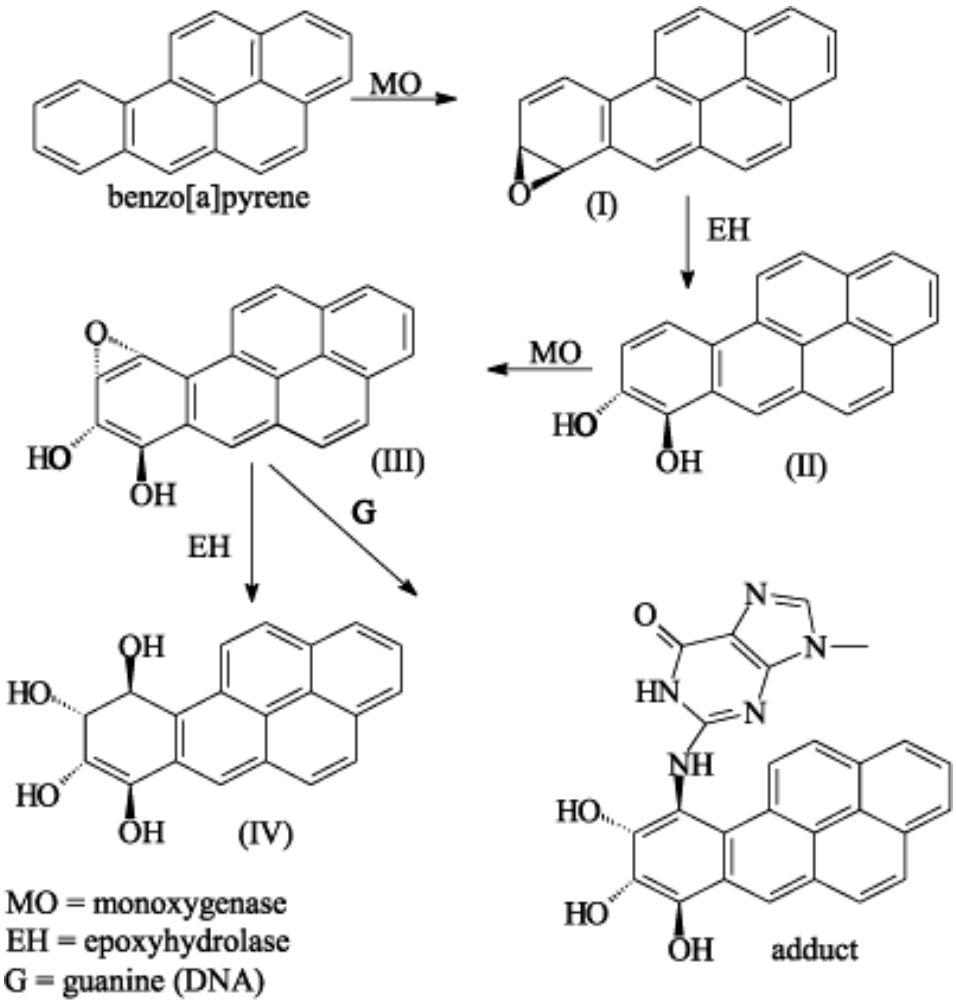
Although many of these genetic changes may occur independently of histological type, their frequency and timing of occurrence with respect to cancer progression are different between SCLC and NSCLC. Furthermore, as described above, a number of genetic and epigenetic differences have been identified between the two main histological types of NSCLC, i.e. SCC and ADC, as well as between smoking and non-smoking related cancers.

In a simplified view of the multistep carcinogenic process, three main stages can be described for all cancers: initiation, promotion and progression. The initiation stage is a rapid phase of interaction between the carcinogenic agent and the target cell DNA. Most carcinogens in tobacco products require metabolic activation before they can react with DNA, although some, such as ethylene oxide, formaldehyde and acetaldehyde, can react directly. The response of the organism to carcinogens is similar to that for any other foreign compound or drug. Many carcinogens are lipophilic compounds that readily cross plasma membranes to accumulate in the cytoplasm and the nucleus.

The metabolism involves phase I reactions, mainly mediated by microsomal oxidases encoded by the cytochrome P450 gene superfamily, however, other enzymes such as epoxide hydrolase 1 are involved as well. Cytochrome P450 enzymes, which are part of the mammalian systems designed to respond to foreign compounds, catalyze the addition of an oxygen atom to the carcinogen, increasing its water solubility and converting it to a metabolite that is more readily excretable (Guengerich 2003). This “metabolic detoxification” process is further assisted by phase II enzymes to convert the oxygenated carcinogen to a form that is highly soluble in water. As far as this process is efficient, the organism will be protected. However, some of the intermediates formed by the interaction of cytochrome P450 enzymes with carcinogens are in fact quite reactive. Such intermediates or metabolites generally possess an electrophilic center and can react with nucleophilic guanines in DNA, resulting in the formation of DNA adducts (Figure 7). The overall process is known as metabolic activation and converts an un-reactive chemical (benzo(a)pyrene) into an intermediate (diol epoxide) that covalently binds to DNA.

The equilibrium between metabolic activation and detoxification varies between individuals; detoxification constitutes the most effective way of preventing cancer, by blocking the genotoxic damage at the early stages of carcinogenesis. If DNA adducts are bypassed by a DNA polymerase during the process of detoxification, the resulting G:A and G:G mismatches cause G>T and G>C transversion mutations. This phase is relatively long and marked by the establishment and replication of mutated cells. Somatic mutations derived from DNA adducts can contribute to cancer promotion and represent more specific endpoints than DNA damage. Mutations can occur in reporter genes, such as *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) i.e., in genes not related to cancer development but used as surrogates because they are relatively easily evaluated, or can occur more specifically, e.g. in oncogenes or tumour suppressor genes.

Figure 7: ***In vivo*** **oxidative metabolic pathway of benzo(a)pyrene** ***via*** **hydrophilic intermediates (I-IV) and formation of DNA adducts with guanine base**



From Formenton et al. 2005

It has been established conclusively that PAH-DNA adducts derived from cigarette-smoke carcinogens cause specific mutations, most frequently G>T, that lead to miscoding (Pfeifer and Denissenko 1998, Hecht 2003). If growth controlling genes are involved, these somatic mutations strongly contribute to cellular transformation and the development of tumours. Recently, it has been demonstrated (Anna et al. 2009) that carriers of G to T transversions also had a high level of bulky DNA adducts in non-tumorous lung tissue, thus providing

evidence for tobacco-related carcinogenesis in lung cancer development. The mutated cell(s) are selected *in vivo* because of their growth advantage (e.g. loss of contact inhibition, loss of apoptotic pathways), which correlates with increased genetic instability (i.e. the potential to acquire further advantageous genetic changes) and with capability to metastasize (Beerenwikel et al. 2007). As a result, proto-oncogenes (e.g. *KRAS*) and tumour suppressor genes (e.g. *TP53* and *CDKN2A*) in particular, but also all genes involved in cell-cycle regulation, tumour cell invasion, DNA repair, chromatin remodelling, cell signalling, transcription, and apoptosis, are critical targets of mutations by carcinogens.
Damaged cells may be removed by apoptosis but if uncontrolled cell growth persists, premalignant cells will gradually develop into neoplastic ones. This is the final stage of tumorigenesis, the progression phase, where cells bear some or all of the “hallmarks of cancer”. Six “hallmarks of cancer” cells have been originally described by Hanahan and Weinberg. These hallmarks included persistent proliferative signalling (e.g. through disrupted negative-feedback mechanisms that attenuate proliferative signalling), insensitivity to growth suppressors, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and activated tissue invasion and metastasis (Hanahan and Weinberg 2000). A recent re-assessment (Hanahan and Weinberg 2011) has added 4 additional “hallmarks” (inducing genome instability and mutation; avoiding immune destruction; deregulating cellular energetic; tumour-promoting inflammation). These functions allow cancer cells to survive, to proliferate and to disseminate, and they are acquired in different tumour types via distinct mechanisms and at various times during the course of multistep tumorigenesis.

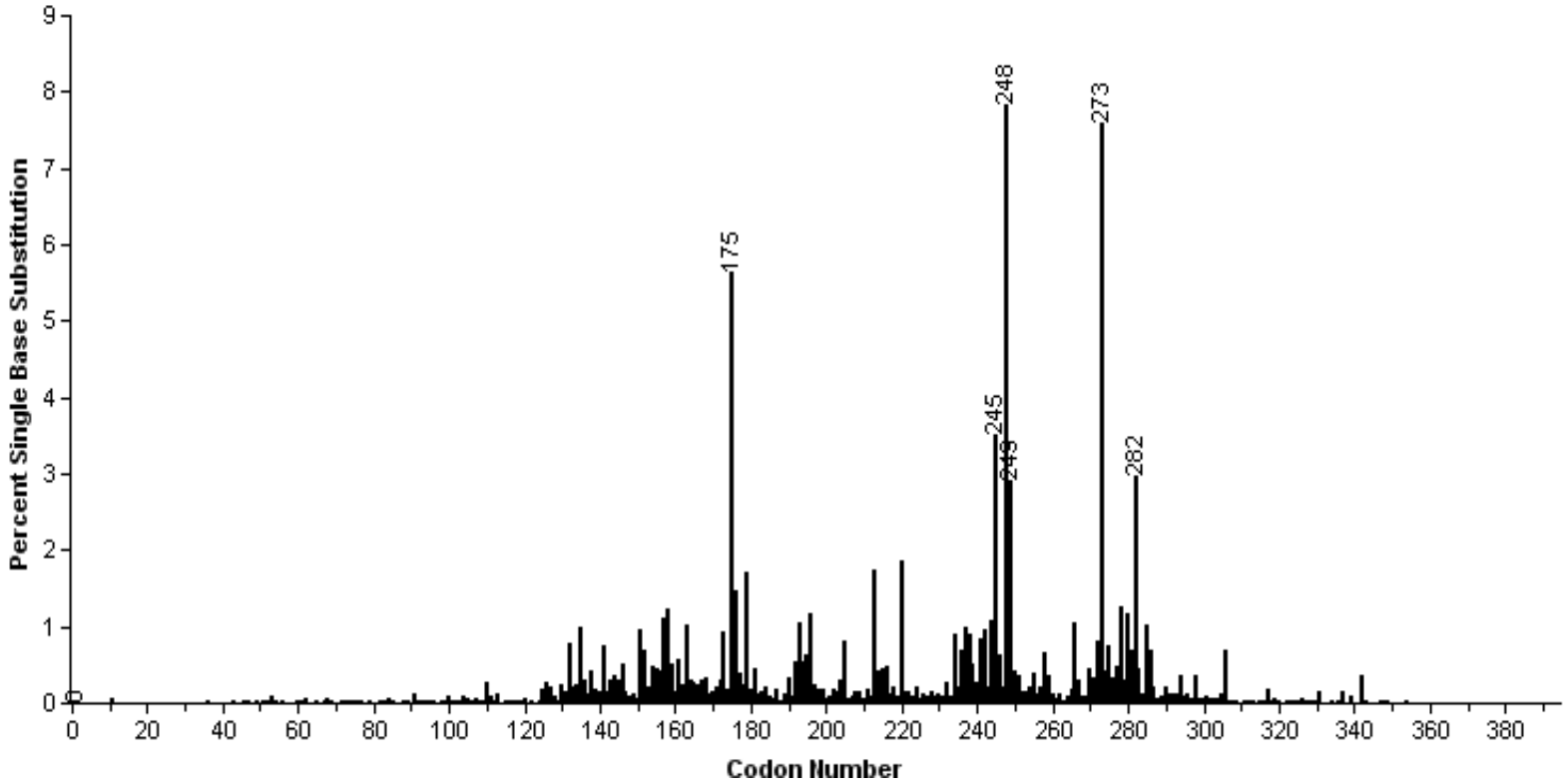
Nutrition and other lifestyle-related factors that influence cellular processes may also interfere with the *hallmarks*’ development at many levels. Of course they are critical in directly influencing energy metabolism and interconnected mechanisms leading to inflammation. At a more subtle level, they may also have a direct effect on the tumour micro-environment, on the control of the production of potentially

damaging reactive oxygen or nitrogen species and on carcinogen metabolism or detoxification reactions.

Somatic mutations that are causally related to environmental carcinogen exposure and that may activate downstream proliferative pathways are valuable candidates both as biomarkers of exposure and biomarkers of effect. A carcinogenic mutational spectrum shows distinctive features, a sort of molecular "signature" of carcinogenesis that traces the causative carcinogenic agent. The PAH-DNA adduct positions are known today as the major mutational hotspots of *TP53* in human lung cancer (Greenblatt et al. 1994). A *TP53* Mutation Database has been maintained and developed at the International Agency for Research on Cancer in Lyon, France, since 1994. The database compiles all *TP53* mutations that have been reported in the published literature since 1989 (Petitjean et al. 2007). This database is highly informative concerning the mutational spectra in *TP53* with respect to interactions with mutagenic chemicals and other environmental carcinogens.

TP53 encodes an all-round tumour suppressor transcription factor, p53, which mediates multiple anti-proliferative effects in response to a variety of stresses, including in particular DNA damage. Most known mutations fall within the DNA-binding domain and inactivate the suppressor activity by preventing DNA binding and transactivation (Figure 8). Mutations at five major "hotspots" account for about 30% of all known mutations. These codons are R175, G245, R248, R249, R273 and R282. The apparent "hypermutability" of these sites is due to two factors that suggest interplay between selection and mutagenesis. First, these residues play important roles at the surface of contact between p53 and target DNA. Thus, substitution of these residues results in a protein with decreased affinity for DNA, which has lost the capacity to suppress proliferation (Cho et al., 1994). Second, G to T transversion mutations induced by benzo(a)pyrene diol epoxide adducts from tobacco smoke in *TP53* have been shown to occur preferentially at methylated CpG (cytosine-phosphate-guanine) sites, thus strengthening a link between PAHs present in cigarette smoke, lung cancer mutations and epigenetic marks (Yoon et al. 2001).

Figure 8: Codon distribution of *TP53* mutations



From IARC *TP53* database, November 2010

Epigenetic changes can precede and drive genetic alterations, thus contributing to attenuate maintenance of the integrity of the genome. In this sense epigenetic instability could be the counterpart of genetic instability. The most studied epigenetic change that in some cases is associated with a genetic one is gene promoter hypermethylation driving somatic point mutations, which in turn may activate oncogenes and inactivate tumour suppressor genes. As an example, a significant association between epigenetic silencing by promoter methylation of O^6-methylguanine-DNA methyltransferase (*MGMT*) and G:C-to-A:T transition mutations in *TP53* and in *KRAS* at codon 12, has been found (Watanabe et al. 2005; Esteller et al., 2000).

DNA methylation is a postreplication modification almost exclusively found in the position 5 of cytosines (5-mC) in a dinucleotide CpG sequence context, and is carried out by methyltransferase enzymes. The CpG dinucleotide is underrepresented in the genome and is generally methylated, except for CpG-rich clusters of approximately 1-2 Kb length (so called CpG islands) found in the promoter region and first exons of many genes. Methylation of CpG islands is important for the establishment and maintenance of cell-type and/or tissue-specific gene expression. It is often associated with the inhibition of gene expression, but not necessarily with gene silencing, and it changes slowly with age and in response to environmental effects such as diet.

It is not clearly understood why certain CpG islands are hypermethylated in cancer cells while others remain methylation free, but it can be hypothesized, as it has been done in the case of genetic mutations, that a particular gene is preferentially methylated with respect to others in certain tumour types because its inactivation confers a selective clonal advantage. Another hypothesis concerns the role played by the environment and nutrition, since the most hypermethylated tumour types are those of the gastrointestinal tract (Esteller, 2007) probably because they are more exposed to external carcinogenic agents. Evidence of a causal role of the environment in epigenetic modifications came first from observing that epigenetic patterns of monozygotic twin pairs diverged

as they became older (Fraga et al., 2005). It is thus quite reasonable to assume that external factors such as smoking, physical activity or diet, among others, together with internal factors can influence the hypermethylation status of specific tumour suppressor genes.

Major epigenetic changes include DNA methylation, post-translational modifications of histone proteins (affecting mainly chromatin folding) and non-coding RNAs (playing a role in heterochromatin formation, DNA methylation targeting and gene silencing). Epigenetic mechanisms affect functional DNA regulation without changing the DNA structure and they can be seen as acting at the interface between genome and environmental signals. The list of genes altered by epigenetic mechanisms and observed to be associated with cancer is rapidly expanding due to next-generation sequencing techniques and to various initiatives in the field of epigenetics, such as the Human Epigenome Project. The epigenome is extremely sensitive to endogenous and exogenous (environmental) stimuli in cancer. It is characterized by a gradual and reversible acquisition of tumour-specific alterations that are implicated in virtually every step of tumour development and progression (Jones and Baylin 2002), thus acting as ideal intermediate biomarkers. In particular, several studies have provided evidence that DNA promoter hypermethylation detected in tumour cells as well as surrogate cancerous tissues (blood or plasma DNA) could be exploited as a source of diagnostic and prognostic biomarkers for lung cancer.

Even if the exact causal relationship is still poorly understood, it has been suggested that gene promoter hyper-methylation could: (i) contribute to enhance the binding of carcinogens, (ii) increase the mutability of methylated cytosines and (iii) silence tumour-suppressor genes and DNA repair genes facilitating tumourigenesis (Sawan et al. 2008). Aberrant DNA methylation may also precede genetic changes and possibly trigger them in the course of tumour development. Mouse models of lung cancer development have shown that even a few weeks of exposure to tobacco smoke or tobacco smoke condensate can increase the methylation levels of numerous genes prior to any overt

histopathological changes (Phillips and Goodman 2009). Anyway, the mechanisms in humans through which smoking inactivates genes involved in DNA repair, detoxification, cell cycle regulation, and apoptosis are still not clearly understood. In exposed humans, tobacco smoking has been shown to influence DNA methylation levels of specific genes (e.g. *MTHFR*) whereas methylation levels of other genes investigated (including *CDKN2A* and *RASSF1A*) were not associated with smoking status (Vaissière et al., 2009; Vineis et al., 2011). These interesting results indicate that tobacco smoke may target specific genes for promoter methylation and point out that DNA methylation changes may alter the expression of genes with weak or no tumour suppressing activity, including genes with other cellular functions such as DNA repair or carcinogen detoxifying activity.

The search for predictive epigenetic biomarkers could then be reasonably expanded beyond the traditional panel of tumour suppressor genes. To date, only few studies have investigated overall 5-mC content (e.g. LINE-1: repetitive elements often used as indicators of global DNA methylation) and smoking and they failed to find a clear association, at least in adulthood. However, global hypo-methylation co-exists with gene-specific promoter hyper-methylation in most human cancers (Herceg 2007) and it is thought to re-express proto-oncogenes or imprinted genes, to activate latent viral and parasitic transposons, thus contributing to genomic chromosomal instability. It has been suggested that tobacco smoke chemicals that affect epigenetic marks in adulthood could have a larger impact when exposure occurs *in utero*, i.e. during establishment of epigenetic profiles. Evidences of altered epigenetic patterns in the offspring of women who smoked during pregnancy (Breton et al. 2009) may suggest a role of epigenetic mechanisms in developmental disease.

In summary, epigenetic mechanisms can be viewed as an interface between the environment and the genome; their deregulation may disrupt key cellular processes and ultimately result in oncogenic transformation and tumour development. Environmental factors such as tobacco smoke may leave

epigenetic fingerprints that could be exploited as biomarkers for risk assessment and prevention. In contrast to the genome itself, epigenetic status is more dynamic and recent studies suggested that the establishment and maintenance of epigenetic patterns might be particularly sensitive to environmental influences during specific stages (such as in utero development). Also, epigenetic alterations are reversible and typically acquired in a gradual manner, thus offering opportunities for prospective investigations on exposed individuals who develop alterations over time. However, still much remains unknown and future studies need to establish epigenomes in normal tissues and specific cancers as well as to identify environmental factors associated with epigenetic changes. There is also the need to elucidate the molecular mechanisms by which environmental factors deregulate normal epigenetic patterns and how these events relate to cancer development. Finally, it remains to be established how epigenetic deregulation induced by exposures in early life could influence cancer risk in adulthood. Remarkable conceptual advances in epigenetics and the emergence of powerful technologies that allow the analysis of epigenetic events in high throughput and genome-wide settings as well as the availability of unique prospective and population-based cohorts should facilitate this task. A genome-wide approach using surrogate samples such as peripheral tissue samples for epigenetic risk factors may provide important information for the discovery of new tissue- and cell- specific epigenetic biomarkers.

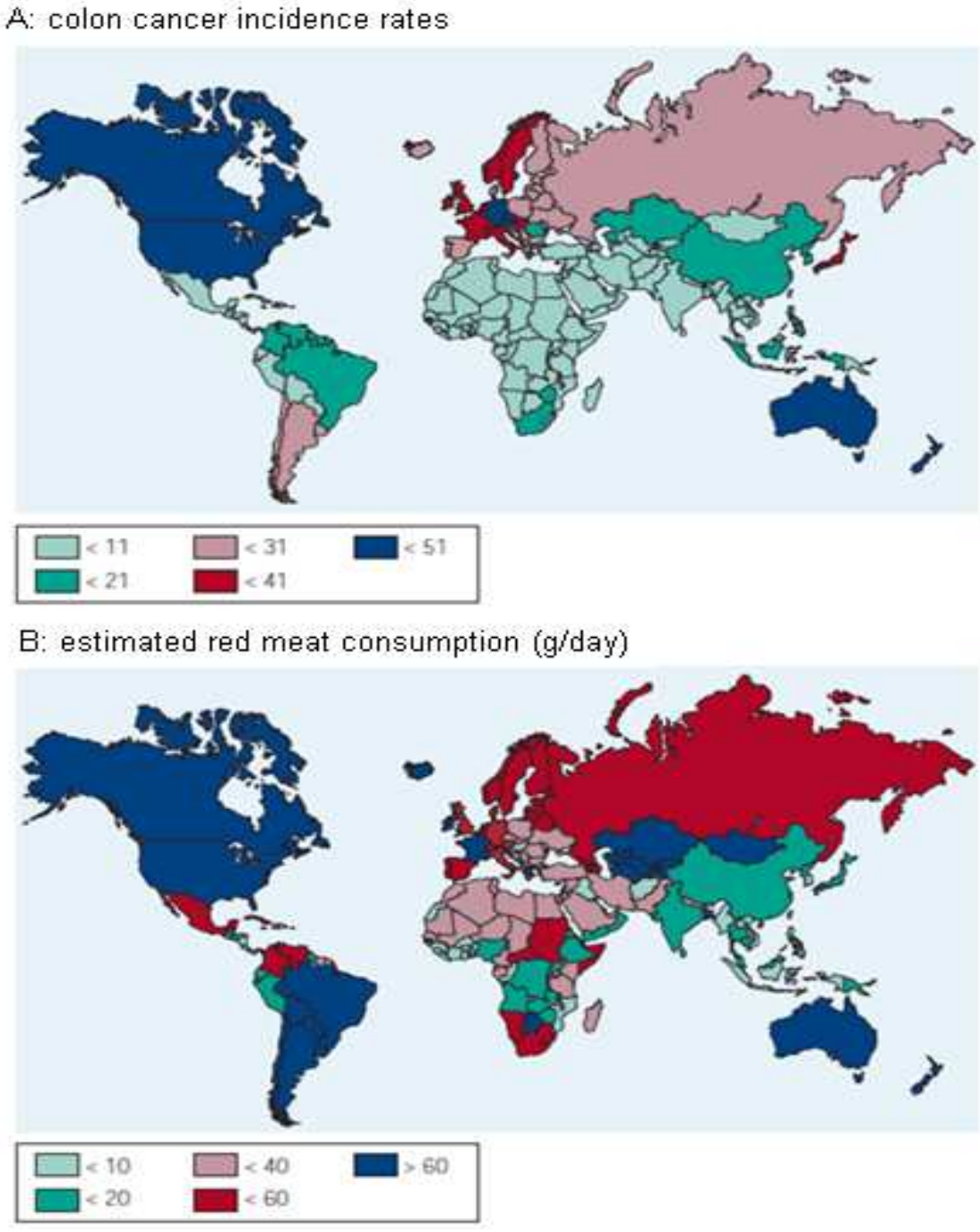
Protective effect of diet against cancer development

The close relationship between nutrition and health has always been clear to humans, as witnesses this ancient statement of Hippocrates in 400 B.C.: "…to the human body it makes a great difference whether the bread be fine or coarse; with or without the hull, whether mixed with much or little water, strongly wrought or scarcely at all, baked or raw…. Whoever pays no attention to these things, or, paying attention, does not comprehend them, how can he understand the diseases which befall man?"

The field of nutrition started to gain interest in the mid 1900, and in 1975 the results from a small prospective study suggested that people with a low intake of vitamin A from foods were at increased risk for lung cancer (Bjelke 1975). The differences in cancer rates between countries in relation to diet were also investigated at that time and it was suggested that various dietary factors, including plant foods, might have important effects on cancer risk (Armstrong and Doll 1975). The number of epidemiological studies on nutrient intake and cancer then started to increase, and in 1992 a review of 156 studies concluded that 'for most cancer sites, persons with low fruit and vegetable intake experience about twice the risk of cancer compared to those with a high intake, even after control for potentially confounding factors' (Block et al. 1992). Figure 9 suggests a role for red meat intake in colon cancer development, as the countries with the highest incidence (North-America and Australia in Panel A) correlate well with the countries consuming the most red-meat (Panel B). Epidemiological evidence started also to be supported by mechanistic results showing the potential protective effects of various micro-nutrients, such as the reduction of oxidative DNA damage or an increased activity of carcinogens detoxifying enzymes (Steinmetz and Potter 1991). In 1997, the First World Cancer Research Report on Diet and Cancer (World Cancer Research Fund/American Institute for Cancer Research 1997) established that there was *convincing* evidence that high intakes

of fruit and/or vegetables may decrease the risk of cancers of mouth, pharynx, oesophagus, stomach, colon and lung.

Figure 9: Comparison between colon cancer incidence and red meat consumption



From Bingham et al. 2004

The same report, 10 years after, updated the previous evidence from convincing to either *probable* or *limited-suggestive* (World Cancer Research Fund/American Institute for Cancer Research 2007), highlighting the difficulty to extrapolate epidemiological findings to humans. This report is the most comprehensive critical review to date on diet and cancer and includes an updated process to continuously incorporate new evidence, perform meta-analyses and to revise judgements as necessary. The association of fruit and vegetable consumption with smoking-related cancer incidence was evaluated in the large European Prospective Investigation into Cancer and Nutrition (EPIC) and found to significantly decrease lung cancer risk (Linseisen et al. 2007). In contrast, other studies showed no beneficial effects (Tsubono et al. 2001) or failed to find a positive association for a reduced risk of lung cancer (Wright et al. 2008). IARC evaluation (IARC, Handbook of Cancer Prevention Vol. 9, 2004b) concluded that there was *limited* evidence that eating cruciferous vegetables reduces lung cancer risk and *inadequate* evidence to assess the independent effects on human cancer risk of specific micronutrients (e.g. isothiocyanates) as opposed to their combined effects with other compounds.

Clearly, a huge challenge encountered by nutritional epidemiology is an accurate estimate of exposure's levels to identify which dietary components, at what doses and over what time periods, enhance risk or protect against cancer development (Jenab et al. 2009). In many epidemiological studies, exposure is assessed from food frequency questionnaires giving rise to a number of methodological issues such as problems of misclassification, confounding and recall bias. The difficulty in exposure assessment is due to the poor use of validated diet assessment instruments and there is a need for development and application of dietary biomarkers.

Consumption of foods varies by season, dietary habits, age and environment. The best approach is to estimate dietary intake by combining information on intake with measured concentrations of the micronutrient under study. A biomarker or set of biomarkers (e.g. urinary isothiocyanates) better reflect the

intake of a food, that contains a specific pattern of substances, and may also provide insights into biological mechanisms. Moreover, biomarkers of dietary exposure offer objectivity and accuracy and overcome differences in cooking and dietary habits.

In conclusion, the use of biomarkers in nutrition is very attractive although very few dietary biomarkers have been validated in humans so far. *In vitro* studies are very promising and agents that decrease oxidative DNA damage have already been proven to decrease the subsequent development of cancer. On the other hand, the actual mechanisms in humans of these chemopreventive agents are very complex and a biomarker will need to account for the actual dietary intake, different bioavailabilities (different metabolites in tissue compared to plasma/urine), high inter-individual variability in metabolic processes, lifestyle variables (e.g. smoking, physical activity).

Protective mechanisms of polyphenols and isothiocyanates: epigenetic modulation

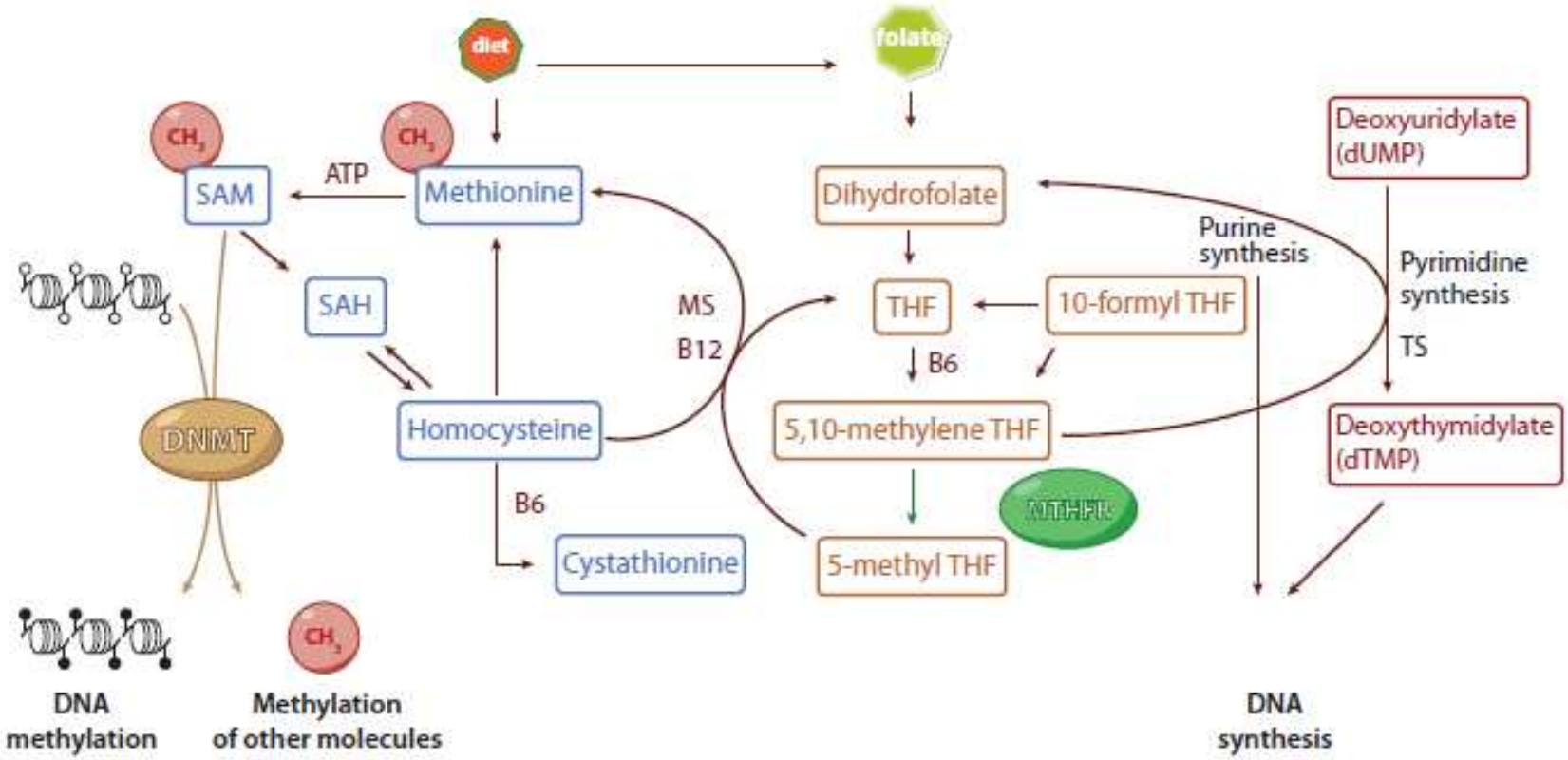
A new research field in nutrition which carries the promise of elucidating mechanism pathways is the study of epigenetic regulation by diet. The molecular link between epigenetics and nutrition can be exemplified by the one-carbon metabolic pathway whereby dietary compounds such as vitamin B12 or folic acid are implicated in the regulation of the cytosine methylation pathway (Figure 10).
Given that S-adenosylmethionine (SAM) is the donor of methyl groups during DNA methylation, dietary sources of methyl groups, including folate and methionine, are primary candidates as potential modulators of DNA methylation. More broadly, dietary factors that interact with the one-carbon metabolism include B vitamins that act as coenzymes (e.g. vitamins B6 and B12) and are also modulators of DNA methylation.

It is not clearly understood why certain CpG islands are hypermethylated in cancer cells while others remain methylation free. The environment and nutrition could modulate this local hypermethylation, since the most hypermethylated tumour types are those of the gastrointestinal tract that are more exposed to external carcinogenic agents. Moreover, if we take into consideration the study from Fraga et al. (Fraga et al. 2007) that reports that patterns of epigenetic modifications of monozygotic twin pairs diverge as they become older, it is not surprising that external factors such as smoking habits, or diet, together with internal factors, can influence the hypermethylation status of specific tumour suppressor genes. Another proof of principle that dietary exposures affect epigenetic marks comes from studies of the adult offspring of women exposed to famine during their pregnancy. Adults who were exposed periconceptionally to famine during the Dutch Hunger Winter of 1944–1945 had lower methylation levels of the imprinted gene *insulin-like growth factor-2* (a gene critical for tumourigenesis) compared with their unexposed, same-sex siblings (Heijmans et al. 2008).

Even if these studies had several limitations including lack of knowledge of the specific dietary habits linked with the DNA methylation changes, they demonstrate the impact of dietary changes on epigenetic marks.

Nowadays, a growing body of literature demonstrates that some micronutrients, constituents of food and herbs, may have a great influence on DNA methylation patterns. Studies of effect of natural compounds indicate that they are able to prevent or reverse promoter hypermethylation-induced silencing of key tumour suppressor genes and inhibit cancer development (Lee et al. 2005; Fang et al. 2003).

