Facoltà di Scienze Matematiche Fisiche e Naturali
Dipartimento di Biologia

Dottorato di ricerca in Biologia
(Ph. D. in Biology)

XX° Ciclo
(A.A. 2006/2007)

"DNA damage repair and genetic polymorphisms: assessment of individual sensitivity and repair capacity"

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CONTENTS

ABBREVIATIONS

- 5'dRP: 5'-deoxyribose-5-phosphate
- 6-4PPs: (6-4) photoproduct
- 8-OH-G: 8-oxoguanine
- AP: Apurine/Apyrimidinic
- APE1: AP-Endonuclease 1
- BER: Base Excision Repair
- BNMN: Micronucleated Binucleated Cells
- BPDE: Benzo(a)Pyrene diol epoxide
- BRCT: BRCA C-Terminal
- BrdU: Bromo-deoxy-Uridine
- BSA: Bovine Serum Albumin
- CAs: Chromosomal Aberrations
- CPDs: Cyclobutane Pyrimidine Dimers
- CS: Cockaine's Syndrome
- CsA: Chromosome Aberration
- CSA: Cockaine's Syndrome Complementation Group A
- CSB: Cockaine's Syndrome Complementation Group B
- CtA: Chromatid Abberration
- DDB: Damaged DNA Binding Protein
- DMSO: Di-Methyl Sulfoxide
- DNA-PK: DNA-dependent Protein-Kinase
- DSBR: DSBs Repair
- DSBs: Double Strand Breaks
- DTIC: Decarbazine
- ERCC: Excision Repair Cross Complementing Group
- FEN1: Flap Endonuclease 1
- FITC: Fluoresceinisothiocyanate
- FPG: Formaamidopyrimidine-DNA-glycosylase
- GCR: Global Genomic Repair
- GSH: Glutathione
- GSTs: Glutathione S-Transferases
- GTBP: GT-Binding Protein
- HDR or HR: Homologous (Directed) Recombination
- HeF2: Human Homologue of Factor 2
- HNPCC: Hereditary Non-Polyposis Colorectal Cancer
- IR: Ionizing Radiation
- MMR: Mismatch Repair
- MN: Micronuclei
- MPG: Methyl-Purine-Glycosylase
- MSH: Mismatch Repair Protein
- NER: Nucleotide Excision Repair
- NHEJ: Non Homologous End-Joining
- NS: Non-Smokers
- OGG1: 8-oxoG-DNA-Glycosylase
- PAHs: Polyciclyc Aromatic Hydrocarbons
- PARP1: Poly(ADP-ribose)-polymerase 1
- PBS: Phosphate Buffered Salne
- PCNA: Proliferating Cell Nuclear Antigen
- PCR: Polymerase Chain Reaction

- PI: Propidium Iodide
- Pol β: Polymerase β
- Pol δ: Polymerase δ
- Pol: ε: Polymerase ε
- RD: Residual DNA Damage
- RF-C: Replication Factor-C
- RFLP-PCR: Restriction Fragment Length Polymorphism
- ROS: Reactive Oxygen Species
- RPA: Replication Protein A
- RT: Radiotherapy
- S: Smokers
- SCGE: Single Cell Gel Electrophoresis
- SNP: Single Nucleotide Polymorphism
- SSBs: Single Strand Breaks
- TCR: Transcription Coupled Repair
- TD: Tail DNA
- TFIIH: Transcription Factor IIH
- TTD: Trichothiodystrophy
- UV: Ultraviolet
- UVSS: UV-sensitive syndrome
- XP: Xeroderma Pigmentosum
- XPB: Xeroderma Pigmentosum Group B
- XPC:Xeroderma Pigmentosum Group C
- XPD: Xeroderma Pigmentosum Group D
- XPE: Xeroderma Pigmentosum Group E
- XPG: Xeroderma Pigmentosum Group G
- XRCC1: X-ray Cross Complementing Group 1
- XRCC2: X-ray Cross Complementing Group 2
- XRCC3: X-ray Cross Complementing Group 3
- XRCC4: X-ray Cross Complementing Group 4
- XRCC5: X-ray Cross Complementing Group 5
- XRCC7: X-ray Cross Complementing Group 7

I. INTRODUCTION

Mammalian cells are constantly exposed to ubiquitous environmental and endogenous genotoxic agents. The biologic consequences of mutations and persisting lesions range from the onset of carcinogenesis, genetic disorders, and apoptosis to general malfunctioning of cells that contribute to aging.

Distinct mechanisms have evolved to repair different types of DNA damage and to maintain genomic integrity. Investigators have demonstrated that persons showing severely compromised repair capacity have increased mutation rates, genomic instability, and increased risk of cancer (Berwick and Vineis, 2000).

In general, healthy subjects also differ in their intrinsic capacity to repair DNA damage (Setlow, 1983), and this variation may result from alterations in gene expression or could be a result of the polymorphisms of genes involved in different repair pathways. The repair mechanisms that have particular relevance are those that remove minor changes in helical structures, like base excision repair (BER) and nucleotide excision repair (NER), or those that participate in DNA double-strand breaks (DSBs) resolution, like homology-directed repair (HDR) and non-homologous end-joining (NHEJ) repair (Figure 1).

One of the most recurrent types of DNA damage is caused by oxidative stress. Oxidative stress describes a condition in which cellular antioxidant defences are insufficient to keep the levels of reactive oxygen species (ROS) below a toxic threshold. This may be due either to excessive production of ROS, or to loss of antioxidant defences or to both. Anti-oxidant enzymes and DNA repair proteins are two major mechanisms by which cells counteract the deleterious effects of ROS. There are many types of ROS which exist in the cellular environment and differ in their formation site, their physiological functions and reactivity. Hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical (OH*) are thought to be more important than the others but the OH* is the most active of all the ROS.

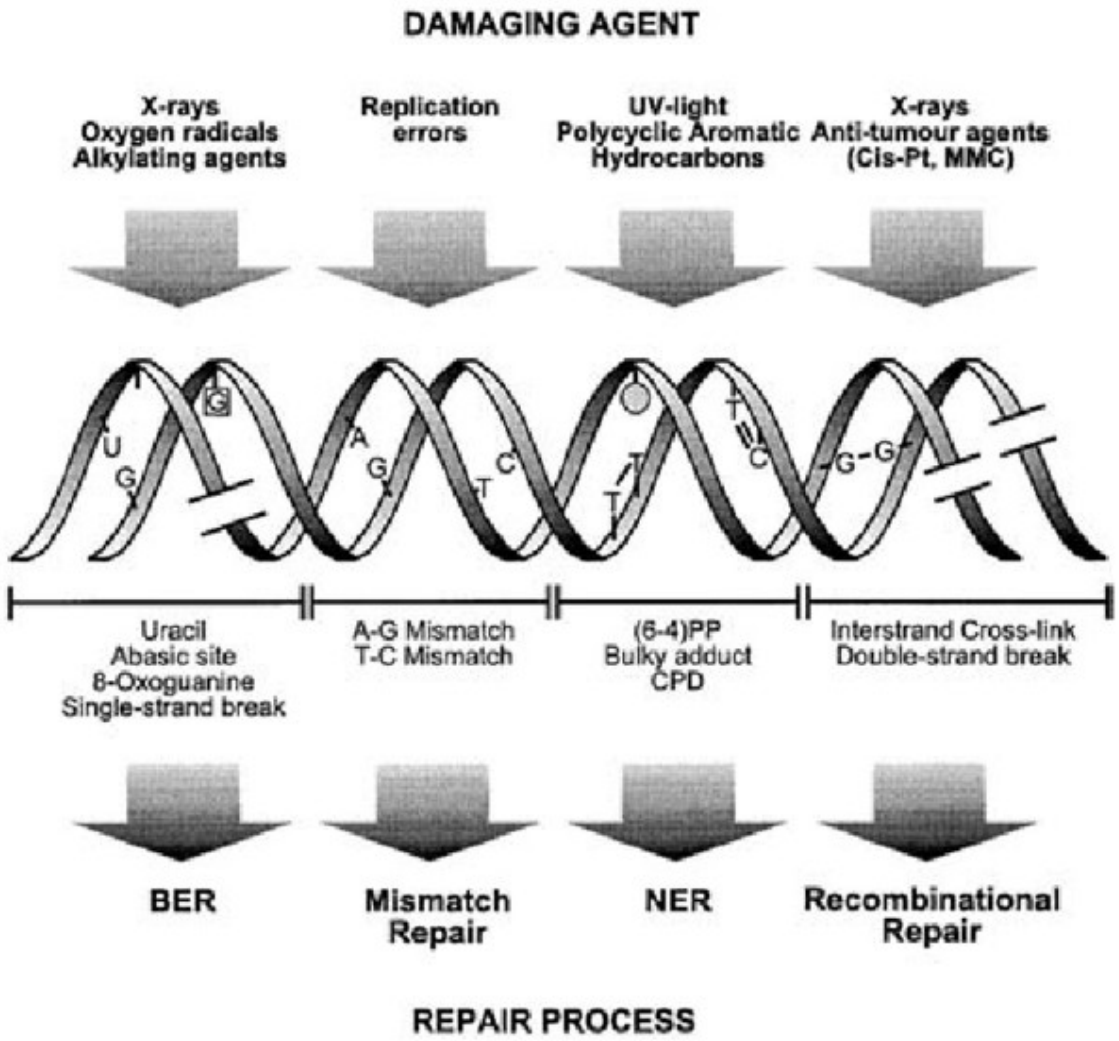


Figure 1 - A simplified schema of the most important DNA repair pathways (uemweb.biomed.cas.cz)

Glutathione (GSH) is an important intracellular antioxidant molecule that protects cells against ROS, but an age–related decline in GSH has been observed suggesting that this phenomenon may represent the onset of various diseases. Therefore, given the role of Glutathione S-transferases (GSTs) to catalyse the conjugation between electrophiles and GSH, polymorphisms in genes encoding the GSTs may influence the individual capacity of preventing oxidative damage.

In the last years a great deal of interest has been focused on the susceptibility genes which are low penetrance genes. Several genes involved in xenobotics metabolism (i.e. GSTs) and DNA repair belong to this category. These genes are often polymorphic and these variant alleles are commonly present in human populations.

DNA repair gene polymorphisms and GSTs gene polymorphisms can be considered genetic susceptibility biomarkers, a measure of an inherited characteristic that could modulate organism responses to environmental exposure.

Interactions between susceptibility genes and environmental factors could modulate cancer risk in exposed populations.

1. Genetic susceptibility

It has been well recognized for a long time that significant variations exist in the human population regarding response to environmental mutagenic agents and in the subsequent development of long-term health problems such as cancer. Although many factors can contribute to these events, understanding the genetic basis for these variations is of major interest because knowledge can lead to a more precise prediction of human health problems and to more effective prevention of disease.

The discovery of polymorphisms in genes for chemical metabolisms and for DNA repair has generated tremendous interest in understanding the phenomenon of genetic susceptibility in the population (Norppa, 2003).