Figure 10: Methyldonor through one-carbon metabolism



Methionine reacts with ATP to form S-adenosyl methionine (SAM) which is the methyl (-CH3) donor for DNA methylation. Folate in the form of tetrahydrofolate (THF) participates in one-carbon transfer reactions. Vitamin B12 acts as an essential co-enzyme in the transfer of the methyl group from 5-methyl THF to methionine. Vitamin B6 serves as a co-enzyme in other reactions (TS, thymidylate synthase; MS, methionine synthase). From Lamprecht and Lipkin 2003.

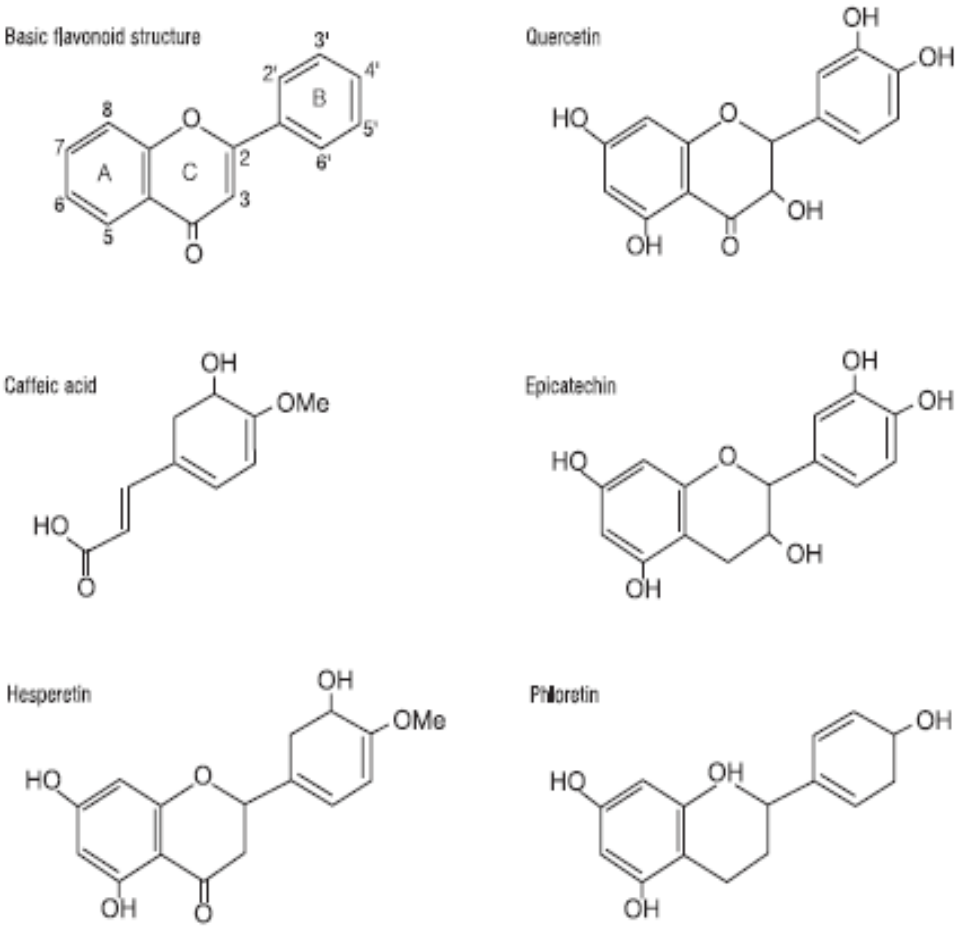
Among the many plant phytochemicals, tea polyphenols (e.g. catechin, epicatechin, (-)-epigallocatechin-3-gallate), bioflavonoids (e.g. quercetin, fisetin, myricetin), genistein from soybean and coffee polyphenols (e.g. caffeic acid, chlorogenic acid) have received much attention for their health benefits. It was recently shown that they have the ability to inhibit DNA methyltransferase, thus DNA methylation levels, and eventually provide chemopreventive and anticancer properties (Li and Tleefsbol 2010). It was recently proposed (Guarrera et al. 2007) that a flavonoids-rich diet might influence the gene expression of DNA repair genes and thus possibly be implicated in decreasing tumour development.

Flavonoids have been reported to have multiple biological effects, depending on their structure and characteristics, including scavenging of oxidative agents, anti-inflammatory action, inhibition of platelet aggregation and antimicrobial activity (Rhodes and Price. 1997, Yang et al. 2001, Yoon and Baek 2005; Shen et al. 2005).

The numerous *in vitro* and *in vivo* evidences of chemopreventive and therapeutic effects of these phytochemicals have encouraged several clinical trials looking for evidence of cancer prevention. In vitro studies have shown that tea polyphenols and bioflavonoids inhibit DNMT-1-mediated DNA methylation in a dose-dependent manner (Lee et al. 2005). This effect appears to be due to increased synthesis of S-adenosylhomocysteine (SAH), a non-competitive inhibitor of trans-methylation reactions, by decreasing methionine synthase activity. Polyphenols are present in fruits, vegetables, seeds and drinks (e.g. green tea) and are regularly consumed in a healthy diet.

Several thousand polyphenolic molecules have been identified (i.e. with a common structure of diphenylpropanes C_6-C_3-C_6), providing a large panel of diverse structures that influence polyphenols' bioavailability, biological properties and health effects (Figure 11).

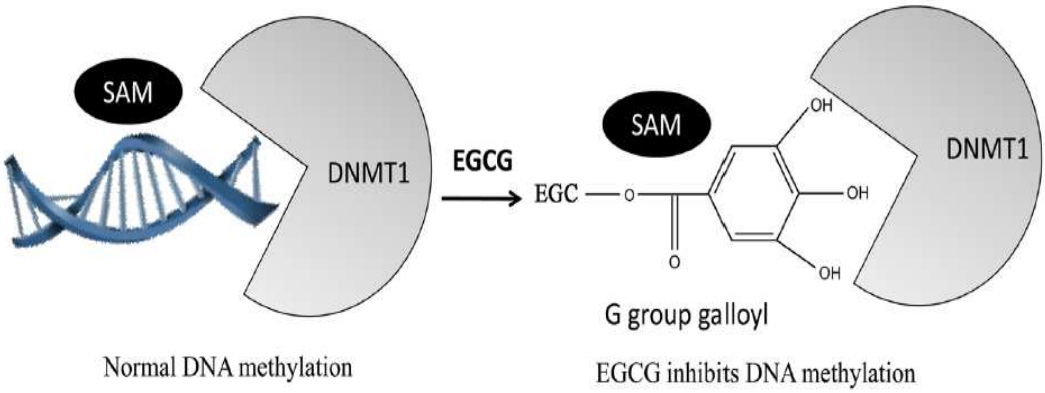
Figure 11: The basic structure of flavonoids and the chemical structure of selected polyphenols



From Manach et al. 2004

In particular, it has been reported that epigallocatechin gallate (EGCG; a polyphenol contained in green tea) inhibited DNA methyltransferase activity dose-dependently in several types of cancer cells, resulting in transcriptional reactivation (increased protein expression) of the methylation-silenced genes *CDKN2A* and *MLH1* (a gene involved in DNA mismatch repair) (Fang et al. 2003). The proposed mechanism of action of EGCG is a competitive inhibition of DNA methyltransferase 1 (DNMT1) through interaction with its catalytic site (Figure 12).

Figure 12: Interaction of EGCG with DNMT1

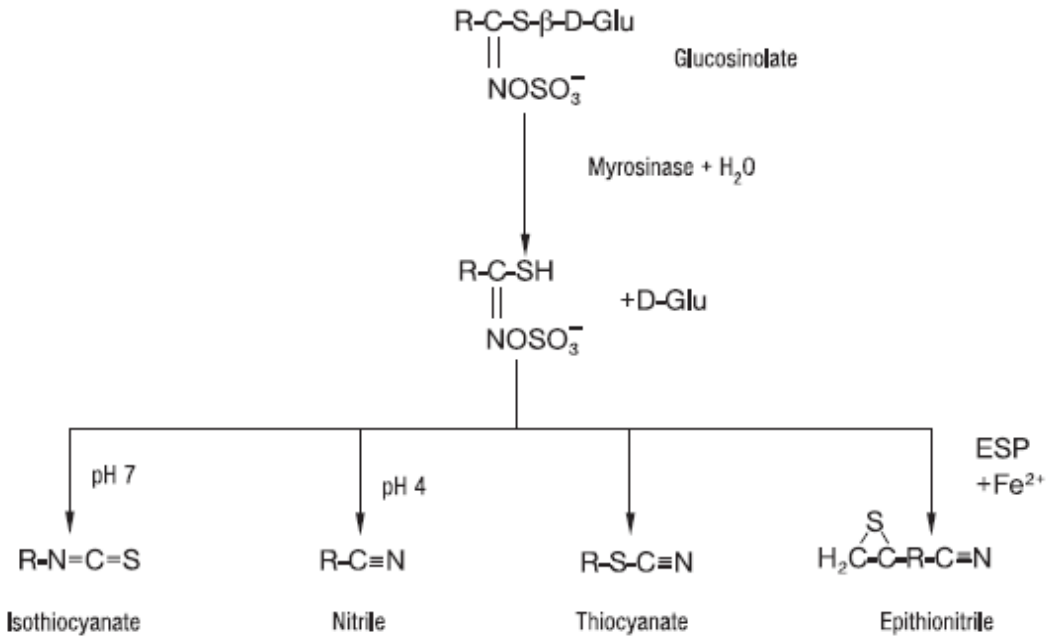


From Li and Tleefsbol 2010

However, it is important to note that the gene expression regulation seems to be cell specific (based on *in vitro* differences among cells from the same tissue), dose- (opposite effects for the same polyphenol depending on dose level) and time-dependent (Ramos 2008). Moreover, polyphenols markedly differ from one another in their bioavailability and intestinal metabolism.

Another class of highly promising cancer-preventive dietary agents, which possibly act through epigenetic mechanisms, is the isothiocyanates (ITCs). ITCs are metabolites of glucosinolates stored in plants such as cruciferous vegetables (e.g., broccoli, cabbage, cauliflower, Brussels sprouts). Cruciferous vegetables belong to the large botanical family of Brassicaceae, which count about 3000 species. The petals of plants of this family have a distinctive cruciform arrangement, which is the origin of the term “cruciferous”. Upon wounding of the vegetable, for example during harvesting, during freeze-thawing, during food preparation, or during chewing whilst eating, myrosinase is released from the “myrosin” cells and is able to hydrolyse glucosinolates within the damaged plant. The outcome of the reaction with myrosinase depends on the nature of the aglycone, as well as the reaction temperature and the pH (Figure 13).

Figure 13: Hydrolysis of glucosinolates



At high or neutral pH the formation of isothiocyanates is favoured while at low pH the formation of nitriles is favoured. From Hayes 2008.

Hydrolysis of glucosinolates with aliphatic or aromatic side chains gives rise primarily to ITCs at neutral pH (Hayes et al. 2008). The bioavailability of ITCs greatly depends upon myrosinase activity, which can be partially inactivated by heat during cooking or steaming, and by the length of time the vegetable is chewed.

Many of the biological properties of these compounds are determined by the chemical structure of the side-chain, which, in turn, is determined by the structure of the parent glucosinolate molecule. In view of the diverse spectrum of chemicals generated from glucosinolates, it is not surprising that a number of distinct cancer chemopreventive mechanisms have been proposed for cruciferous vegetables. *In vitro* studies suggested that ITCs may induce apoptosis, cell-cycle arrest and Phase II carcinogen-detoxifying enzymes (Zhang 2004; Seow et al. 2005). The xenobiotic-metabolizing phase II enzymes are among the most important of the defence systems. They modulate the access of

intermediate chemical carcinogens to DNA in target tissues, usually by reducing their biological activity and by accelerating their excretion. ITCs may inhibit the bioactivation of pro-carcinogens found in tobacco smoke such as PAH (Hecht 2000), enhancing excretion of carcinogenic metabolites before they can damage DNA. Recent epidemiological evidence of the protective effect of ITCs against cancer comes from studies on lung cancer risk and consumption of either total or specific cruciferous vegetables (Lam et al. 2009, IARC 2004b). It has also been suggested that ITCs may indirectly activate transcription factors such as Nrf2 (nuclear factor E2-related factor 2) and NF-kB (nuclear factor-kappaB), whose signalling pathways cover a variety of protective cellular events (Shen et al. 2005). Finally, the isothiocyanate sulforaphane has been shown to inhibit histone deacetylases (HDACs) (Myzak et al. 2006), possibly altering gene expression and having then implications for cell fate by altering tumourigenesis. HDAC enzymes may prevent gene transcription by favouring chromatin's coiled structure. In pre-cancerous and cancerous cells, tumour suppressor genes are associated with deacetylated histones, resulting in the inactivation of these genes. Inhibition of HDACs may prevent the removal of acetyl moieties from histones, thus allowing transcription of the tumour suppressor genes.

Many other food compounds, including food toxins, alcohol and folate, may influence DNA methylation. Folate is a vitamin B that participates in the one-carbon metabolism and affects methyl-group availability; alcohol is known to interfere with folate absorption and supply to tissue (Hillman and Steinberg 1982). Moreover, recently, alcohol and folate were shown to be significantly and independently associated with methylation profiles in breast tumours (Christensen et al. 2010).

Several studies on nutrition and cancer show highly encouraging results from both *in vitro* and *in human* evidence, but several questions still remain to be addressed. Since the pathways of nutrient metabolism are encoded in the genes it is essential to understand the influence of an individual's genetic make-up on the metabolism of nutrients and of nutrients on gene regulation.

Numerous bioactive compounds have been isolated and identified, and their potential health-promoting effects evaluated extensively, both *in vitro* and *in vivo*. An important aspect that could be advocated is that purified phytochemicals not necessarily have the same beneficial health effect as when their source is a food or a complete diet. There is a growing body of evidence suggesting that the actions of phytochemicals administered as dietary supplements may fail to provide the health benefits that have been observed when following diets rich in fruits, vegetables or whole grains.

Compounds contained in cruciferous vegetables could affect cancer risk by several mechanisms and relatively high doses of single bioactive agents may show potent anti-carcinogenic effects. As a note, the cancer-preventive effects that certain whole foods and diets were shown to have can better be explained in terms of synergistic interactions between the different dietary ingredients involved. In conclusion the field of diet influence on cancer risk is a very complex and challenging one, where new biomarkers of exposure and effect are most needed and where emerging mechanistic hypotheses should be tested in appropriately designed randomised controlled trials.

Biomarkers of environmental exposure: genetic and epigenetic approaches

Many studies on exposure have focused on environmental factors that induce measurable biomarkers in exposed subjects ahead of disease development.

This category of biomarkers of exposure is very wide and includes DNA or protein adducts of carcinogens, measurement of viral loads, of immune response, of the accumulation of toxins, of inflammatory cytokines, etc. In recent years, studies on the early steps of carcinogenesis have helped identify early genetic changes that are detectable primarily in cancer tissues but are thought to occur well before development of the tumours. Two of these early changes are mutations in “master” cancer genes such as *TP53*, and modifications of

epigenetic patterns in the promoter region of specific genes involved in cell cycle, apoptosis or DNA repair. Evidence that these changes may occur and be detectable in normal tissue as the result of exposure to specific cancer-causing agents is still scarce due to practical and ethical difficulties in conducting experimental prospective studies in a human setting.

Nevertheless, mutations and methylation in several genes that are good candidate markers of exposure to carcinogens have been studied in some details in tobacco-induced lung carcinogenesis. With respect to *TP53* mutation patterns, it has been shown that some tobacco carcinogens induce specific mutations that are rare in cancers associated with exposures other than tobacco (including lung cancers of never-smokers). With respect to methylation patterns, several studies using bronchial tumour and matched, non tumour tissues have uncovered the presence of high levels of methylation in the promoter of genes such as *CDKN2a* or *RASSF1*.

Not only tobacco but also diet may alter DNA methylation in tissues. Recent *in vitro* evidences showed the capacity of specific micronutrients (i.e. polyphenols) to inhibit DNA methyltransferase activity in several cancer cells, thus transcriptionally reactivating methylation-silenced tumour suppressor genes or genes involved in cell-cycle regulation. However, the mechanisms through which these epigenetic changes occur is still poorly understood and effects in humans are controversial.

Thus, while it is legitimate to consider that these modifications occur as a direct or indirect result of exposure, whether such mutation or methylation changes can be used as biomarkers of exposure in molecular epidemiology is still very questionable.

Chapter II: Research Objectives

In this thesis, I have explored the application of either DNA methylation changes or *TP53* mutations to the study of exposure to tobacco smoking in two different settings, i.e. observational and experimental. The main objective was to identify and solve logistical, technical, data analysis and interpretation problems related to the application of these molecular parameters as biomarkers. Moreover, the prognosis impact of mutations (i.e. *TP53*, *KRAS* or *EGFR*) on the risk of lung cancer recurrence or metastasis was explored, evaluating their use as biomarkers of disease progression, and the use of DNA methylation as biomarker of exposure to diet.

The project was articulated in two parts:

A: *Study of the effects of a calibrated, defined dietary intervention on DNA methylation patterns of specific genes, in heavy smokers*

In a randomized intervention trial, 90 heavy smoking volunteers were assigned to different dietary regimens for one month. Peripheral blood was collected at inclusion in the study and at the end of the intervention period, and patterns of methylation were analysed by pyrosequencing. We have measured levels of gene-promoter methylation as biomarker of exposure to tobacco and as biomarker of intermediate effect of specific micronutrients (i.e. cruciferous vegetables and isothiocyanates). An intervention trial design was adopted since DNA methylation has not been yet established or validated as a biomarker of dietary exposure. In this study, the relationship between levels of DNA methylation (both global and gene-specific) and the response to particular micronutrients was analysed retrospectively. We analysed promoter methylation of genes involved in cell-cycle regulation or 1-carbon metabolism and of repetitive elements dispersed throughout the genome (used as indicators of global methylation).

B: *Study of the pattern, types and distribution of somatic mutations and polymorphisms in an international series of lung cancer tissues*

Defined cases of lung cancers were recruited prospectively in several hospitals in Western Europe. Case-case comparisons were performed to identify differences in mutation patterns in relation with histology and history of exposure to carcinogens as well as to other factors for which data were collected at recruitment. The target material was DNA extracted from primary lung cancer tissue collected in a clinical setup. We have extended the analysis to test retro-prospectively the biomarkers' predictive value for lung cancer recurrence in order to identify whether an individual's prognosis can be based on their status.

The detailed design, methods, results and discussion are presented for each of these approaches and the results summarized in a general discussion underlining the challenges of using these parameters as biomarkers of exposure and early effects in cancer research.

Chapter III:
Methylation patterns in sentinel genes in peripheral blood cells of heavy smokers: influence of cruciferous vegetables in an intervention study

Working Hypothesis

In this randomized intervention study, the methylation patterns of selected genes were analysed before and after a 4-week intervention of protective diets in a heavy-smokers population. To date, there have been few studies that have examined the effects of dietary (and other environmental) factors on epigenetic marks in intervention studies in humans. Current available evidence is derived from either observational studies (which however may carry uncertainties about causality and difficulties in characterizing exposure) or animal studies (where, in some cases, experimental conditions and/or exposure doses may be difficult to translate to humans). In humans, tobacco smoking has been shown to increase DNA methylation of cancer-associated genes such as *RASSF1A* (encoding a modulator of RAS signalling), *MTHFR* (a regulator of folate metabolism) and *CDKN2A/ARF* (encoding the suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$) (Vaissière et al. 2009). Moreover, in a cohort of smokers, Stidley et al. observed that folate and other nutrients were associated with decreased gene promoter methylation levels in cells exfoliated from the aerodigestive tract (Stidley et al. 2010). We have observed a similar association in a previous study using DNA extracted from peripheral white blood cells (WBC). In a nested case-control study on lung cancer within the EPIC cohort, serum methionine levels were associated with decreased smoking-associated hyper-methylation in *CDKN2A*, *RASSF1A* and *MTHFR* (Vineis et al 2011).

A randomized intervention trial was initially undertaken to investigate the ability of different diets to increase urinary anti-mutagenicity and to inhibit the formation of DNA adducts in exfoliated bladder cells of heavy smokers (Malaveille et al 2004;

Talaska et al. 2006). These studies showed an increased anti-mutagenicity but no consistent effect on DNA adducts. Here we have extended this trial to measure patterns of methylation levels in DNA extracted from WBC of heavy smokers. The rationale for the project was that tobacco carcinogens can affect DNA methylation of certain key genes and dietary components may regulate this process. Therefore, we have analysed 5 genes proposed to be frequent targets of methylation in lung cancer or involved in the DNA methylation process itself, and with distinct endogenous methylation patterns (Table 2).

Table 2: Epigenetic study: list of genes and their putative biomarker function

Gene	Gene card	Putative biomarker characteristic
LINE-1	Multi-copy, retrotransposable element evenly distributed throughout the genome	Provide a marker for overall genome methylation; their expression may be associated with increased retrotransposition
RASSF1A	Tumour suppressor gene regulator of RAS signalling	Very commonly hypermethylated and down-regulated in tobacco-induced lung cancers
CDKN2As: ***$p14^{ARF}$***, ***$p16^{INK4a}$***	Locus encoding two critical suppressor factors regulating cell cycle and apoptosis	Often hypermethylated in tobacco-induced cancers
MLH1	Gene involved in DNA mismatch repair and therefore in the control of genetic stability	Its down-regulation may be linked to a “mutator phenotype” by which cells may acquire cancer-causing mutations at a high rate
MTHFR	Gene coding a folate-metabolism enzyme	Critical regulator 1C-metabolism with multiple effects on energy metabolism and, in particular, on precursors of DNA methylation and on the biosynthesis of nucleic acids

Materials and Methods

Study design

The study was designed according to the CONSORT guidelines (http://www.consort-statement.org). This blind randomized controlled trial was conducted in Torino, Italy, among a population of healthy blood-donor volunteers, all heavy smokers.

An introduction phase (run-in), lasting 1 month, preceded the trial and consisted in a qualifying visit during which 120 consenting volunteers were asked to complete a questionnaire on lifestyle and medical conditions. Healthy (on the basis of a medical questionnaire) men, aged 52 years on average, with self-reported history of heavy smoking (i.e. at least 15 cigarettes/day over the last 10 years) and with balanced dietary habits were included (vegetarians were excluded). Inclusion data were verified by medical and epidemiological staff and qualifying volunteers were asked to provide informed consent for participation into the intervention trial.

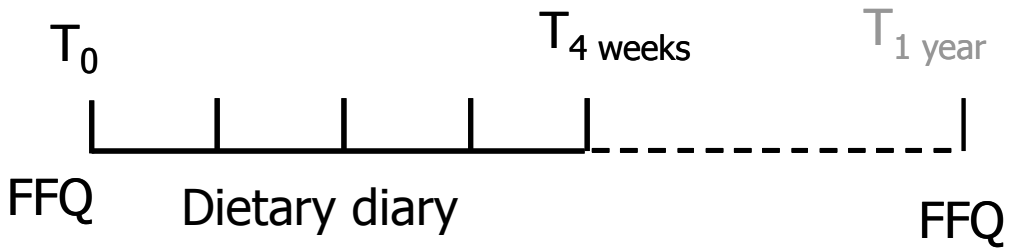
A total of 90 volunteers complied with all requirements and provided a non-fasting blood sample at the beginning and at the end of the trial. A sequential number was assigned to each eligible volunteer. Computer-assisted randomization was conducted to assign the participants to each of the intervention groups. All subsequent sample management and analyses were conducted in a manner blinded as to the status of the participants.

The participants were given an induction course by a professional cook on how to prepare diets according to their assigned intervention group. Participants were invited to substitute their regular diet with the intervention diet and to report daily compliance using an intervention diary. Group 1 was assigned a ‘normal diet’, consisting of an isocaloric diet balanced in fruits and vegetables (according to the recommendation of the World Cancer Research Fund). Group 2 was assigned an ‘enriched diet’, including polyphenols- and isothiocyanates-rich foods such as cruciferous vegetables; and Group 3 was assigned a ‘supplemented diet’, based

on supplementation of the normal diet with polyphenols in the form of green tea and soy products.

The trial lasted 4 weeks, starting the day after the induction course. All participants filled a food frequency questionnaire (FFQ) at inclusion and a dietary diary for the duration of the intervention. A FFQ was filled again 1 year after the end of the trial and smoking habits were recorded again (Figure 14).

Figure 14: Schema representing the dietary recordings during the project



Intakes of micronutrients (flavonoids, several vitamins and folate) were estimated through the self-administered diary, checked weekly and abstracted by a dietician who developed a food-nutrient-intake matrix specifically focused on flavonoids, to quantitatively assess their intake.

Statistical analysis

For each gene and each individual, methylation levels were expressed as the average percentage of methylation at all the CpGs included in the DNA domain analysed. Methylation levels in each intervention group were expressed as medians and interquartile range. We tested differences in methylation levels between T0 and T4 using the non-parametric Wilcoxon signed rank test. Equality of variance was tested using the folded form F statistic.

To assess these differences, we did not include in this comparison samples with a methylation value of 0 at both T0 (inclusion) and T4 (end of intervention). These negative samples included 25 pairs (T0/T4) for *MLH1* and 10 pairs for *ARF*. Similarly, an outlier value for LINE1 was not included in the final analysis. This outlier was observed at T4 for patient 67 (normal diet: T0=72.05; T4=66.23).

The Kruskal-Wallis test was used for comparison of differences in methylation across the three dietary regimes.

All analyses were performed using STATA 11 and SAS V9.2. All tests were two sided and statistical significance was assessed at the level of 0.05.

Laboratory Methods

DNA methylation analysis

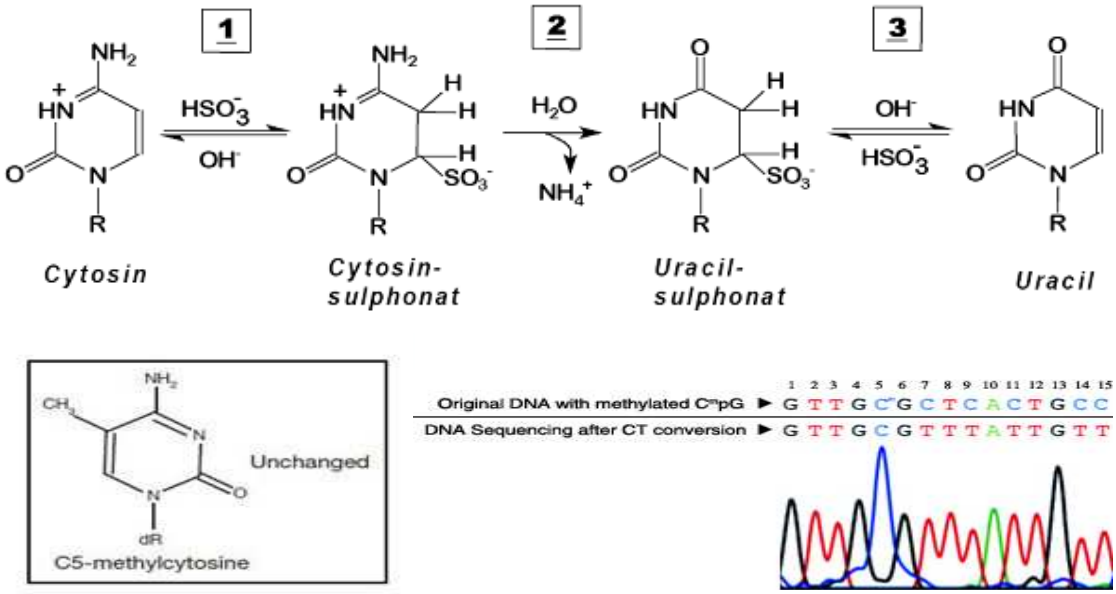
To identify gene-specific and global DNA methylation (methylation of repetitive elements interspersed throughout the genome) of genomic DNA from blood lymphocytes, we relied on the well validated pyrosequencing technique. Sensitive and quantitative methods are needed to detect even subtle changes in the degree of methylation as biological samples often represent a heterogeneous mixture of different cells.

The analysis involved several steps: (1) the bisulfite treatment to discriminate the methylation status of the sample, (2) the PCR amplification of the sample, (3) sample preparation for pyrosequencing analysis and (4) analysis in the pyrosequencing instrument.

Bisulfite treatment of genomic DNA

Bisulfite treatment of genomic DNA samples results in the hydrolytic deamination of nonmethylated cytosines (C) to uracils (U), whereas methylated cytosines (mC) are resistant to conversion (Figure 15). After PCR, U is amplified as thymine (T), and mC is amplified as C. In the resulting pyrogram, mC and C are therefore represented as C (former methylayed cytosine) and T (former nonmethylated cytosine) peaks, respectively, and can be analysed as a virtual C/T polymorphism in the bisulfite-treated DNA. Probably the most critical step in the bisulfite conversion is denaturing the DNA since only single-stranded DNA is accessible to chemical modification.

Figure 15: Bisulfite treatment and example of DNA sequencing product



The methylated cytosine at nucleotide position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 are converted into uracil and detected as thymine following PCR. Adapted from Zymo Research website.

0.5-1µg of genomic DNA from blood lymphocytes were treated with EZ DNA methylation-Gold Kit™ (D5007, Zymo Research, Orange, USA), according to the manufacturer protocol which allows a conversion efficiency >99% and DNA recovery >75%. 130µl of CT conversion reagent was added to 20µl of each DNA sample in a conversion plate. DNA was denaturated (at 98°C for 10min), incubated (at 64°C for 2.5h) and stored (at 4°C up to 20h). After desulphonation, single-stranded DNA was washed and desulphonated. 30µl were recovered with an elution buffer and stored at -20°C.

PCR amplification

DNA treatment with sodium bisulfite converts the four-letter genetic code into a three-letter alphabet. As the former complementary DNA strands are differentially modified, 2 populations of DNAs are available for primer design. DNA was quantified by PicoGreen (*Pico*fluor™ Fluorometer and PicoGreen dsDNA

Quantitation Reagent, supplied by Molecular Probes, Inc., Eugene, Oregon) and diluted to 20-25ng/µL. Bisulfite-treated DNA was amplified in 50 µL using specific PCR conditions (Table 3) and primers (Table 4). PCR reactions involved a 15-min polymerase activation at 95°C, 50 cycles of denaturation (95°C, 30s), primer annealing (30s at 51°C for *MLH1*, 55°C for *RASSF1A* and *ARF*, 56°C for *MTHFR*, 58°C for *LINE-1*, 64°C for *CDKN2A*), and extension (72°C, 30s), followed by a final 10-min extension at 72°C. 10µL of PCR products were checked on agarose gel previous to pyrosequencing analysis.

Table 3: PCR mixtures

MLH1 – RASSF1A – MTHFR – LINE-1
5u/µl GoTaq Hot Start (Biomega), 5X buffer, 25mM $MgCl_2$, 8mM each dNTP, 10µM each primer
ARF – CDKN2A
5u/µl Hotstar Taq (Qiagen), 10X buffer, 8mM each dNTP, 10µM each primer

Purification and preparation

PCR products were converted into single-stranded DNA, one strand was isolated (through labelling with biotin) and used as template in the pyrosequencing reaction. dNTP were removed from the PCR mixture to allow for controlled addition of single nucleotides (PyroGold Reagent kit, Biotage AB, Uppsala, Sweden). 3µl of Streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden) were added to 40µl binding buffer (10 mM Tris-HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixed with 40µl PCR product for 10 min at room temperature. The beads containing the immobilized templates were captured on the filter probes after the vacuum was applied and then washed with 70% ethanol for 10s, denaturation solution (0.2 M NaOH) for 10s, and washing buffer (10 mM Tris-acetate, pH 7.6) for 10s. The vacuum was then released, and the beads were released into a PSQ 96 Plate Low (Biotage AB) containing 40µl annealing buffer (20 mM Tris-acetate, 2 mM $MgAc_2$, pH 7.6) and 0.5 µM sequencing primer.