Currently, major research activities have been focused on polymorphisms in DNA repair genes as an important component of the individual susceptibility phenomenon because DNA repair activities are critically involved with the protection of the genome and with prevention of cancer. This interest is further stimulated by studies which showed positive association between these polymorphic genes and cancers in lung, head and neck, and bladder, etc. Polymorphism in the XRCC3 gene (exon 7, codon 241) has been implicated in the increased risk of melanoma (Winsey et al, 2000) and several types of cancer have been related to polymorphism in XRCC1 (X-Ray Cross Complementing Group 1) gene (Divine et al, 2001)(Hsieh et al, 2003). Case-control studies on a SNP present in a coding region of hOGG1 (8-oxoG-DNA-glycosylase) suggest that this polymorphism may be a risk factor for adenocarcinoma (Ito et al, 2002) and stomach cancer (Takezaki et al, 2002). One study describes a possible SNP (Single Nucleotide Polymorphism) contribution in the XPC (Xeroderma Pigmentosum Group C) gene to the risk of developing bladder cancer (Sanyal et al, 2004).

Several studies have suggested that genetic polymorphisms in genes coding for xenobiotic metabolizing enzymes are associated with increased cancer incidence. Associations have been found between deletions of the GSTM1 and GSTT1 genes (null genotypes) and susceptibility to bladder, colon, skin and lung cancer, and between GSTP1 polymorphism and risk of oral cancer (Rebbeck, 1997}{Park et al, 1999}.

Based on these data we can conclude that specific metabolic and DNA repair gene variants can affect cancer risk. Predisposition to hereditary cancer syndromes is dominated by the strong effects of some high-penetrance tumor susceptibility genes, while predisposition to sporadic cancer is influenced by the combination of multiple low-penetrance genes. Before translating these findings into clinical use and application for public health measures, large population-based studies and validation of the results will be required.

1.1 Radiosensitivity

DNA has been widely considered to be the main target for the induction of cell death following exposure to ionising radiation (IR). Ionising radiation may disrupt chemical bonding in the DNA molecule, including both single-strand breaks (SSBs) and double-strand breaks (DSBs) either by depositing energy directly (direct effect) or by generating free radicals which, in turn, will attack the DNA molecule (indirect effect). A protein kinase cascade connects the detection of DNA damage to the implementation of an appropriate response: DNA repair, cell cycle arrest or cell death (Schmidt-Ulrich et al, 2000)(Dent et al, 2003) (Figure 2).

Deficiencies in DNA repair pathways and cell death regulation may result in higher vulnerability to ionizing radiation (IR). Cases of hypersensitivity to IR have been well known to radiation oncologists for many years. Around 5-7% of cancer patients develop adverse side-effects to external radiation therapy (RT) in normal tissue within the treatment field.

Several patient- and treatment-related factors are known to influence the variability of these side-effects, but up to 70% of cases remain unexplained (Turreson et al, 1996).

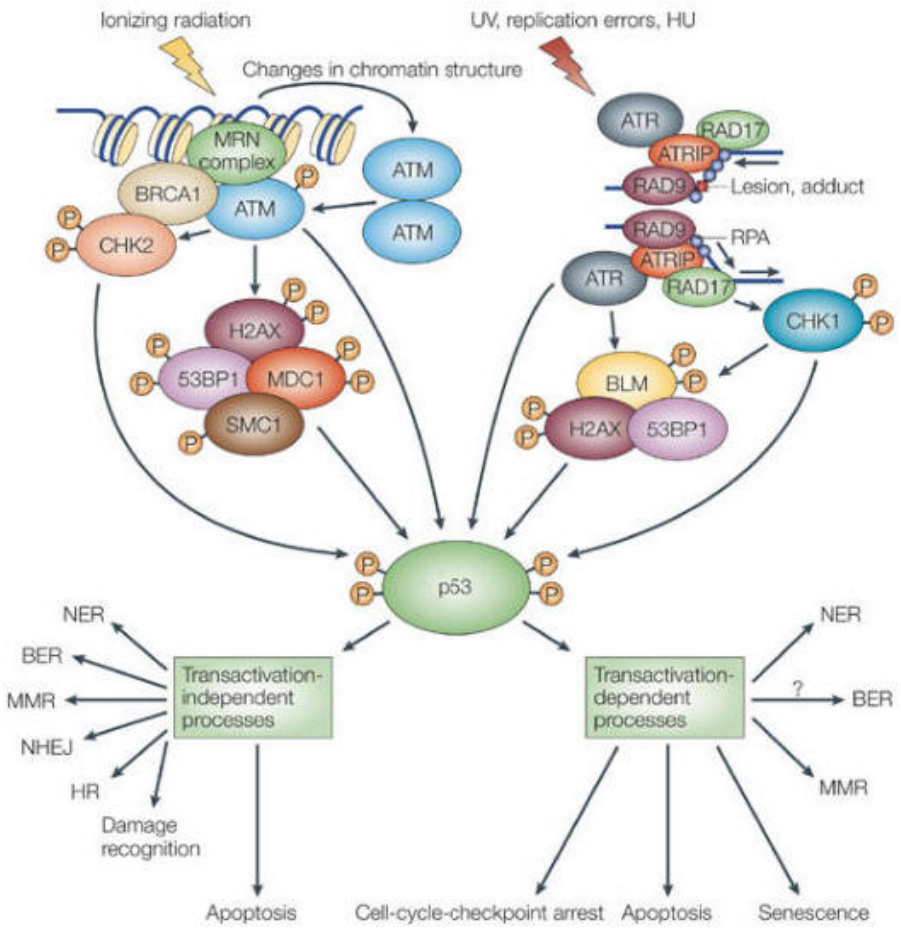


Figure 2 - DNA damage detection pathways. ATM (ataxia telangiectasia mutated); ATR–ATRIP (ataxia-telangiectasia-and-RAD3-related–ATR-interacting-protein) complex; MRN complex [which comprises RAD50, MRE11 (meiotic-recombination-11) and NBS1 (Nijmegen breakage syndrome-1)]; BRCA1 (breast-cancer-susceptibility protein-1); H2AX (histone-2A family, member X), 53BP1 (p53-binding protein-1), MDC1 (mediator of DNA-damage checkpoint protein-1); SMC1 (structural maintenance of chromosomes-1); (ss)DNA–RPA (replication protein A) complex; BLM (Bloom syndrome protein); HU, hydroxyurea; UV, ultraviolet light (Sengupta and Harris, 2005)

In recent years, much effort has been made to improve the basic knowledge of radiation effects on normal and neoplastic cells, looking for an integration of classical radiation biology with new emerging concepts from the fields of genetics and molecular biology. Advance has been substantial, with definition and clear description of several signalling pathways involved in cellular radiation response, but genetic determinants and molecular mechanism of clinical therapeutic radiosensitivity are still poorly understood. Strong evidence in favor of a genetic basis of radiation response originates from studies on patients with rare genetic syndromes such as ataxia telangiectasia, Fanconi's anemia, Nijemegen Breakage syndrome and Bloom's syndrome. A

small number of the case reports of patients affected by these diseases showed patterns of enhanced cellular and clinical radiosensitivity and increased susceptibility to cancer development (Alter, 2002)(Rogers et al, 2000). These syndromes are clearly related to germ line mutations regarding genes involved in detection of DNA damage or DNA repair (Gatti, 2001). Mutations in repair genes have also been detected in patients with reported extreme radiosensitivity, even if not affected by any of these syndromes (Riballo et al, 1999)(Severin et al, 2001). All of the cited genetic syndromes are very rare, characterized by mendelian inheritance and probably of limited importance when addressing the issue of clinical radiosensitivity in a population of unselected cancer patients. However, as pointed out by different authors (Fernet and Hall, 2004)(Andreassen et al, 2002), they could be considered as a "proof of principle" that clinical radiosensitivity is in fact determined by genetic factors. The possible association between *in vitro* cell radiosensitivity and clinical patterns of sensitivity among unselected cancer patients is an experimental approach which should be considered. Studies investigating the correlation between *in vitro* radiosensitivity (with a variety of biological end points) and clinical radiosensitivity in breast cancer patients have led to contradictory results or have not been found to predict radiation toxic effects on normal tissues (Dikomey et al, 2003)(Twardella and Chang-Claude, 2002). Twardella and Chang-Claude reviewed (2002) 25 studies published between 1990 and 2000, with the aim of identifying which tests should be considered most promising. *In vitro* assays 4 groups were classified in (Figure 3):

- testing the ability to survive after exposure to radiation;
- cytogenetic tests evaluating the frequency of specific chromosomal aberrations (CAs) in irradiated cells;
- a cell's ability to repair radiation-induced damages;
- other radiation-induced end points like apoptosis.

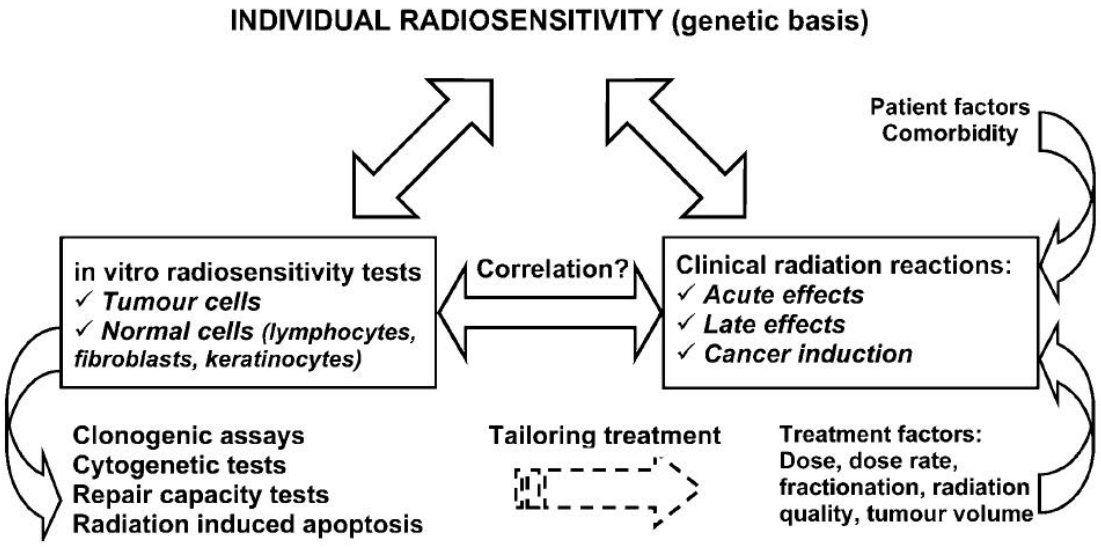


Figure 3 - Relationship between in vitro radiosensitivity and clinical radiosensitivity, considering factors that influence side effects. The way forward lies on the development of predictive assays which permit tailoring patient's treatment (Bourguignon et al, 2005)

Fernet and Hall (2004) observed that the possibility of identifying an *in vitro* assay showing sufficient sensitivity and specificity in the detection of individuals who will probably develop acute or late clinical radiation toxicity is highly debatable. In any case, getting closer to the point of the genetic basis of clinical radiosensitivity, the fact that the weak correlation sometimes demonstrated between in vitro data and clinical data seems likely to depend on genetic factors should be taken into consideration. This hypothesis is supported by the current knowledge of biological events (DNA repair or apoptosis) which can be now clearly regarded as genetically controlled complex cellular phenomena (Filippi et al, 2006).

Among the possible biological endpoints, chromosomal aberrations (Scott, 2000)(Smart et al, 2003) and repair of induced-DNA damage (DSBs and SSBs) (Muller et al, 2002)(Popanda et al, 2003)(Vodicka et al, 2007) have been measured comparing the data obtained with different clinical side effects.

Since studies analysing breast cancer patients with hypersensitivity to radiation suggest that DNA repair mechanisms are involved (Fernet and Hall, 2004), the characterization of DNA repair capacity in lymphocytes might be a suitable approach to predict clinical radiation reaction. The alkaline single cell gel electrophoresis (SCGE) or Comet assay has been shown to be useful for the assessment of DNA damage and repair within epidemiologic studies. It is a

reliable, sensitive and rapid method of detecting direct DNA damage at single cell level, with respect to SSB, DNA interstrand cross-link and base damages that appear as endonuclease-sensitive sites (Tice et al, 2000). DNA repair can be monitored by incubating cells after treatment with damaging agent and measuring the damage remaining at successive time intervals (cellular repair assay) (Olive P, 1999)(Cornetta et al, 2006) **(see INTRODUCTION, section 4.2, page 42)**.

Significant differences in DNA repair kinetics were detected between patients with severe skin reactions and patients with normal reactions to radiation when the Comet assay was used (Alapetite et al, 1999)(Oppitz et al, 1999).

More recently Popanda and co-workers (2003) have shown that the reduced DNA repair capacity measured by the Comet assay in breast cancer patients corresponded only partially to the occurrence of acute radiation sensitivity. Moreover the initial DNA damage as well as the residual damage after a lapse of repair time may provide valuable information with regard to individual levels of radiosensitivity (Roos et al, 2000)(Vodicka et al, 2007).

On the whole, evidence is now emerging that normal tissue radiation effects are due to a genetically determined individual variability. In view of the importance of DNA repair in cell and tissue response, the genes responsible for DNA damage signalling and repair pathways are suitable candidates in the search for the genetic basis of clinical radiosensitivity. Tacking into account that IR generates a wide range of DNA damage (base and sugar modifications, SSBs and DSBs, interstrand crosslinks), genes involved in the removing of minor changes in helical structure (like BER and NER) or those participating in DNA DSBs resolution (HR and NHEJ) are all of interest in assessing the genetic basis of radiosensitivity (Muller et al, 2001)(Andreassen et al 2002).

1.2 Genetic polymorphisms

In two randomly selected human genomes 99.9% of the DNA sequence is identical. The remaining 0.1% is thought to include some differences or variations in the genome between individuals. This variation, called polymorphism, arises because of mutations.

Genetic polymorphism is the existence of variants with respect to a gene locus (alleles), a chromosome structure (e.g., size of centromeric heterochromatin), a gene product (variants in enzymatic activity or binding affinity), or a phenotype. The term DNA polymorphism refers to a wide range of variations in nucleotide base composition, length of nucleotide repeats, or single nucleotide variants. These differences in sequence can result from mutations involving a single nucleotide or from deletions or insertions of variable numbers of contiguous nucleotides. DNA polymorphisms are important as genetic markers to identify and distinguish alleles at a gene locus and to determine their parental origin (Hartwell et al, 2004).

The exact definition of a genetic polymorphism is: the occurrence in the same population of multiple discrete allelic states of which at least two have high frequency (conventionally 1% or more) (Table 1).

Table 1 - Main differencies between genetic polymorphisms and mutations

	Genetic Polymorphism	**Mutation**
Frequency	*High (> 1%)*	*Low (< 1%)*
Individual risk	*Low*	*High*
Population risk	*Appreciable*	*Low*
Research	*Cohort and Case-Control Studies*	*Familiar Analysis Studies*

Polymorphisms based on insertion or deletion of one or more nucleotides can be divided into those with multiple alleles (multiallelic) and those with only two alleles (diallelic). Nearly all of the multiallelic polymorphisms are based on tandem repeats (STR – Simple Tandems Repeats), also called “microsatellites” (Hartwell et al, 2004). This kind of polymorphism has been the predominant type of DNA sequence variation used in human genetic studies since about 1990 (Weber et al, 2002)

Diallelic polymorphisms often arise during meiotic recombination due to an unequal crossing-over between non-homologous sites of homologous chromosomes. This is the case for GSTM1 and GSTT1 deletion polymorphisms **(see INTRODUCTION, section 3, page 38)**, which consist in a removal of the entirely coding exons, giving rise to a null genotype (McCarrol et al, 2006).

The simplest form of genetic polymorphisms is the substitution of one single nucleotide with another, termed SNP. SNPs are more common than other types of polymorphisms and occur at a frequency of approximately 1 in

1000 base pairs (Brookes, 1999) throughout the genome (promoter region, coding sequences, and intronic sequences). These simple changes in DNA sequence, most of which are probably located in intergenic spacers, are believed to be stable and not deleterious to organisms. SNPs that do not change encoded amino acids are called synonymous and are not subject to natural selection (Kimura, 1983). On the other hand, non-synonymous SNPs alter amino acids and might be subject to natural selection. SNPs can be observed between individuals in a population, may influence promoter activity or DNA and pre-mRNA conformation, and play a direct or indirect role in phenotypic expression (Lohrer and Tangen, 2000}.

Because some SNPs are functional, comparative studies on identical twins, fraternal twins, and siblings suggest that genetic variation is one of the factors associated with susceptibility to many common diseases as well as all other human traits such as height or curly hair (Martin et al, 1997}.

Therefore, it may be possible to understand why some individuals are susceptible to common disorders by using the human genome sequence and the variations between individuals.

2. DNA repair

The human genome, like other genomes, encodes information to protect its own integrity (Lindahl and Wood, 1999). DNA repair enzymes continuously monitor chromosomes to correct damaged nucleotide residues generated by exposure to carcinogens and cytotoxic compounds. The damage is partly a consequence of environmental agents such as ultraviolet (UV) light from the sun, inhaled cigarette smoke, or incompletely defined dietary factors (Wood et al, 2001).

However, a large proportion of DNA alterations are caused unavoidably by endogenous weak mutagens including water, ROS and metabolites that can act as alkylating agents. Genome instability caused by the great variety of DNA damaging agents would be an overwhelming problem for cells and organisms (Wood et al, 2001).

The sequencing of the human genome (Venter et al, 2001) yielded a first overview of the large number of proteins involved in the protection of the genome. Recently, two papers compiled data of ~130 human DNA repair genes, which were cloned and sequenced. Not all of them, however, have been characterised yet as to their function (Ronen and Glickman, 2001)(Wood et al, 2001).

DNA repair genes can be sub-grouped into genes associated with signalling and regulation of DNA repair on the one hand and on the other into genes associated with distinct repair mechanisms such as mismatch repair (MMR), BER, NER, DNA DSBs repair (DSBR). However, recent experimental data have shown a cross-talk between the DNA repair pathways (Figure 4). The functional association between XRCC1 (BER) and DNA-PK (DNA-dependent protein kinase, DSBR) in response to ionizing radiation have provided the first evidence for their involvement in a common DSBR pathway (Levy et al, 2006). The use of DNA glycosylases (BER) may be lesion specific, mediate synergistic parallel repair pathways, or can be called in action in response to genotoxic stimulus. In all of these cases, DNA glycosylases have multiple roles, and are used to alter the composition and function of different repair machines (MMR and NER) (Kovtun and McMurray, 2007). XPC-HR23B complex (NER) acts as cofactor in base excision repair of 8-hydroxyguanine (8-OH-G), by stimulating the activity of its specific DNA glycosylase OGG1 (D'Errico et al, 2006). In vitro

experiments suggest that the mechanism involved is a combination of increased loading and turnover of OGG1 by XPC-HR23B complex.

What is clear, however, is that the cell takes no “one size fits all” strategy to repair its DNA, and removal of most lesions will be context dependent. Thus, it is probable that the assembly of the most efficient repair machine will be sensitive to the type and size of the lesion, the replication and transcriptional status of the DNA, the DNA sequence context, and the cell type (Kovtun and McMurray, 2007).

Mutations in genes involved in DNA repair are responsible for the development of tumors and various hereditary diseases characterised by complex metabolic alterations.

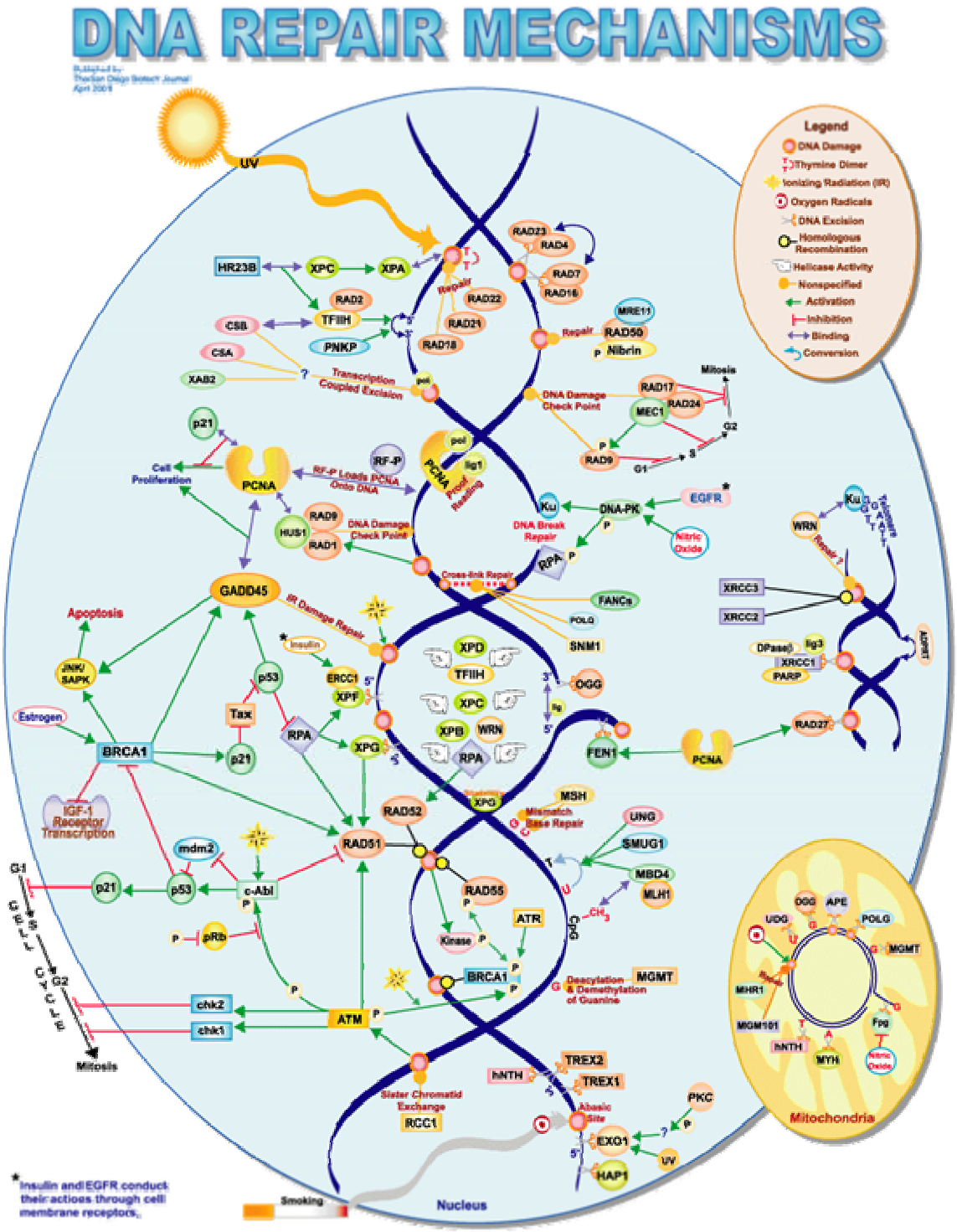


Figure 4 - DNA Damage Response. DNA damage is caused by a variety of sources. The cellular response to damage may involve activation of a cell cycle checkpoint, commencement of transcriptional programs, execution of DNA repair, or when the damage is severe, initiation of apoptosis (The San Diego Biotech Journal, 2001)

2.1 DNA repair pathways

Base Excision Repair

BER is responsible for removing DNA-damaged bases, which can be recognised by specific enzymes, the DNA glycosylases. The main lesions subjected to BER are oxidised DNA bases, arising spontaneously within the cell, during inflammatory responses, or from exposure to exogenous agents, including ionising radiation and long-wave UV light. Another main source of lesions repaired by BER is DNA alkylation induced by exogenous carcinogens such as nitrosamines. Also, various anticancer drugs such as decarbazine (DTIC) and temozolomide induce alkylation lesions repaired by BER.