Table 4: List of primers used in the pyrosequencing assay

Gene	Amplification primers (5'→3')	Sequencing primers (5'→3')	Modified sequence/corresponding unmodified sequence analysed (5'→3')
$p14^{ARF}$ (F)	TTGAGGGTGGGAAGATGGT	GGAGGGAGAGGAA	**Y**G**Y**GGGTTTTGAGT**Y**GTT**Y**G**Y**G**Y**G**Y**G**Y**G
$p14^{ARF}$ (R)	biotin-CCCRAACCTCCAAAATCTC		**CGCG**GGCCCTGAGC**CG**CC**CGCGCGCGCG**
RASSF1A (F)	AGTTTGGATTTTGGGGGAGG	GGGTTAGTTTTGTGGTTT	**YG**TT**YG**GTT**YGYG**TTTGTTAG**YG**TTTAAAGTTAG**YG**
RASSF1A (R)	biotin-CAACTCAATAAACTCAAACTCCCC		**CG**CC**CG**GCC**CGCG**CTTGCTAG**CG**CCCAAAGCCAG**CG**
LINE-1 (F)	biotin-TAGGGAGTGTTAGATAGTGG	AACTCCCTAACCCCTTAC	**R**CCCTACTT**CR**ACT**CRCR**CA**CR**ATA**CR**
LINE-1 (R)	AACTCCCTAACCCCTTAC		**G**CCCTGCTT**CG**GCT**CGCG**CA**CG**GTG**CG**
MLH1 (F)	TTTAGGAGTGAAGGAGGT	GTTTTGAYGTAGAYGTTTT ATTAGGGT	**Y**G**Y**G**Y**GTT**Y**GT**Y**GTT**Y**GTTATATAT**Y**GTT**Y**GTAGTATT
MLH1 (R)	biotin-CCCTATACCTAATCTATC		**CGCGCG**CT**CG**C**CG**TC**CG**CCACATAC**CG**CT**CG**TAGTATT
MTHFR (F)	TTTTAATTTTTGTTTGGAGGGTAGT	GGGTTTGGATTTTGAG	**Y**GGTATGAGAGATTT**Y**GGGAGAAGATGAGG**Y**GG**Y**GATTG
MTHFR (R)	biotin-AAAAAAACCACTTATCACCAAATTC		**CG**GCATGAGAGACTC**CG**GGAGAAGATGAGG**CG**G**CG**ATTG
$p16^{INK4a}$ (F)	GAGGGGTTGGTTGGTTATTAGA	TGGTTATTAGAGGGTG	GGG**Y**GGAT**Y**G**Y**GTG**Y**GTT**Y**GG**Y**GGTTG**Y**GGAGAGG
$p16^{INK4a}$ (R)	biotin-TACAAACCCTCTACCCACCTAAAT		GGG**CG**GAC**CGCG**TG**CG**CT**CG**G**CG**GCTG**CG**GAGAGG

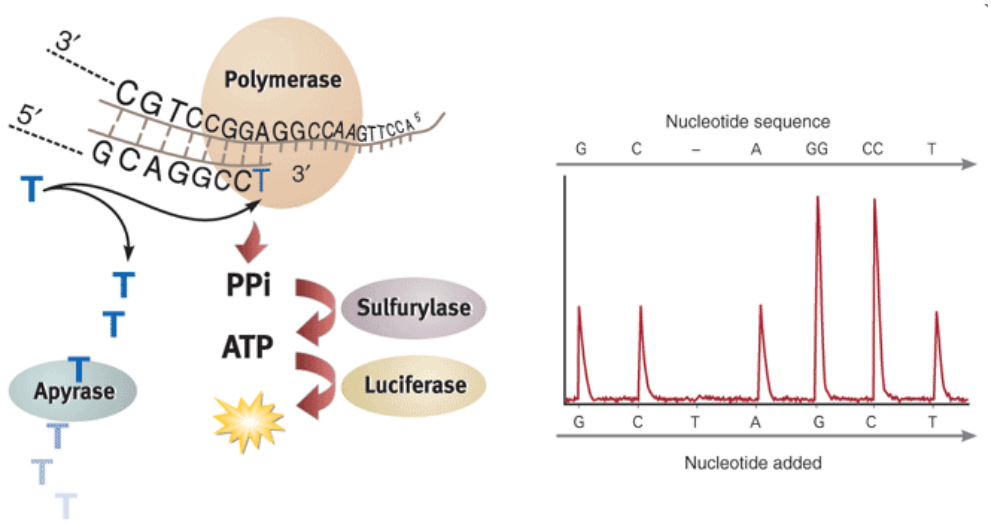
Pyrosequencing reaction

Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit (Biotage AB), which contained the enzyme, substrate, and nucleotides. The PCR involved two amplification primers that are specific to the sequence of interest, one of which is a biotinylated primer (please refer to Table 4 for details). During the PCR, the biotin tail is incorporated into the amplicon sequence. Biotin-labeled amplicons are captured by binding to streptavidin-coated Sepharose beads, and DNA is denatured to produce ssDNA template for the pyrosequencing assay. The ssDNA is released and is combined with the sequencing primer, which is extended during the pyrosequencing reaction to provide the sequence of the template DNA.

The sequential incorporation of every nucleotide is converted to light, which is detected by the PSQ 96 instrument, enabling the sequence of the template strand to be determined. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand if it is complementary to the base in the template strand. Each incorporation event releases a pyrophosphate, which is converted to ATP by a sulfurylase (Figure 16). This ATP then drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. ATP and unincorporated dNTPs are continuously degraded by apyrase. The light is switched off, and the next dNTP is added. As the process continues, the complementary DNA strand is built up.

The light signal is detected via CCD camera and is converted into a quantitative signal in the program which allows determination of the nucleotide sequence. The resulting pyrograms are converted to numerical values for peak heights using the instrument’s software.

Figure 16: The principle of pyrosequencing and the output pyrogram



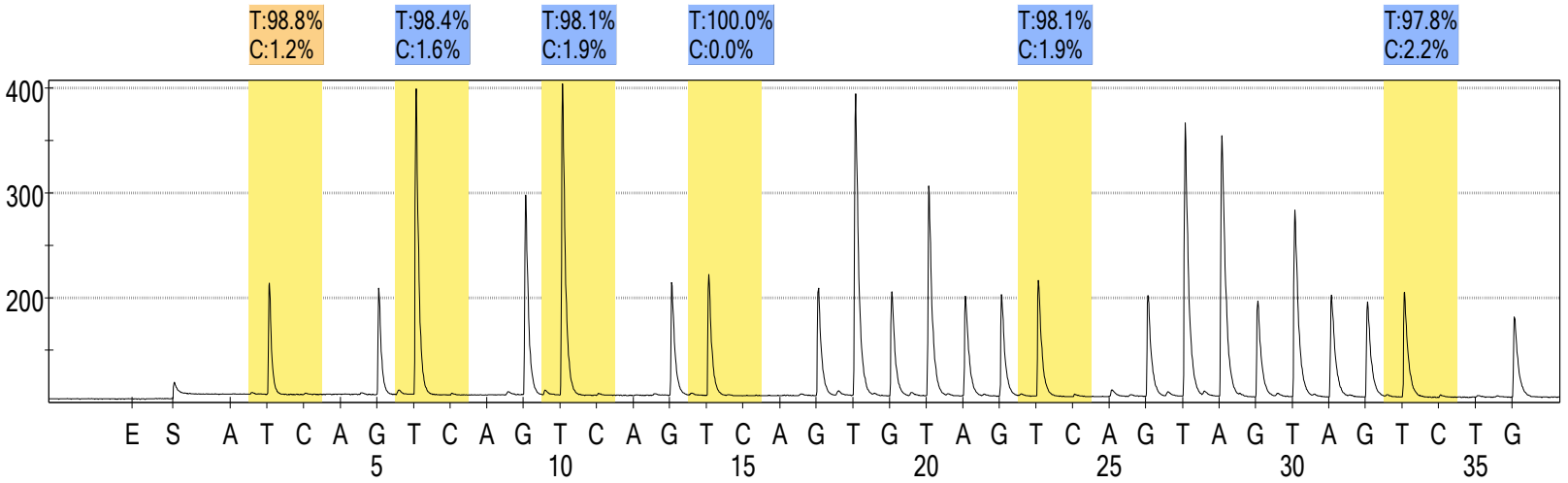
Double peak heights indicate incorporation of two nucleotides in a row. Adapted from Qiagen website.

The degree of methylation at a *single CpG* is calculated as allele frequency:

$$\text{Methylation \%} = \left(\frac{\text{peak height methylated}}{\text{peak height methylated + peak height non-methylated}} \right) * 100$$

Figure 17 gives an example of pyrogram showing the percentage at each CpG site interrogated in the gene (for each CpG dinucleotide interrogated, *C* represents the methylated allele and *T* represents the un-methylated allele converted during the bisulfite treatment).

Figure 17: *RASSF1A* pyrogram of promoter methylation in sample 1 at the end of the trial (T4 of supplemented diet)



The methylation levels for each gene at the target CpGs were finally expressed as mean percentage of methylation of all CpG sites analysed.
Pyrosequencing is a highly reliable and quantitative method for the analysis of DNA methylation at multiple CpGs and it provides a quantitative estimate of the level of DNA methylation at defined CpG sites with reference to built-in internal controls (Tost et al. 2003) to minimize PCR bias.

We analysed promoter methylation of p16*INK4A*, p14*ARF*, *MTHFR*, *RASSF1A* and *MLH1*. *INK4A* assay interrogated 7 CpGs; *ARF* assay interrogated 8 CpGs; *MTHFR* assay interrogated 6 CpGs; *RASSF1A* assay interrogated 6 CpGs and *MLH1* assay interrogated 8 CpGs. LINE-1 assay interrogated 6 CpG sites.

Results

Life-style profiles in the different dietary groups

Table 6 shows dietary habits (data from diary at the end of the study) among the three groups. The regimens were successful in increasing the intake of isothiocyanates (i.e. cruciferous vegetables), particularly in the group following the enriched diet, and of polyphenols (i.e. green tea and soya products) in the group receiving flavonoids supplementation. Moreover, a previous publication on the same study (Table 5) showed that blood protein adducts levels of dietary isothiocyanates (i.e. SFN-Lys: sulforaphane lysine) correlated with cruciferous vegetables intake in the enriched group as determined by the dietary questionnaire (Kumar et al. 2010). Adducts levels of sulforaphane mirrored ITCs bioavailability during the 4-week intervention (T0: baseline and T4: end of study).

Table 5: SFN-Lys and cruciferous vegetable intake by trial arm at T0 and T4

Dietary group (n)	SFN-Lysine (%)		Cruciferous vegetables (g/day)	
	T0	T4	T0	T4
Control (29)	17.2	10.3	2.4	6.3
Enriched (29)	13.8	31.0	4.9	68.8
Supplemented (27)	44.4	29.6	4.5	38.7

Adapted from Kumar et al. 2010

Table 6: **Mean (g/day averaged on the 4-weeks intervention) and standard deviation (SD) of selected food items for each dietary group**

Dietary items	Normal diet (n=29)	Enriched diet (n=30)	Supplemented diet (n=29)	*P*-value
Other foods	13.90 (32.03)	27.95 (18.00)	20.96 (12.55)	<0.0001
Soft drinks	13.91 (22.49)	19.42 (34.33)	30.21 (67.05)	0.81
Butter	2.31 (3.97)	1.78 (2.00)	1.42 (1.32)	0.89
Coffee	140.10 (68.08)	140.35 (54.46)	130.27 (61.14)	0.63
Meat	111.97 (52.36)	86.20 (36.70)	112.58 (67.39)	0.06
Cereals	393.43 (157.28)	373.18 (98.68)	373.01 (116.40)	0.93
Sweets	64.27 (51.60)	66.01 (44.43)	58.63 (33.95)	0.87
Cheese	41.42 (25.31)	40.99 (25.32)	33.86 (16.79)	0.35
Fruits	317.14 (192.01)	459.69 (147.23)	359.57 (167.10)	0.002
Nuts	2.01 (2.36)	3.59 (5.26)	0.83 (1.65)	0.002
Milk	87.13 (190.17)	64.86 (69.65)	73.33 (74.31)	0.59
Legumes	22.52 (19.94)	42.16 (22.93)	36.93 (20.91)	0.002
Margarine	0.04 (0.12)	0.01 (0.04)	0.06 (0.25)	0.48
Oil	26.52 (8.98)	25.20 (7.17)	23.06 (10.14)	0.17
Potatoes	32.04 (21.22)	33.68 (23.35)	43.46 (19.93)	0.07
Fish	56.49 (32.04)	52.83 (21.82)	45.74 (21.48)	0.42
Spices	0.45 (1.46)	0.18 (0.24)	0.26 (0.35)	0.35
Soya and derivatives	0.45 (1.20)	**2.62 (7.71)**	**3.54 (7.34)**	0.05
Green tea	6.10 (22.39)	1.42 (6.10)	**56.23 (61.13)**	**<0.0001**
Eggs	12.38 (6.46)	9.42 (5.76)	11.78 (8.08)	0.24
Vegetables	227.70 (103.26)	298.72 (125.76)	253.64 (93.45)	0.06
Cruciferous	6.30 (9.43)	**68.80 (46.43)**	**38.73 (32.24)**	**<0.0001**
Flavonoids (mg/day)	61.71 [59.73]	217.47 [77.83]	229.23 [84.00]	< 0.0001

There is evidence that age, alcohol, and folate may influence DNA methylation. Age is associated with methylation in non-pathological tissues (Ozanne and Constancia 2007), folate is a vitamin B that participates in the one-carbon metabolism and affects methyl-group availability, and alcohol is known to interfere with folate absorption and supply to tissues (Hillman and Steinberg 1982). We studied the levels of these variables by type of diet and found no association (see Table 7) for folate, age or alcohol. Consequently we think that these factors should not confound the association between methylation and diet.
On the other hand, participants in the normal-dietary group appeared to smoke slightly more than in the experimental groups. Consequently all comparisons are adjusted by number of cigarettes smoked at the time of blood collection.

Table 7: **Age, selected lifestyle and dietary habits by dietary group on the 4-week intervention, mean (SD)**

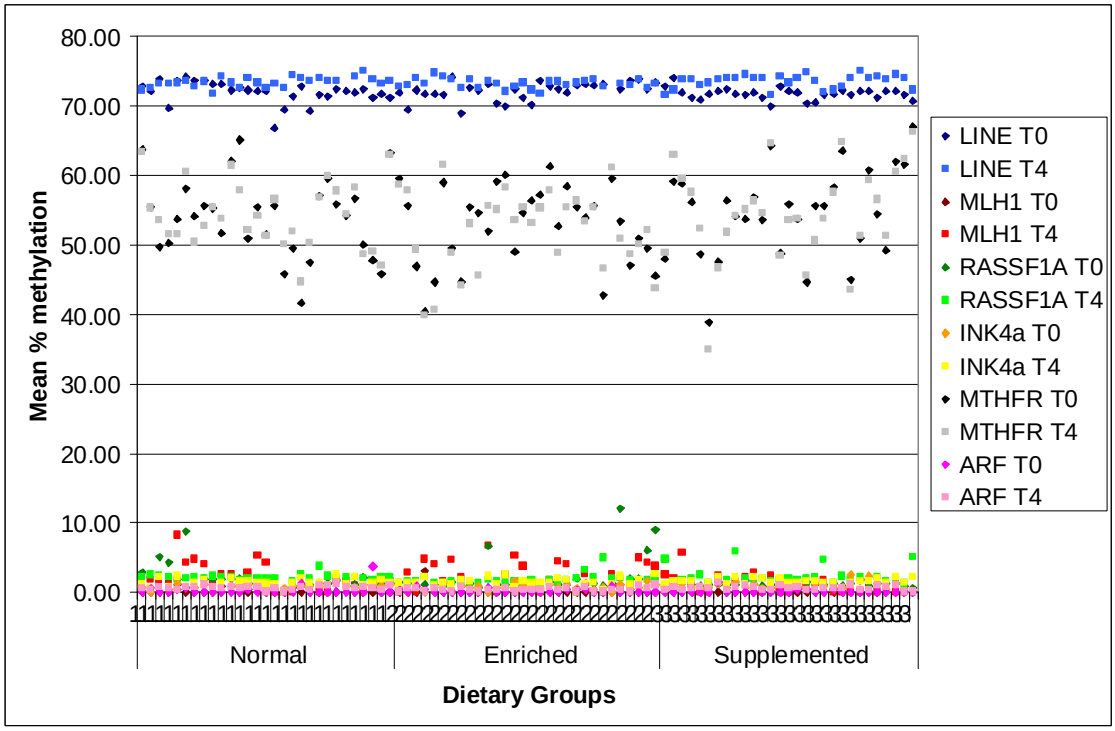
Variable	Normal diet (n=29)	Enriched diet (n=30)	Supplemented diet (n=29)	*P*-value
Age (years)	51.19 (7.02)	53.65 (6.99)	52.39 (6.15)	0.40
Alcohol (mg/day)	303.56 (246.07)	242.28 (255.78)	228.48 (126.31)	0.53
Folate intake (g/day)	212.40 (66.13)	199.86 (6.79)	200.60 (8.56)	0.34
Smoking (cigs/day)	27.28 (9.52)	20.43 (5.48)	20.28 (5.94)	0.005

Methylation patterns in individual markers

Three distinct patterns of methylation were distinguishable (Figure 18). LINE-1 was highly methylated, with over 70% of all CpGs consistently methylated in all subjects at both time points. *MTHFR* showed an intermediate methylation pattern with an average of over 50% methylated sites. The two *CDKN2A* genes and *MLH1* showed a low constitutive methylation level (between 0% and 5%); *p14ARF* showed methylation below the detection levels in 11% of individuals and *MLH1* in 29%, at both T0 and T4. *RASSF1A* methylation values were between 5% and 15% for 16% of individuals at T0 and 5% at T4.

The high methylation level of LINE-1 is in agreement with the hypothesis that these nuclear elements, which are distributed across the genome, may reflect on average the whole genome methylation changes. Previous studies have reported a similar level of methylation of LINE-1 in other groups of subjects, including cancer cases and controls. The intermediate methylation status of *MTHFR* suggests that this *locus* has a mechanism of methylation control distinct from whole genome methylation. Methylation levels of the cancer-related genes *CDKN2As* (*p16*^{INK4a} and *p14*^{ARF}), *RASSF1A* and *MLH1* were very low and had a skewed distribution (with a large number of null values), at both the beginning and the end of the 4-week dietary intervention.

Figure 18: Plots of % methylation distribution in the selected genes and LINE1 sequences, by three dietary regimes



Influence of a 4-week dietary intervention

In Table 8, the difference between methylation levels at T0 and T4 (*p*-value[1]) and the equality of variance in the two populations (*p*-value[2]) are presented.

The comparison between methylation patterns at T0 and T4 across all dietary regimes shows small but significant changes for LINE-1, but not for *MTHFR*. For the latter gene, no changes were detected between T0 and T4 within any intervention group or between the three intervention groups.

Methylation levels of the cancer-related genes were very low (<5%) both before and after dietary intervention. Consequently, changes in methylation, although statistically significant, are unlikely to impair gene expression but could reflect changes in peripheral blood cells population.

Table 8: Median and Inter Quartile Range (IQR) of percentage methylation levels, by gene, dietary group and time point (T0 and T4)

Gene	Diet	T0 Median [IQR]	T4 Median [IQR]	*P*-value[1]	*P*-value[2]
LINE-1	Normal	72.1 [69.5-73.7]	73.4 [72.6-74.3]	<0.0001	<0.0001
	Enriched	72.4 [69.7-73.7]	73.2 [72.4-74.0]	0.0009	<0.0001
	Supplem.	71.9 [70.5-72.8]	74.0 [71.9-74.5]	<0.0001	0.36
MTHFR	Normal	54.2 [46.0-63.2]	53.7 [48.7-61.4]	0.85	0.34
	Enriched	54.3 [44.6-59.5]	53.3 [44.0-58.4]	0.59	0.99
	Supplem.	55.6 [44.9-63.6]	54.2 [45.5-64.6]	0.98	0.73
p16^{INK4a}	Normal	0.8 [0.0-1.2]	1.3 [0.6-2.1]	0.0002	0.12
	Enriched	0.7 [0.0-1.1]	1.3 [0.5-2.3]	0.0001	0.07
	Supplem.	0.8 [0.4-1.5]	1.5 [1.0-2.1]	<0.0001	0.14
p14^{ARF}	Normal	0.2 [0.0-1.3]	0.7 [0.4-1.0]	<0.0001	<0.0001
	Enriched	0.2 [0.0-0.6]	0.5 [0.2-0.7]	0.0002	0.22
	Supplem.	0.0 [0.0-0.4]	0.5 [0.4-0.8]	<0.0001	0.08
RASSF1A	Normal	1.6 [0.9-16.1]	2.0 [1.8-14.9]	0.25	0.02
	Enriched	1.1 [0.8-11.7]	1.7 [1.3-2.1]	0.005	<0.0001
	Supplem.	1.1 [0.9-1.4]	1.9 [1.4-4.7]	<0.0001	<0.0001
MLH1	Normal	0.1 [0.1-0.8]	1.6 [0.6-4.2]	<0.0001	<0.0001
	Enriched	0.1 [0.0-0.1]	2.7 [0.9-5.4]	<0.0001	<0.0001
	Supplem.	0.0 [0.0-0.0]	2.0 [1.0-4.2]	0.05	<0.0001

LINE-1 interquartile range at T4 appears to be much smaller than at T0, with statistically significant differences in the variances at T0 and T4, respectively (also in Figure 19). This result suggests a decrease of inter-individual methylation levels of LINE1 after dietary intervention.

Figure 19: % methylation of LINE-1 and *MTHFR* by diet

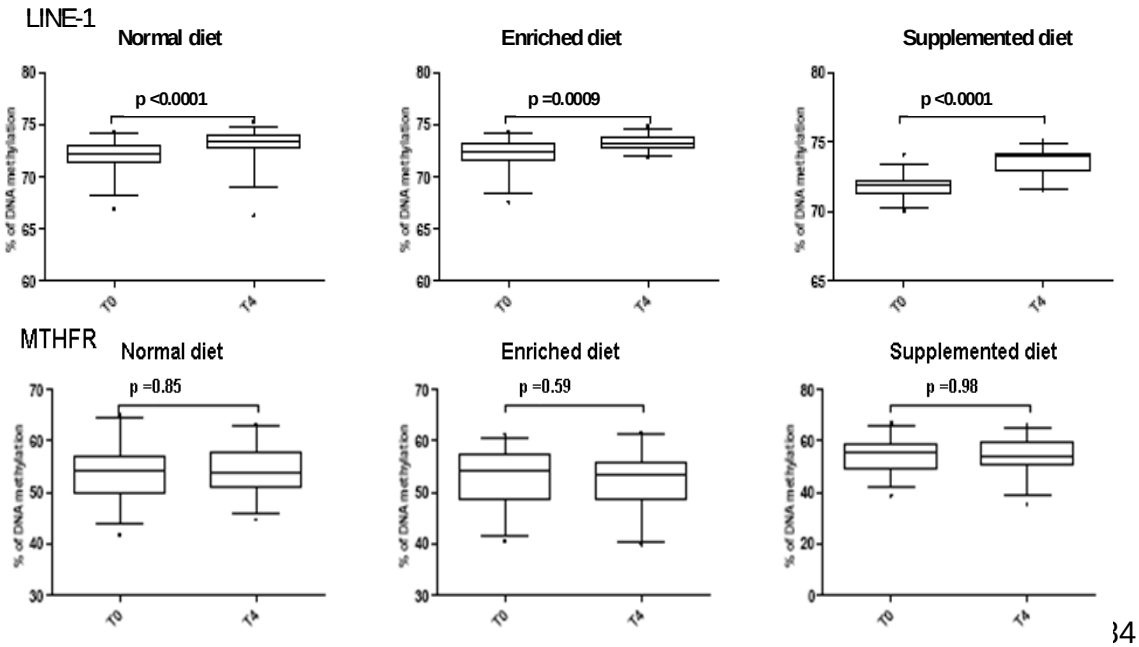
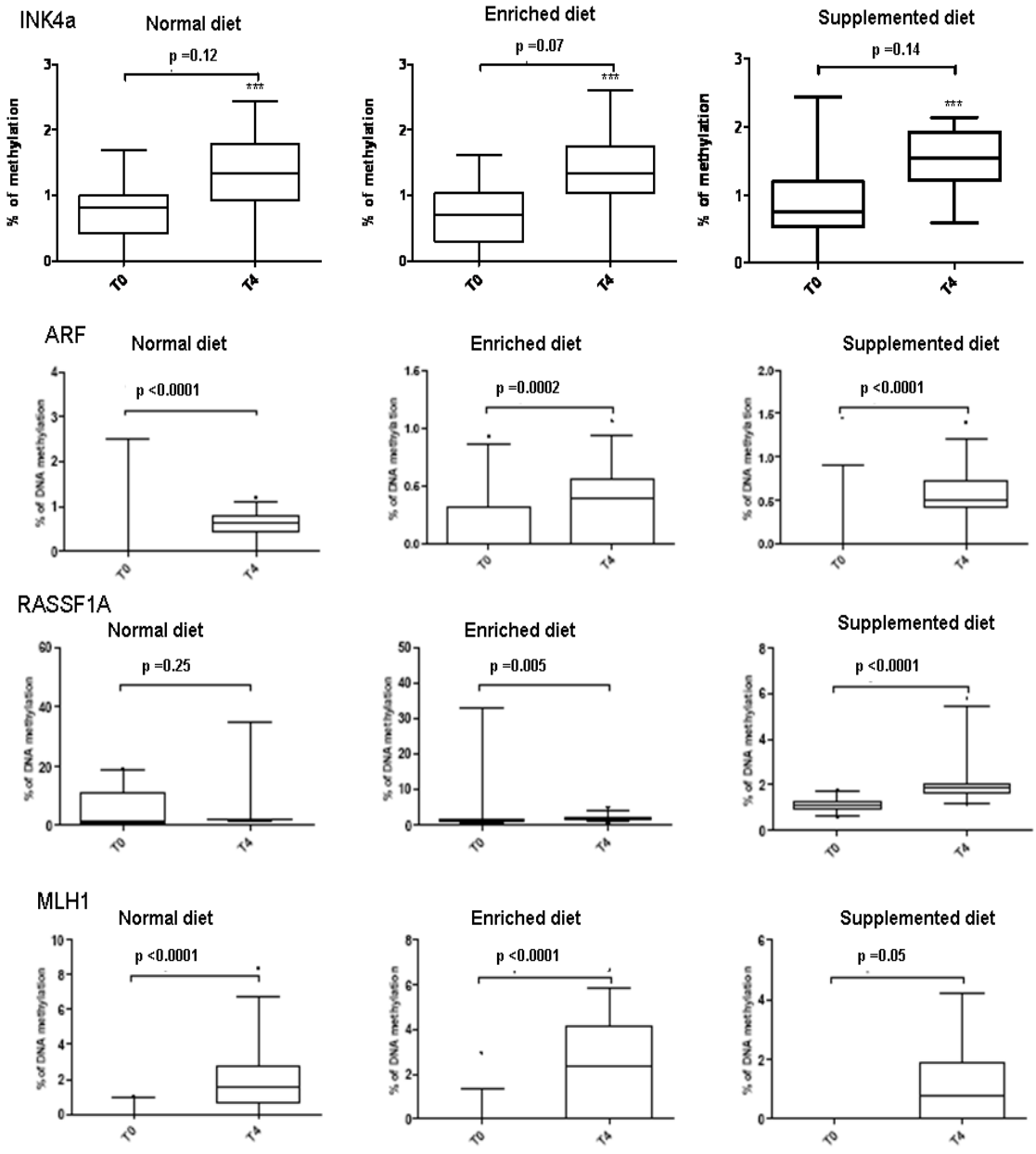


Figure 20 shows the difference between methylation levels at T0 and T4 in the remaining genes analysed.

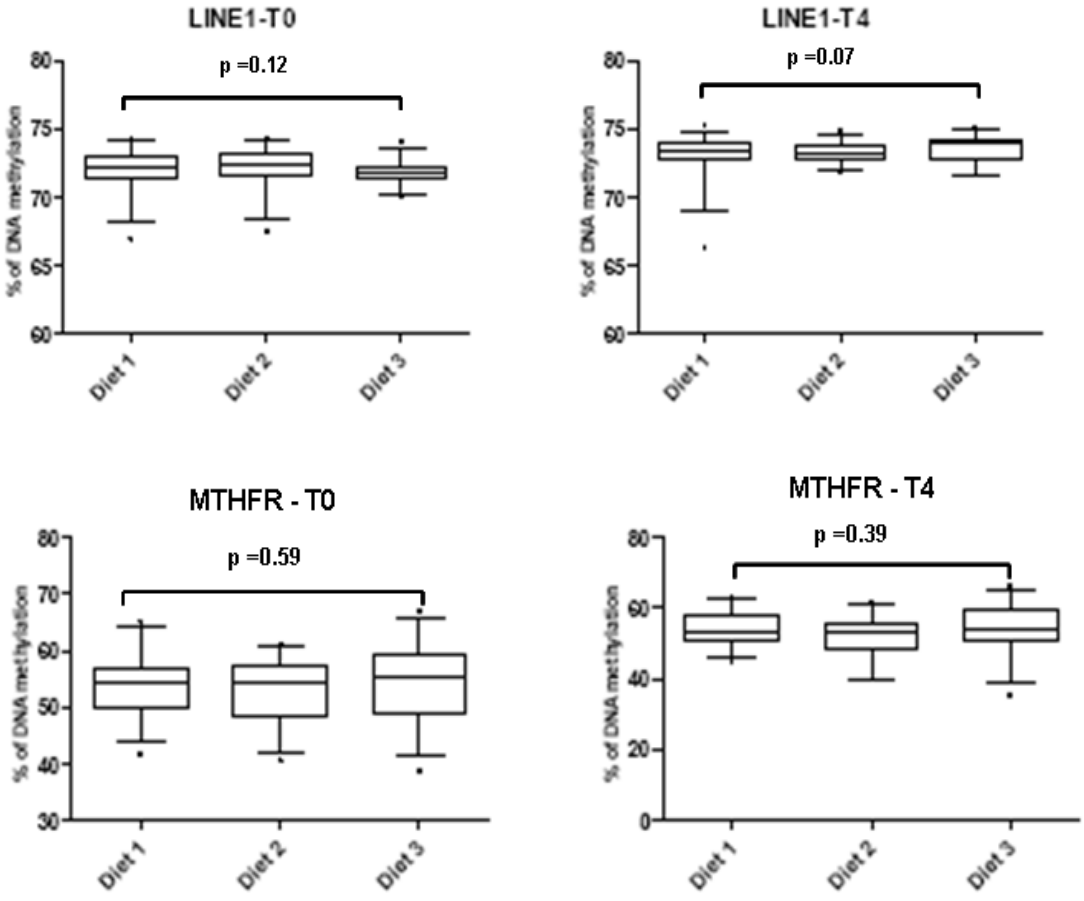
Figure 20: % methylation of *INK4A*, *ARF*, *RASSF1A* and *MLH1* by diet



Kruskal-Wallis test was performed for comparing differences in methylation across the three dietary regimes at each time point (T0 and T4). Results are shown in Figure 21 (diet 1=normal diet, diet 2= enriched diet, diet 3= supplemented diet).

We judged the measured changes for genes other than LINE-1 and *MTHFR* to be too unstable for any meaningful comparison.

Figure 21: LINE-1 and *MTHFR* % methylation at T0 and T4 for each diet



Discussion

As described previously, specific pyrosequencing primers were designed to focus on a series of five to eight CpG dinucleotides in the promoter region of the cyclin-dependent kinases inhibitor 2A (p16*INK4A* and p14*ARF*), the methylenetetrahydrofolate reductase (*MTHFR*), the Ras-association domain family 1 isoform A (*RASSF1A*), the mutL homolog 1 (*MLH1*) gene and for the LINE-1 (long interspersed nuclear elements) repetitive sequence. Pyrosequencing has been extensively used to measure DNA methylation in our laboratory (Vaissire et al. 2009) and the food frequency questionnaire was previously validated by biological measures of nutrients intake (Kumar et al. 2010). Nevertheless we cannot rule out the possibility to have encountered PCR bias due to preferential amplification of one allele and a laboratory drift due to a batch effect. PCR bias may result in a large sequence difference between completely methylated and non-methylated template after bisulfite treatment. Moreover, even if we obtained good standard deviation from repeating a subset of samples with the two time points from the same subject in the same batch, we have run them in first instance in different batches and here we have presented results from this first experiment.

The observed inter-individual differences (often an order of magnitude) in all genes do not appear to be a result of a technical artifact attributable to pyrosequencing, because similar large inter-individual differences for many genes have been validated in other studies by quantitative RT-PCR. These changes could be linked to substantial variation in allele-specific methylation at each locus. Intra-individual differences between the baseline and the end of the dietary intervention were observed in particular for LINE-1 and *MTHFR*; the remaining genes showing negligible differences. These differences may reflect a dietary impact on global methylation levels and on one-carbon metabolism. Moreover, since there are evidences that different CpG sites within a genomic island may be methylated at different level, and that smoking may affect the

methylation status of some (but not other) CpG sites in the same gene, it would be of great interest to perform single CpG analyses, rather than regional, and evaluate the associated mRNA promoter expression in response to diet.

Loss of methylation of LINE-1 has been considered as a risk factor for cancer, neurological and cardiovascular diseases (Baccarelli et al. 2009). LINE-1 are a group of transposable elements distributed throughout the genome. While most LINE-1 sequences are methylated and silent, a small subset of them is actively transcribed and can be retrotransposed within the human genome. We observed a high methylation level for LINE-1 (median above 72%), in agreement with methylation levels reported in cancer-free individuals in peripheral blood cells (Choi et al. 2009, Zhang et al. 2011). We also observed a small but significant increase in global methylation of LINE-1 among the participants, consistent with the role of DNA methylation in controlling retrotransposon mobility by lowering their activities and thereby stabilizing the genome. Moreover, the inter-individual methylation levels of LINE-1 decreased after dietary intervention, while this effect was not obvious for the selected genes. Specifically, the Interquartile Range appears to be much smaller at the end of the intervention, with statistically significant differences in the variances with respect to the baseline. This result suggests that intervention with a controlled diet may have beneficial impact at individual level by stabilizing the basal patterns of DNA methylation levels distributed over the genome, narrowing inter-individual epigenetic variations and thus reducing biodiversity. Together, the increase in LINE-1 methylation and the decrease of dispersion in the distribution of individual LINE-1 methylation levels may reflect a form of increased epigenetic stability after dietary intervention. In the case of LINE-1, this epigenetic stability may have an impact on the expression and retrotransposition of these multi-copy, mobile elements, thus controlling their capacity to modify genetic and chromatin landscapes. Epigenetic stability could be the counterpart of genetic stability and play a role in cancer prevention, since it has been shown that loss of genetic stability may promote tumour progression. More precise evaluation of these changes will require a

better understanding of temporal changes in methylation patterns in subjects who did not change their diet.

Methylation levels of cancer-related genes analysed in the cancer-free individuals were extremely low (<5%) and we did not observe the aberrations potentially associated with smoking in cancer cells such as promoter hyper-methylation of cancer-related genes (Vaissière et al. 2009). Also, average levels of *MTHFR* methylation were 50% as reported in tumours of never-smokers. The mechanisms through which smoking might trigger epigenetic changes are still not clearly understood and our patterns of methylation do not seem to suggest exposure to tobacco smoking.

We observed no major difference between the intervention groups, suggesting that the effects detected at T4 were not driven by differences in the nature of the dietary intervention. We have evidence from questionnaires (Malaveille et al. 2004, Talaska et al. 2006) that subjects did alter their dietary habits when entering the study. Correspondingly, we observed a change in methylation levels after intervention in each group, including in the normal isocaloric diet group. It is likely that the dietary changes induced in group 1, which forms the basis of the diet received by the two other groups, superseded those of specific supplementation at this particular time point. Shorter as well as longer time points would be necessary to conclude whether the supplementation in groups 2 and 3 may have a specific effect on methylation patterns in addition to those of the isocaloric diet (designed following dietary international recommendations).