Lesions removed from DNA by BER include incorporated uracil, fragmented pyrimidines, N-alkylated purines (7-methylguanine, 3-methyladenine, 3-methylguanine), 8-oxo-7,8-dihydroguanine (8-OxoG) and thymine glycol and many others. The major oxidized purine, 8-Oxo-G, is highly mutagenic because of mispairing with adenine. N-Alkylpurines are vulnerable to spontaneous hydrolysis of the N-glycosylic bond, giving rise to apurinic/apyrimidinic (AP) sites, which are one of the most frequent (>10^4 formed per day per cell) (Lindahl, 1990) and potent lethal (Loeb, 1985} lesions.

BER mechanism proceeds in the following steps (Figure 5):

- *Recognition, base removal and incision*: the first step in BER is carried out by specific DNA glycosylases which recognize and remove damaged or incorrect (e.g. uracil) bases by hydrolyzing the N-glycosidic bond (Scharer and Jiricny, 2001). In mammalian cells, 11 different glycosylases have been found characterised by different substrates specificities and modes of action. These DNA glycosylases are subgrouped into type I and type II glycosylases, the first leave an AP site in DNA (e.g. methyl-purine-glycosylase, MPG), whereas type II enzymes give rise to a single strand break (e.g. OGG1).

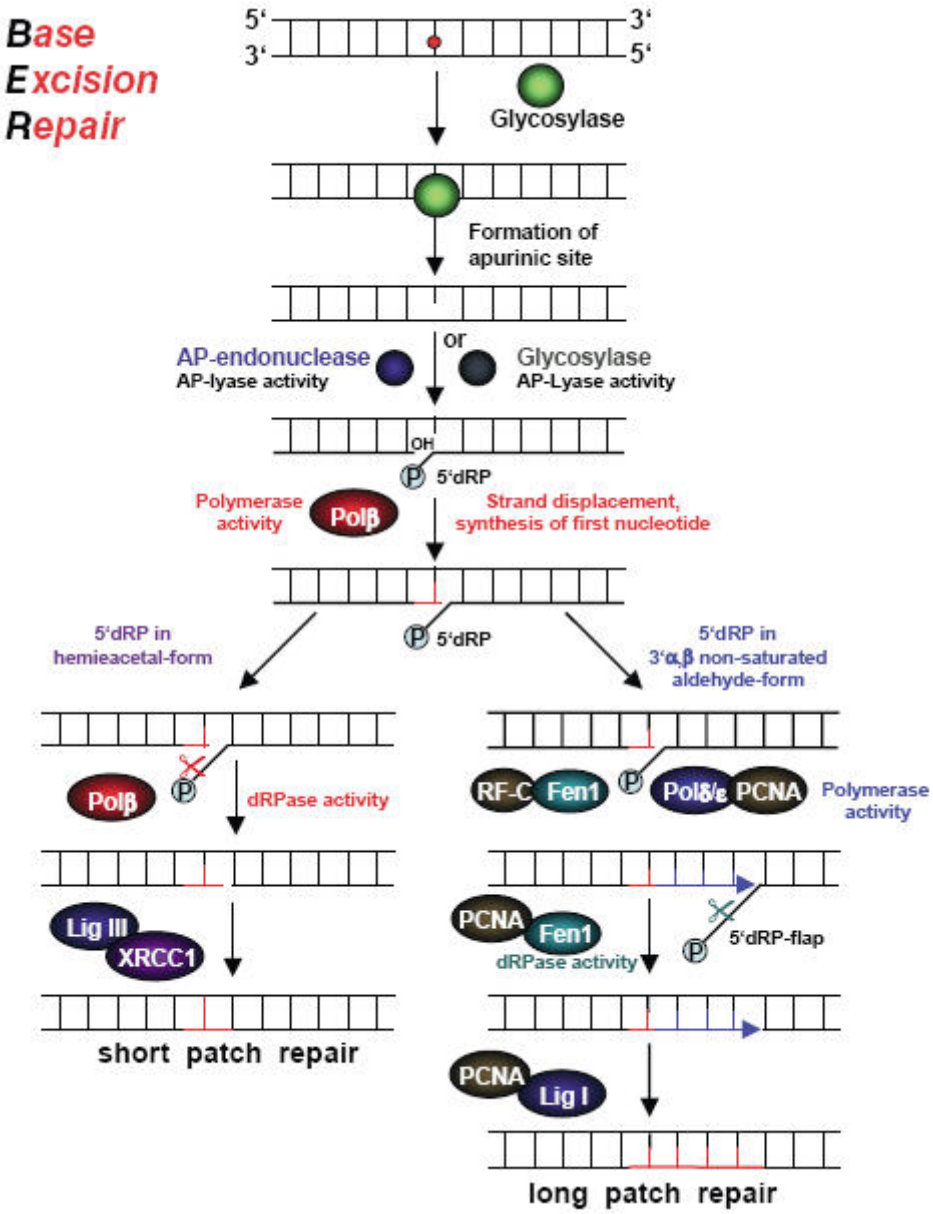


Figure 5 - Mechanism of base excision repair (BER). Recognition of the DNA lesion occurs by a specific DNA glycosylase which removes the damaged base by hydrolyzing the N-glycosidic bond. The remaining AP site is processed by APE. Depending on the cleavability of the resulting 5'dRP by Polβ, repair is performed via the short or long patch BER pathway (Christmann et al, 2003)

- *Nucleotide insertion*: the insertion of the first nucleotide is not dependent on the chemical structure of the AP site. During short-patch BER, 5'dRP is displaced by DNA-polymerase β (Pol β), which inserts a single nucleotide (Wiebauer and Jiricny, 1990). Pol β is also involved in long-patch BER (Dianov et al, 1999), inserting the first nucleotide at reduced AP sites (Podlutsky et al, 2001).

- *Decision between short- and long-patch repair:* The critical step in the decision between short- and long-patch BER is the removal of 5'dRP upon the insertion of the first nucleotide. Besides polymerisation activity, Pol β also exerts lyase activity and is thereby able to catalyze the release of the hemiacetal form of 5'dRP residues from incised AP sites by β-elimination (Prasad et al, 1998). In contrast, oxidised or reduced AP sites, 3'-unsaturated aldehydes or 3'-phosphates are resistant to β-elimination by Pol β (Nakamura et al, 2000). Upon dissociation of Pol β from damaged DNA, further processing occurs by PCNA (Proliferating Cell Nuclear Antigen)-dependent long-patch repair (Matsumoto et al, 1999). For example, the removal of 8-oxoG occurs mainly via short-patch BER; only 25% of lesions are repaired via the long-patch repair pathway (Dianov et al, 1998).
- *Strand displacement and DNA-repair synthesis by long-patch BER:* in contrast to short-patch repair, in which single base insertion by Pol β the DNA backbone is directly sealed, several additional steps occur during long-patch repair. After dissociation of Pol β, strand displacement and further DNA synthesis is accomplished by Pol ε or Pol δ together with PCNA and RF-C (Replication Factor-C) (Stucki et al, 1998), resulting in longer repair patches of up to 10 nucleotides. The removal of the deoxyribophosphate flap structure (5'dRPflap) is executed by flap endonuclease FEN1 stimulated by PCNA (Klungland and Lindahl, 1997).
- *Ligation:* the ligation step is performed by DNA ligases I and III (Tomkinson et al, 2001). Ligase I interacts with PCNA and Pol β and participates mainly in long-patch BER (Prasad et al, 1996). DNA ligase III interacts with XRCC1, Pol β and PARP-1 [poly(ADP-ribose) polymerase-1] and is involved only in short-patch BER (Kubota et al, 1996).

Nucleotide Excision Repair

Bulky DNA adducts, such as UV-light-induced photolesions [(6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs)], intrastrand cross-links, large chemical adducts generated from exposure to aflatoxine, benzo[*a*]pyrene and other genotoxic agents are repaired by NER (Friedberg, 2001). In NER about 30 proteins are involved. Cells defective in NER belong to different complementation groups and UV-hypersensitive disorders such as xeroderma pigmentosum (XP), Cockayne's syndrome (CS), trichothiodystrophy (TTD), UV-sensitive syndrome (UVSS) and a ariety of UV-hypersensitive rodent lines, in which the defect can be complemented by human genes belonging to the excision repair cross-complementing group (ERCC) (Vermeulen et al, 1997).

NER consists of two distinct pathways termed global genomic repair (GGR) and transcription-coupled repair (TCR) (Figure 6).

GGR is thought to be largely transcription-independent and removes lesions from the non-transcribed domains of the genome and the non-transcribed strand of transcribed regions.

TCR removes different RNA-polymerase-blocking lesions from the transcribed strand of active genes (Mellon et al, 1987).

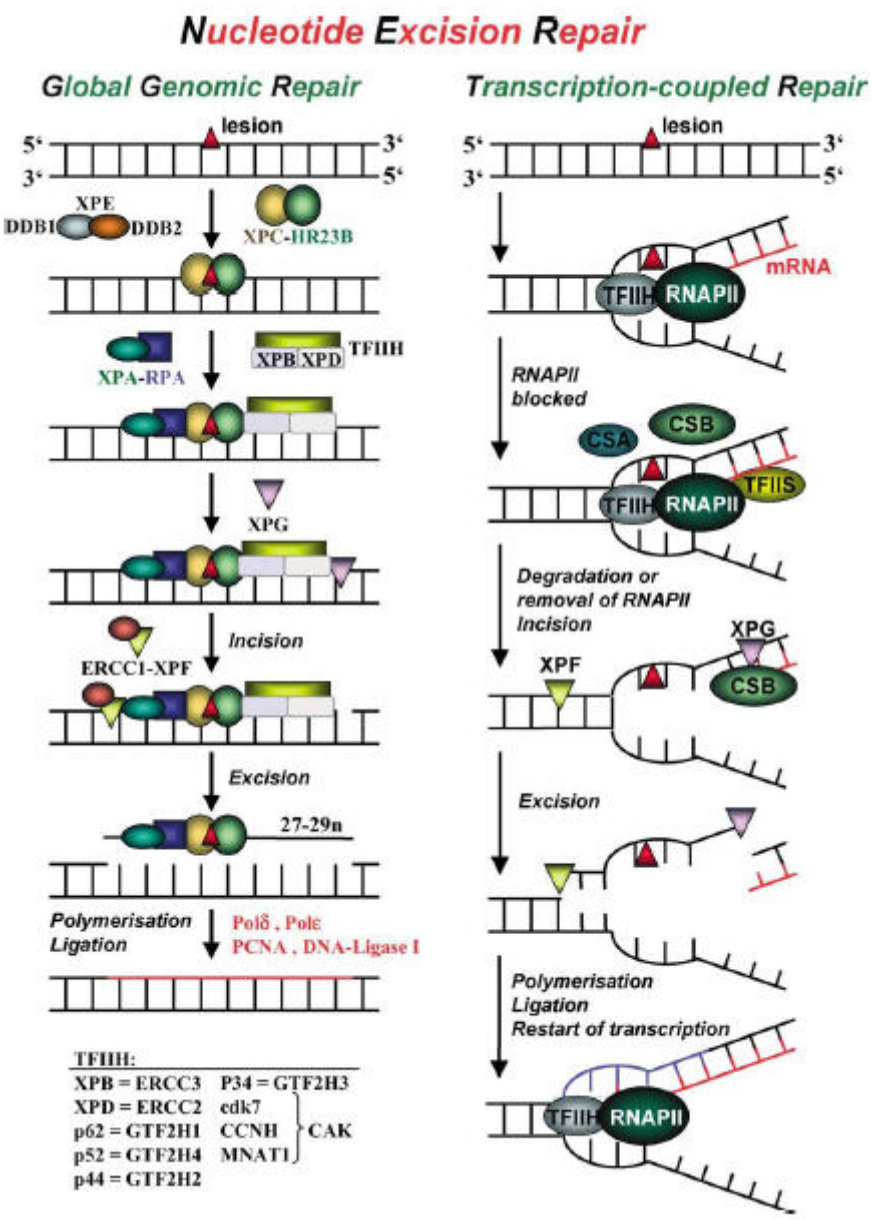


Figure 6 - Mechanism of nucleotide excision repair (NER). During global genomic repair (GGR), recognition of the DNA lesion occurs by XPC–HR23B, RPA–XPA or DDB1–DDB2. DNA unwinding is performed by the transcription factor TFIIH, and excision of the lesion by XPG and XPF–ERCC1. Finally, resynthesis occurs by Polδ or Polε and ligation by DNA ligase I. During transcription-coupled repair (TCR) the induction of the lesion results in blockage of RNAPII. This leads to assembly of CSA, CSB and/or TFIIS at the site of the lesion, by which RNAPII is removed from the DNA or displaced from the lesion, making it accessible to the exonucleases XPF–Ercc1 and XPG cleaving the lesion-containing DNA strand. Resynthesis again occurs by Polδ or Polε and ligation by DNA ligase I (Christmann et al, 2003)

In contrast to TCR, the mechanism of GGR has been elucidated in great detail. It proceeds as follows:

- *DNA-damage recognition:* the XPC-HR23B and RPA-XPA complexes identify DNA lesions. The XPC-HR23B complex recognizes UV-induced 6-4PPs with high specificity (Hey et al, 2002); it does not recognize CPDs, 8-oxoguanine or O^6-methylguanine (Kusumoto et al, 2001). In contrast, the RPA-XPA complex recognizes 6-4PPs and DNA treated with cisplatin (Vasquez et al, 2002). Not solved yet is the question of which complex is first in the sequential assembly of the NER proteins. Some authors claim that XPC-HR23B binds first to a helix distortion, which is verified by RPA-XPA and TFIIH (Sugasawa et al, 2001). Others suggest that RPA-XPA is the first DNA-damage recognition factor (Wakasugi and Sancar, 1999). Another important factor involved in the UV damage recognition process is the 'damaged DNA binding protein' (DDB), a heterodimer of two polypeptides DDB1 (p127) and DDB2 (p48) that belong to the XPE-complementation group (Tang and Chu, 2002). XPC is also inducible by ionizing radiation and alkylating agents (Amundson et al, 2002), and by treatment with benzo[*a*]pyrene diol epoxide (Wang et al, 2003).
- *DNA unwinding:* after recognition of the lesion, the transcription factor TFIIH consisting of seven different proteins (XPB, XPD, GTF2H1, GTF2H2, GTF2H3, GTF2H4, CDK7, CCNH and MNAT1) is recruited to the site of DNA damage. This recruitment is most likely mediated by the XPC-HR23B complex (Yokoi et al, 2000). TFIIH harbours DNA helicase activity, which is exerted by its helicase subunits XPB (Schaeffer et al, 1993) and XPD (Schaeffer et al, 1994). It is responsible for unwinding the DNA around the lesion (Evans et al, 1997a).
- *Excision of the DNA lesion:* after damage recognition and the formation of an open complex, excision of the lesion is carried out by dual incisions at defined positions flanking the DNA damage (Evans et al, 1997b). 3'-incision is performed by XPG (Habraken et al, 1994), and 5'-incision by the XPF-ERCC1 complex (Sijbers et al, 1996).
- *Repair synthesis:* the arising DNA gap is filled in by Pol δ and Pol ε and sealed by DNA ligase I and accessory factors (Araujo et al, 2000).

Mismatch Repair

The MMR system is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, methylation and replication errors (Modrich and Lahue, 1996). The main targets of MMR are base mismatches such as G/T (arising from deamination of 5-methylcytosine), G/G, A/C and C/C (Fang and Modrich, 1993).

MMR not only binds to spontaneously occurring mismatches but also to various chemically induced DNA lesions such as alkylation-induced O^6-methylguanine paired with cytosine or thymine (Duckett et al, 1996), 1,2-intrastrand (GpG) cross-links generated by cisplatin (Yamada et al, 1997), UV-induced photoproducts (Wang et al, 1999) purine adducts of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxides (Wu et al, 1999), 2-aminofluorene or N-acetyl-2-aminofluorene (Li et al, 1996) and 8-oxoguanine (Colussi et al, 2002). The importance of MMR in maintaining genomic stability and reducing mutation load is clearly illustrated by MMR deficiency syndromes such as Hereditary Non-Polyposis Colorectal Cancer (HNPCC) (Aaltonen et al, 1993).

The steps by which MMR proceeds are as follows (Figure 7):

- *Recognition of DNA lesions:* the recognition of mismatches or chemically modified bases is performed by the so-called MutSα complex, which binds to the lesions. MutSα is composed of the MutS homologous proteins MSH2 (Fishel et al, 1993) and MSH6 (also known as GT-binding protein, GTBP) (Palombo et al, 1995). MSH2 can also form a complex with the mismatch repair protein MSH3. This complex is designed MutSβ (Palombo et al, 1996). Depending on the binding partner, the heterodimers have different substrate specificities and, therefore, play a different role in mismatch repair. Thus, the MutSα complex is able to bind to base-base mismatches and to insertion/deletion mismatches (Umar et al,1994), whereas MutSβ is only capable of binding to insertion/deletion mismatches (Genschel et al, 1998).

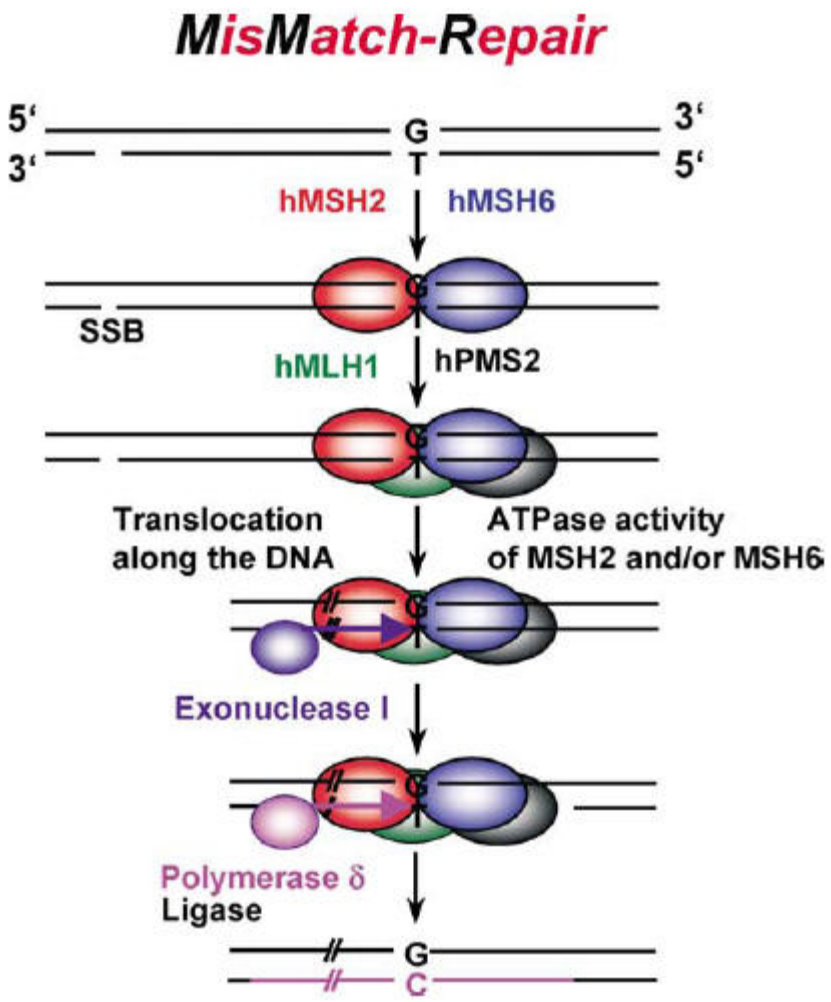


Figure 7 - Mechanism of mismatch repair (MMR). Recognition of DNA lesions occurs by MutSα (MSH2–MSH6). According to the molecular switch model, binding of MutSα–ADP triggers ADP → ATP transition, stimulates intrinsic ATPase activity, and provokes the formation of a hydrolysis-independent sliding clamp, followed by binding of the MutLα complex (MLH1–PMS2). According to the hydrolysis-driven translocation model, ATP hydrolysis induces translocation of MutS α along the DNA. After formation of a complex composed of MutS α and MutL α, excision is performed by ExoI and repair synthesis by Polβ (Christmann et al, 2003)

- *Strand discrimination:* presently, it is not clear how MMR discriminates between the parental and the newly synthesised DNA strand. It is supposed that the daughter strand is identified by non-ligated SSBs arising during replication (Thomas et al, 1991). The problem with this model is that the SSBs and the mismatch can be separated from each other by a great distance.

- *Excision and repair synthesis:* upon binding to the mismatch, MutSα associates with another heterodimeric complex (MutLα), consisting of

the MutL homologous mismatch repair protein MLH1 and PMS2 (Li and Modrich, 1995). The excision of the DNA strand containing the mispaired base is performed by exonuclease I (Genschel et al, 2002) and the new synthesis by Pol δ (Longley et al, 1997). Whether or not MMR is inducible by genotoxic stress is still a matter of debate. Both transcriptional and post-translational mechanisms appear likely to be involved in the regulation of MMR.