Epigenetic regulation of gene expression by dietary polyphenols is most probably cell specific, dose- and time-dependent (Ramos 2008). Moreover, the mechanisms in humans is further complicated by the actual dietary intake (particularly of methyl donors), different bioavailabilities (different metabolites in tissue compared to plasma/urine) and high inter-individual variability in metabolic processes. Consequently, our results raise the question of whether peripheral

blood may be an adequate source of DNA for monitoring variations in methylation patterns induced by environmental exposures or by dietary interventions. Peripheral blood cells mainly comprise quiescent or post-mitotic cells, in which DNA methylation levels might be extremely stable as compared to those of actively dividing cells. On the other hand, the lifetime of peripheral blood cells varies from a few hours to a few weeks suggesting that in the present study design (extending over a 4-week period), most cells may have been completely renewed at least once, if not several times.

It would be interesting to extend the intervention described here in two directions. First, it would be important to use shorter time intervals to better monitor the patterns of DNA methylation changes that may occur within the lifetime of a given blood cell population (e.g., over one week). Second, it would be interesting to separate different cell populations and assess their methylation profiles in relation to their proliferative capacity (Wu et al. 2011). The use of a combination of markers in flow cytometry may allow the isolation of a small subpopulation of cells with hematopoietic stem cell (HSC) characteristics in which intervention-induced methylation changes may be of much greater amplitude and relevance to cancer than whole peripheral blood cells. In the present study design, such variations may be masked by the stability of methylation patterns of the post-mitotic cells that form the overwhelming population of cells present in buffy coat. Further studies to calibrate methylation in different populations of blood cells would be useful to determine how to best use this biomarker in intervention studies.

Chapter IV:
Prevalence and prognostic value of *TP53 KRAS* and *EGFR* mutations in NSCLC: the EUELC cohort

Working Hypothesis

In the multicenter study somatic mutations in tumour suppressor genes, which were most probably the consequence of a long process involving the effect of exposure to tobacco carcinogens, were analysed prior to NSCLC recurrence. At early stages of NSCLC, three genes appear commonly mutated: *TP53* (in both ADC and SCC), *EGFR* and *KRAS* (mostly, if not exclusively, occurring in ADC and in a mutually exclusive manner). The biological impact of these mutations is relatively well understood: while inactivation of *TP53* removes a central mechanism of growth suppression in response to DNA damage, activating mutations in *EGFR* or *KRAS* constitutively stimulate one of the main growth and survival promoting signalling pathways.

Somatic mutations may be induced by exposure to a variety of mutagens occurring in the external environment. The most common sequence changes are base substitutions, transitions or transversion, which usually involve replacement of a single base. Transitions (pyrimidine C or T replaced by a pyrimidine, or purine A or G replaced by a purine) are commoner than transversions (pyrimidine replaced by a purine or conversely). The excess of transitions over transversions is at least partly due to the high frequency of C>T transitions resulting from cytosine methylation and subsequent spontaneous deamination in the CpG dinucleotide (Shen et al. 1992). These transitions may occur in the absence of direct mutagen attack onto DNA. In contrast, transversions (e.g. G >T) are often the consequence of covalent damage to DNA (e.g. bulky DNA adducts).

Functionally, *TP53* mutations may differ according to their nature and position, as well as to the presence of a common polymorphism at codon 72 in the mutant allele (Bergamaschi et al. 2003). Knowing *TP53* mutation status has potential applications for cancer prognosis (Schneider et al. 2000, Samowitz et al. 2002) and early diagnosis (Sidransky 2002), identification of mutagen "fingerprints" (Greenblatt et al. 1994), and prediction of therapeutic outcomes (Borresen et al. 1995). Most known mutations fall within the DNA-binding domain and inactivate the tumour suppressor by preventing DNA binding and transactivation.

In addition, the *TP53* gene is highly polymorphic and there is evidence that mutations may occur at different rates on different *TP53* alleles. We have analysed the distribution of 3 common polymorphisms located within a 312 bp region of the TP53 gene encoding the N-terminus of p53, in relation with *TP53* mutation status. These three polymorphisms are located in intron 2 (PIN2, rs.1642785: G to C), intron 3 (PIN3, rs.17878362: 16bp duplication) and in exon 4 (PEX4, rs.1042522: non-silent G to C). Many studies have investigated the associations of *TP53* polymorphisms with increased risk for cancers. The P72R polymorphism in exon 4 is the most extensively studied both in experimental and population studies. In lung cancer, the Pro/Pro genotype in exon 4 could predict for shorter progression-free survival (Han et al. 2008, Liu et al. 2011). The current consensus from a large number of studies is that the alleles of rs1042522 in *TP53* that encode arginine (G-allele) or proline (C-allele) at codon 72 have different apoptotic capacities, and that R72 is more effective in inducing apoptosis than P72, which in turn associates with accelerated smoking-related decline in lung function (Hancox et al. 2009).
P72R is in partial linkage disequilibrium with the duplication of a 16 base pairs in intron 3 (PIN3) that was found to be weakly associated with increased lung cancer risk (Wu et al. 2011, Hu et al. 2010). PIN3 may require the presence of Pro codon 72 variant for stronger prognostic effect (Boldrini et al. 2008). The mechanism trough which PIN3 may increase the risk of developing cancer remains to be elucidated, but the 16 base pair insertion might influence

alternative splicing of p53 protein, leading to unstable transcripts or proteins with altered activities (Gemignani et al. 2004).
The possible biological impact of PIN2 variant in intron 2 is a matter for speculation. This polymorphism has been much less studied than PEX4 and PIN3 and there is no strong data on its possible association with cancer risk. Nevertheless, it is in almost complete linkage disequilibrium with PEX4, so that many of the associations reported for PEX4 might as well be due to PIN2 (or to the combination of both polymorphisms). Preliminary experimental evidence suggests that PIN2 may regulate the stability of p53 pre-mRNA, with consequences that remain to be explored (Hainaut, personal communication).

Given the complex polymorphic structure of *TP53*, haplotypes may provide more relevant information than individual polymorphisms. We have also analysed *TP53* haplotypes by combining the three polymorphisms. As reference we have defined GNA haplotype as the presence of **G** allele in intron 2 (rs.1642785), **N**on-duplication in intron 3 (rs.17878362) and G allele coding for **A**rginine in exon 4 (rs.1042522); and CDP haplotype as the presence of PIN2 **C** allele, PIN3 **D**uplication and PEX4 C allele coding for a **P**roline.

EGFR gene encodes a transmembrane receptor for Epidermal Growth Factor and related ligands, which contains an intracellular tyrosine kinase domain (important for signal transduction). Deregulation of human epidermal growth factor receptor pathways by over-expression or constitutive activation can promote tumour processes including angiogenesis and metastasis and is associated with poor prognosis, in particular in a certain fraction of NSCLCs (Marks et al. 2008). Somatic mutations of *EGFR* gene are found almost exclusively in adenocarcinomas of never-smoking women and cluster in domains of the kinase that constitutively induce its activity and signal transduction (Tokumo et al. 2005). *EGFR* is a valid lung cancer marker that is well known to correlate with clinical response to tyrosine kinase inhibitor's therapy (Paez et al. 2004).

KRAS gene encodes GTP/GDP exchange factor, acting as a downstream effector of *EGFR* signalling that mediates the activation of growth promoting signalling cascades of kinases. Mutations mostly fall at codon 12, located in the GTP binding pocket and preventing its hydrolysis. *KRAS* status is a valid biomarker to predict clinical response to Cetuximab treatment in colorectal cancer patients (Van Cutsem et al. 2009). There is evidence that *TP53* or *KRAS* transversion mutations in NSCLC of smokers occur prevalently at G bases and are commonly the sites of adduct formation by metabolites of polycyclic aromatic hydrocarbons (Denissenko et al. 1996; Hainaut and Pfeifer 2001; Hussain et al. 2001).

These observations suggest that at least some of these mutations may occur as the consequence of exposure to tobacco smoke and precede the development of cancer (Table 9), and therefore have an impact on molecular and biological patterns of lung carcinogenesis, but their impact on clinical lung cancer prognosis remains a matter of debate. The central hypothesis behind the project is that by detecting and treating lung cancer at an early stage, patient mortality can be lowered. The purposes of the study were to assess if mutations in *TP53*, *KRAS* or *EGFR* genes may act as biomarkers of tobacco exposure and to investigate their use as biomarkers of risk for lung cancer recurrence or metastasis.

Table 9: Genetic study: list of genes and their putative biomarker function

Gene	Gene card	Putative biomarker characteristic
TP53	Tumour suppressor gene	Mutations are strongly associated with tobacco-induced cancers and polymorphisms
KRAS	Oncogene	Activating mutations and expression is common in a subset of tobacco-induced cancers
EGFR	Coding a receptor tyrosine kinase	Over-expression is common in multiple cancers; mutations occur in a subset of lung adenocarcinoma in never-smokers

Materials and Methods

Study Design

The EBTB and EUELC databases

We took advantage of the European Early Lung Cancer (EUELC) project and biobank (Cassidy et al. 2009; Field et al. 2009) to collect patients with good quality frozen tissues. The EUELC project is a collaborative effort among 12 centres in France, Germany, Italy, the Netherlands, Spain and the United Kingdom. The study was funded under the European Union Framework V Programme, which sought to promote greater research integration, co-ordination and exchange among European research institutions.

The European Bronchial Tissue Bank (EBTB) central database was set up at the University of Liverpool (Liverpool, UK) to optimise standardisation, preservation and use of clinical specimens. Specimens were collected by a technician employed by each participating centre for this purpose. Standard operating procedures were developed to ensure uniformity in the collection and labelling of specimens. All specimens (lung cancer biopsies, sputum, bronchial lavage and blood) were processed and archived at the EBTB.

The EUELC web-based database was created in SQL Server 2000 in partnership with InferMed. The database contained all specimens' details and allowed an interactive access from all study centres to directly enter lifestyle and medical data during each follow-up visit. This approach ensured controlled data entry for each centre with data editing being restricted to the data manager and to the principal investigators.

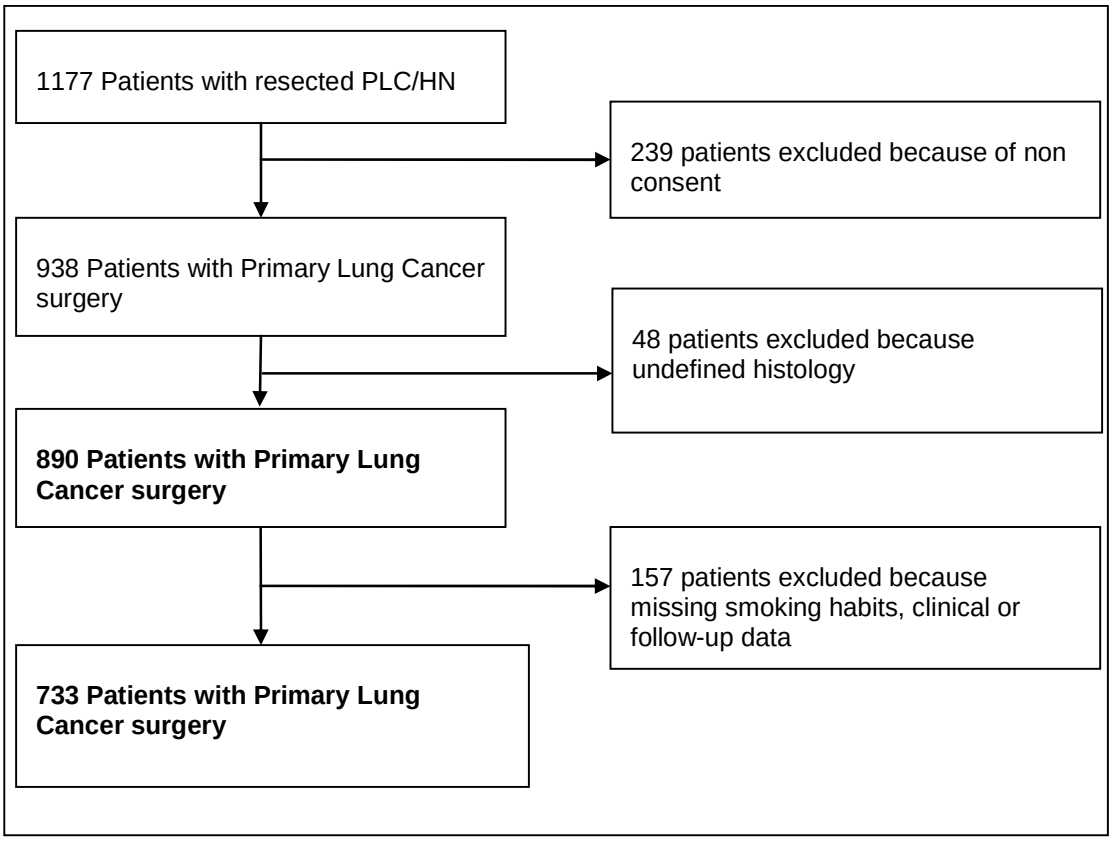
Selection of patients

Beginning in 2002 and continuing through 2006 were selected 1,177 patients from participating hospitals with surgically resected primary NSCLC with limited cancer stages (such as T_1N_0; confirmed either by histological or cytological analysis). The patients were considered at very high risk of developing progressive lung cancer (i.e. Second Primary Lung Cancer (SPLC) and/or

metastasis) and were all Caucasians. Finally 733 patients with primary lung cancer surgery and complete data from life-style questionnaire were selected (Figure 22). Patients were followed-up every 6 months up to 2011 with a median follow-up period of about 48 months. The different statuses available to define patient's health were: Alive and Well, Alive with the disease, Died from other causes, Died from the disease, Metastatic recurrence, SPLC, Treatment with chemotherapy, with radiotherapy, or with both.

Progressive Disease (PD) was defined as the development of a SPLC or recurrence/metastasis in individuals with a history of a completely resected primary lung or head & neck cancer. Disease Free (DF) was defined as the absence of Progressive Disease after surgery at the time of the last follow-up.

Figure 22: Selection of patients from the EUELC database



Life-style questionnaire

Instructions for interviewing and coding were developed and training to research interviewers was carried out in each centre. All lifestyle questionnaires were translated to ensure consistency across European partners. After obtaining written consent, all participants completed a 45-min in-person interview. The questionnaire collected detailed information on socioeconomic and demographic characteristics, medical history, family history of cancer, history of tobacco consumption and occupational exposure to asbestos. Extensive information about tobacco smoking was elicited for all participants including their age at start/end of consumption and the number of cigarettes smoked per day. Individuals who had smoked at least 100 cigarettes in their lifetime were considered as ever-smokers. A former smoker quit smoking at least 1 year before diagnosis while a current smoker smoked in the last 2 years before interview. Information on history of cancer among first-degree relatives (i.e. parents, siblings and biological children) was recorded, including age at diagnosis and site of cancer.

Selection of tumour samples

Immunohistochemistry of p53 protein was first tested on 306 lung cancer frozen tissues by a collaborative centre in France. Our laboratory received 273 p53 positive samples ready for DNA extraction and for laboratory analysis of *TP53*, *KRAS* and *EGFR* genes. Only samples with known follow-up of the corresponding patient were included in the statistical analysis (Figure 23).

Our series (Table 10) included 11 never-smokers (<100 cigarettes smoked in a lifetime), 86 former smokers (smoking cessation ≥ 2 years before diagnosis) and 152 current smokers, and 1 patient without informed smoking status. There were 110 SCC, 133 ADC and 7 patients recorded as “other histologies” (large cell carcinoma or mixed histologies).

Figure 23: Selection of samples for mutational analysis

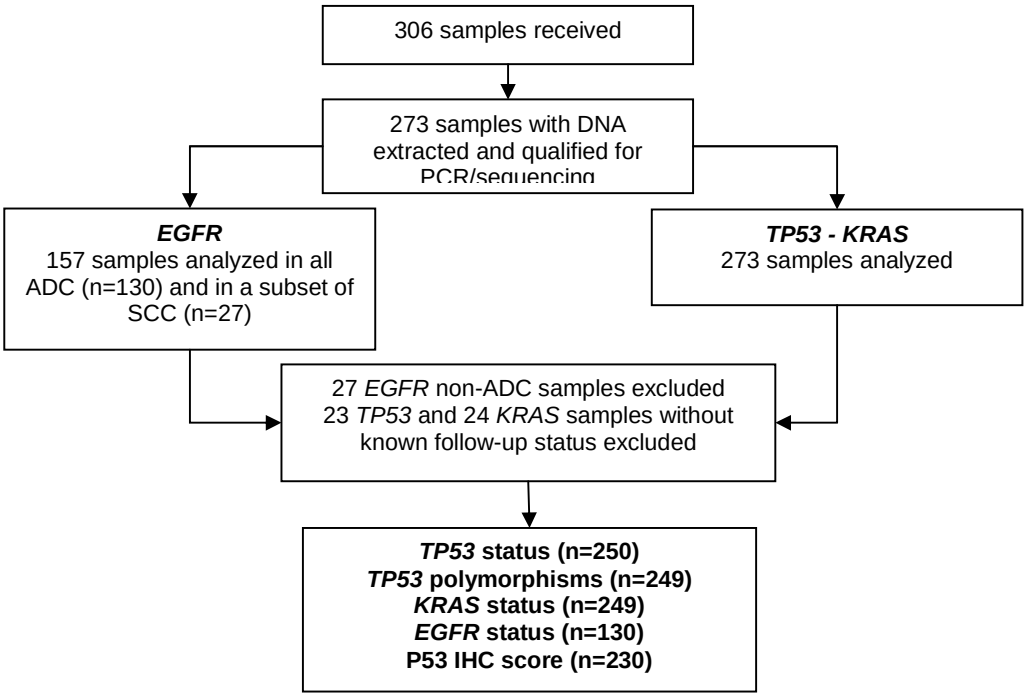


Table 10: Characteristics of patients included in the analysis

Variable (missing)	Items	n
Gender	Male	210
	Female	40
Age	< 60	89
	[60-65[	82
	[65-70[	30
	≥ 70	49
Histology	ADC	133
	SCC	110
	Others	7
Asbestos exposure (2)	None	191
	Yes	57
Tumour score (1)	T1	76
	T2	150
	T3	15
	T4	8
Nodal score (1)	N0	173
	N1	65
	N2	2
	NX	9
Past pulmonary illness (2)	No	110
	Yes	138
Smoking status (1)	Current smoker	152
	Former smoker	86
	Never-smoker	11
Total		250

Statistical analysis

250 patients with known follow-up status were selected for statistical analysis of *TP53* mutations; 249 samples for *KRAS*; 130 ADC samples for *EGFR* and 249 samples for *TP53* polymorphisms. As a data quality check, Hardy Weinberg equilibrium was tested for all polymorphisms studied using the χ^2 test.

The Mantel-Haenszel χ^2 test, stratified by centre, was used to test both the association of clinical parameters with biomarkers and between biomarkers (e.g. mutation status with polymorphisms). Bootstrap analysis was performed to obtain non-parametric confidence intervals for risk estimates. This method creates multiple samples of patients through a process of random selection and performs the analysis on each dataset to calculate a mean risk with its non-parametric confidence intervals.

KRAS mutations were independently analysed from another EUELC partner using a different laboratory assay. Results were analysed for reproducibility between the two centres using the Kappa test statistic for measure of agreement beyond chance.

The Kappa coefficient is a measure of inter-agreement and has been computed by SAS software as:

$$K = (Pa-Pe) / (1-Pe)$$

Pa = [(wild-types + mutants) / total samples] and Pe = [P(mutant) + P(wild-type)]. In this case, the K coefficient measures the agreement between two or more judges who have coded a qualitative variable. A value of 0 denotes an agreement due to chance while a value of 1 means a perfect agreement. Agreement is considered important beyond a value of 0.60.

A Cox proportional hazard model was used to test how certain biomarkers affect survival, both overall and specifically from lung cancer. Clinical parameters associated with mortality were additionally adjusted for. The Fine & Gray (F&G)

model was applied to estimate association of biomarkers and clinical variables with the progression of primary lung cancer. The F&G model takes into account the presence of “competing risks” for patients who died from causes other than lung cancer. The aim of the regression analysis was to estimate lung cancer-specific recurrence probabilities, by censoring failures due to competing risks.

Univariate F&G analysis was carried out by computing unadjusted matched HR to compare Progressive Disease (PD) subjects and Disease Free (DF) subjects for each variable of interest. PD patients were individually matched with DF patients on centre, sex, age at surgery (±3 years), histology, nodal stage and follow-up time (at least as long as the event time for matched PD subjects). Biomarkers were assessed one at a time in a multivariate F&G model adjusted for T and N cancer scores (of the TNM classification system, Beasley et al. 2004) for potential risk factors of cancer recurrence identified from the univariate analysis. Multivariable analysis was conducted to identify risk factors that were independently associated with Progressive Disease. The criteria for selection of these variables for possible inclusion in the multivariable analysis were based on both biological importance and a *p* value of less than 0.10 in the univariate analysis. The analysis was stratified by centre to account for differences in the number of patients recruited. According to the distribution of follow up duration we censored the analysis at 48 months. Cumulative incidence plots were performed to illustrate the risk of disease progression through time according to the mutation status of the genes analysed.

All statistical analyses were performed using SAS 9.1.3 (SAS Institute, NC). The F&G model was used to compute Hazard Ratio (HR) and 95% confidence interval (CI). Statistical significance was assessed at the level of 0.05.

Laboratory Methods

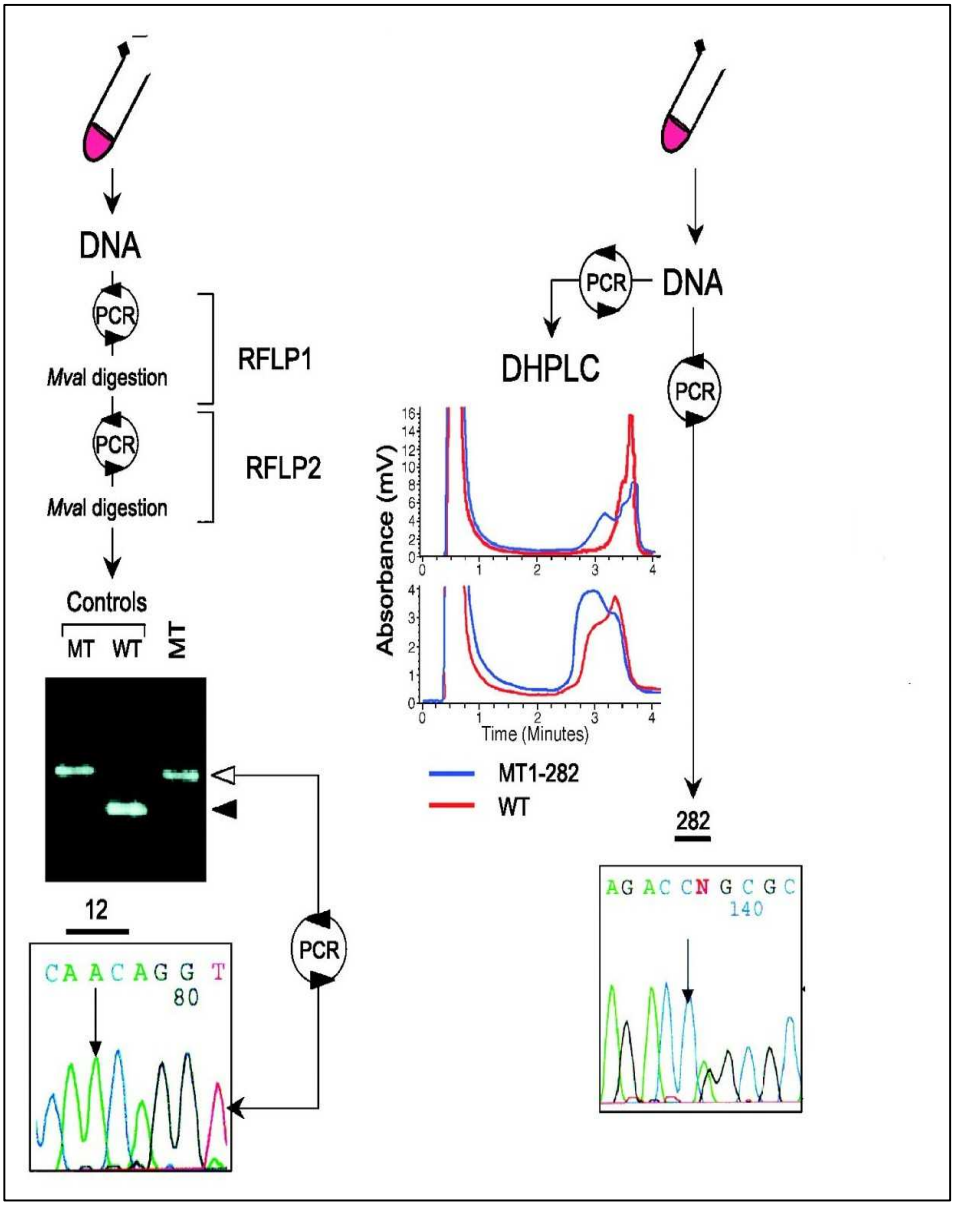
Analysis of somatic mutations

DNA previously extracted from frozen tissue was received from the European Bronchial Tissue Bank and analysed for *TP53* (exons 4-10 including flanking splice sites, i.e. residues 52 to 364) mutations by pre-screening with denaturing high-pressure liquid chromatography (dHPLC) followed by a second PCR and bi-directional sequencing. Specimen with matched dHPLC and sequencing results were considered as containing a mutation. Finally, *TP53* mutation's type was analysed with reference to the IARC mutation database.

KRAS mutations at codon 12 were analysed by mutant-enriched PCR as described by Gormally et al. (Gormally et al. 2006). To avoid false-positive results generated during successive PCR rounds, all analyses were repeated twice. After digestion with MvaI enzyme, the mutant PCR product is excised, amplified and sequenced. *KRAS* codon 12 ME-PCR was able to detect up to 0.1% of mutant DNA in wild-type DNA (Figure 24).

EGFR mutations were detected using PCR-based direct sequencing of the four exons of the TK domain (exons 18–21) as described by Pao et al. (Pao et al. 2004). All sequencing reactions were performed in both forward and reverse directions, and all mutations were confirmed by an independent PCR amplification.

Figure 24: Flow chart illustrating the main steps of the procedure for *TP53* and *KRAS* analysis



From Le Calvez et al. 2005

Detection of TP53 mutations by dHPLC and sequencing

It has been previously demonstrated (Le Calvez et al. 2005) that pre-screening by dHPLC represents a sensitive method with detection levels of mutant DNA ranging between 3% and 12% depending on mutation type and sequence context (Table 11). In comparison, direct sequencing does not detect most mutations in samples containing less than 25%–30% of mutated DNA (Rosenblum et al. 1997; Ahrendt et al. 1999).

Table 11: Detection limit of percent mutant DNA by dHPLC

Cell line	Exon	Codon	Mutation	dHPLC
Hs578T	5	157	GTC>TTC	12.50
T47D	6	194	CTT>TTT	3.12
TE11	7	237	ATG>ATT	3.12
TE6	7	248	CGG> CAG	6.25
TE1	8	272	GTG>ATG	3.12
MDA-MB 231	9	280	AGA>AAA	6.25

dHPLC is a conformation-based method of mutation detection that relies on the fact that DNA fragments analysed are a mixture of wild-type and mutant DNA. DNA that contains a sequence alteration (most commonly occurring in heterozygous form) has differential mobility under partially denaturing conditions during reverse-phase ion-exchange HPLC (Keller et al. 2001), resulting in heteroduplexes eluting first from the column. To allow for heteroduplex formation PCR products were heated at 95℃ for 4min and then cooled at room temperature for one hour.

5 to 10µl of the PCR products were then injected into a preheated reverse-phase column (DNASep Column, Transgenomic) equilibrated by an ion pairing agent TEAA 0.1M (triethylammonium acetate). DNA was removed from the column at a constant flow rate of 0.9ml/min by a linear acetonitrile gradient, achieved by mixing a buffer A (TEAA 0.1M) with a buffer B (TEAA 0.1M and acetonitrile 25%).

Acetonitrile (CH_3CN) rose of 2% per min during 2.5 min from an initial concentration at T_0.

The temperature for optimum separation of heteroduplex from homoduplex was calculated by Transgenomic software in order to maintain almost 75% of PCR product in a double stranded form. The assay was run at three temperatures (except for exon 7) to attain maximal sensitivity.

dHPLC, whilst very sensitive, will not distinguish between pathogenic and non-pathogenic sequence variants and consequently it was always performed injecting both positive and negative internal controls for each screening temperature (Table 12).

Table 12: dHPLC conditions used for *TP53* screening

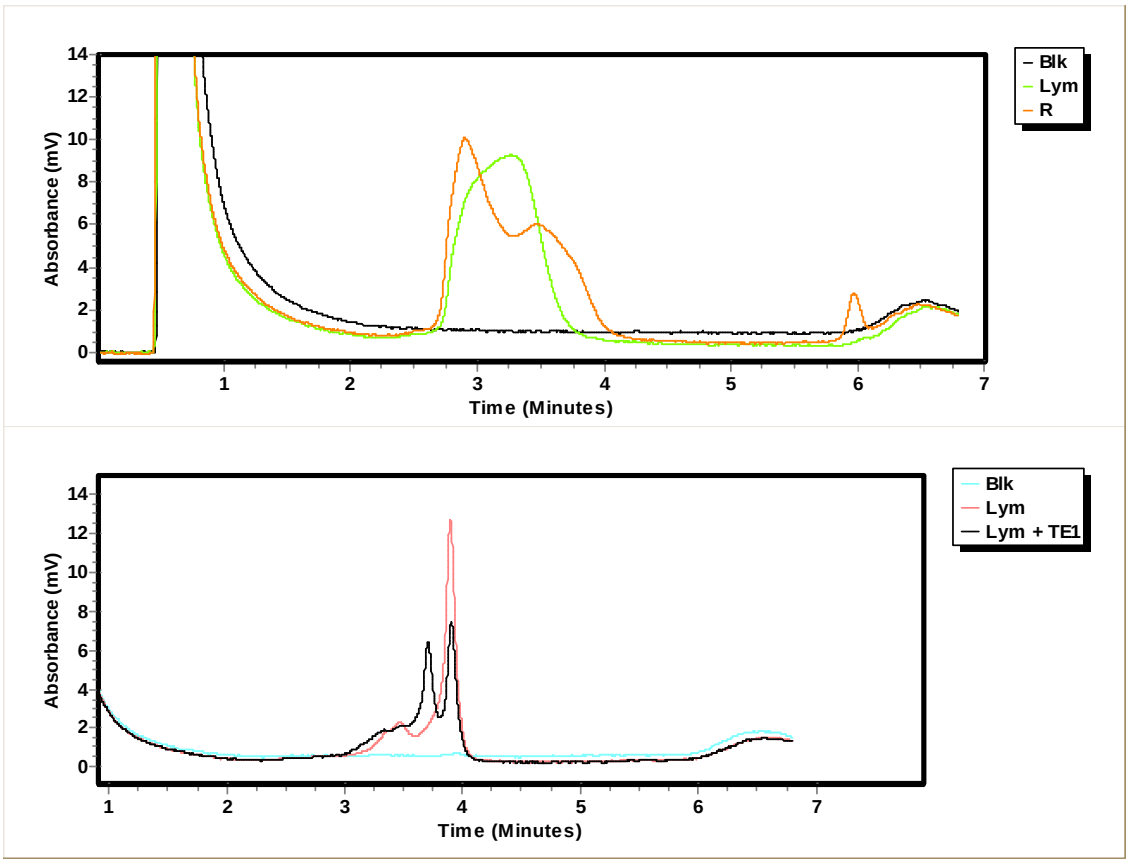
Exon	Temperature (℃)	(%CH_3CN) T_0-$T_{2.5}$	Cell line	Mutation	Cancer
4	62	50-58	IGR191	(cd 36: CC**G**-CC**A**)	Squamous Cell
	65	50-55	IGR2	(cd 91: TG**G**-TG**A**)	Carcinoma
	68	45-50	Raji (R)	(cd 72: C**G**C-C**C**C)	Burkitt lymphoma
5-6	62	59-64	T47D	(cd 194: **C**TT-**T**TT)	Breast
	66	53-58	YL38	(cd 144: **C**AG-**T**AG)	Oesophagus
	68	50-55	Hs578T	(cd 157: **G**TC-**T**TC)	Breast
7	64	53-58	TE6	(cd 248: C**G**G-C**A**G)	Oesophagus
			TE11	(cd 237: AT**G**-AT**T**)	
8-9	60	58-63	TE1	(cd 272: **G**TG-**A**TG)	
	62	56-61	TE1	(cd 272: **G**TG-**A**TG)	
	65	50-55	MDA-MB231	(cd 280: A**G**A-A**A**A)	Breast

The eluted DNA was detected at 260nm. Figure 25 shows chromatogram of a blank sample (Blk), of a negative control (e.g. lymphocytes – Lym-) and of a positive control bearing a known mutation (e.g. TE1 or R).

The positive controls consisted either of DNA isolated from cell lines that contained a mutation diluted with equal quantity of wild-type DNA (e.g. TE1 +

lymphocytes) to enable heteroduplex formation (because only mutant sequences were present in our cell lines); or of DNA isolated from tumour samples (e.g. R) that contained mutant sequences and wild-type sequences from neighbouring non-tumour cells.

Figure 25: Negative and positive dHPLC controls



PCR reactions for exons 4, 5-6 and 8-9 of *TP53* gene involved a 2-minute polymerase activation at 94°C, 20 cycles of denaturation (94°C, 30s), primer annealing (63°C, 45s), and extension (72°C, 60s), followed by 30 cycles of denaturation (94°C, 30s), primer annealing (60°C, 45s), and extension (72°C, 60s) and a final 10-minute extension at 72°C.

PCR reaction for exon 7 involved a 15-minute polymerase activation at 95°C, 50 cycles of denaturation (94°C, 30s), primer annealing (60°C, 30s), and extension (72°C, 30s), followed by a final 10-minute extension at 72°C.

Table 13 lists the amplification conditions and primers (HotStar Taq and Taq Platinum both from Invitrogen, Paisley, UK).