DNA Double-Strand Break Repair

DSBs are highly potent inducers of genotoxic effects (chromosomal breaks and exchanges) and cell death (Pfeiffer et al, 2000)(Lips and Kaina, 2001). In higher eukaryotes a single non-repaired DSB inactivating an essential gene can be sufficient for inducing cell death via apoptosis (Rich et al, 2000). There are two main pathways for DSB repair, HR and NHEJ, which are error-free and error-prone, respectively (Figure 8-9).

The usage of NHEJ and HR depends on the phase of the cell cycle. NHEJ occurs mainly in G0/G1, whereas HR occurs during the late S and G2 phases (Takata et al, 1998).

The NHEJ system ligates the two ends of a DSB without the requirement of sequence homology between the DNA ends (Critchlow and Jackson, 1998). The first step in NHEJ is the binding of a heterodimeric complex consisting of the proteins Ku70 (Reeves and Sthoeger, 1989) and Ku80 (XRCC5) (Jeggo et al, 1992) to the damaged DNA, thus protecting the DNA from exonuclease digestion. Following DNA binding, the Ku heterodimer associates with the catalytic subunit of DNA-PK (XRCC7, DNA-PKcs) (Sipley et al, 1995) thereby forming the active DNA-PK holoenzyme (Smith and Jackson, 1999). DNA-PKcs is activated by interaction with a single-strand DNA at the site of DSB (Martensson and Hammarsten, 2002) and displays Ser/Thr kinase activity (Kim et al, 1999). One of the targets of DNA-PKcs is XRCC4 (Leber et al, 1998), which forms a stable complex with DNA ligase IV. The XRCC4-ligase IV complex binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but not-ligatable ends (Lee et al, 2003).

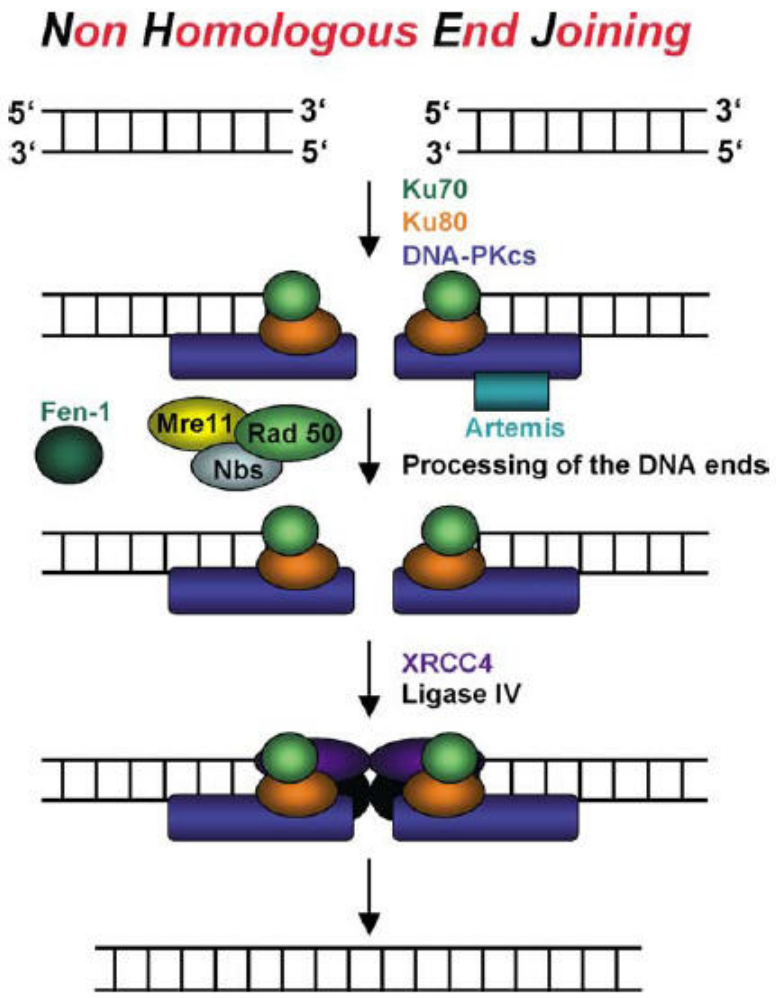


Figure 8 - Mechanism of non-homologous end joining (NHEJ). Recognition of and binding to damaged DNA occurs by the Ku70–Ku80 complex. Thereafter, the Ku heterodimer binds to DNA–PKcs, forming the DNA–PK holoenzyme. DNA–PK activates XRCC4–ligase IV, which links the broken DNA ends together.Before re-ligation by XRCC4–ligase IV, the DNA ends are processed by the MRE11–Rad50–NBS1 complex, presumably involving FEN1 and Artemis (Christmann et al, 2003)

Processing of DSBs is mainly performed by the MRE11-Rad50-NBS1 complex (Nelms et al, 1998), wich displays exonuclease, endonuclease and helicase activity (Paull and Gellert, 1999) and removes excess DNA at 3' flaps. One candidate responsible for removal of 5' flaps is the flap endonuclease 1 (FEN1). Deficiency of this protein leads to a strong reduction in the usage of the NHEJ pathway (Wu et al, 1999). Another protein involved in processing overhangs during NHEJ is the protein Artemis, which acts in a complex with DNA-PK (Moshous et al, 2001). Artemis displays single-strand-specific exonuclease activity. Upon forming a complex with and being phosphorylated by DNA-PKcs, Artemis acquires endonuclease activity, degrading single-strand

overhangs and hairpins, which seems to be necessary for processing 5' and 3' overhangs during NHEJ (Jeggo and O'Neill, 2002).

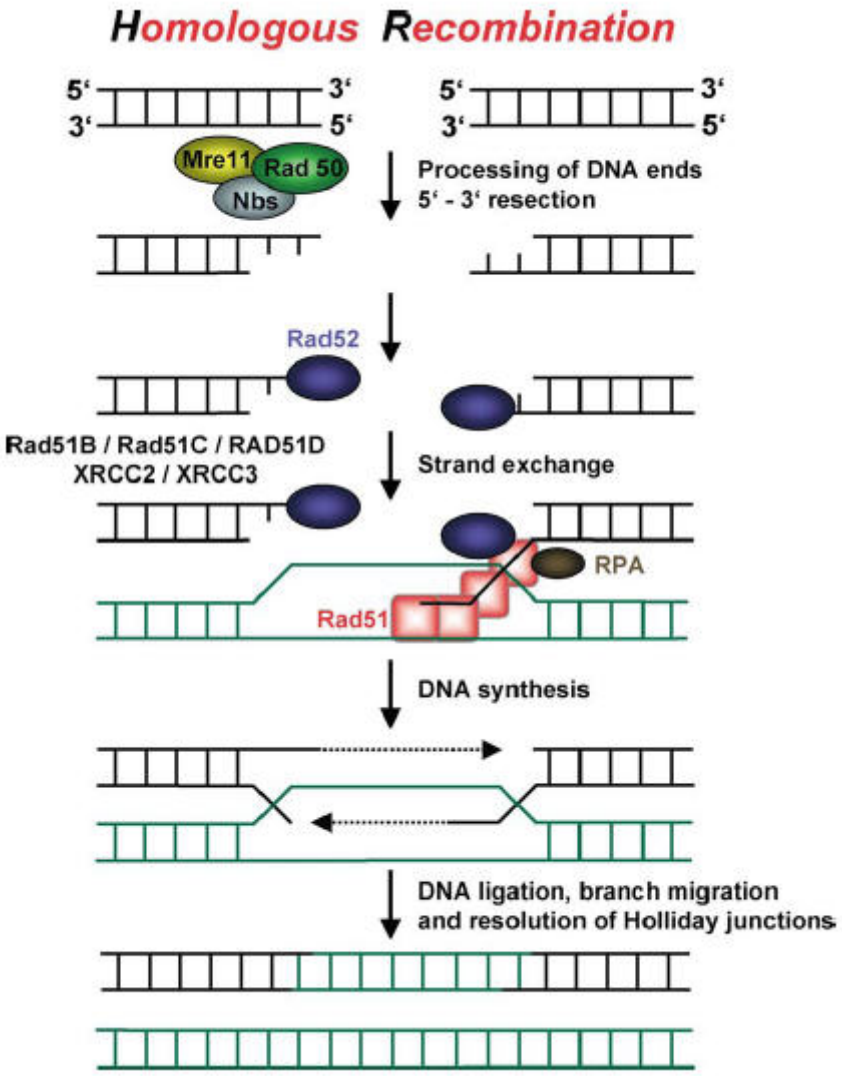


Figure 9 - Mechanism of homologous recombination (HR). Homologous recombination starts with nucleolytic resection of the DSB in the 5' → 3' direction by the MRE11–Rad50–NBS1 complex, forming a 3' single-stranded DNA fragment to which Rad52 binds. Rad52 interacts with Rad51, provoking a DNA strand exchange with the undamaged, homologous DNA molecule. Assembly of the Rad51 nucleoprotein filament is facilitated by different Rad51 paralogues (such as Rad51B, Rad51C and Rad51D, XRCC2 and XRCC3). After DNA synthesis, ligation and branch migration, the resulting structure is resolved (Christmann et al, 2003)

During HR, the damaged chromosome enters into physical contact with an undamaged DNA molecule with which it shares sequence homology and which is used as template for repair (Sonoda et al, 2001). HR is initiated by a nucleolytic resection of the DSB in the 5'-3'direction by the MRE11-Rad50-

NBS1 complex. The resulting 3' single-stranded DNA is thereafter bound by a heptameric ring complex formed by Rad52 proteins (Stasiak et al, 2000), which protects against exonucleolytic digestion. Rad52 competes with the Ku complex for the binding to DNA ends. This may determine whether the DSB is repaired via the HR or the NHEJ pathway (Van Dyck et al, 1999). Rad52 interacts with Rad51 and RPA (Replication Protein A) stimulating DNA strand exchange activity of Rad51 (New et al, 1998). The human Rad51 protein is the homologous of the *E.Coli* recombinase RecA. It forms nucleofilaments, binds single- and double-stranded DNA and promotes ATP-dependent (Benson et al, 1994) and RPA-stimulated (SIgurdsson et al, 2001) interaction with homologous region on an undamaged DNA molecule. Thereafter Rad51 catalyzes strand-exchange events with the complementary strand in which the damaged DNA molecule invades the undamaged DNA duplex , displacing one strand as D-loop (Gupta et al, 1998). The assembly of the Rad51 nucleoprotein filament is facilitated by five different paralogues of Rad51 (Rad51B, C and D; and XRCC2 and XRCC3) which could play a role during pre-synapsis (Masson et al, 2001). Another important protein that interacts with Rad51 is RPA (Golub et al, 1998). It is supposed that RPA stabilizes RAD51-mediated DNA pairing by binding to the displaced DNA strand (Eggler et al, 2002). After DSB recognition and strand exchange performed by Rad proteins, the resulting structures are resolved according to the classical model for DNA cross-over first proposed by Robin Holliday in 1964 (Holliday, 1964).

2.2 DNA repair gene polymorphisms

XRCC1

XRCC genes are a component of several different DNA damage recovery pathways, while XRCC proteins do not show similarity in biochemical functions.

A lot of information has been derived from mutant mammalian (rodent and human) cell lines showing a particular sensitivity not only to X-ray but also to other DNA damaging agents (Thacker and Zdienicka, 2003).