Table 13: PCR conditions for *TP53* exons 4 to 9

PCR conditions		Exon 4
Primers (5'3')	Forward	tgaggacctggtcctctgac
	Reverse	agaggaatcccaaagttccA
Mix		Taq Platinum (0.8U/20ul mix), 1.5mM $MgCl_2$, 0.2mM each dNTP, 1µM each primer
		Exon 5-6
Primers (5'3')	Forward	tgttcacttgtgccctgact
	Reverse	ttaacccctcctcccagaga
Mix		Taq Platinum (0.8U/20ul mix), 1.5mM $MgCl_2$, 0.2mM each dNTP, 0.4µM each primer
		Exon 7
Primers (5'3')	Forward	cttgcccacggtctccccaa
	Reverse	aggggtcagcggcaagcaga
Mix		HotStar Taq (0.8U/20ul mix), 1.5mM $MgCl_2$ 0.2mM each dNTP, 0.4µM each primer
		Exon 8-9
Primers (5'3')	Forward	ttgggagtagatggagcct
	Reverse	agtgttagactggaaacttt
Mix		Taq Platinum (0.8U/20ul mix), 2mM $MgCl_2$, 0.2mM each dNTP, 0.4µM each primer

Bidirectional sequencing of PCR products was used as a final step of the mutation scanning procedure, both confirming and identifying the sequence alteration. Sequencing was performed on an independent PCR product to confirm presence of a mutation.

Conventional unidirectional sequencing of PCR products by fluorescent di-deoxy terminators has a sensitivity of about 95% (i.e. may miss 1 in 20 mutations) and a

low but finite false positive rate due to base mis-incorporation and other sequencing, optical or polymer artefacts.

Prior sequence analysis, PCR products were purified with the enzyme ExoSap-IT (USB) for 15min at 37℃ and 15min at 80℃. PCR prod ucts were analysed by a 16-capillary automated sequencer (ABI PRISM® 3100 Genetic Analyser, Applied Biosystems), based on the Sanger method (see principle at: http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Obenrader/sanger_method_page.htm). Sequencing reaction was done with 1.5µl BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA) on 7µl of purified PCR product, adding 1.25µl buffer and 0.5µl primer at 10µM (same primers as those used for PCR amplification reactions).

The PCR reaction involved 30 cycles of 10-min denaturation at 96°C, primer annealing (50°C, 5s), and extension (60°C, 4min). Sequences were imported in a sequence-analysis software using the reference sequence NC_000017.9 from Genbank (http://www-p53.iarc.fr/TP53sequence_NC_000017-9.html) to allow visual inspection of chromatograms. Variations were finally checked with the mutation validation tool available at IARC (http://www-p53.iarc.fr) to distinguish between a known polymorphism and a mutation as well as to obtain frequency and functional data.

Detection of EGFR mutations by bidirectional sequencing

EGFR mutations were detected using PCR-based direct sequencing. All sequencing reactions were performed in both forward and reverse directions, and all mutations were confirmed by PCR amplification of an independent DNA isolate. The PCR mix contained 1.5mM $MgCl_2$, 0.8µM of each primer, 200µM of each dNTP, 1.5U of Taq platinum polymerase. PCR reactions involved a 2-minute Taq platinum polymerase activation at 94°C, 50 cycles of denaturation (94°C, 30s), primer annealing (59°C, 45s), and extension (72°C, 45s), followed by a final 10-minute extension at 72°C. After bidirectional sequencing, occurrence of mutations was assessed with reference to sequences in the COSMIC mutation database (www.sanger.ac.uk/genetics/CGP/cosmic/).

Detection of KRAS mutations by ME-PCR

Exon 1 of *KRAS* gene was amplified with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and with primers forward (1F) 5'-actgaatataaacttgtggtagtgggacct-3' and reverse (3R) 5'-ggtgcaggaccattctttgatacagat-3'. SW480 (human colon adenocarcinoma cell line containing mutation at *KRAS* codon 12, CCA>CAA, Glycine> Valine) was used as PCR internal positive control.

The PCR mix contained 1.5mM $MgCl_2$, 0.4µM of each primer, 200µM of each dNTP, 1.5U of polymerase, 10mM Tris-HCl and 50mM KCl. PCR reaction involved a 6-minute polymerase activation at 95°C, 50 cycles of denaturation (94°C, 30s), primer annealing (58°C, 30s), and extension (72°C, 30s), followed by a final 10-minute extension at 72°C. PCR product was checked on a 2% agarose gel.

10µl of the amplified product (157 bp) were digested with restriction endonuclease Mva I (Roche Applied Science) at 37°C overnight. The enzyme recognises a CC/TGG sequence, which has been created between codons 11 and 12 by primer 1F and between codons 48 to 50 by primer 3R. Wild type sequence at codon 12 gives 3 digestion products of 29, 114 and 14 bp whereas mutant sequence at any of the 2 first positions of codon 12 abolishes the restriction site introduced by primer 1F giving two restriction products of 143bp and 14 bp. Digested products were checked on a 3% agarose gel. Enrichment of the mutant DNA was performed from 1µl of the first PCR product, which was re-amplified by semi-nested PCR using primers 1F and 2R 5'-gaggtaaatcttgttttattatgcatatta-3' under the same conditions. The 135 bp amplification product was digested with Mva I. Only mutant re-amplified fragments (and uncompleted wild-type digested product) contain restriction sites and are cut with Mva I. This second step allows detection of mutations in small quantities of mutated DNA.

Detection of TP53 polymorphisms

Exon 4 was pre-screened by dHPLC; abnormal chromatograms were additionally analysed by RFLP on an independent PCR product. A new PCR (for primers and conditions please refers to Table 14) and digestion of 7µl of PCR product were performed. The BstUI restriction endonuclease (BioLabs) was used to cut within the GC|CG sequence encompassing codon 72 (CGC). Mutant fragments were visualised on 3% agarose gel stained with ethidium bromide, eluted, re-amplified by PCR and sequenced.

PIN2 and PIN3 were analysed by bi-directional sequencing and each reaction was repeated at least twice. Exons 2-3 of *TP53* gene were amplified with primers forward 5'-tctcatgctggatccccact-3' and reverse 5'-agtcagaggaccaggtcctc-3'. The PCR mix contained Taq Platinum (0.8U/20µl mix), 1.5mM MgCl2, 0.2mM of each dNTP, 0.4µM of each primer (p559 forward and pE3Ri reverse). PCR reaction involved a 2-minute polymerase activation at 94°C, 50 cycles of denaturation (94°C, 30s), primer annealing (61°C, 45s), and extension (72°C, 45s), followed by a final 10-minute extension at 72°C. PCR products were sequenced and procedure was repeated to confirm the presence of the polymorphism.

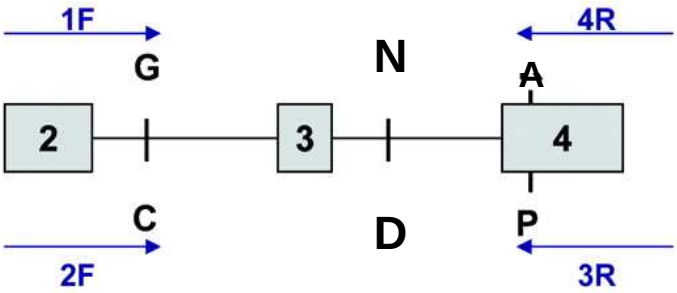
Detection of TP53 haplotypes (PIN2-PIN3-PEX4)

To determine the haplotypes defined by the three *TP53* polymorphisms, we used a method developed at IARC (Marcel et al. 2009), based on the amplification refractory mutation system (ARMS) and involving four different allele-specific PCR to identify haplotypes directly on agarose gel prior confirmation by bidirectional sequencing.

Figure 26 represents primers location on *TP53* gene for amplification of the two PIN2 alleles (1F, <u>G allele</u>: 5'-aagggcaggccaggagggg**G**-3' and 2F, <u>C allele</u>: 5'-aagggcaggccaggagggg**C**-3') and the two PEX4 alleles (3R, <u>allele coding a P</u>: 5'-tgctggtgcaggggccacg**G**-3' and 4R, <u>allele coding an R</u>: 5'-tgctggtgcaggggccacg**C**-3').

The PCR mix contained Taq Platinum (0.5U/20µl mix), 0.8mM MgCl2, 0.5mM of each dNTP, 0.2µM of each primer. PCR reaction involved 2-minute polymerase activation at 94°C, 35 cycles of denaturation (94°C, 30s) and extension (72°C, 1m30s), followed by a final 5-minute extension at 72°C.

Figure 26: Schematic representation of primers location on *TP53* gene



TP53 gene fragment

Haplotypes were identified directly on an agarose gel. The difference in PIN3 status was determined by difference in the electrophoretic mobility of the two bands on a 3% agarose gel, the ‘‘G-A’’ band migrating faster than the ‘‘C-P’’ band, consistent with the presence of a repeat of the 16 bp of PIN3 in the ‘‘C-P’’ allele (D). This difference was assessed with positive internal controls from the oesophageal cellular lines TE1 (GNA haplotype) and TE3 (CDP haplotype). The presence of the expected haplotype was independently confirmed by bidirectional sequencing for the three *TP53* variants.

Results

Mutation prevalence

Table 14 shows the mutation prevalence in the selected genes in the EUELC cohort. 48.4% of 250 patients bore a *TP53* mutation (including 5 silent mutations). A total of 18.5% *KRAS* mutations at codons 12 and 13 were detected. *EGFR* mutations were initially analysed in 157 cases (140 ADC and 17 non-ADC). Since earlier reports suggest that this gene is rarely mutated in NSCLC types other than ADC, and since we found only 1 mutation (exon 19, codon 742, Val>Leu) in non-ADC cases, we performed the statistical analysis on a selection of 130 ADC cases with known follow-up status and we detected 13.1% *EGFR* mutations.

Eighteen patients had mutations in two genes (Table 15), including 11 patients with *TP53* mutations among 46 with *KRAS* mutations, and 6 patients with *TP53* mutations among 17 with *EGFR* mutations. One patient with *KRAS* mutation also had a silent mutation in exon 21 of *EGFR* (codon 836, CGC>CGT Arg>Arg). No patient had mutations in the 3 genes.

Table 14: Mutation prevalence in EUELC patients

Gene	Status	n	%
TP53 (n=250)	Wild-type	129	51.6
	Mutant (exons 4-9)	121	48.4
KRAS (n=249)	Wild-type	203	81.5
	Mutant (codon 12)	46	18.5
EGFR (n=130)	Wild-type	113	86.9
	Mutant	17	13.1

Table 15: Prevalence of cases with mutations in more than one gene

Mutations		n	%
KRAS (n=46)	*TP53*	11	23.9%
EGFR (n=17)	*TP53*	6	35.3%
	KRAS	1	5%

TP53 mutations

Functionally, *TP53* mutations may differ according to their nature and position. Knowing *TP53* mutation status has potential applications for identification of mutagen "fingerprints" (Greenblatt et al. 1994) and early diagnosis (Sidransky 2002).

Patterns and distribution

DNA extracted from tumour material was analysed for *TP53* mutations covering exons 4 to 9 including flanking splice sites. Based on data in the IARC *TP53* mutation database, these regions contain over 90% of all mutations ever reported in lung cancer. DNA was analysed for mutations in *TP53* by a two-step approach, with first pre-screening by dHPLC followed by an independent analysis by direct sequencing of all DNA fragments that gave an abnormal chromatogram. Figure 27 gives an example of mutated sample identified by an abnormal chromatogram (id: 04-053-00-TfD in Panel B) with a profile equivalent to that of a positive (mutated) internal standard (cell line TE1 in Panel A).

Patterns of *TP53* mutations are shown in Figure 28. This mutation pattern is dominated by a high prevalence of G:C to T:A transversions (33%), with an overall prevalence of transversions at G:C and A:T base pairs (49%) and G:C to A:T transitions at CpG dinucleotides rare (6%). This pattern is compatible with the known profile of *TP53* mutations in lung cancers of smokers. The codon distribution was also in agreement with the known smoking-patterns; i.e. with G:C to T:A transversions and hotspots at codons 157 and 158 (Figure 28).

Figure 27: Panel A: Chromatogram of internal standards for *TP53* exons 8-9 (wild-type: lymphocyte, mutant: TE1); Panel B: example of positive sample (id: 04-053-00-TfD)

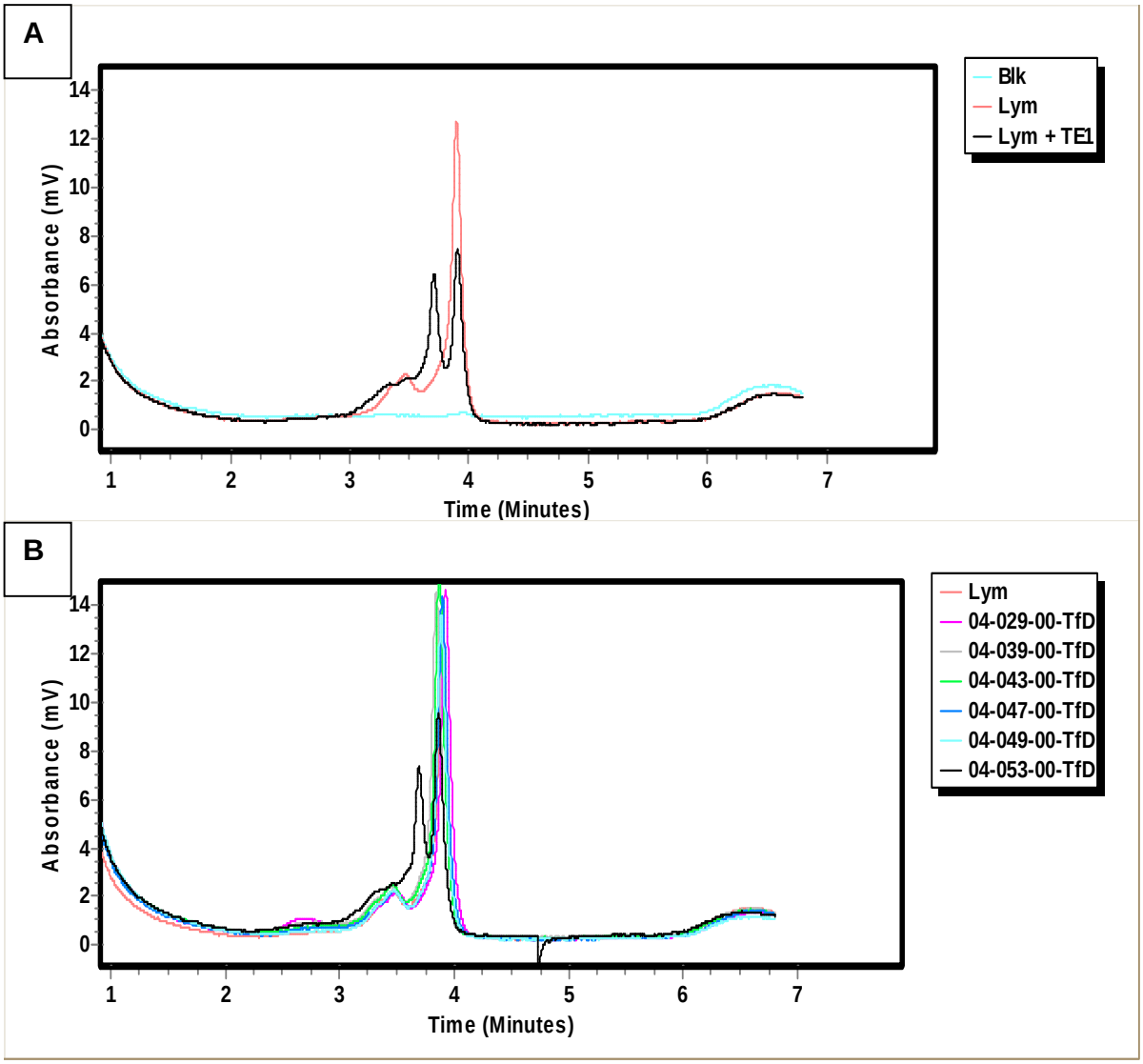
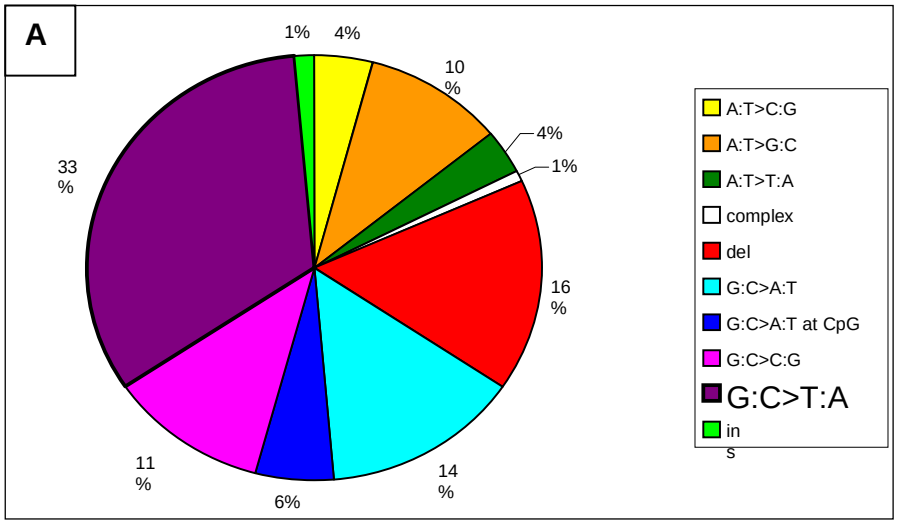
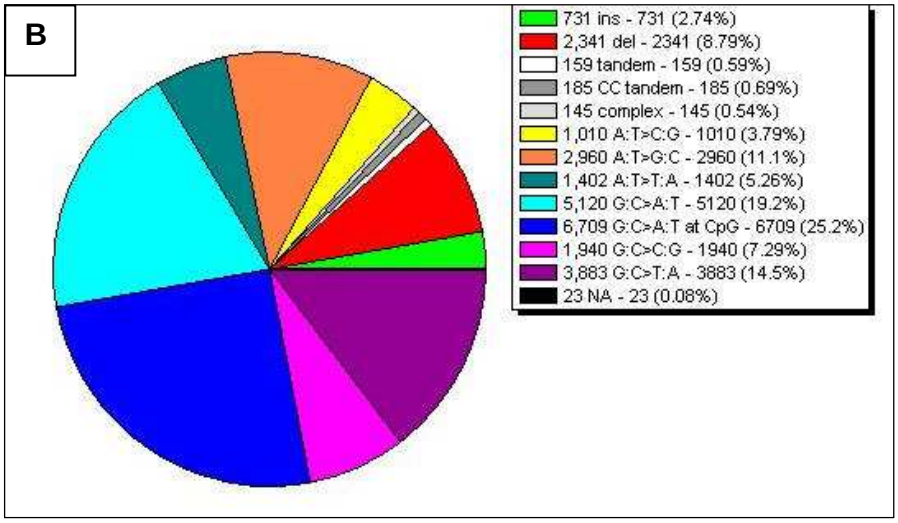


Figure 28: Patterns of *TP53* mutations broken down by type of base substitution: Panel A: EUELC; Panel B: *TP53* database



Mutation pattern / 121 mutations / EUELC project



Mutation pattern / 26608 mutations / IARC TP53 Database, November 2010

Figure 29a shows the distribution of mutations along the coding sequence of *TP53*. Codons with the highest mutation prevalence were 157, 158, 249, 273 and 282. These sites correspond to the codons reported as “hotspots” in lung cancers. Minor mutation spots were observed at codons 234, 244 and 285. All hotspots mutations are categorized as “deleterious” in the IARC *TP53* mutation database, i.e. predicting loss of p53 transactivation function.

Figure 29b shows the specific location of the codons that carried G to T transversions. Transversions at codon 157, 158, 273 are typical mutations following DNA damage by metabolites of PAH. In contrast, codon 249 has not been described as a major site of adduction for such compounds. Rather, it is specific to aflatoxin adducts. Nevertheless, an excess of G to T transversions at codon 249 has already been noted in lung cancers, in smokers as well as in non-smokers. Unconfirmed reports have suggested a possible role of radon as inducer of these mutations.

Figure 29a: *TP53* mutations distribution at exons 4 to 9

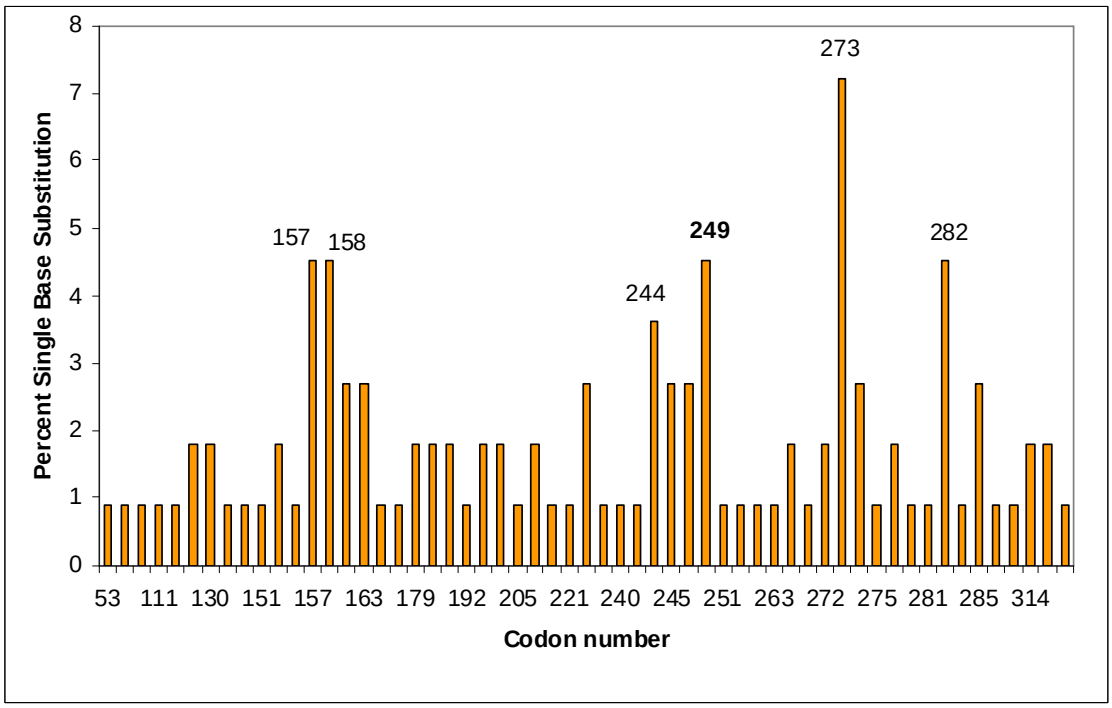
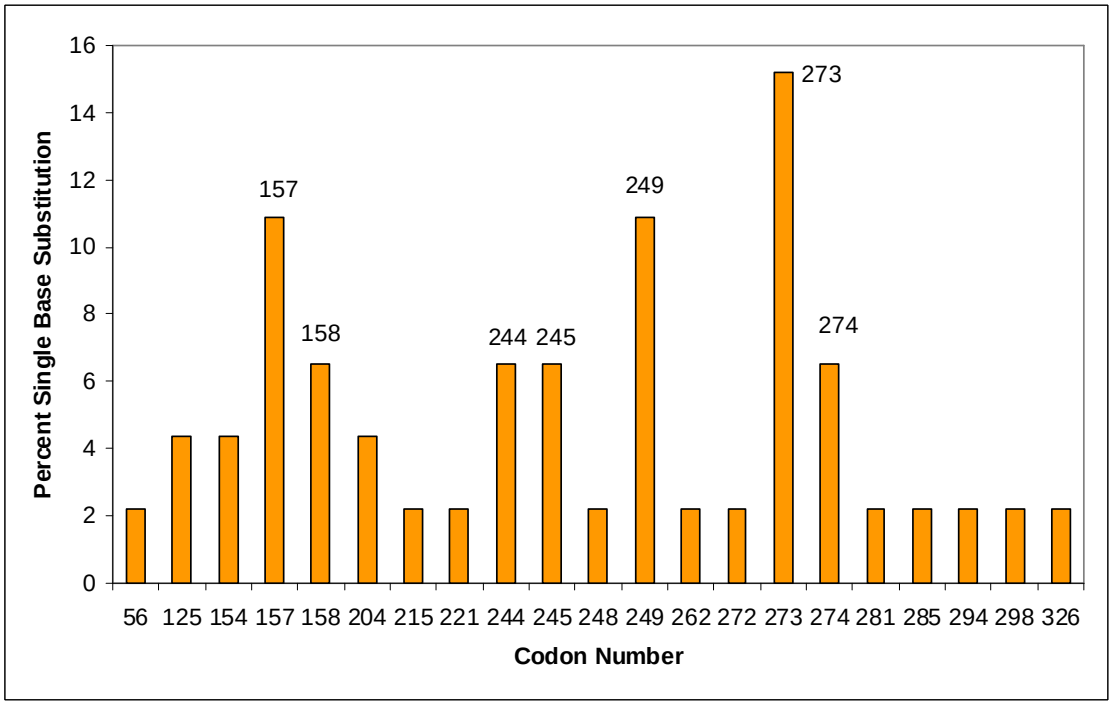


Figure 29b: *TP53* G>T mutations distribution at exons 4 to 9



Association with clinical/individual parameters

The mutations were classified by effect and type according to their predicted capacity to modify protein sequence (Effect Group classification) and were grouped into categories based on the predicted effect on the p53 protein (Table 16).

The **Effect Group** classification was as follows:

1. Missense mutations in DNA-binding motif
2. Missense mutations outside DNA-binding motif
4. Non-missense (including nonsense, insertion, deletion, splice site)
0. Silent mutations, no mutation, synonymous variation, intronic variation, outside splice site and wild-type.

The categories for *TP53* mutations were as follows:

Category: CONSERVATION

- Deleterious = 1
- Neutral = 2
- NA = 4 or 0 (i.e. silent mutant and wild-type)

Based on evolutionary protein conservation, groups 2 and 0 are predicted to not affect protein function while groups 1 and 4 are predicted to affect it.

Category: TRANSACTIVATION

- Non-functional = 1
- Functional/Partially functional = 2
- NA = 4 or 0 (=silent mutant and wild-type)

In yeast assays, groups 2 and 0 retain transactivation capacity while groups 1 and 4 loose it.

Category: STRUCTURE

- Same values as Effect Group

Category: CLINICAL IMPACT

- Deleterious = 1 (Effect Group 4 and 1)
- Non-deleterious = 2 (Effect Group 2 and functional mutations)
- Wild-type samples and silent mutations = 0

Group 1 relates to very bad clinical outcome while group 2 relates to intermediate clinical outcome. If a sample contained more than one mutation, we considered the more deleterious one for statistical analysis.

Table 16: ***TP53* mutation distribution by effect and type grouped into categories for predicted effect on the protein**

***TP53* Categories**	**Mutation effect**	**n**	**%**
Conservation	0 – Silent (and WT)	133	54.1
	1 – Deleterious	78	31.7
	2 – Neutral	1	0.4
	4 – Non missense	34	13.8
Transactivation	0 – Silent (and WT)	133	54.1
	1 – Non Functional	73	29.7
	2 – Functional/Partially functional	6	2.4
	4 – NA	34	13.8
Structure	0 – Silent (and WT)	133	54.7
	1 – Missense mutations in DNA-binding domain	44	18.1
	2 – Missense mutations outside DNA-binding domain	32	13.2
	4 – Non-missense	34	14.0
Type	0 – Wild Type	130	53.3
	1 – CpGs sites	6	2.5
	2 – A:T > xxx	24	9.8
	3 – All G>T	36	14.8
	4 – G>A or G>C not a CpG sites	27	11.1
	5 – Other	21	8.6
Clinical impact	0 – Silent (and WT)	133	53.8
	1 – Deleterious	82	33.2
	2 – Non-deleterious	32	13.0

Tables 17a to d classify *TP53* mutations into different categories and smoking variables. We found no association between smoking exposure and *TP53* mutations broken down by predicted effect on conservation, transactivation, and structure of p53 protein or clinical impact.

Table 18 shows the association between the prevalence of *TP53* G>T transversion and exposure to tobacco smoking. We did not find any association.

Table 17a: *TP53* mutations classified into conservation categories in relation to smoking

Variable (missing)	Items	CONSERVATION categories (n=246)						*P* value
		2+0		1		4		
		n	%	n	%	n	%	
Smoking status (1)	Current smoker	80	60.2	43	55.1	25	73.5	0.49
	Former smoker	46	34.6	32	41.0	8	23.5	
	Never-smoker	7	5.3	3	3.8	1	2.9	
Age at smoking initiation (1)	< 16	51	40.5	28	37.8	12	36.4	0.25
	[16 – 18[	21	16.7	14	18.9	5	15.2	
	[18 – 20[	24	19.1	11	14.9	12	36.4	
	≥ 20	30	23.8	21	28.4	4	12.1	
Years of smoking (2)	< 30	16	12.8	16	21.6	1	3.0	0.18
	[30 – 40[	37	29.6	19	25.7	12	36.4	
	[40 – 50[	34	27.2	26	35.1	12	36.4	
	≥ 50	38	30.4	13	17.6	8	24.2	
Pack-years (3)	< 20	21	15.9	13	16.9	3	8.8	0.80
	[20 – 40[	43	32.6	32	41.6	12	35.3	
	[40 – 50[	23	17.4	12	15.6	8	23.5	
	> 50	45	34.0	20	26.0	11	32.4	
Cigarette type (4)	Filter	54	43.6	33	45.2	16	48.5	0.85
	Mixed	39	31.5	20	27.4	7	21.2	
	Non filter & rolled	31	25.0	20	27.4	10	30.3	
Years since quit smoking	[2 – 6[	10	21.7	2	6.3	1	12.5	0.58
	[6 – 13[	14	30.4	10	31.3	4	50.0	
	[13 – 20[	8	17.4	11	34.4	2	25.0	
	≥ 20	14	30.4	9	28.1	1	12.5	

Table 17b: *TP53* mutations classified into transactivation categories in relation to smoking

Variable (missing)	Items	TRANSACTIVATION categories (n=245)						*P* value
		2+0		1		4		
		n	%	n	%	n	%	
Smoking status (1)	Current smoker	83	60.1	40	54.8	25	73.5	0.50
	Former smoker	48	34.8	30	41.1	8	23.5	
	Never-smoker	7	5.1	3	4.1	1	2.9	
Age at smoking initiation (1)	< 16	52	39.7	27	39.1	12	36.4	0.15
	[16 – 18[	21	16.0	14	20.3	5	15.2	
	[18 – 20[	27	20.6	8	11.6	12	36.4	
	≥ 20	31	23.7	20	29.0	4	12.1	
Years of smoking (2)	< 30	17	13.1	15	21.7	1	3.0	0.10
	[30 – 40[	39	30.0	17	24.6	12	36.4	
	[40 – 50[	34	26.2	26	37.7	12	36.4	
	≥ 50	40	30.8	11	15.9	8	24.2	
Pack-years (3)	< 20	22	16.1	12	16.7	3	8.8	0.89
	[20 – 40[	46	33.6	29	40.3	12	35.3	
	[40 – 50[	23	16.8	12	16.7	8	23.5	
	> 50	46	33.6	19	26.4	11	32.4	
Cigarette type (4)	Filter	56	43.4	31	45.6	16	48.5	0.81
	Mixed	41	31.8	18	26.5	7	21.2	
	Non filter & rolled	32	24.8	19	27.9	10	30.3	
Years since quit smoking	[2 – 6[	10	20.8	2	6.7	1	12.5	0.59
	[6 – 13[	15	31.3	9	30.0	4	50.0	
	[13 – 20[	8	16.7	11	36.7	2	25.0	
	≥ 20	15	31.3	8	26.7	1	12.5	

Table 17c: *TP53* mutations classified into structure categories in relation to smoking

Variable (missing)	Items	STRUCTURE categories (n=243)								*P* value
		0		1		2		4		
		n	%	n	%	n	%	n	%	
Smoking status (1)	Current smoker	80	60.6	25	56.8	17	53.1	25	73.5	0.59
	Former smoker	45	34.1	18	40.9	13	40.6	8	23.5	
	Never-smoker	7	5.3	1	2.3	2	6.3	1	2.9	
Age at smoking initiation (1)	< 16	51	40.8	19	45.2	9	30.0	12	36.4	0.31
	[16 – 18[	20	16.0	8	19.1	6	20.0	5	15.2	
	[18 – 20[	24	19.2	6	14.3	4	13.3	12	36.4	
	≥ 20	30	24.0	9	21.4	11	36.7	4	12.1	
Years of smoking (2)	< 30	16	12.9	7	16.7	9	30.0	1	3.03	0.11
	[30 – 40[	36	29.0	12	28.6	8	26.7	12	36.4	
	[40 – 50[	34	27.4	13	31.0	12	40.0	12	36.4	
	≥ 50	38	30.7	10	23.8	1	3.3	8	24.2	
Pack-years (3)	< 20	21	16.0	7	16.3	6	18.8	3	8.8	0.67
	[20 – 40[	42	9.2	19	44.2	13	40.6	12	35.3	
	[40 – 50[	23	17.6	4	9.3	7	21.9	8	23.5	
	> 50	45	34.4	13	30.2	6	18.8	11	32.4	
Cigarette type (4)	Filter	53	43.1	21	51.2	13	43.3	16	48.5	0.86
	Mixed	39	31.7	10	24.4	9	30.0	7	21.2	
	Non filter & rolled	31	25.2	10	24.4	8	26.7	10	30.3	
Years since quit smoking	[2 – 6[	9	20	0	0	3	23.1	1	12.5	0.60
	[6 – 13[	14	31.1	6	33.3	3	23.1	4	50.0	
	[13 – 20[	8	17.8	6	33.3	4	30.8	2	25.0	
	≥ 20	14	31.1	6	33.3	3	23.1	1	12.5	

Table 17d: *TP53* mutations classified into clinical impact categories in relation to smoking

Variable (missing)	Items	CLINICAL IMPACT categories (n=247)						*P* value
		0		1		2		
		n	%	n	%	n	%	
Smoking status (1)	Current smoker	80	60.6	54	65.9	17	53.1	0.71
	Former smoker	45	34.1	26	31.7	13	40.6	
	Never-smoker	7	5.3	2	2.4	2	6.3	
Age at smoking initiation (1)	< 16	51	40.8	31	39.2	9	30.0	0.69
	[16 – 18[	20	16.0	14	17.7	6	20.0	
	[18 – 20[	24	19.2	19	24.1	4	13.3	
	≥ 20	30	24.0	15	19.0	11	36.7	
Years of smoking (2)	< 30	16	12.9	10	12.7	9	30.0	0.08
	[30 – 40[	36	29.0	26	32.9	8	26.7	
	[40 – 50[	34	27.4	25	31.7	12	40.0	
	≥ 50	38	30.7	18	22.8	1	3.3	
Pack-years (3)	< 20	21	16.0	11	13.6	6	18.8	0.84
	[20 – 40[	42	32.1	33	40.7	13	40.6	
	[40 – 50[	23	17.6	13	16.0	7	21.9	
	> 50	45	34.4	24	29.6	6	18.8	
Cigarette type (4)	Filter	53	43.1	38	48.7	13	43.3	0.71
	Mixed	39	31.7	18	23.1	9	30.0	
	Non filter & rolled	31	25.2	22	28.2	8	26.7	
Years since quit smoking	[2 – 6[	9	20.0	1	3.9	3	23.1	0.60
	[6 – 13[	14	31.1	10	38.5	3	23.1	
	[13 – 20[	8	17.8	8	30.8	4	30.8	
	≥ 20	14	31.1	7	26.9	3	23.1	

Table 18: *TP53* smoking-related mutations in relation to smoking

Variable (missing)	Items	Other (n = 130)		All G > T (n = 36)		*P* value
		n	%	n	%	
Smoking status (1)	Current smoker	78	60.5	25	69.4	0.60
	Former smoker	44	34.1	10	27.8	
	Never-smoker	7	5.4	1	2.8	
Age at smoking initiation (1)	< 16	51	41.8	14	41.2	0.29
	[16 – 18[	20	16.4	3	8.8	
	[18 – 20[	22	18.0	4	11.8	
	≥ 20	29	23.8	13	38.2	
Years of smoking (2)	< 30	15	12.4	6	17.7	0.67
	[30 – 40[	36	29.8	11	32.4	
	[40 – 50[	33	27.3	12	35.3	
	≥ 50	37	30.6	5	14.7	
Pack-years (3)	< 20	20	15.6	4	11.4	0.24
	[20 – 40[	41	32.0	18	51.4	
	[40 – 50[	22	17.2	5	14.3	
	> 50	45	35.2	8	22.9	
Cigarette type (4)	Filter	53	44.2	16	45.7	0.78
	Mixed	38	31.7	9	25.7	
	Non filter & rolled	29	24.2	10	28.6	
Years since quit smoking	[2 – 6[	9	20.5	1	10.0	0.54
	[6 – 13[	14	31.8	3	30.0	
	[13 – 20[	8	18.2	5	50.0	
	≥ 20	13	29.6	1	10.0	

TP53 mutations and p53 immunodetection

Since missense *TP53* mutations may lead to nuclear accumulation of mutant p53 protein we tested their association in our series. Information on both mutation status and IHC was available for a subset of 230 patients.

P53 immunostaining was expressed as a score summing up intensity and distribution as follows: sum of 0, no staining (= score 0); sum of 1 to 3, slight staining (= score 1); sum of 4 to 5, moderate staining (= score 2); and sum of 6 to 7, marked staining (= score 3).

We found a strong correlation between mutation status and p53 IHC ($p<0.0001$). Among tumours with mutations, 62% were highly positive for p53 protein. Surprisingly, also 25% of tumours with wild-type *TP53* had high expression of p53 across the tumour (Table 19). This result suggests that p53 may be consistently expressed in a subset of lung cancers without missense mutations in the DNA binding domain.

Table 19: p53 expression in association with *TP53* status

p53 expression score	*TP53* status		Total
	Wild Type	Mutated	
0,1,2	87 37.8 %	43 18.7 %	130 56.5 %
3	29 12.6 %	71 30.9 %	100 43.5 %
Total	116 50.4 %	114 49.6 %	230 100 %

TP53 polymorphisms

The *TP53* gene is highly polymorphic and there is evidence that mutations may occur at different rates on different *TP53* alleles. We have analysed the distribution of 3 common polymorphisms located within a 312 bp region of the *TP53* gene encoding the N-terminus of p53, in relation with *TP53* mutation status. These three polymorphisms are located in intron 2 (PIN2, rs.1642785: G

to C), intron 3 (PIN3, rs.17878362: 16bp duplication) and in exon 4 (PEX4, rs.1042522: non-silent G to C).

Data (Table 20) show that *TP53* mutations occurred preferentially (p=0.05) in subjects who were homozygous for the rare PEX4 allele (i.e. CC subjects). *TP53* mutations in PEX4 CC samples were 85.7%, as compared to 43.9% and 46.6% in G-C heterozygous and G-G homozygous respectively. The two other polymorphisms did not appear to be associated with significant differences in mutation prevalence.

Table 20: ***TP53* status and polymorphisms**

***TP53* polymorphism (n= 245)**	***TP53* allele**	***TP53* status**				***P* value**
		Wild type (n = 128)		**Mutated (n = 117)**		
		n	**%**	**n**	**%**	
PIN2	CC	7	5.5	15	12.8	0.21
	GC	58	45.3	44	37.6	
	GG	63	49.2	58	49.6	
PIN3	DD	4	3.1	8	6.8	0.16
	ND	43	33.6	29	24.8	
	NN	81	63.3	80	68.4	
PEX4	CC	2	1.6	12	10.3	0.05
	CG	55	43.0	43	36.8	
	GG	71	55.5	62	53.0	

The G allele in PIN2 was more frequent in men than women (p=0.0019). Tobacco exposure variables (i.e. “pack-years” and “cigarette type”) were differently distributed among individuals with different PIN2 alleles (Table 21a).

Borderline association was observed between the 16bp duplication and the individual history of pulmonary illness (p=0.047, Table 21b).

Previous studies (Marcel et al. 2009) showed linkage disequilibrium between *TP53* PIN2 and *TP53* PEX4. Similarly to PIN2, there were significantly more men than women with a PEX4 GG homozygous genotype (Table 21c).

Table 21a: *TP53* PIN2 and clinical variables

Variable (missing)	Items	PIN2 (n=249)				*P* value
		CC/CG		GG		
		n	%	n	%	
Gender	Male	97	77.0	111	90.2	**0.0019**
	Female	29	23.0	12	9.8	
Age	< 60	47	37.3	40	32.5	0.35
	[60-65[	45	35.7	37	30.1	
	[65-70[	12	9.5	19	15.5	
	≥ 70	22	17.5	27	22.0	
Past pulmonary illness (2)	No	59	47.2	50	41.0	0.30
	Yes	66	52.8	72	59.0	
Asbestos exposure (2)	None	102	81.6	89	73.0	0.12
	Yes	23	18.4	33	27.1	
Nodal score (1)	N0	85	68.0	89	72.4	0.47
	N1,N2,NX	34	27.6	40	32.0	
Tumour score (1)	T1	42	33.6	35	28.5	0.40
	T2, T3, T4	83	66.4	88	71.5	
Histology	SCC / Others	60	48.8	54	42.9	0.50
	ADC	63	51.2	72	57.1	
Smoking status (1)	Current smoker	74	58.7	78	63.9	0.32
	Former smoker	44	34.9	41	33.6	
	Never-smoker	8	6.4	3	2.5	
Years of smoking (2)	< 30	22	19.0	14	11.8	0.10
	[30-40[	38	32.8	30	25.2	
	[40-50[	29	25.0	44	37.0	
	≥ 50	27	23.3	31	26.1	
Age at smoking initiation (1)	< 16	43	36.8	46	38.7	0.42
	[16-18[	24	20.5	18	15.1	
	[18-20[	18	15.4	28	23.5	
	≥ 20	32	27.4	27	22.7	
Pack-years (3)	< 20	26	21.0	13	10.7	**0.04**
	[20-40[	41	33.1	47	38.5	
	[40-50[	26	21.0	18	14.8	
	≥ 50	31	25.0	44	36.1	
Years since quit smoking	[2 – 6[	7	15.9	6	14.6	0.88
	[6 – 13[	12	27.3	14	34.2	
	[13 – 20[	11	25.0	10	24.4	
	≥ 20	14	31.8	11	26.8	
Cigarette type (4)	Filter	59	51.3	44	37.3	**0.036**
	Mixed	32	27.8	35	30.0	
	Non Filter & Rolled	24	20.9	39	33.1	
Family history of Lung/HN cancer (6)	No Lung/HN cancer	94	77.1	86	71.1	0.32
	Lung/HN cancer	28	23.0	35	28.9	
Personal history of Lung/HN cancer (2)	No Lung/HN cancer	114	91.2	110	90.2	0.93
	Lung/HN cancer	11	8.8	12	9.8	

Table 21b: *TP53* PIN3 and clinical variables

Variable (missing)	Items	PIN 3 (n=249)				*P* value
		NN		DD/ND		
		n	%	n	%	
Gender	Male	141	86	67	78,8	0.16
	Female	23	14	18	21,2	
Age	< 60	56	34,1	31	36,5	0.07
	[60-65[	48	29,3	34	40	
	[65-70[	20	12,2	11	12,9	
	≥ 70	40	24,4	9	10,6	
Past pulmonary illness (2)	No	64	39,3	45	53,6	**0.047**
	Yes	99	60,7	39	46,4	
Asbestos exposure (2)	None	127	77,9	64	76,2	0.53
	Yes	36	22,1	20	23,8	
Nodal score (1)	N0	119	72,6	55	65,5	0.20
	N1,N2,NX	45	27.4	29	34.5	
Tumour score (1)	T1	45	27,4	32	38,1	0.47
	T2, T3, T4	119	72.6	52	61.9	
Histology	SCC / Others	82	50	53	62,4	0.33
	ADC	82	50	32	37.6	
Smoking status (1)	Current smoker	101	62	51	60	0.96
	Former smoker	55	33,7	30	35,3	
	Never-smoker	7	4,3	4	4,7	
Years of smoking (2)	< 30	30	18,4	17	20,5	0.40
	[30-40[	41	25,2	27	32,5	
	[40-50[	52	31,9	21	25,3	
	≥ 50	40	24,5	18	21,7	
Age at smoking initiation (1)	< 16	60	38,5	29	36,3	0.45
	[16-18[	29	18,6	13	16,3	
	[18-20[	33	21,2	13	16,3	
	≥ 20	34	21,8	25	31,3	
Pack-years (3)	< 20	25	15,3	14	16,9	0.19
	[20-40[	57	35	31	37,3	
	[40-50[	24	14,7	20	24,1	
	≥ 50	57	35	18	21,7	
Years since quit smoking	[2 – 6[	7	12,7	6	20	0.57
	[6 – 13[	19	34,5	7	23,3	
	[13 – 20[	15	27,3	6	20	
	≥ 20	14	25,5	11	36,7	
Cigarette type (4)	Filter	64	41,6	39	49,4	0.26
	Mixed	43	27,9	24	30,4	
	Non Filter & Rolled	47	30,5	16	20,3	
Family history of Lung/HN cancer (6)	No Lung/HN cancer	114	70,8	66	80,5	0.11
	Lung/HN cancer	47	29,2	16	19,5	
Personal history of Lung/HN cancer (2)	No Lung/HN cancer	147	90,2	77	91,7	0.96
	Lung/HN cancer	16	9,8	7	8,3	

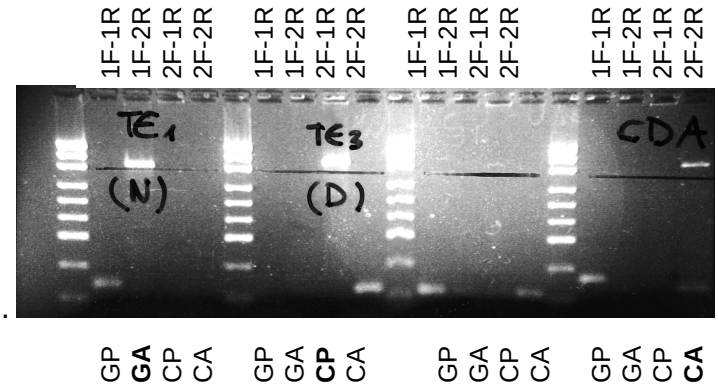
Table 21c: *TP53* PEX4 and clinical variables

Variable (missing)	Items	PEX4 (n=249)				*P* value
		GG		CG/CC		
		n	%	n	%	
Gender	Male	120	88,2	88	77,9	**0.0079**
	Female	16	11,8	25	22,1	
Age	< 60	43	31,6	44	38,9	0.39
	[60-65[	20	14,7	11	9,7	
	[65-70[	30	22,1	19	16,8	
	≥ 70	43	31,6	39	34,5	
Past pulmonary illness (2)	No	56	41,5	53	47,3	0.39
	Yes	79	58,5	59	52,7	
Asbestos exposure (2)	None	101	74,8	90	80,4	0.32
	Yes	34	25,2	22	19,6	
Nodal score (1)	N0	97	71,3	77	68,8	0.66
	N1,N2,NX	39	28.7	35	31.3	
Tumour score (1)	T1	38	27,9	39	34,8	0.17
	T2, T3, T4	98	72.1	73	65.2	
Histology	SCC / Others	67	49.3	47	41.6	0.42
	ADC	69	50,7	66	58,4	
Smoking status (1)	Current smoker	86	63,7	66	58,4	0.38
	Former smoker	45	33,3	40	35,4	
	Never-smoker	4	3	7	6,2	
Years of smoking (2)	< 30	21	15,6	26	23,4	0.15
	[30-40[	32	23,7	36	32,4	
	[40-50[	46	34,1	27	24,3	
	≥ 50	36	26,7	22	19,8	
Age at smoking initiation (1)	< 16	50	38,2	39	37,1	0.88
	[16-18[	21	16	21	20	
	[18-20[	28	21,4	18	17,1	
	≥ 20	32	24,4	27	25,7	
Pack-years (3)	< 20	16	11,9	23	20,7	0.08
	[20-40[	51	37,8	37	33,3	
	[40-50[	20	14,8	24	21,6	
	≥ 50	48	35,6	27	24,3	
Years since quit smoking	[2 – 6[	7	15,6	6	15	0.92
	[6 – 13[	15	33,3	11	27,5	
	[13 – 20[	11	24,4	10	25	
	≥ 20	12	26,7	13	32,5	
Cigarette type (4)	Filter	50	38,5	53	51,5	0.10
	Mixed	40	30,8	27	26,2	
	Non Filter & Rolled	40	30,8	23	22,3	
Family history of Lung/HN cancer (6)	No Lung/HN cancer	97	72,9	83	75,5	0.71
	Lung/HN cancer	36	27,1	27	24,5	
Personal history of Lung/HN cancer (2)	No Lung/HN cancer	122	90,4	102	91,1	0.69
	Lung/HN cancer	13	9,6	10	8,9	

TP53 haplotypes

Haplotypes were analysed by ARMS. Figure 30 shows an example of “CDA” haplotype (i.e. PIN2-C allele, PIN3-Duplication and PEX4-G allele coding Arginine) charged on a 3% gel. Four different PCR mix were prepared to allow identification of PIN2 and PEX4 alleles. Positive controls allowed identification of PIN3: TE1 cell line was used as control for GNA haplotype (bearing Non-duplication in PIN3) and TE3 cell line was used as control for CDP haplotype (bearing Duplication in PIN3).

Figure 30: CDA haplotype sample charged on a 3% gel



The different distribution of *TP53* status among the haplotypes proved to be not statistically significant (Table 22).

When *TP53* haplotypes were analysed in relation to individual and clinical variables (Table 23) the GNA allele appeared to be significantly associated with male gender, no exposure to asbestos and an intermediate number of cigarettes smoked (in pack-years).

The GNA allele frequency was of 66.85%; the following most represented alleles were CDP with a frequency of 16.12% and CNP with an allele frequency of 9.16%.

Table 22: ***TP53* status among haplotypes**

***TP53* haplotypes (n= 245)**	***TP53* status**				***P* value**
	Wild type (n = 128)		**Mutated (n = 117)**		
	n	**%**	**n**	**%**	
GNA-CDP	35	63.6	20	36.4	0.38
GNA-CNP	16	50.0	16	50.0	
GNA-GNA	56	50.0	56	50.0	
Others	21	45.7	25	54.4	

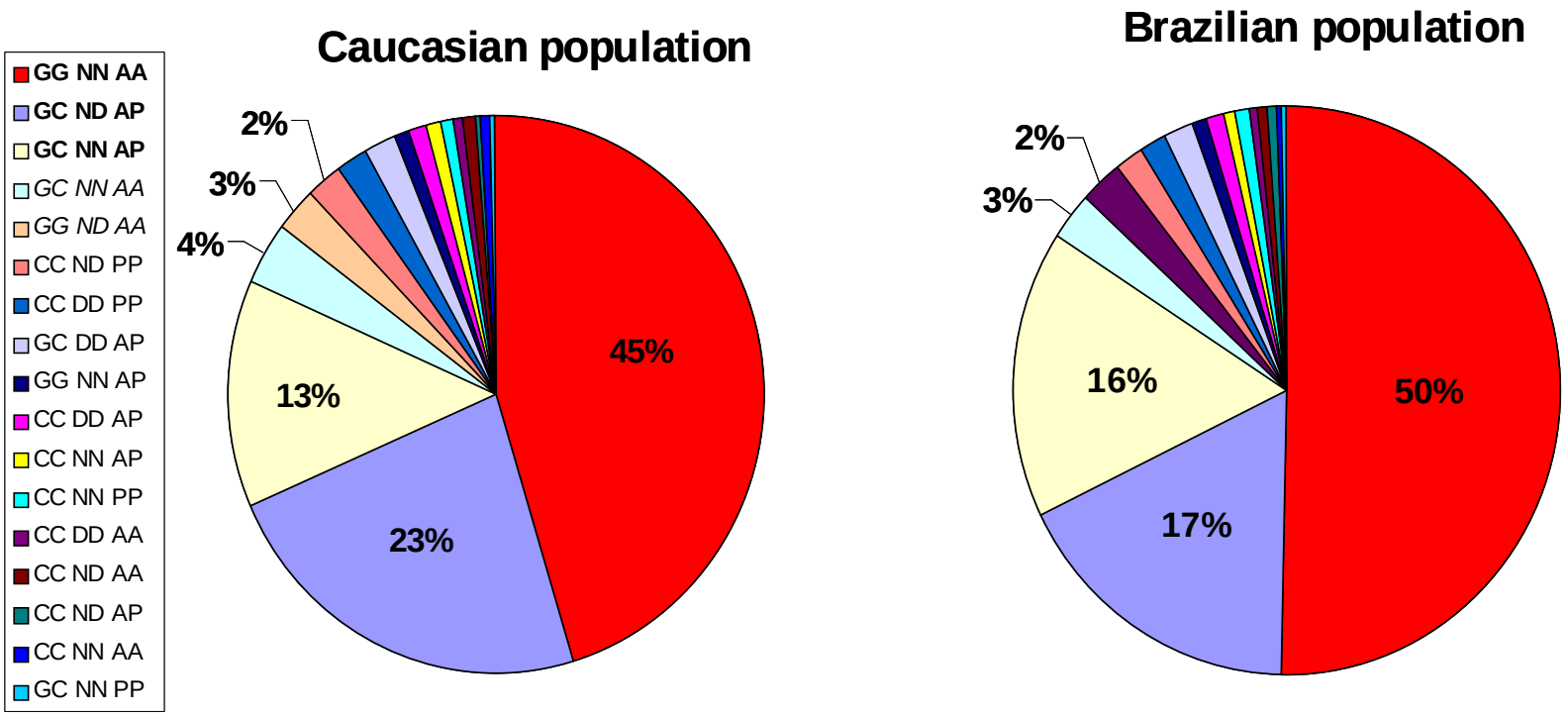
Table 23: ***TP53* haplotypes and clinical variables**

Variable (missing)	**Items**	***TP53* haplotypes (n= 249)**								***P* value**
		GNA-CDP		**GNA-CNP**		**GNA-GNA**		**Others**		
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	
Gender	Male	43	76.8	24	75.0	102	89.5	39	83.0	**0.046**
	Female	13	23.2	8	25.0	12	10.5	8	17.0	
Age	< 60	21	37.5	12	37.5	38	33.3	16	34.0	0.27
	[60-65[	6	10.7	1	3.13	15	13.2	9	19.2	
	[65-70[	6	10.7	9	28.1	27	23.7	7	14.9	
	≥ 70	23	41.7	10	31.3	34	29.8	15	31.9	
Past pulmonary illness (2)	No	29	52.7	13	40.6	44	38.9	23	48.9	0.41
	Yes	26	47.3	19	59.4	69	61.1	24	51.1	
Asbestos exposure (2)	None	47	85.5	28	87.5	85	75.2	31	66.0	**0.02**
	Yes	8	14.6	4	12.5	28	24.8	16	34.0	
Nodal score (1)	N0	37	67.3	24	75.0	84	73.7	29	61.7	0.52
	N1,N2,NX	18	32.7	8	25.0	30	26.3	18	38.3	
Tumour score (1)	T1	25	45.5	10	31.3	32	28.1	10	21.3	0.11
	T2, T3, T4	30	54.6	22	68.8	82	71.9	37	78.7	
Histology	SCC/Others	19	33.9	16	50.0	58	50.9	21	44.7	0.36
	ADC	37	66.1	16	50.0	56	49.1	26	55.3	
Smoking status (1)	Current	33	58.9	19	59.4	71	62.8	29	61.7	0.65
	Former	20	35.7	10	31.3	39	34.5	16	34.0	
	Never	3	5.4	3	9.4	3	2.7	2	4.3	
Years of smoking (2)	< 30	7	13.5	7	24.1	13	11.8	9	20.5	0.61
	[30-40[	18	34.6	8	27.6	29	26.4	13	30.0	
	[40-50[	14	26.9	7	24.1	38	34.6	14	31.8	
	≥ 50	13	25.0	7	24.1	30	27.3	8	18.2	
Age at smoking initiation (1)	< 16	17	32.1	12	41.4	40	36.4	20	45.5	0.65
	[16-18[	10	18.9	6	20.7	18	16.4	8	18.2	
	[18-20[	10	18.9	5	17.2	27	24.6	4	9.1	
	≥ 20	16	30.2	6	20.7	25	22.7	12	27.3	
Pack-years (3)	< 20	9	16.4	11	34.4	12	10.6	7	15.2	**0.01**
	[20-40[	24	43.6	6	18.8	45	39.8	13	28.3	
	[40-50[	10	18.2	6	18.8	15	13.3	13	28.3	
	≥ 50	12	21.8	9	28.1	41	36.3	13	28.3	

Years since quit smoking	[2 – 6[	4	20.0	1	10.0	5	12.8	3	18.8	0.85
	[6 – 13[	6	30.0	3	30.0	14	35.9	3	18.8	
	[13 – 20[	3	15.0	4	40.0	10	25.6	4	25.0	
	≥ 20	7	35.0	2	20	10	25.6	6	37.5	
Cigarette type (4)	Filter	25	48.1	14	48.3	41	37.6	23	53.5	0.29
	Mixed	15	28.9	7	24.1	32	29.4	13	30.2	
	NonFilter/Rolled	12	23.1	8	27.6	36	33.0	7	16.3	
Family history of Lung/HN cancer (6)	No LC/HNC	41	75.9	22	68.8	81	72.3	36	80.0	0.73
	LC/HNC	13	24.1	10	31.3	31	27.7	9	20.0	
Personal history of Lung/HN cancer (2)	No LC/HNC	51	92.7	29	90.6	101	89.4	43	91.5	0.71
	LC/HNC	4	7.3	3	9.4	12	10.6	4	8.5	

Figure 31 shows patterns of *TP53* genotypes distribution in the EUELC population. The three major genotypes found were GGNNAA, GCNDAP and GCNNAP. As a comparison we show the genotypes distribution in a Brazilian population (results from Marcel et al. 2009).

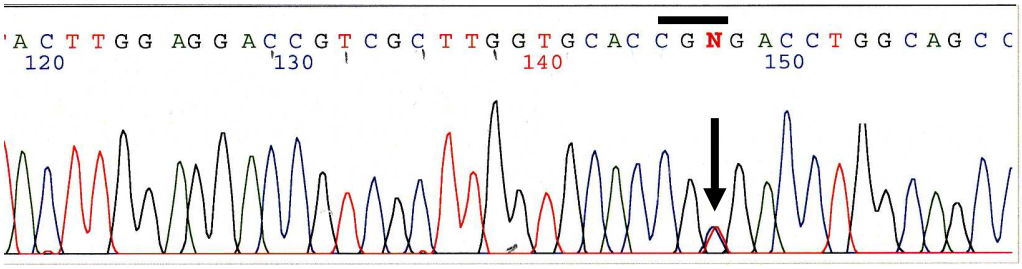
Figure 31: Patterns of *TP53* genotype distribution in the EUELC population and in a Brazilian population



EGFR mutations

Deregulation of human epidermal growth factor receptor pathways by over-expression or constitutive activation can promote tumour processes including angiogenesis and metastasis and is associated with poor prognosis, in particular in a certain fraction of NSCLCs (Marks et al. 2008). Somatic mutations of *EGFR* gene cluster in domains of the kinase that constitutively induce its activity and signal transduction (in exons 18 to 21). We found 13.07% *EGFR* mutations, spread among the 4 exons tested (4% in exon 18, 3% in exon 19, 3% in exon 20 and 5% in exon 21). 30 % of mutations were deletion in exon 19 and the single-point mutation at position 858 (L858R) in exon 21. All *EGFR* mutations were reported by the COSMIC mutation database. Figure 32 shows a silent *EGFR* mutation (in a patient who also carried a *KRAS* mutation).

Figure 32: *EGFR* mutation: exon 21, codon 836, CGC>CGT, Arg>Arg

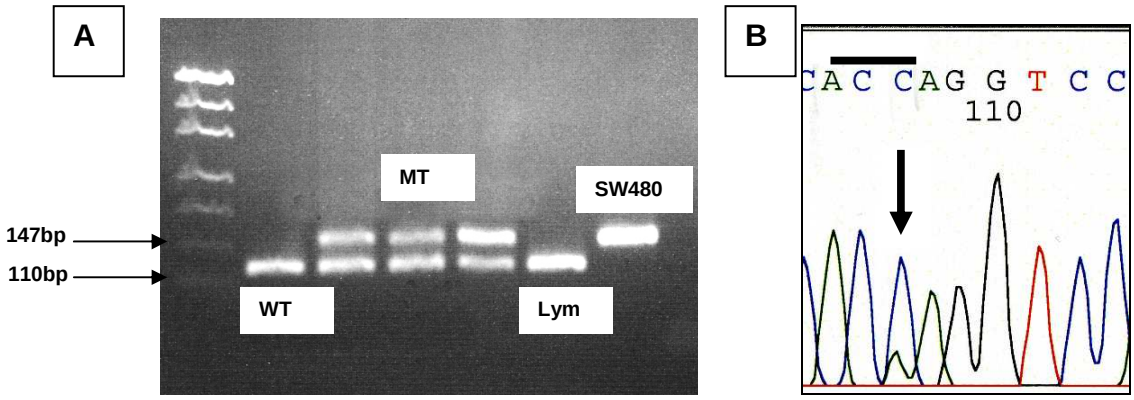


KRAS mutations and Reproducibility of *KRAS* mutational analysis

A total of 18.5% *KRAS* mutations at codon 12 and 13 were detected. Missense G>T transversions represented 98% of *KRAS* mutations. Transversions were either **G**GT>**T**GT (Gly>Cys) or G**G**T>G**T**T (Gly>Val) at codon 12. *KRAS* and *EGFR* mutations were mutually exclusive except for one tumour containing both mutations (an extremely rare occurrence according to the literature). Interestingly, in this tumour the *EGFR* mutation was a silent mutation (codon 836 CGC>CGT Arg>Arg) and was thus not supposed to lead to tyrosine kinase activation.

Figure 33 shows digestion products of *KRAS* samples (panel A). Negative (i.e. lymphocyte) and positive (i.e. SW480 cell line, *KRAS* mutated at codon 12) controls were also loaded on gel. A sequence variation at any of the 2 first positions of codon 12 gives two restriction products of 143bp and 14bp (non visible band of mutant sample). Panel B is an example of sequence that revealed *KRAS* mutations at codon 12 **C**CA>**A**CA→**G**GT>**T**GT: Gly>Cys.

Figure 33: Panel A: wild type and mutant *KRAS* samples charged on a 3% gel; Panel B: sequence of the mutant sample



A EUELC partner in the Netherlands analysed *KRAS* mutations by point EXACCT method (Thunissen et al. 2011); we tested the two methods (ME-PCR and microarray) for reproducibility. We observed clear reproducibility of *KRAS* mutations from both EUELC partners (Kappa coefficient = 0.94 and 95%CI= [0.88 – 1.00]; Table 24).

Table 24: *KRAS* status in the two centres

***KRAS* (the Netherlands)**	***KRAS* (IARC)**		**Total**
	Wild Type	**Mutated**	
Wild Type	181 81.2 %	4 1.8 %	185 83.0 %
Mutated	0 0.0 %	38 17.0 %	38 17.0 %
Total	181 81.2 %	42 18.8 %	223 100 %

Mutation prevalence and distribution in association with individual and pathological parameters

Tables 25a, b and c show the associations between mutations in *TP53*, *KRAS*, *EGFR* and pathological, demographic or exposure variables (Mantel Haenszel χ^2 test stratified by centre). None of these mutations were associated with either T or N score (of TNM classification of tumours) of our samples.

TP53 mutations were significantly less frequent in ADC (39.7%) than in SCC (57%) with p<0.0001 (Table 25a). Similarly, *KRAS* mutations were preferentially found in ADC (89.1%) than in SCC (10.9%) with p<0.0001 (Table 25b).

TP53 mutations were marginally more common in subjects who reported a personal past history of pulmonary illness or a family history of lung cancer, but these associations were not significant (p=0.1505 and p=0.1620, respectively). Exposure to tobacco smoking or asbestos (i.e. “smoking status” and “history of exposure to asbestos” variables) did not associate with either *TP53* or *KRAS* mutations. Similarly, there was no significant association with variables related to tobacco smoking exposure (i.e. smoking duration, age at smoking initiation, consumption in pack-years, time since quitting smoking and cigarette type). Nevertheless, *TP53* and *KRAS* mutations tended to be more common in lung cancers of ever- than former- or never-smokers: 62%, 34.7% and 3.3%, respectively, for *TP53* and 52.2%, 45.7% and 2.2 %, respectively, for *KRAS*.

When analyzing *EGFR* status in association with smoking variables we found that *EGFR* mutations were significantly more present in never-smoking women (Table 25c). Somatic mutations of *EGFR* gene are found almost exclusively in adenocarcinoma of never-smoking women and cluster in domains of the kinase that constitutively activate its activity and signal transduction (Tokumo et al. 2005).