Common polymorphisms have been described in several XRCC genes and attempts to link them to cancer are being actively pursued.

To date, three polymorphisms have been identified in the XRCC1 gene, which result in non-conservative amino acid substitutions (Shen et al, 1998). The gene is involved in BER coding for a scaffolding protein for other repair factors.

The XRCC1 protein interacts with OGG1, PARP, DNA ligase III, and DNA polymerase β (Figure 10) to rejoin DNA strand breaks and repair gaps left during BER (Caledecott, 2003)(Brem and Janet, 2005). The protein encompasses two BRCA C-terminal (BRCT) motifs with independent and important roles. The interaction of XRCC1 and ligase III is mediated by the BRCT 2 domain, which is required in a cell cycle stage-specific pathway (Cappelli et al, 1997). The central region (amino acids 315-403) named BRCT 1, the most evolutionary conserved motif, interacts with PARP-1 and Pol β.

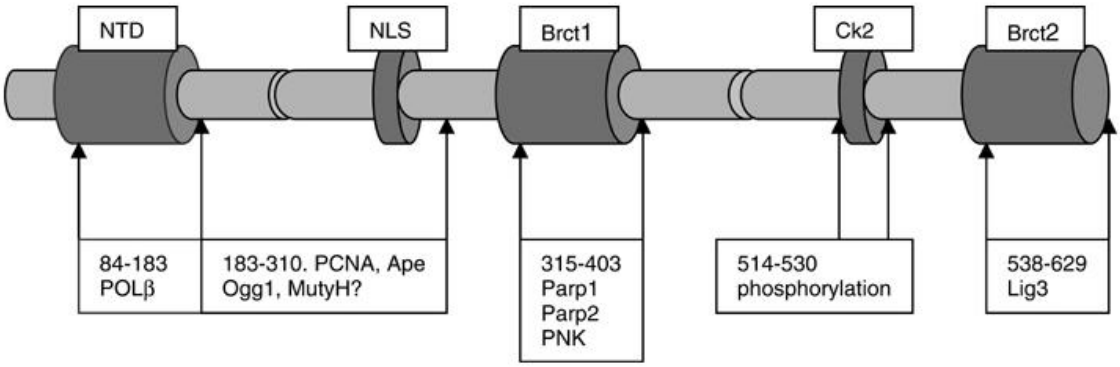


Figure 10 - Human XRCC1 domains and locations of binding sites with interactive protein partners (Ladiges WC, 2006)

A requirement for PARP for efficient repair of SSBs induced by ionizing radiation and alkylating agents has been described (Dantzer et al, 2000) and XRCC1 is recruited within seconds to the sites of DNA strand breakage. Recently it was shown that XRCC1 is phosphorylated at the BRCT I domain at Ser 371 by DNA-dependent protein kinase in response to DNA damage (Levy et al, 2006). The most frequent XRCC1 polymorphism (exon 10 codon 399, Arg to Gln) occurs in the interaction site with PARP. This may lead to a modification in repair activity, and XRCC1 Gln399 allele has been reported as a risk factor for different types of cancer (Table 2) including head-neck (Sturgis et al, 1999), bladder (Stern et al, 2001), lung (Divine et al, 2001), and glioma (Wang et el, 2004).

OGG1

OGG1, also involved in BER, has both DNA glycosylases and apurinic/apyrimidinic lyase activities to excise 8-oxoguanine (8-OH-G) opposite cytosine and hydroxyl-formamidopyrimidine. OGG1 is phosphorylated in vivo and in vitro by kinases (Cdk4 and c-Abl) but the functional consequences of these modifications are only partially characterized (Hu et al, 2005). Increased OGG1 expression has been shown to correlate with increased repair activity of 8-OH-G (Kondo et al, 2000). Several SNPs of OGG1 with amino acids substitutions have been described, and Ser326Cys is the most common. Case-control studies on this polymorphism suggest that OGG1Cys326 may be a risk factor for a variety of human cancers (Table 2)(Ito et al, 2002)(Takezaki et al, 2002), even though this polymorphic amino acid is located outside the domains conserved among DNA glycosylases. The functional differences between the 2 polymorphic OGG1 proteins in human cells still remain unclear. Yamane et al (2004) suggested that OGG1-Cys326 protein had a lower ability to suppress mutation than wild-type protein in human cells in vivo.

XPC

SNPs have been also found in 5 (XPA, XPC, XPD, XPF, and XPG) of the 7 NER genes of XP complementation groups (Shen et al, 1998). NER proteins repair oxidative damage, abasic sites, C-C mismatches, and bulky adducts.

XPC proteins may complex with HR23B to function as a damage sensor and repair recruitment factor in the NER pathway. DNA damage is recognized by the XPC-HR23B complex, followed by recruitment of the TFIIH complex of proteins (Sugasawa et al, 1998). Although many of the polymorphisms found in XP genes do not change the amino acid sequence and are poorly characterized at present, one polymorphism in XPC (exon 15 codon 939) leads to a Lys to Gln substitution and, even if its functional relevance has not been determined (Khan et al, 2000), it was associated with reduced repair of radiation-induced DNA damage (Vodicka et al, 2004). A case-control study has described a possible contribution from this polymorphism to the risk of developing head and neck cancer (Shen et al, 2001)(Table 2).

XRCC3

XRCC3 protein is a member of a family of Rad 51-like proteins (Liu et al, 1998) that participate in homologous recombination to maintain chromosome stability and repair DNA double strand-breaks (Pierce et al, 1999). The substitution Thr to Met in codon 241 (due to a transition C>T) does not reside in the ATP-binding domains, which are the only functional domains identified in the protein at this time (Shen et al, 1998). No association has been found between the variant allele and the development of lung cancer (David-Beabes et al, 2001), while a statistically significant increase in variant allele frequency was reported in melanoma skin cancer and bladder cancer (Matullo et al, 2001)(Table 2).

Table 2 – List of some case-control studies involving DNA repair gene SNPs and the increased risk of developing cancer

SNP	Cancer	Reference
XRCC1 399 G>A	Bladder	*Stern et al, 2001; Matullo et al, 2001*
	Breast	*Duell et al, 2001*
	Head-Neck	*Sturgis et al, 1999*
	Lung	*Divine et al, 2001*
	Skin	*Nelson et al, 2002; Sturgis et al, 1999*
	Glioma	*Wang et al, 2004*
OGG1 326 C>G	Oesophagus	*Xing et al, 2001*
	Lung	*Wilkman et al, 2000; Ito et al, 2001*
	Stomach	*Takezaki et al, 2002*
	Prostate	*Xu et al, 2002*
XPC 939 A>C	Bladder	*Sanyal et al, 2004*
	Head-Neck	*Shen et al, 2001*
XRCC3 241 C>T	Lung	*Butkiewicz et al, 2001*
	Skin	*Matullo et al, 2001*

3. GLUTATHIONE S-TRANSFERASES

Human GSTs belong to a multi-gene family of four different classes of detoxification isozymes (α, μ, π, θ) (Hayes et al, 2005), which are involved in detoxification of xenobiotics by conjugating a wide range of different chemicals with reduced GSH (Table 3). GST M1 (μ, mu class), T1 (θ, theta), P1 (π, pi) and A1 (α, alpha) are known to be polymorphic.

Table 3 - Substrate preferences of human glutathione-S-transferases (modified from Haynes JD et al, 2005)

Class Enzyme	Substrate of reaction *
Alpha, A1-1	*Δ⁵-ADD, BCDE, BPDE, Busulfan, Chlorambucil, DBADE, DBPDE, BPhDE, N-a-PhIP*
Alpha, A2-2	*CuOOH, DBPDE, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole*
Alpha, A3-3	*Δ⁵-ADD, Δ⁵-pregnene-3,20-dione, DBPDE*
Alpha, A4-4	*COMC-6, EA, 4-hydroxynonenal, 4-hydroxydecenal*
Alpha, A5-5	*CDDO-Im*
Mu, M1-1	*trans-4-phenyl-3-buten-2-one, BPDE, CDE, DBADE, trans-stilbene oxide, styrene-7,8-oxide*
Mu, M2-2	*COMC-6, 1,2-dichloro-4-nitrobenzene, aminochrome, dopa O-quinone, PGH2→PGE2*
Mu, M3-3	*BCNU, PGH2→PGE2*
Mu, M4-4	*CDNB*
Mu, M5-5	*low for CDNB*
Pi, P1-1	*acrolein, base propenals, BPDE, CDE, Chlorambucil, COMC-6, EA, Thiotepa*
Theta, T1-1	*BCNU, butadiene epoxide, CH2Cl2, EPNP, ethylene oxide*
Theta, T2-2	*CuOOH, menaphthyl sulfate*

*Abbreviations: **Δ⁵-ADD**, Δ⁵-androstene-3,17-dione; **BCDE**, benzo[g]chrysene diol epoxide; **BCNU**, 1,3-bis(2-chloroethyl)-1-nitrosourea; **BPDE**, benzo[a]pyrene diol epoxide; **BPhDE**, benzo[c]phenanthrene diol epoxide; **CDE**, chrysene- 1,2-diol 3,4-epoxide; **COMC-6**, crotonyloxymethyl-2-cyclohexenone; **DBADE**, dibenz[a,h]anthracene diol epoxide; **DBPDE**, dibenzo[a,l]pyrene diol epoxide; **EA**, ethacrynic acid; **EPNP**, 1,2-epoxy-3-(pnitrophenoxy)propane; **N-a-PhIP**, N-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; **CDDO-Im**, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; **CDNB**, chloro-dinitro benzene

The GSTM1 and GSTT1 genes are involved in the detoxification of diol epoxides derived from the metabolism of polycyclic aromatic hydrocarbons (PAH). There is evidence that a GSTM1 deletion, present in both alleles, affecting 50% of Caucasians and causing a total lack of enzyme activity, is weakly associated with lung cancer in smokers (Rebbeck, 1997) and that the risk could be higher in GSTM1-null females than in males (Tang et al, 1998).

However, recent meta-analysis and pooled analysis did not confirm these results (Ye et al, 2006).

A similar deletion polymorphism is also present in the GSTT1 gene, and recent studies indicated that GSTT1-null genotype was associated with an increased risk of prostate cancer among smokers (Kelada et al, 2000). A recent pooled analysis (Kirsch-Volders et al, 2006) indicated that GSTT1-null subjects have lower micronucleus (MN) frequencies than their GSTT1-positive counterparts.

Two other GSTs, GSTP1 and GSTA1, which are abundant in lung and liver, respectively (Rowe et al, 1997), are important catalysts for glutathione conjugation with benzo(*a*)pyrene diol epoxide (BPDE) and other PAH-derived diol epoxides. The GSTP1 gene has two different SNPs in the coding region, which produce four different alleles: the wild-type allele *A (codon 105 Ile, codon 114 Ala) and the variant alleles *B (105 Val, 114 Ala), *C (105 Val, 114 Val) and *D (105 Val, 114 Val) (Ali-Osman et al, 1997). These isoforms have different efficiencies in conjugating and metabolizing tobacco-smoke substrates, with GSTP1*C and *B being the most efficient (Sundberg et al, 1998).

The polymorphism in the GSTA1 gene contains three linked base-substitutions in the promoter region, at positions -567, -69 and -52, which results in a differential expression (Coles et al, 2001) with a lower transcriptional activation reported for the GSTA1 variant allele than for the GSTA1 wild-type allele (Morel et al, 2002). In a recent study it has been found that different GSTA1 genotypes are related to breast cancer among current smokers (Ahn et al, 2006).