Table 25a: ***TP53* status in association with clinical and smoking variables**

Variable (missing)	Items	*TP53* status				*P* value
		Wild type (n = 129)		Mutated (n = 121)		
		n	%	n	%	
Gender	Male	107	82.9	103	85.1	0.89
	Female	22	17.1	18	14.9	
Age	< 60	40	31	49	40.5	0.60
	[60-65[	16	12.4	14	11.6	
	[65-70[	24	18.6	25	20.7	
	≥ 70	49	38	33	27.3	
Marital Status (2)	Unaccompanied	39	30.2	26	21.5	0.10
	Accompanied	90	69.8	95	78.5	
Education level (39)	No/Primary Level	93	76.2	88	74.6	0.72
	High educated	29	23.8	30	25.4	
Past pulmonary illness (2)	No	50	39.4	60	49.6	**0.15**
	Yes	77	60.6	61	50.4	
Asbestos exposure (11)	No	100	78.7	91	75.2	0.80
	Yes	27	21.3	30	24.8	
Nodal score (4)	**N0**	89	69.5	84	69.4	0.37
	N1	30	23.4	35	28.9	
	N2	2	1.6	0	0	
	NX	7	5.5	2	1.7	
Tumour score (4)	**T1**	39	30.5	37	30.6	0.98
	T2	76	59.4	74	61.2	
	T3	8	6.3	7	5.8	
	T4	5	3.9	3	2.5	
Histology (1)	**ADC**	85	65.9	48	39.7	**<.0001**
	SCC	41	31.8	69	57	
	Others	3	2.3	4	3,3	
Smoking status (1)	Current smoker	77	60.2	75	62	0.83
	Former smoker	44	34.4	42	34,7	
	Never-smoker	7	5.5	4	3,3	
Years of smoking (14)	< 30	15	12.5	20	17.2	0.37
	[30-40[	35	29.2	35	30.2	
	[40-50[	33	27.5	39	33.6	
	≥ 50	37	30.8	22	19.0	
Age at smoking initiation (5)	< 16	50	41.3	41	35.3	0.78
	[16-18[	20	16.5	21	18.1	

	[18-20[	22	18.2	26	22.4	
	≥ 20	29	24.0	28	24.1	
Pack-years (18)	< 20	20	15.8	18	15.0	0.76
	[20-40[	41	32.3	48	40.0	
	[40-50[	22	17.3	22	18.3	
	≥ 50	44	34.7	32	26.7	
Years since quit smoking	[2 – 6[	9	20.5	4	9.5	0.71
	[6 – 13[	14	31.8	14	33.3	
	[13 – 20[	8	18.2	13	31.0	
	≥ 20	13	29.6	11	26.2	
Cigarette type (33)	Filter	52	43.7	52	45.2	0.55
	Mixed	38	31.9	29	25.2	
	Non filter & rolled	29	24.4	34	29.6	
Family history of Lung/HN cancer (32)	No Lung/HN cancer	96	76,8	83	69,7	**0.16**
	Lung/HN cancer	29	23,2	36	30,3	
Personal history of Lung/HN cancer (3)	No Lung/HN cancer	118	92,9	107	88,4	0.44
	Lung/HN cancer	9	7,1	14	11,6	

Table 25b: *KRAS* status in association with clinical and smoking variables

Variable (missing)	Items	*KRAS* status				*P* value
		Wild type (n = 203)		Mutated (n = 46)		
		n	%	n	%	
Gender	Male	172	84.7	38	82.6	0.81
	Female	31	15.3	8	17.4	
Age	< 60	73	36	14	30.4	0.77
	[60-65[	26	12.8	5	10.9	
	[65-70[	38	18.7	12	26.1	
	≥ 70	66	32.5	15	32.6	
Past pulmonary illness (2)	No	88	43.6	21	46.7	0.77
	Yes	114	56.4	24	53.3	
Asbestos exposure (2)	No	154	76.2	36	80	0.82
	Yes	48	23.8	9	20	
Nodal score (1)	N0	140	69.3	33	71.7	0.60
	N1, N2, NX	62	30.7	13	28.3	
Tumour score (1)	T1	68	33.7	9	19.6	0.10
	T2, T3, T4	134	66.3	37	80.4	
Histology	SCC / Others	110	54.2	5	10.9	**<.0001**
	ADC	93	45.8	41	89.1	
Smoking status (1)	Current smoker	128	63.4	24	52.2	0.08
	Former smoker	64	31.7	21	45.7	
	Never-smoker	10	5	1	2.2	
Years of smoking (14)	< 30	27	14.1	9	20.0	0.29
	[30-40[	54	28.3	14	31.1	
	[40-50[	63	33.0	10	22.2	
	≥ 50	47	24.6	12	26.7	
Age at smoking initiation (5)	< 16	75	39.1	17	37.8	0.95

	[16-18[	32	16.7	9	20.0	
	[18-20[	36	18.8	9	20.0	
	≥ 20	49	25.5	10	22.2	
Pack-years (18)	< 20	28	13.9	10	21.7	0.64
	[20-40[	74	36.8	15	32.6	
	[40-50[	36	17.9	8	17.4	
	≥ 50	63	31.3	13	28.3	
Years since quit smoking	[2 – 6[	11	17.2	2	9.5	0.71
	[6 – 13[	22	34.4	6	28.6	
	[13 – 20[	15	23.4	5	23.8	
	≥ 20	16	25.0	8	38.1	
Cigarette type (4)	Filter	79	42	24	53.3	0.23
	Mixed	58	30.9	9	20	
	Non Filter & Rolled	51	27.1	12	26.7	
Family history of Lung/HN cancer (5)	No Lung/HN cancer	146	73.4	33	73.3	0.92
	Lung/HN cancer	53	26.6	12	26.7	
Personal history of Lung/HN cancer (2)	No Lung/HN cancer	183	90.6	41	91.1	0.49
	Lung/HN cancer	19	9.4	4	8.9	

Table 25c: *EGFR* status in association with clinical and smoking variables

Variable (missing)	Items	*EGFR* status				*P* value
		Wild type (n = 113)		Mutated (n = 17)		
		n	%	n	%	
Gender	Male	92	81.4	11	64.7	0.35
	Female	21	18.6	6	35.3	
Age	< 60	41	36.3	5	29.4	0.21
	[60-65[	15	13.3	2	11.8	
	[65-70[	19	16.8	6	35.3	
	≥ 70	38	33.6	4	23.5	
Past pulmonary illness	No	55	48.7	8	47.1	0.72
	Yes	58	51.3	9	52.9	
Asbestos exposure	No	89	78.8	14	82.4	0.93
	Yes	24	21.2	3	17.7	
Nodal score (1)	N0	83	73.5	11	68.8	0.95
	N1, N2, NX	30	26.6	5	31.3	
Tumour score (1)	T1	36	31.9	5	31.3	0.87
	T2, T3, T4	77	68.1	11	68.8	
Smoking status	Current smoker	64	56.6	8	47.1	0.11
	Former smoker	43	38.1	5	29.4	
	Never-smoker	6	5.3	4	23.5	
Years of smoking (1)	< 30	21	19.8	4	30.8	0.49
	[30-40[	33	31.1	2	15.4	
	[40-50[	29	27.4	3	23.1	
	≥ 50	23	21.7	4	30.8	
Age at smoking initiation	≥ 16	59	55.1	8	61.5	0.87
	< 16	48	44.9	5	38.5	
Pack-years (1)	≤ 40	61	54.5	13	76.5	0.15
	> 40	51	45.5	4	23.5	
Years since quit smoking	≥ 13	24	55.8	3	60.0	0.96

	< 13	19	44.2	2	40.0	
Cigarette type (3)	Filter	48	45.7	5	41.7	0.97
	Mixed	31	29.5	4	33.3	
	Non Filter & Rolled	26	24.8	3	25.0	
Family history of Lung/HN cancer (2)	No Lung/HN cancer	80	71.4	10	62.5	0.34
	Lung/HN cancer	32	28.6	6	37.5	
Personal history of Lung/HN cancer	No Lung/HN cancer	103	91.2	16	94.1	0.79
	Lung/HN cancer	10	8.9	1	5.9	
Gender / Smoking status	Others	109	96.5	13	76.5	**0.0067**
	Never-smoker female	4	3.5	4	23.5	

Update of follow-up status for EUELC patients

The statistical analysis in the present report is based on the 2011 version of the EUELC database. Laboratory analyses were completed in 2007 but the overall median follow-up time per centre at that time was of only 16.6 months. This short follow-up could have biased both the selection of Progressive Disease (PD) and Disease Free (DF) subjects. Moreover, clinical data appeared to be incomplete for several important variables (e.g. tobacco smoking) potentially related to somatic mutations in *TP53*, *KRAS* and *EGFR* genes. In 2011 we decided that we were not able to exploit the laboratory results in full and consequently we asked the 12 centres involved in the EUELC project to provide us with clinical update. These centres were: Liverpool, Leicester, Belfast, Edinburg and Dublin (UK), Nancy and Grenoble (France), Nijmegen and Amsterdam (the Netherlands), Milan (Italy), Heidelberg (Germany) and Pamplona (Spain). Table 26 provides details of missing values for each centre as recorded in the EUELC database 2007 version.

Table 26: Percentage of missing values in clinical variables by participating centre in 2007

Variable	Centres											
	Amsterdam	Belfast	Dublin	Edinburgh	Grenoble	Heidelberg	Leicester	Liverpool	Milan	Nancy	Nijmegen	Pamplona
Gender							0,1					
Date of interview	0,1	1,9		0,1			0,2	1,9			0,4	
Date of birth							0,1				0,1	
Marital status		2,1		0,1			0,2		0,1		1,2	
Age at end of education	0,4	3,5	0,1	0,4	0,4	2,9	0,2	2	0,1	0,3	1,6	0,8
Highest education level	0,2	3,4		0,4	0,2		0,2	1,8	0,1		1,6	
Smoking status		1,9		0,4			0,3		0,1		1,5	
Pack-years*	0,2	2,5	0,1	0,7		0,5	0,5	0,1	0,1		1,8	0,4
Age at smoking initiation*		2,3		0,5		0,1	0,4		0,1		1,5	0,1
Time since quit smoking*		2		0,5			0,4		0,1		1,5	
Smoking duration*	0,2	2,3		0,6		0,5	0,5	0,1	0,1		1,8	0,4
Type of cigarette*	0,2	3,6		0,7	1,5	0,5	0,5	0,4	0,1		1,8	0,2
Past cancerous malignant growth / tumour	0,1	1,6	0,1	0,6			0,3				1,5	
Location of past cancerous malignant growth	0,5	7,1	0,5	2,5	1		1,5		0,5		7,6	
Asbestos exposure	0,1	2,3		0,8			0,4	0,6			1,5	
Family history of lung/head&neck cancer	0,2	3,6		2,6		0,1	0,3	1,1	0,1		1,9	0,1
Family history of any cancer	0,2	3,6		2,6		0,1	0,3	1,1	0,1		1,9	0,1
Primary lung cancer status						0,1	0,1					
Date of Surgery PLC												
Histology PLC												
TNM PLC				0,2		0,1	0,6	0,1				
Head and neck cancer status				0,2			0,1		1		0,1	
Date of surgery HN		2,9		8,6			2,9		25,7		5,7	
Histology HN				8,6	2,9		2,9		25,7		2,9	
TNM HN				8,6			2,9		25,7	2,9	2,9	
Total average missing values per patient	0,42	5,39	0,06	1,95	0,28	0,43	1,65	0,89	0,48	0,04	3,41	0,20

* Percentage computed on former and current smokers

The update- work plan involved the following steps:

1. Identification of a Minimal Variable List corresponding to the information we determined as both useful and practically feasible;
2. Personal contact and individual arrangements with each of the major participating centres to develop data retrieval, taking into account the specificities of each centre;
3. Final collation of results into the EUELC database.

The Minimal Variable list for completeness of follow-up status included: “Alive and Well”, “Alive with disease” (no treatment), “Died from other causes”, “Died from the disease”, “Metastatic relapse/reoccurrence”, “SPLC” (Second Primary Lung Cancer), “Treatment with chemotherapy”, “Treatment with radiotherapy”, “Treatment with both chemotherapy and radiotherapy”. The clinical status in 2007 and the updated one in 2011 are shown in Table 27. According to follow-up status we censored the analysis at 48 months and the overall free median follow-up rose to 29 months.

Table 27: Clinical status of EUELC patients before and after update of the database

Clinical status	Year: 2007	Year: 2011
Progressive Disease (%)	22.1%	26.4%
Disease Free (%)	77.9%	73.6 %
Disease Free median follow up (months)	18	29
Overall median follow up (months)	16.6	25
Died of other causes (%)	5.7%	21.7 %
Died of the disease (%)	14.7%	17.5 %

Prognostic significance of somatic mutations

Patients were grouped into two categories according to disease evolution status until September 2011. In the univariate analysis, those who developed a second primary lung cancer, a recurrence or metastasis, or who died of the disease were grouped as progressive disease (PD). PD represented 26.4% of EUELC patients. Patients who were alive and asymptomatic for the disease and who were not undergoing treatment by chemotherapy and/or radiotherapy, were classified as disease-free (DF) and represent 73.6% of our series.

We could identify that a more severe PLC tumour (i.e. tumour showing higher T and N scores) was associated to an increased risk of disease progression (respectively p=0.0014 and p=0.0006, Table 28). Surprisingly, smoking duration longer than 40 years was more frequent among DF patients than among PD patients (56.1% *versus* 43.3%).

Table 28: Clinical risk factors of disease progression

Variable (missing)	Items	DF (n=162)		PD (n=99)		P value
		n	%	n	%	
Gender (1)	Female	23	14,2	18	18,4	0.1285
Age	> 65	81	50	51	51,5	0.5066
Education duration (22) in years	> 16	54	35,8	37	41,6	0.7591
Professional exposure (6)	Asbestos	35	22	21	21,9	0.5629
Past pulmonary illness (6)	At least one	83	52,2	61	63,5	0.0991
Family history of HN/Lung cancer (10)	HN/Lung cancer	44	28	22	23,4	0.4807
Family history of cancer (10)	Any cancer	79	50,3	47	50	0.7761
Smoking status (1)	Current smoker	36	22,2	13	13,3	0.0634
Cigarette type (10)	Non Filtered	82	55,4	52	56,5	0.7210
Years since quit smoking	< 5	68	42	43	43,4	0.7059
Age at smoking initiation	< 16 years old	74	45,7	37	37,4	0.5271
Pack-years (8)	> 40	79	50,3	42	43,3	0.1196
Smoking duration in years (8)	**>40**	**88**	**56,1**	**42**	**43,3**	**0.0224**
PLC Histology	ADC	89	54,9	54	54,5	0.5583
	SCC	70	43,2	40	40,4	0.4535
Tumour score (1)	T2, T3, T4 *vs* T0, T1	99	61,1	82	83,7	**0.0014**
Nodal score (1)	N1, N2, Nx *vs* N0	33	20,4	44	44,9	**0.0006**
Tumour Stage (1+ 9 Nx*)	Stages II & III *vs* Stage I	34	21,4	42	45,2	**0.0038**

** 9 stages unknown because of undetermined pN*

TP53, *KRAS* or *EGFR* mutation status, however, were not associated with disease evolution. There were 45.4% patients with *TP53* mutation in the PD category versus 50.7% in the DF category (p=NS). No prognostic value was found when mutations were grouped into different categories according to their predicted effects on p53 protein structure or function (Table 29). G to T transversions were marginally more common among PD patients than DF but this effect was not statistically significant (adjusted HR: 1.46 [0.89 – 2.41], p=0.14). Likewise, p53 IHC positive status was not associated with prognosis (p=NS).

Table 29: **Associations between biomarkers and disease progression**

Variable	Items	DF		PD		HR (95% CI)	P*	Adj HR (95% CI)	P$
		n	%	n	%				
TP53 status (n=250)	Wild Type	70	49.3	59	54.6	1	0.45	1	0.64
	Mutated	72	50.7	49	45.4	0.86 (0.59 – 1.27)		0.91 (0.62 – 1.40)	
Conservation (n=246)	0 – Silent	74	51.7	60	58.3	1	0.28	1	0.56
	1 – Deleterious	44	30.8	34	33	0.74 (0.93 – 0.61)		1.01 (0.66 – 1.56)	
	4 – Non missense	25	17.5	9	8.7	0.56 (0.28 – 1.14)		0.69 (0.34 – 1.40)	
Transactivation (n=246)	0 – Silent mt / wt/ Functional / Partially functional	77	53.8	62	60.2	1	0.28	1	0.56
	1 – Non Functional	41	28.7	32	31.1	0.93 (0.60 – 1.44)		0.99 (0.64 – 1.54)	
	4 – Non missense	25	17.5	9	8.7	0.56 (0.28 – 1.14)		0.68 (0.33 – 1.39)	
Structure (n=243)	0 – Silent and WT	73	51.8	60	58.8	1	0.37	1	0.65
	1 – Missense in DNA-binding domain	23	16.3	21	20.6	1.01 (0.61 – 1.67)		1.05 (0.63 – 1.74)	
	2 – Missense outside DNA-binding domain	20	14.2	12	11.8	0.79 (0.42 – 1.49)		0.83 (0.44 – 1.56)	
	4 – Non missense	25	17.7	9	8.8	0.55 (0.27 – 1.12)		0.67 (0.33 – 1.36)	
Structure	0/4 – Silent and WT / Non missense	98	69.5	69	67.6	1	0.95	1	0.93
	1/2 – Missense	43	30.5	33	32.4	1.01 (0.67 – 1.54)		1.02 (0.67 – 1.55)	
Type (n=244)	0 – Others	124	88.6	84	80.8	1	0.19	1	**0.14**
	1 – All G > T	16	11.4	20	19.2	1.4 (0.9 – 2.3)		**1.46 (0.89 – 2.41)**	
Deleterious	0 – Wild Type and silent mutations	73	51.4	60	57.1	1	0.66	1	0.82
	1 – Deleterious	49	34.5	33	31.4	0.86 (0.56 – 1.32)		0.95 (0.61 – 1.47)	
	2 – Non deleterious	20	14.1	12	11.4	0.79 (0.42 – 1.48)		0.82 (0.43 – 1.54)	
KRAS status	Wild Type	118	84.3	85	78	1	0.26	1	0.46
	Mutated	22	15.7	24	22	1.30 (0.82 – 2.06)		1.19 (0.75 – 1.90)	

KRAS / *TP53*	Otherwise	138	97.9	102	93.6	1		1	
	Both Mutated	3	2.1	7	6.4	2.08 (0.95 – 4.57)	0.07	1.67 (0.74 – 3.77)	0.21
KRAS / *TP53*	Otherwise	52	36.4	40	37.7	1		1	
	At least one Mutated	91	63.6	66	62.3	0.92 (0.62 – 1.37)	0.70	0.97 (0.65 – 1.44)	0.87
PIN2	CC	13	9.2	9	8.4	0.98 (0.48 – 2.02)		0.97 (0.47 – 2.00)	
	CG	59	41.5	45	42.1	1.11 (0.74 – 1.67)	0.86	1.15 (0.76 – 1.74)	0.77
	GG	70	49.3	53	49.5	1		1	
PIN3	DD	5	3.5	7	6.5	1.46 (0.66 – 3.23)		1.39 (0.62 – 3.10)	
	ND	44	31	29	27.1	1.04 (0.67 – 1.62)	0.65	1.08 (0.68 – 1.70)	0.71
	NN	93	65.5	71	66.4	1		1	
PEX4	CC	9	6.3	5	4.7	0.79 (0.31 – 2.01)		0.83 (0.32 – 2.14)	
	CG	57	40.1	42	39.3	1.07 (0.71 – 1.60)	0.85	1.09 (0.72 – 1.65)	0.83
	GG	76	53.5	60	56.1	1		1	
P53 Haplotype	GNA-CDP	33	23.6	23	21.1	1.07 (0.64 – 1.76)		1.16 (0.69 – 1.96)	
	GNA-CNP	17	12.1	15	13.8	1.15 (0.63 – 2.07)	0.95	1.52 (0.63 – 2.11)	0.93
	OTHERS	65	46.4	49	45	1.15 (0.69 – 1.92)		1.11 (0.66 – 1.89)	
	GNA-GNA	25	17.9	22	20.2	1		1	
EGFR status (only ADC)	Wild Type	62	87.3	51	86.4	1		1	
	Mutated	9	12.7	8	13.6	1.31 (0.62 – 2.80)	0.48	0.97 (0.67 – 1.38)	0.68
p53 expression	0 , 1 , 2	96	53.6	67	52.8	1		1	
	3	83	46.4	60	47.2	1.00 (0.70 – 1.43)	1.00	0.98 (0.68 – 1.41)	0.91
p53 / TP53	Otherwise	88	67.2	71	71.7	1		1	
	p53 = 3 and TP53 = mutated	43	32.8	28	28.3	0.87 (0.56 – 1.35)	0.53	0.95 (0.60 – 1.49)	0.81

* F&G model with centre stratification
$ F&G model with centre stratification adjusted on T and N scores.

Since there were important disparities in patient recruitment between countries and centres, we repeated these analyses on the largest homogenous subgroup, that is on the 103 patients from the French centres (Nancy and Grenoble; Table 30).

Table 30: **Number of patients with available *TP53* mutation status, by country**

Country	N° patients	Percentage
France	**103**	**41.20**
Italy	47	18.80
UK	37	14.80
Germany	31	12.40
Spain	22	8.80
The Netherlands	10	4.00

Similarly, neither *KRAS* nor *TP53* mutations had prognostic value in this subgroup (Table 31, F&G model with centre stratification adjusted on T and N scores). However, patients with tumours that carried both *KRAS* and *TP53* mutations had a marginally significantly higher risk of developing a SPLC, lung cancer recurrence or metastasis (adjusted HR: 3.26 [1.07-9.90], p=0.038).

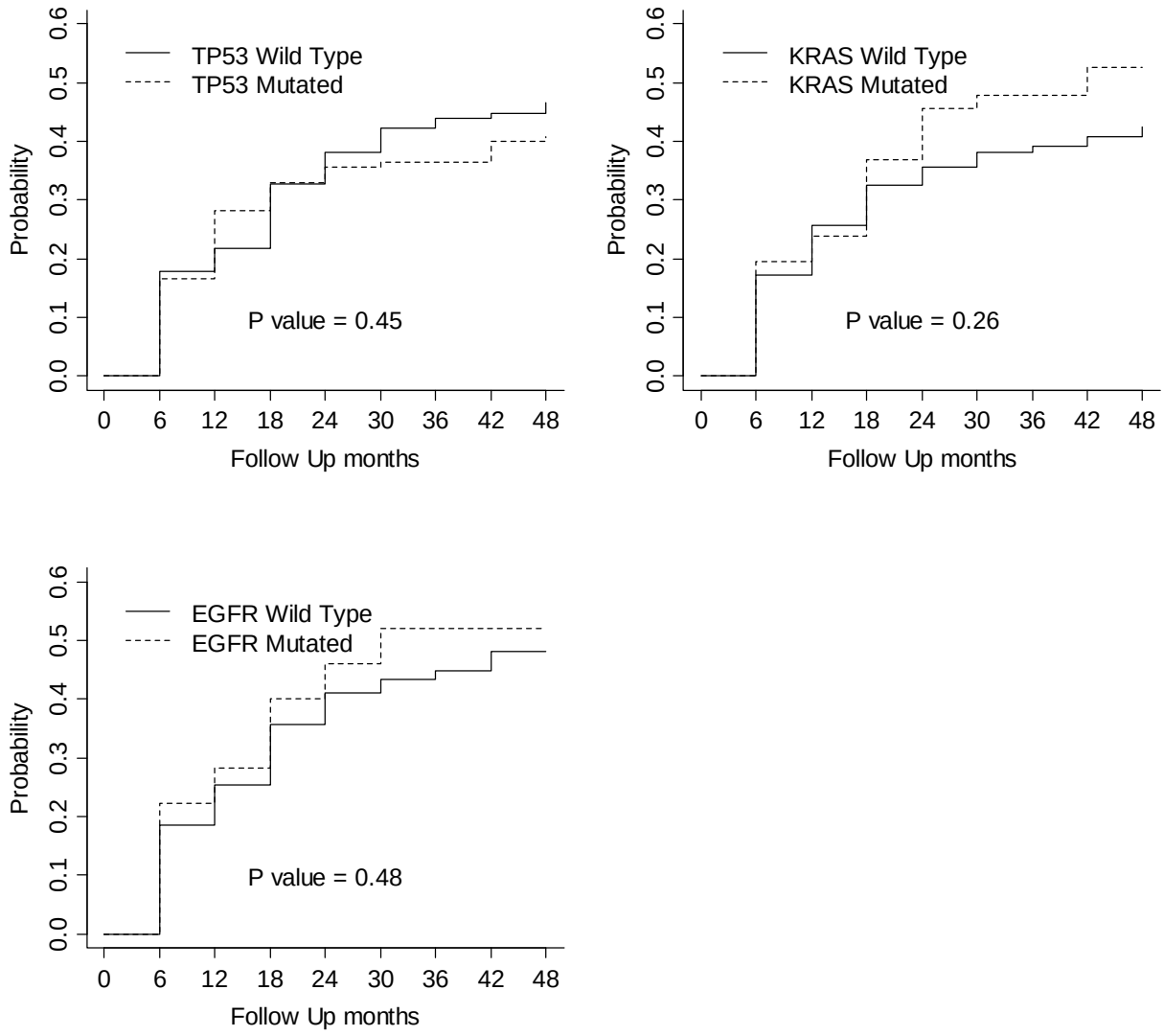
Table 31: **Association between biomarkers and disease progression in the French subgroup**

Variable	Items	DF		PD		Adj HR (95% CI)	P
		n	%	n	%		
TP53 status	Wild Type	22	38.6	22	47.8	1	0.61
	Mutated	35	61.4	24	52.2	0.86 (0.48 – 1.54)	
Conservation	0 – Silent	23	41.1	23	53.5	1	0.59
	1 – Deleterious	22	39.3	15	34.9	0.90 (0.47 – 1.74)	
	4 – Non missense	11	19.6	5	11.6	0.60 (0.22 – 1.61)	
Transactivation	0 – Silent mt / wt / Functional / Partially functional	24	42.9	24	55.8	1	0.58
	1 – Non Functional	21	37.5	14	32.6	0.87 (0.45 – 1.70)	
	4 – Non missense	11	19.6	5	11.6	0.59 (0.22 – 1.59)	
Structure	0 – Silent and WT	23	42.6	23	53.5	1	0.75
	1 – Missense in DNA-binding domain	9	16.7	8	18.6	1.07 (0.47 – 2.42)	
	2 – Missense outside DNA-binding domain	11	20.4	7	16.3	0.90 (0.38 – 2.10)	
	4 – Non missense	11	20.4	5	11.6	0.60 (0.22 – 1.61)	
Structure	0/4 – Silent and WT / Non missense	34	63	28	65.1	1	0.81
	1/2 – Missense	20	37	15	34.9	1.08 (0.57 – 2.06)	
Type	0 – Others	47	85.5	34	77.3	1	0.18
	1 – **All G > T**	8	14.5	10	22.7	**1.64 (0.80 – 3.40)**	
Deleterious	0 – Wild Type and silent mutations	23	41.8	23	50	1	0.94
	1 – Deleterious	21	38.2	16	34.8	0.91 (0.47 – 1.75)	

	2 – Non deleterious	11	20	7	15.2	0.88 (0.38 – 2.06)	
KRAS status	Wild Type	52	89.7	38	80.9	1	0.26
	Mutated	6	10.3	9	19.1	1.53 (0.73 – 3.22)	
KRAS* / *TP53	Otherwise	57	98.3	43	91.5	1	**0.038**
	Both Mutated	1	1.7	4	8.5	**3.26 (1.07 – 9.90)**	
KRAS / *TP53*	Otherwise	17	29.8	17	37	1	0.62
	At least one Mutated	40	70.2	29	63	0.86 (0.47 – 1.57)	
PIN2	CC	7	12.3	5	10.6	0.72 (0.26 – 2.04)	0.82
	CG	25	43.9	17	36.2	1.02 (0.54 – 1.91)	
	GG	25	43.9	25	53.2	1	
PIN3	DD/ND	36	63.2	34	72.3	0.85 (0.44 – 1.64)	0.62
	NN	21	36.8	13	27.7	1	
PEX4	CC	6	10.5	4	8.5	0.75 (0.25 – 2.22)	0.87
	CG	24	42.1	17	36.2	0.97 (0.52 – 1.80)	
	GG	27	47.4	26	55.3	1	
P53 Haplotype	GNA-CDP	13	22.8	8	17	1.03 (0.45 – 2.38)	0.82
	GNA-CNP	8	14	7	14.9	1.06 (0.45 – 2.52)	
	OTHERS	23	40.4	23	48.9	0.70 (0.32 – 1.56)	
	GNA-GNA	13	22.8	9	19.1	1	
EGFR status (only ADC)	Wild type	24	85.7	21	100	1	-
	Mutated	4	14.3	0	0	Not computable	
P53 Expression	0 , 1 , 2	37	56.9	29	59.2	1	1.00
	3	28	43.1	20	40.8	1.00 (0.56 – 1.80)	
P53 / TP53	Otherwise	35	62.5	31	68.9	1	0.76
	P53 = 3 and TP53 = mutated	21	37.5	14	31.1	0.91 (0.48 – 1.72)	

Cumulative incidence plots were performed (Figure 34, *P* values are from univariate Fine & Gray model) to illustrate the risk of disease progression through time according to the mutation status of the 3 genes, namely *TP53*, *KRAS* and *EGFR*. It appeared that the risk to develop a PD was higher (but not statistically significant) for patients with *KRAS* or *EGFR* mutation.

Figure 34: Cumulative incidence plots of the Progressive Disease risk for *TP53*, *KRAS* and *EGFR* mutation



Discussion

Many studies have investigated the prognostic value of *TP53* or *KRAS* mutations in lung cancer. There is evidence that both the pattern and frequency of mutations vary according to the risk factors. However, it remains unclear whether mutations are associated with increased risk of rapid disease progression and of unfavourable outcome. Here we have used the setup of a large European collaborative study, EUELC, to assess *TP53*, *KRAS* and *EGFR* mutations value as biomarkers of exposure to tobacco smoke and their prognostic value in a structured series of NSCLC cases. We have assessed *EGFR* mutations in a subgroup of 130 adenocarcinomas, as mutations in this gene have been reported to be rare in other histological types of lung cancers (Shigematsu et al. 2005, Yatabe and Mitsudomi 2007). We have also analysed the relationships between *TP53* mutations and several common *TP53* polymorphisms.

Our data show that *TP53, KRAS* and *EGFR* mutations allow discrimination between the two main lung cancer histological subtypes, since the mutations occurred at different rates among SCC and ADC cases. *TP53* mutations were detected in 57% of SCC, versus 39.7% of ADC. In contrast, *KRAS* mutations were detected in 89.1% of ADC and were rarer in SCC (10.9%). Of 110 ADC analysed, 13.1% were positive for *EGFR* mutation in contrast to none in SCC. Distribution of *TP53*, *KRAS* and *EGFR* mutations among NSCLC histologies were in agreement with previous studies in Caucasians.

Mutation patterns clearly reflected exposure to tobacco smoking, thus enabling their use as biomarkers of exposure to tobacco. The *TP53* mutation prevalence and pattern were in agreement with previous publications and with the IARC *TP53* Mutation Database. For both *TP53* and *KRAS* genes, the codon distribution showed a higher proportion of G to T transversions, in agreement with the well-documented prevalence of this mutation type in lung cancers of smokers. Moreover, as shown in other case series, *KRAS* and *TP53* mutations tended to be more common in lung cancers of ever-smokers than in former- or never-

smokers. *EGFR* mutations were significantly associated with never-smoking status but not restricted to NSCLC of never-smokers, since detected in about 10% of former (5/48) or current (8/72) smokers. One tumour was found to contain both *EGFR* and *KRAS* mutation, an extremely rare occurrence according to the literature. Interestingly, the *EGFR* mutation in this tumour was a silent one (codon 836 CGC>CGT Arg>Arg) and thus would not lead to tyrosine kinase activation.

We did not encounter major technical limitations. All the laboratory techniques used were previously well validated for high sensitivity and specificity and we showed high reproducibility for *KRAS* analyses with a collaborative centre. Moreover, the EUELC database provided us with good quality frozen tissues and warranties that data collection from life-style questionnaires was the most homogeneous among centres as possible.

In the present case series, mutation of none of the three genes analysed seems to carry a significant prognosis value in the cohort as a whole or in specific histological subgroups. Given the multi-centric character of the study, and the possibility of a bias due to recruitment centre, we performed a separate analysis on the largest and most homogeneous subgroup (French centres) that revealed a borderline effect in patients carrying both *TP53* and *KRAS* mutations (HR=3.26 [1.07-9.90], p=0.038) but not in patients carrying either of these mutations.

Similar to our results, a study on Japanese patients with surgically resected ADC did not identify any prognostic implication for *TP53* or *KRAS* mutations (Kosaka et al.2009). The authors detected a significant association between *EGFR* mutation and longer survival while none of the gene mutations appeared to be an independent prognosis marker. Of note, in this Japanese series 49% of the patients had *EGFR* mutations, a much higher rate than in the present Caucasian series (13.1%). It is well documented that mutations in *EGFR* are associated with never-smoking status, female gender and Asian ethnicity (Mounawar et al. 2007, Shigematsu et al. 2005,) then the relatively low prevalence of *EGFR* mutations in our series may reflect the characteristics of the patients recruited in EUELC, i.e. Caucasian, 84% males and 95.2% ever smokers. Other studies on Caucasian

populations reporting higher prevalence of *EGFR* mutations (Rosell et al. 2009) also show a more important proportion of never-smokers and/or of women in their series. The low number of non-smokers could have biased the mutation prevalence in our series as well as the association of mutations with smoking status.

Based on these results, the conservative conclusion is that mutation status does not predict short-term outcomes in completely resected lung cancers. However, given the overall poor prognosis of lung cancer over a period of 5 to 8 years, the short length of follow-up time was a main impairing factor to the full assessment of prognostic significance of the genes. It remains to be determined whether mutation status may be a prognosis factor for longer-term outcomes. The absence of short-term prognosis value does not preclude that mutations have significance as predictors of response to specific forms of therapies. Mutations in *EGFR*, for example, are predictors of response to Tyrosine Kinase Inhibitors (Paez et al. 2004). From a biological viewpoint, *TP53* and *KRAS* mutations may represent very early events in lung carcinogenesis, occurring before tumour onset as the result of genetic damage by tobacco carcinogens. Although these mutations do participate in launching bronchial cells on the path to transformation and progression, it is likely that the tumour behaviour may be dictated by specific, additional events that occur after their initiation. The fact that tumours carrying both *TP53* and *KRAS* mutations might have a worse prognosis can have two explanations. First, these patients may have particularly high exposure to tobacco carcinogens, or second, they are particularly susceptible to their mutagenic effects. These patients may thus have increased risk of acquiring additional mutations which, in turn, may be responsible of their poorer prognosis. Thus, presence of both *TP53* and *KRAS* mutations in the same lesion may identify a small group of tumours that are genetically unstable and prone to the accumulation of mutations accelerating disease progression and/or escape from therapy.

Molecular diagnosis of EUELC patients was based on bronchial biopsies which showed a high heterogeneity among the primary lung cancers. We could have taken advantage of this wide clinical population by analysing somatic mutation according to different stages of the tumours. Since the T and N scores of TNM tumours classification were associated with disease progression, it would be valuable to analyse the prognostic value of our biomarkers in the more common subpopulation of primary lung cancers (i.e. T1+T2, N0+N1). Moreover, SPLC should be distinguished from recurrence in the definition of disease progression since they show very different histopathological features and accordingly they may show very different mutator phenotypes and clinical outcome. In addition, *TP53* mutations are used for tumor response to cysplatin-based therapy, tumors carrying *EGFR* mutations may be sensitive to lung cancer therapy with *EGFR* inhibitors and our results may suggest that tumors carrying both *KRAS* and *TP53* mutations might respond differently to therapeutic intervention. Since we have knowledge that in EUELC the progressive-diseases were treated by adjuvant therapy (both radiotherapy and chemotherapy), it would be extremely valuable to analyse the status of our biomarkers according to the different chemotherapeutic intervention that patients undergone, thus effectively translating our findings.
Other candidate markers may involve genes with activating mutations, making it possible to treat these cancers using selective pharmacological inhibitors (Sharma et al. 2010), and epigenetic changes in DNA methylation patterns and in microRNA expression which may distinguish different NSCLC subgroups (Voortman et al. 2010).

We have additionally analysed *TP53* polymorphisms and showed their potential to modulate lung cancer pathways. Our data show that *TP53* mutations tend to occur at different rates on different *TP53* alleles. Although the group of patients was small, patients with two C alleles of PEX4 (encoding Proline instead of Arginine at codon 72) tended to have more frequently a mutation in *TP53* than patients with at least one G allele. Thus, the C allele of *TP53* may be intrinsically more “mutable” than the G allele, perhaps as a result of subtle differences in the

functional properties of p53 proteins with either Arginine or Proline at position 72. Experimental studies have identified such functional differences, including a greater ability to induce apopotosis for 72P than for 72A (Dumont et al. 2003). This observation is in agreement with results from Mechanic et al. (Mechanic et al. 2007) who found that common genetic variation in *TP53* could modulate lung cancer pathways, as suggested by the association of *TP53* codon 72 polymorphism with lung cancer in African Americans and with somatic *TP53* mutation frequency in lung tumours. Thus, in future studies, it will be important to take into account both *TP53* mutation and *TP53* haplotypes in assessing the prognosis and predictive significance of *TP53* gene status in lung cancer.

Chapter V: General Discussion and Future Perspectives

In this project two approaches have been investigated to advance the use of biomarkers of exposure and intermediate effect in molecular epidemiology.
The potential advantages of using biomarkers in molecular epidemiological studies are commonly reported as the possibility to improve the accuracy of exposure measurement, to identify intermediate health effects and to identify subpopulations with increased susceptibility to develop health effects in the presence of a carcinogen (Gallo et al. 2011). Examples of the potential opportunities for exposure and risk assessment of incorporating biomarkers in cancer epidemiological studies are the use of patterns of *TP53* mutations to distinguish tobacco-related lung cancer from non tobacco-related lung cancer (Hainaut and Pfeifer 2001), of *EGFR* and *KRAS* status to distinguish those individuals who will clinically respond positively to therapy (Marks et al. 2008, Van Cutsem et al. 2009) and more recently, of DNA methylation to trace exposure to tobacco and diet (Vaissière et al. 2009, Vineis et al 2011).

The actual value of a biomarker largely depends on its validity. While the list of biomarkers is growing fast, the validation of new biomarkers is lagging behind. In this thesis we have used well validated techniques of laboratory analysis, such as dHPLC and pyrosequencing, for all our experiments.

The first part of the project is an intervention study *in vivo* to investigate the usefulness of epigenetic patterns as biomarkers of modifying effects of diet on tobacco-related methylation changes. This approach encompasses two successive hypotheses as to the amplitude of the changes that may occur in actual biomarker levels. First, we put forward the hypothesis that methylation levels in DNA from blood lymphocytes are significantly altered by smoking. Second, we formulate the subsequent hypothesis that specific dietary

intervention may at least partially reverse these methylation changes and restore methylation patterns similar (or close) to those detectable in never-smokers. Thus, one of the intrinsic difficulties of this study was that, within the proposed design, we were not in a position to assess each of these two hypotheses separately. We lacked a control group of never-smokers to establish whether methylation levels were different (and to which extent) from those of our smoking population. Therefore, we could only speculate about the amplitude of the expected effect of dietary intervention. These difficulties are compounded by the limited knowledge of the dynamics of methylation patterns in DNA from peripheral blood lymphocytes.

Despite these limitations, our study has identified several features that may pave the way to future studies using methylation patterns as biomarkers for monitoring the effects of dietary intervention. In particular, one of the most striking results is that, in subjects receiving polyphenols supplementation, overall methylation levels as measured through LINE-1 methylation patterns showed an important reduction in their variations from one participant to the other, although the actual difference in average levels themselves was only very minor. This type of biological modification makes sense: providing participants with a homogeneous, calibrated diet, we may have reduced the impact of normal variability in the distribution of the biomarker. It follows that the intervention has somehow stabilized individual methylation patterns. This, in turn, is an interesting observation with respect to the concept of “epigenetic stability”. Indeed, it could be proposed that the main beneficial effect of a calibrated dietary intervention may stand not in increasing or decreasing methylation patterns (depending upon the particular gene under study) but in stabilizing the baseline pattern. Stabilizing baseline methylation patterns may indeed make them less prone to variations and consequently more resistant to modifications by reactive substances such as oxyradicals. In some aspects, this concept of “biomarker stabilization” is related to the concept of “biodiversity” used in ecological studies, with, however, a major difference. In the ecological conceptual model it is assumed that the larger the diversity, the better the impact at population level. A large dispersion and

diversity in the biomarker distribution is supposed to help the population to cope with a wide range of environmental changes. In contrast, for “epigenetic biomarker stabilization”, the beneficial impact at individual level would stem from a narrower range of variations, due to re-enforcements of the mechanisms that control and “repair” the biomarker status. It would be of great interest to take this concept into further biomarker studies and examine its usefulness, in particular in monitoring preventive interventions.

The second part of the project is a prospective study designed to assess whether somatic mutations can act as biomarkers of exposure to tobacco smoke and of risk of lung cancer recurrence. Our approach is based on a standard model of lung carcinogenesis, which implies the accumulation of mutations in key genes whose patterns reflect exposure to tobacco smoke. While this model is well established in both experimental and animal studies, for obvious reasons it has not been as such demonstrated in smokers. No one would accept taking biopsies in pre-cancerous and cancerous tissues of patients and keep them under observation to quantify the accumulation of molecular changes. Thus, our interpretation of the significance of the biomarkers is constrained by the limitations of this concept. For example, there is excellent observational and experimental evidence that PAHs from tobacco smoke can induce G to T transversions in *TP53* gene. Yet in our analysis, we did not detect a significant association between tobacco smoking status and presence of G to T transversion. The lack of this expected association can be, in part, due to insufficient precision in estimating tobacco consumption through questionnaires. It may also be the consequence of the fact that tobacco contains numerous carcinogens other than PAH capable of inducing a wide pattern of mutations. Thus, measuring tobacco consumption is by no means identical to measuring levels of PAH that may form specific DNA-adducts in target tissues. Moreover, the individual susceptibility to DNA damage induced by tobacco carcinogens is under strong control by genetic and epigenetic factors, which as such were not measured in our model.

Aside from possible associations with exposure to tobacco smoke, mutations in *TP53* or *KRAS* were strongly expected to reveal information on tumour prognosis. We formulated indeed the hypothesis that tumours with a defined, measurable detrimental mutation may have a worse biological behaviour than tumours without that specific mutation. However, this view overlooks the fact that tumours lacking a defined mutation may carry even worse genetic (or epigenetic) alterations in another, not measured, biomarker. In many instances, the biomarker may be a factor belonging to the same pathway (e.g. a factor regulating p53 expression, stability or activity), thus having the same overall effect as the measured mutation. In such circumstances, it would indeed be impossible to identify different prognostic effects by studying a specific mutation. Rather than assessing a particular form of damage in a gene, it might have been necessary to assess the overall function of the gene product.

In line with the notions above, it is quite remarkable that studies on lung cancer as well as on other cancers where *TP53* mutations are frequent have failed to assign a clear prognostic value to the mutations. We suggest that, in a manner comparable to the effects of a calibrated diet as discussed above, tobacco may also operate as a factor that "homogenizes" genetic and epigenetic patterns. Rather than generating a large diversity of changes (which would be expected to lead to widely different clinical behaviour of cancers), tobacco may act by targeting specific pathways with a high load of mutagens, thus breaking these pathways at different points with functionally equivalent effects. Interestingly, a strong prognostic effect has been assigned to *TP53* mutations in breast cancer. In this latter cancer type, mutations are detected in only about 20-25% of the cases and there is strong molecular evidence that breast cancer occurs in distinct types and subtypes. Those cancers with *TP53* mutation might well then develop according to mechanisms very different than those without mutations.

In conclusion, our work further highlights the difficulties of implementing biomarkers in epidemiological cancer studies. In addition to the technical, logistical and statistical considerations, the main constraints are the study design

and the conceptual model on which the hypotheses on the significance of the biomarker are generated. Our work shows that it is very difficult to master these two aspects even in the context of studies of limited size and with biomarkers of limited complexity. These problems are compounded to a large scale when using “omics” approaches, in which the multiplicity and complexity of data points representing biomarkers may be even more difficult to harness within a single study design and conceptual model.

References

1. Abu-Asab M, Chaouchi M, Alesci S, et al. Biomarker in the age of omics: time for a system biology approach. Omics 2011; 15(3): 105-12.
2. Anna L, Holmila R, Kovács K, Gyorffy E, Gyori Z, Segesdi J, Minárovits J, Soltész I, Kostic S, Csekeo A, Husgafvel-Pursiainen K, Schoket B. Relationship between TP53 tumour suppressor gene mutations and smoking-related bulky DNA adducts in a lung cancer study population from Hungary. Mutagenesis 2009; 24(6):475-80.
3. Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990; 62:503-14.
4. Ahrendt SA, Halachmi S, Chow JT, Wu L, Halachmi N, Yang SC, et al. Rapid p53 sequence analysis in primary lung cancer using an oligonucleotide probe array. Proc Natl Acad Sci U S A 1999; 96:7382–7.
5. Aquilina NJ, Delgado-Saborit JM, Meddings C, et al. Environmental and biological monitoring of exposures to PAHs and ETS in the general population. Environ Int 2010; 36(7): 763-71.
6. Armstrong B, Doll R. Environmental factors, cancer incidence and mortality in different countries with special reference to dietary practices. Int J Cancer 1975; 15(4): 617–31.
7. Balassiano K, Lima S, Jenab M, et al. Aberrant DNA methylation of cancer-associated genes in gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST). Cancer Lett 2011; 311(1):85-95.
8. Baccarelli A, Wright RO, Bollati V et al. Rapid DNA methylation changes after exposure to traffic particles. AJRCCM 2009; 179:572-78.
9. Banerjee HN, Verma M. Epigenetic mechanisms in cancer. Biomark Med 2009; 3(4):397-410.
10. Beasley MB, Brambilla E, Travis WD. The 2004 World Health Organization classification of lung tumours. Semin Roentgenol 2005; 40(2): 90–7.
11. Beerenwinkel N, Antal T, Dingli D, et al. Genetic progression and the waiting time to cancer. PLoS Comput Biol 2007; 3 (11): 225.
12. Bergamaschi D, Gasco M, Hiller L, Sullivan A, Syed N, Trigiante G, et al. p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell 2003; 3:387-402.
13. Bjelke E. Dietary vitamin A and human lung cancer. Int J Cancer 1975; 15(4): 561–65.
14. Biomarkers Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001; 69(3):89-95.

15. Bingham S, Riboli E. Diet and cancer—the European Prospective Investigation into Cancer and Nutrition. Nat Rev Cancer 2004; 4(3):206-15.
16. Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer 1992; 18(1): 1–29.
17. Boccia S, Boffetta P, Brennan P, Ricciardi G, Gianfagna F, Matsuo K, van Duijn CM, Hung RJ. Meta-analyses of the methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and risk of head and neck and lung cancer. Cancer Lett 2009; 273(1):55-61.
18. Boldrini L, Gisfredi S, Ursino S, et al. Effect of the p53 codon 72 and intron 3 polymorphisms on non-small cell lung cancer (NSCLC) prognosis. Cancer Invest 2008; 26(2): 168-72.
19. Borresen AL, Andersen TI, Eyfjord JE, Cornelis RS, Thorlacius S, Borg A, et al. TP53 mutations and breast cancer prognosis: particularly poor survival rates for cases with mutations in the zinc-binding domains. Genes Chromosomes Cancer 1995; 14:71-75.
20. Brey RL, Cote SA, McGlasson DL, et al. Effects of repeated freeze-thaw cycles on anticardiolipin antibody immunoreactivity. Am J Clin Pathol 1994; 102:586.
21. Brennan P, Hainaut P, Boffetta P. Genetics of lung-cancer susceptibility. Lancet Oncology 2011; 12 (4): 399-408.
22. Breton HM, Byun M, Wenten F, et al. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. Am J Respir Crit Care Med 2009; 180:462-7.
23. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glöckner S, Piantadosi S, Gabrielson E, Pridham G, Pelosky K, Belinsky SA, Yang SC, Baylin SB, Herman JG. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med 2008; 358(11):1118-28.
24. Caboux E, Hainaut P, Gormally E. Biological Resource Centers in Molecular Epidemiology Studies: Collecting, Storing and Analyzing Biospecimens. Molecular Epidemiology of Chronic Diseases. Vineis P, Garte S, Wild C. 2008; 267-78.
25. Cassidy A, Balsan J, Vesin A, et al. Cancer diagnosis in first-degree relatives and non-small cell lung cancer risk: Results from a multi-centre case-control study in Europe. Eur J Cancer 2009; 45(17): 3047-53.
26. Chan DW, Semmes OJ, Petricoin EF, et al. National Academy of Clinical Biochemistry Guidelines: The Use of MALDI-TOF Mass Spectrometry Profiling to Diagnose Cancer. American Association for Clinical Chemistry 2006.
27. Cho Y, Gorina S, Jeffrey PD, et al. Crystal structure of a p53 tumour suppressor-DNA complex: understanding tumourigenic mutations. Science 1994; 265: 346-55.

28. Choi JY, James SR, Link PA, et al. Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis 2009; 30(11):1889-97.
29. Christensen BC, Kelsey KT, Zheng S, et al. Breast cancer DNA methylation profiles are associated with tumour size and alcohol folate intake. PLoS Genet 2010; 6(7): e1001043.
30. COSMIC Database. www.sanger.ac.uk/genetics/CGP/cosmic/. Last Modified Mar 23 2011.
31. Denissenko MF, Pao A, Tang MS, et al. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspot in P53. Science 1996; 274: 430–32.
32. Diamandis EP, Fritche H, Lilja H, et al. Tumour Markers: Physiology, Pathobiology, Technology, and Clinical Applications. Washington, DC: AACC Press 2002.
33. Diamandis EP, Schmitt M, van der Merwe D. National Academy of Clinical Biochemistry Guidelines: The Use of Microarrays in Cancer Diagnostics. American Association for Clinical Chemistry 2006.
34. Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. Bmj 2004; 328:1519.
35. Downs JA. Chromatin structure and DNA double-strand break responses in cancer progression and therapy. Oncogene 2007; 26: 7765–72.
36. Dumont P, Leu JI, Della Pietra AC, et al. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 2003; 33(3): 357-65.
37. ECNIS 2006. Biomarkers of carcinogen exposure and early effects. Nofer Institute of Occupational Medicine. Farmer PB, Emeny JM, editors.
38. ECNIS 2007. Epidemiological concepts of validation of biomarkers for the identification/quantification of environmental carcinogenic exposures. Vineis P, Gallo V. editors.
39. Esteller M., Toyota M., Sanchez-Cespedes M., et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumourigenesis. Cancer Res 2000; 60: 2368–2371. PMID: 10811111.
40. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev 2007; 8:286-98.
41. Esteban M; Castano A. Non-invasive matrices in human biomonitoring: A review. Environ Int 2009; 35: 438-49.
42. Epigenetics and human health. Linking hereditary, environmental and nutritional aspects. Edited by Haslberger AG and Gressler S. 2009.

43. Fang MZ, Wang Y, Ai N, et al. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003; 63, 7563–70.
44. Field JK, Liloglou T, Niaz A, et al. EUELC project: a multi-centre, multipurpose study to investigate early stage NSCLC, and to establish a biobank for ongoing collaboration. ERJ 2009; 34(6): 1477-86.
45. Figueiredo JC, GrauMV, Wallace K, et al. Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. CEBP 2009; 18: 1041-49.
46. Formenton-Catai AP, Pereira R, Lanças FM, et al. Solid-Phase Purification of Deoxyguanosine-benzo[a]pyrene Diol Epoxide Adducts from Genomic DNA Adduct Synthesis. J Braz Chem Soc 2005; 16(4): 808-14.
47. Fraga MF, Ballestar E, Paz MF, et al. Epigenetic differences arise during the lifetime of monozygotic twins. Proc. Natl. Acad. Sci. USA 2005; 102:10604-10609. PMID: 16009939.
48. Gallo V, Egger M, McCormack V, et al. STrengthening the Reporting of OBservational studies in Epidemiology - Molecular Epidemiology (STROBE-ME): An extension of the STROBE statement. Mutagenesis. 2011.
49. Gemignani F, Moreno V, Landi S. et al. A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of TP53 mRNA. Oncogene 2004; 23(10): 1954-6.
50. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide. Ferlay J, Shin HR, Bray F, et al. Available from: http://globocan.iarc.fr
51. Gormally E, Vineis P, Matullo G, et al. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. Cancer Res. 2006; 66(13):6871-6.
52. Greenblatt MS, Bennett WP, Hollstein M, et al. Mutations in the p53 tumour suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 1994; 54: 4855–78.
53. Guengerich FP. Cytochrome P450 oxidations in the generation of reactive electrophiles: epoxidation and related reactions.Arch Biochem Biophys 2003; 409:59-71.
54. Guarrera S, Sacerdote C, Fiorni L, et al. Expression of DNA repair and metabolic genes in response to a flavonoid-rich diet. Br J Nutr 2007; 98:525-33.
55. Han JY, Lee GK, Jang DH, et al. Association of p53 Codon 72 Polymorphism and MDM2 SNP309 With Clinical Outcome of Advanced Nonsmall Cell Lung Cancer. Cancer 2008; 113(4): 799-807.

56. Hancox RJ, Poulton R, Welch D, et al. Accelerated decline in lung function in cigarette smokers is associated with TP53/MDM2 polymorphisms. Hum Genet 2009; 126: 559–565.
57. Hainaut P, Pfeifer GP. Patterns of p53 G–>T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. Carcinogenesis 2001; 22:367-77.
58. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57-70.
59. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646-74.
60. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumour markers in breast cancer. J Clin Oncol 2007 20; 25(33):5287-312.
61. Hayes JD, Kelleher MO, Eggleston IM. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. Eur J Nutr 2008; 47 Suppl 2: 73-88.
62. Hecht SS. Inhibition of carcinogenesis by isothiocyanates. Drug Metab Rev 2000; 32:395–411.
63. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003; 3:733.
64. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci USA 2008; 105: 17046-49.
65. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 2007; 22:91–103.
66. Hillman RS and Steinberg SE. The effects of alcohol on folate metabolism. Annu Rev Med 1982; 33:345-54.
67. Hoffmann D, Djordjevic MV, Hoffmann I. The changing cigarette. Prev Med 1997; 26(4): 427-34.
68. Hu Z, Li X, Qu X, et al. Intron 3 16 bp duplication polymorphism of TP53 contributes to cancer susceptibility: a meta-analysis. Carcinogenesis 2010; 31(4): 643-7.
69. Hussain SP, Amstad P, Raja K, et al. Mutability of p53 hotspot codons to benzo(a)pyrene diol epoxide (BPDE) and the frequency of p53 mutations in nontumourous human lung. Cancer Res 2001; 61: 6350–5.
70. IARC 1986. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Tobacco Smoking Vol. 38:1–421.
71. IARC 1999. Dos Santos Silva I. Cancer epidemiology: principles and methods.
72. IARC 2002. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Aflatoxins Vol 82: 39-267.

73. IARC 2004a. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Tobacco smoke and involuntary smoking. Vol. 83: 1-1438.
74. IARC 2004b. Handbooks of Cancer Prevention Volume 9: Cruciferous vegetables, isothiocyanates and indoles.
75. IARC 2004c.Cancer Pathology and Genetics. Tumours of the Lung, Pleura, Thymus and Heart. International Agency for Research on Cancer.
76. IARC 2004d. Olivier M, Hussain SP, Caron de Fromentel C, et al. TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer. 247-70.
77. IARC 2004e. Hagmar L, Stromberg U, Tinnerberg H, et al. Epidemiological evaluation of cytogenetic biomarkers as potential surrogate endpoints for cancer.:207–15.
78. IARC 2006. Report of the Advisory Group to Review the Amended Preamble to the IARC Monographs.
79. IARC 2007.Cancer Incidence in Five Continents, Vol. 9; 160.
80. IARC 2010. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Volume 100F and 100E. In press.
81. Issaq HJ, Waybright TJ and Veenstra TD. Cancer biomarker discovery; opportunities and pitfalls in analytical methods. Electrophoresis 2011; 32(9):967-75.
82. Jarabek AM, Pottenger LH, Andrews LS, et al. Creating context for the use of DNA adduct data in cancer risk assessment: I. Data organization. Crit Rev Toxicol 2009; 39: 659-78.
83. Jemal A, Siegel R, Ward E, et al. Cancer Statistics 2010; 60:277-300.
84. Jenab M, Slimani N, Bictash M., et al. Biomarkers in nutritional epidemiology: applications, needs and new horizons. Hum Genet 2009; 125:507-25.
85. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3:415-28.
86. Keller G, Hartmann A, Mueller J, et al. Denaturing high pressure liquid chromatography (DHPLC) for the analysis of somatic p53 mutations. Lab Invest 2001; 81(12):1735-7.
87. Key TJ. Fruit and vegetables and cancer risk. BJC 2011; 104: 6-11.
88. Khuder SA. Effect of cigarette smoking on major histological types of lung cancer: a meta-analysis. Lung Cancer 2001; 31:139–48.
89. Kosaka T, Yatabe Y, Onozato R, et al. Prognostic implication of EGFR, KRAS, and TP53 gene mutations in a large cohort of Japanese patients with surgically treated lung adenocarcinoma. J Thorac Oncol 2009; 4(1):22-9.
90. Kumar A, Vineis P, Sacerdote C, et al. Determination of new biomarkers to monitor the dietary consumption of isothiocyanates. Biomarkers 2010; 15:739-45.

91. Kulasingam V and Diamandis P. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nat Clin Pract Oncol 2008; 5 (10): 588-99.
92. Lahiri DK and Schnabel B. DNA isolation by a rapid method from human blood samples: effects of MgCl2, EDTA, storage time, and temperature on DNA yield and quality. Biochem Genet 1993; 31:321-8.
93. Lam TK, Gallicchio L, Lindsley K et al. Cruciferous vegetable consumption and lung cancer risk: a systematic review. Cancer Epidemiol Biomarkers Prev 2009; 18:184-95.
94. Lamprecht SA, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nat Rev Cancer 2003; 3(8):601-14.
95. Le Calvez F, Mukeria A, Hunt J, et al. TP53 and KRAS Mutation Load and Types in Lung Cancers in Relation to Tobacco Smoke: Distinct Patterns in Never, Former, and Current Smokers. Cancer Research 2005; 65:5076-83.
96. Lee WJ, Shim JY and Zhu BT. Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. Mol Pharmacol 2005; 68:1018–30.
97. Li Y and Tleefsbol T. Impact on DNA metylation in cancer prevention and therapy by bioactive components. Curr Med Chem 2010; 17(20): 2141-51.
98. Linseisen J, Rohrmann S, Miller AB, et al. Fruit and vegetable consumption and lung cancer risk: updated information from the European Prospective Investigation into Cancer and Nutrition (EPIC). International Journal of Cancer 2007; 121:1103–14.
99. Liu L, Wu C, Wang Y, et al. Combined Effect of Genetic Polymorphisms in P53, P73, and MDM2 on Non-small Cell Lung Cancer Survival. J Thorac Oncol. 2011 Aug 11.
100. Loeb LA, Bielas JH, Beckman RA. Cancers exhibit a mutator phenotype: clinical implications. Cancer Res 2008; 68: 3551–57.
101. Lopez AD, Collishaw NE, and Piha T. A descriptive model of the cigarette epidemic in developed countries. Tobacco Control 1994; 3: 242-247.
102. Ludwig J and Weinstein J. Biomarkers in Cancer Staging, Prognosis and Treatment Selection. Nature Reviews Cancer 2005; 5: 845-56.
103. Luo K, Liu Z, Karayiannis P. Effect of antiviral treatment on alfa-fetoprotein levels in HBV-related cirrhotic patients: early detection of hepatocellular carcinoma. J Viral Hepat 2010; 17(7).
104. MacMahon B, Trichopoulos D, Brown J, et al. Age at menarche, urine estrogens and breast cancer risk. Int J Cancer 1982; 30(4):427-3.
105. Malaveille C, Fiorini L, Bianchini M, et al. Randomized controlled trial of dietary intervention: association between level of urinary phenolics and anti-mutagenicity. Mutat Res 2004; 561:83-90.

106. Manach C, Scalbert A, Morand C, et al. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004; 79: 727-47.
107. Marcel V, Palmero EI, Falagan-Lotsch P, et al. TP53 PIN3 and MDM2 SNP309 polymorphisms as genetic modifiers in the Li-Fraumeni syndrome: impact on age at first diagnosis. J Med Genet 2009; 46(11):766-72.
108. Martinez J, Georgoff I, Martinez J, Levine AJ. Cellular localization and cell cycle regulation by a temperature sensitive p53 protein. Genes Dev 1991, 5:151-159.
109. Marks JL, Broderick S, Zhou Q, et al. Prognostic and therapeutic implications of EGFR and KRAS mutations in resected lung adenocarcinoma. J Thorac Oncol 2008; 3(2): 111-6.
110. McGuire WL, Horwitz KB, Pearson OH, et al. Current status of estrogen and progesterone receptors in breast cancer. A review. Cancer 1977; 39(6 Suppl):2934-47.
111. Mechanic LE, Bowman ED, Welsh JA, et al. Common genetic variation in TP53 is associated with lung cancer risk and prognosis in African Americans and somatic mutations in lung tumours. Cancer Epidemiol Biomarkers Prev 2007; 16(2):214-22.
112. Medical Research Council. Tobacco smoking and cancer of the lung. British Medical Journal 1957; 1: 1523–24.
113. Melhem MF, Law JC, el-Ashmawy L, Johnson JT, Landreneau RJ, Srivastava S, Whiteside TL. Assessment of sensitivity and specificity of immunohistochemical staining of p53 in lung and head and neck cancers. Am J Pathol. 1995; 146(5):1170-7.
114. Moore HM, Kelly AB, Jewell SD, et al. Biospecimen reporting for improved study quality (BRISQ). J Proteome Res 2011; 10(8):3429-38.
115. Mounawar M, Mukeria A, Le Calvez F, et al. Patterns of EGFR, HER2, TP53, and KRAS Mutations of p14arf Expression in Non–Small Cell Lung Cancers in Relation to Smoking History. Cancer Res 2007; 67(12): 5667-72.
116. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. Carcinogenesis 2006; 27:811–9.
117. Nature Valid concerns. Editorial 2010; 463: 401-402.
118. Ozanne SE and Constancia M. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. Nat Clin Pract Endocrinol Metab 2007; 3(7): 539-46.
119. Paez G, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical responde to gefitinib therapy. Science 2004; 304: 1497-1500.
120. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from ‘‘never-smokers’’ and are associated with sensitivity of tumours to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004; 101:13306-11.

121. Perera FP and Weinstein IB. Molecular epidemiology: recent advances and future directions. Carcinogenesis 2000; 21: 517-24.

122. Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumour phenotype: lessons from recent developments in the IARC TP53 database. Human Mutation 2007; 28(6):622-9. Latest database version (R14, November 2009). http://www-p53.iarc.fr/index.html

123. Peto J. Cancer epidemiology in the last century and the next decade. Nature. 2001; 411:390-5.

124. Pfeifer GP and Denissenko MF. Formation and repair of DNA lesions in the p53 gene: Relation to cancer mutation. Environ Mol Mutag 1998; 31: 197–205.

125. Phillips JM and Goodman JI. Inhalation of cigarette smoke induces regions of altered DNA methylation (RAMs) in SENCAR mouse lung. Toxicology 2009; 260:7–15.

126. Ramos S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Mol Nutr Food Res 2008; 52:507-26.

127. Rhodes M, Price K. Identification and analysis of plant phenolic antioxidants. Eur J Cancer Prev 1997; 6:518-21.

128. Riboli E, Hunt KJ, Slimani N, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002; 5: 1113 – 24.

129. Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009; 361(10):958-67.

130. Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, et al. New dye-labeled terminators for improved DNA sequencing patterns. Nucleic Acids Res 1997; 25:4500–4. 17.

131. Rothman N, Stewart WF, Schulte P. Incorporating biomarkers into cancer epidemiology: a matrix of biomarker and study design categories. CEBP 1995, 4:301-11.

132. Rundle A, Vineis P, Ahsan H. Design options for molecular epidemiology research within cohort studies. CEBP 2005; 14(8):1899-907.

133. Samowitz WS, Curtin K, Ma KN, Edwards S, Schaffer D, Leppert MF, et al. Prognostic significance of p53 mutations in colon cancer at the population level. Int J Cancer 2002; 99:597-602.

134. Sawan C, Vaissière T, Murr R, et al. Epigenetic drivers and genetic passengers on the road to cancer. Mutat Res 2008; 642(1-2):1-13.

135. Schatzkin A, Freedman LS, Schiffman MH, et al. Validation of intermediate end points in cancer research. J Natl Cancer Inst 1990; 82 (22): 1746-52.

136. Schneider PM, Stoeltzing O, Roth JA, Hoelscher AH, Wegerer S, Mizumoto S, et al. P53 mutational status improves estimation of prognosis in patients with curatively resected adenocarcinoma in Barrett's esophagus. Clin Cancer Res 2000; 6: 3153-315.
137. Schulte PA, Perera FP. Molecular Epidemiology: Principles and Practices. San Diego: Academic Press; 1993.
138. Schwartz AG, Prysak GM, Bock CH, et al. The molecular epidemiology of lung cancer. Carcinogenesis 2007; 28:507–18.
139. Seow A, Vainio H, Yu MC. Effect of glutathione-S-transferace M1 polymorphisms on the cancer preventive potential of isothiocyanates: An epidemiological perspective. Mutat Res 2005; 592:1283–91.
140. Sharma SV, Haber DA, Settleman J. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nat Rev Cancer 2010; 10(4):241-53.
141. Shen JC, Rideout WM the 3rd, Jones PA. High frequency mutagenesis by a DNA methyltransferase. Cell 1992; 71:1073-80.
142. Shen G, Jeong W, Hu R, et al. Regulation of Nrf2, NfkB and AP-1 signaling pathways by chemopreventive agents. ARS 2005; 7 (11-12):1648-63.
143. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. JNCI 2005; 97: 339–46.
144. Sidransky D. Emerging molecular markers of cancer. Nat Rev Cancer 2002; 2:210-219.
145. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. Cancer Causes Control 1991; 2(6): 427–42.
146. Stidley CA, Picchi MA, Leng S, et al. Multivitamins, folate and green vegetables protect against gene promoter methylation in the aerodigestive tract of smokers. Cancer Res 2010; 70:568-74.
147. Talaska G, Al-Zoughool M, Malaveille C, et al. Randomized controlled trial: effects of diet on DNA damage in heavy smokers. Mutagenesis 2006; 21:179-83.
148. Thunissen FB, Prinsen C, Hol B, et al.Smoking history and lung carcinoma: KRAS mutation is an early hit in lung adenocarcinoma development. Lung Cancer 2011; in press.
149. Terry MB, Delgado-Cruzata L, Vin-Raviv N, et al. DNA methylation in white blood cells: Association with risk factors in epidemiologic studies. Epigenetics 2011; 6:828-37.
150. Tokumo M, Toyooka S, Kiura K, et al. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. Clin Cancer Res 2005; 11:1167-73.

151. Tost J, Dunker J, Gut IG. Analysis and quantification of multiple methylation variable positions in CpG islands by pyrosequencing. Biotechniques 2003; 35:152–6.

152. Tsubono Y, Nishino Y, Komatsu S, et al. Green tea and the risk of gastric cancer in Japan. N Engl J Med 2001; 344: 632-36.

153. Vaissière T, Hung RJ, Zaridze D, et al. Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. Cancer Res 2009; 69(1):243-52.

154. Van Cutsem E, Kohne CH, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009; 360: 1408-17.

155. Van den Berg L, Rose D. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: the reciprocal system KH2PO4-Na2HPO4-H2O. Arch Biochem Biophys 1959; 81:319- 29.

156. Vineis P, Malats N, Porta M, et al. Human cancer, carcinogenic exposures and mutation spectra. Mutat Res 1999; 436: 185-94.

157. Vineis P, Kogevinas M, Simonato L, Brennan P, Boffetta P. Levelling-off of the risk of lung and bladder cancer in heavy smokers: an analysis based on multicentric case-control studies and a metabolic interpretation. Mutat Res 2000; 463:103-110.

158. Vineis P and Perera F. Molecular Epidemiology and Biomarkers in Etiologic Cancer Research: The New in Light of the Old. CEBP 2007; 16(10):1954-65.

159. Vineis P, Chuang S, Vaissière T, et al. DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study. Epigenetics 2011; 6(2):1-7.

160. Voortman J, Goto A, Mendiboure J, et al. MicroRNA expression and clinical outcomes in patients treated with adjuvant chemotherapy after complete resection of non-small cell lung carcinoma. Cancer Res 2010; 70(21):8288-98.

161. Watanabe T, Katayama Y, Komine C, et al. O6-methylguanine-DNA methyltransferase methylation and TP53 mutation in malignant astrocytomas and their relationships with clinical course. Int J Cancer 2005; 113(4):581-7.

162. Weigelt B, Hu Z, He X, et al. Molecular portraits and 70-gene prognosis signature are preserved throughout the metastatic process of breast cancer. Cancer Res 2005; 65: 9155–9158.

163. WHO 2011. Report on the global tobacco epidemic, http://www.who.int/tobacco/global_report/2011/en/index.html.

164. Wild CP, Jiang YZ, Sabbioni G, et al. Evaluation of methods for quantitation of aflatoxinalbumin adducts and their application to human exposure assessment. Cancer Res 1990; 50: 245-51.

165. Wild CP, Fortuin M, Donato F, et al. Aflatoxin, liver enzymes and hepatitis B virus infection in Gambian children. CEBP 1993; 2: 555-561.
166. Wild C. Completing the Genome with an “Exposome”: The Outstanding Challenge of Environmental Exposure Measurement in Molecular Epidemiology. Cancer Epidemiol Biomarkers Prev 2005; 14: 1847.
167. Wild CP, Vineis P, Garte S. Molecular epidemiology of chronic diseases. 2008.
168. Wild C. Environmental exposure measurement in cancer epidemiology. Mutagenesis 2009; 24:117-125.
169. World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective. AIRC 1997.
170. World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition and the Prevention of Cancer: A Global Perspective. AIRC 2007.
171. Wright ME, Park Y, Subar AF, et al. Intakes of fruit, vegetables, and specific botanical groups in relation to lung cancer risk in the NIH-AARP Diet and Health Study. Am J Epidemiol 2008; 168: 1024-34.
172. Wu HC, Delgado-Cruzata L, Flom JD, et al. Global methylation profiles in DNA from different blood cell types. Epigenetics 2011; 6:76-85.
173. Yang C, Landau J, Huang M, et al. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 2001; 21:381-406.
174. Yatabe Y and Mitsudomi T. Epidermal growth factor receptor mutations in lung cancers. Pathology International 2007; 57: 233–44.
175. Yoon J, Baek S. Molecular targets of dietary polyphenols with anti-inflammatory properties. Yonsei Med J 2005; 46:585-96.
176. Yoon J, Smith L, Feng Z, et al. Methylated CpG Dinucleotides Are the Preferential Targets for G-to-T Transversion Mutations Induced by Benzo[a]pyrene Diol Epoxide in Mammalian Cells : Similarities with the p53 Mutation Spectrum in Smoking-associated Lung Cancers. Cancer Res 2001; 61:7110-17.
177. Youlden DR, Cramb SM, Baade PD. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. J Thorac Oncol. 2008; 3(8):819-31.
178. Zhang Y. Cancer-preventive isothiocyanates; measurement of human exposure and mechanism of action. Mut Res 2004; 555:173-190.
179. Zhang FF, Morabia A, Carroll J, et al. Dietary patterns are associated with levels of global genomic DNA methylation in a cancer-free population. J Nutr 2011; 141(6):1165-71.