4. COMET ASSAY

Over the past decade, the Comet assay, or SCGE has become one of the standard methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair. The assay attracts adherents by its simplicity, sensitivity, versatility, speed and economy.

In the 1970s, Peter Cook and collaborators (1976) developed an approach to investigating nuclear structure based on the lysis of cells with non-ionic detergent and high-molarity sodium chloride. This treatment removes membranes, cytoplasm, and nucleoplasm, and disrupts nucleosomes, almost all histones being solubilized by the high salt. What is left is the nucleoid, consisting of a nuclear matrix or scaffold composed of RNA and proteins, together with the DNA, which is negatively supercoiled as a consequence of the turns made by the double helix around the histones of the nucleosome. The survival of the supercoils implies that free rotation of the DNA is not possible; Cook et al (1976) proposed a model with the DNA attached at intervals to the matrix so that it is effectively arranged as a series of loops, rather than as a linear molecule. When the negative supercoiling was unwound by adding the intercalating agent ethidium bromide, the loops expanded out from the nucleoid core to form a “halo”. A similar effect was seen when ionizing radiation was used to relax the loops – one single-strand break being sufficient to relax the supercoiling in that loop.

The comet assay, too, in its most commonly used form, involves lysis with detergent and high salt, after embedding cells in agarose so that the DNA is immobilized for subsequent electrophoresis. The first demonstration of “comets” was by Ostling and Johanson (1984), who described the tails in terms of DNA with relaxed supercoiling and referred to the nucleoid model of Cook. Essentially, the comet tail seems to be simply a halo of relaxed loops pulled to one side by the electrophoretic field.

The comet assay is most commonly applied to animal cells, whether in culture or isolated from the organism (e.g., whole blood or lymphocytes separated from blood, or cells from disaggregated tissues). However, methods have also been developed to examine damage in the DNA of plant cells. The

cellulose plant cell wall presents a barrier to the release of DNA to form a comet tail, but physically chopping up the tissue with a knife releases nuclei that can be embedded in agarose (Koppen and Angelis, 1998).

The procedures of Ostling and Johanson, who employed a pH of less than 10 for electrophoresis buffer, was not widely adopted.

A few years later, two research groups independently developed preocedures involving treatment at high pH (Singh et al, 1988)(Olive et al, 1990). The use of alkali makes comet tails more pronounced and extends the useful range of damage that can be detected (Collins et al, 1997).

To make the assay more specific as well as more sensitive, an extra step of digesting the nucleoids with an enzyme that recognizes a particular kind of damage and creates a break was introduced. Thus endonuclease III is used to detect oxidized pyrimidines (Collins et al, 1993), formamidopyrimidine DNA glycosylase (FPG) to detect the major purine oxidation product 8-oxoguanine as well as other altered purines (Dusinska and Collins, 1996), T4 endonuclease V to recognize UV-unduced CPDs (Collins et al, 1997), and Alk A incises DNA at 3-methyladenines {Collins et al, 2001}. In each case, the enzyme-sensitive sites converted to additional breaks increase tail intensity.

4.1 Comet Assay and DNA repair

Theoretically, a sound approach to measure repair capacity is to inflict DNA damage on cells and to monitor the speed with which they remove the lesions. Thus, lymphocytes can be treated with ionizing radiation, or H_2O_2, and rejoining of breaks followed; or, after treatment with base-damaging chemicals, and incubation, the remaining lesions can be assayed by use of an appropriate endonuclease on the gel.

Rejoining of DNA strand breaks by most cell types is known to be a rapid process, with a half-time of a few minutes (Frankenberg-Schager, 1989), and these kinetics are seen with the Comet assay, too. Repair of endonuclease III- or FPG-sensitive sites (i.e., oxidized purines and oxidized pyrimidines), by BER, is a slower process, requiring a few hours (Collins and Horvathova, 2001). Repair of UV-induced CPDs by nucleotide excision repair can be detected with the enzyme endonuclease V. This, too, is a relatively slow process (Collins et al, 1997).

Another method to measure DNA repair activity with Comet assay is the in vitro DNA repair assay (Coolins et al, 2001) In this assay the nucleoids are derived from cells with a certain amount of specific DNA damage; they act as a substrate, and are incubated with lymphocyte extract of unknown activity, in place of the purified repair enzymes normally employed. The method has been applied to human lymphocyte samples, and revealed consistent inter-individual differences in repair activity on a DNA substrate containing 8-oxoguanine (Collins et al, 2001). Analogous experiments performed on extract from cultured cells from the *Ogg1*$^{-}$ knockout mouse (Klungland et al, 1999), compared with cells from a wild-type mouse line, indicate that the activity measured is predominantly that of the OGG1 protein.

4.2 Comet Assay and radiosensitivity

Ionizing and UV radiation, as easily quantifiable genotoxins, could be delivered reproducibly to cells without concerns of stability, delivery metabolism or detoxification. Moreover, exposure to one Grey of ionizing radiation was known to produce about 1000 single- and ~30 double-strand breaks per diploid mammalian cell, and the extent of initial damage was regarded as being cell type independent.

The study of radiation effect on living systems encompasses a broad area of research interests including the nature of oncogenic and cytotoxic lesions induced in DNA by ionizing and non-ionizing radiations, factors that affect repair of these lesions, and development of ways to enhance the efficicacy of radiation in treating solid tumors. Many of these areas have been influenced by the development of the Comet assay (Olive, 1999).

There are many methods capable of measuring DNA DSBs and SSBs with good sensitivity. Since the Comet assay is based on the measurement of damage to DNA, an important advantage of this method is that a measure of DNA content is routinely provided by most comet analysis software.

Although exposure to IR generally produces a random distribution of DNA damages within a population of irradiated cells, the absence of oxygen or presence of exogenous thiols can protect cells from this damage. In this case, heterogeneity can be used to calculate the fraction of radiation resistant cells. The initial interest in examining damage and repair in single cells was driven by

the goal to understand the resistance of cells to DNA damage by radiation and etoposide that developed when cells were grown in close three-dimensional cell contact (Olive, 1989).

Another important advantage of analyzing individual cells is that relatively few cells are required. This makes it possible to conduct clinical studies using tumor cells obtained from a single fine needle aspirate biopsy (Olive et al, 1999) or blood cells from a finger prick (Garcia and Mandina, 2005). The small cell number required for this method also makes it pratical to combine various cell sorting technologies with the Comet assay (Olive et al, 1991).

It has been estimated that each mammalian cell under normal growth conditions is subject to several thousand DNA damages per day that include base loss, base alterations, and strand breakage (Loft and Poulsen, 1996). However, unlike single strand breaks and base damages that are repaired as a matter of course by most cells, DNA DSBs and other complex DNA lesions are relatively rare and much more difficult to repair (Olive PL, 1998). Unrejoined DSBs are likely to be lethal, and misrejoined DSBs can cause chromosome aberrations (CAs) and cell death (Fankenberg-Schwager and Frankenberg, 1990) In 1980s, interest moved from detection of SSBs to the development of methods that could detect the DNA lesion associated with lethality and chromosome damage by IR.

Although it is fortunate that DSBs are produced relatively infrequently, this poses the problems for their detection. Twenty to forty times fewer DSBs than SSBs are produced per Gy so that doses in excess of 4 Gy are tipically required to detect these lesions using neutral versions of the Comet assay.

When the population contains a high percentage of cells which are replicating their DNA, sensitivity for detecting DSBs is reduced even further. This occurs because replication bubbles associated with cells in S phase retard migration by a factor of three or more (Olive et al, 1991). Conversely, under alkaline conditions, replication forks in S phase cells behave as single-strand breaks and so more DNA in S phase cells can migrate (Banath et al, 2004). A slight increase in extent of DNA migration from irradiated G1 phase cells compared to G2 phase cells has been observed (Olive and Durand, 2005), probably reflecting differences in chromatin organization. Therefore increases or

decreases in average comet tail moments or percent DNA in tail can occur simply by changing the distribution of cells in the cell cycle.

Exposure to IR, and particularly dense IR like alpha particles and heavy ions, creates multiple DSBs in close proximity. This is likely to explain the greater heterogeneity in comet appearance which is observed after exposure to heavy ions (Testard and Sabatier, 2000). Although clusters of breaks cannot be accurately identified after exposure to dense IR, their reduced ability to be rejoined attests to the complexity of the damage (Stenerlow et al, 2000). Recently, the Comet assay was used to confirm that SSBs were insufficient to activate the DNA repair kinase, ATM, and that DSBs were required (Ismail et al, 2005).

The introduction of histone H2AX phosphorylation as an ultra-sensitive indicator of the presence of DNA DSBs (Rogakou et al, 1998)(sensitivity at the mGy level is possible) has largely supplanted physical methods for the detection of DSBs (Sedelnikova et al, 2003). However, it is important to appreciate that the neutral Comet assay is able to detect the actual DSB and measure its rejoining whereas H2AX is a surrogate of this process which is influenced by other DNA damage signalling events and chromatin events within the cell. The two approaches should therefore be viewed as complementary (Olive, 2007).

DNA DSBs induced by IR can be repaired using the NHEJ pathway or by HR. Lack of NHEJ activity results in inability to rejoin a significant percentage of DNA DSBs, and the Comet assay has been used to confirm this deficiency in end-joining activity (Wojewodzka et al, 2007).

The Comet assay can be useful functional assay of repair capacity that can complement genetic analysis of DNA repair enzymes or gene polymorphisms (Jones et al, 2007)(Cornetta et al, 2006). However, initial DNA damage is dependent primarily on radiation dose, and the initial rate of rejoining of DSBs can appear normal for cell lines that are known to be deficient in HR. This means that the Comet assay may be able to identify only a subset of DNA repair deficiency phenotypes. Using low dose rate radiation exposures can enhance the ability to detect differences in DNA repair capacity (Cassoni et al, 1992).

Several investigators have used the Comet assay to determine whether lymphocytes from patients who experience normal tissue toxicity after irradiation are more likely to be deficient in repair of radiation-induced DNA damage (Popanda et al, 2003). There is no clear consensus on this issue probably because normal tissue toxicity is a complex trait that is likely to involve many factors in addition to DNA repair capacity (Bentzen, 2006). Large differences in radiosensitivity have been associated with relatively small variations in repair capacity (Kasten-Pisula et al, 2005).

IR is also an effective oxidizing agent, producing hundreds of different types of base damages, many of which are substrates for endonuclease III and FPG (Wallace, 1998). The ratio of radiation-induced SSBs to enzyme sensitive cells was about 2:1 (Banath et al, 1999).

Unirradiated cells in proximity to irradiated cells or exposed to their medium can demonstrate an indirect effect of IR, which is termed a bystander effect. Resulting DNA damage, evidenced by MN and gene mutations (Ponnaiya et al, 2004), may be caused by clastogenic factors including cytokines or ROS released by the irradiated cells. A small increase in tail moment and tail length has been demonstrated in bystander cells immediately after irradiation (Przybyszewski et al, 2004).

II. AIM OF THE RESEARCH

The aim of this research was to investigate the genetic basis of individual response to DNA damage due to exogenous exposures. Among the agents potentially affecting individual response both physical (IR) and chemical agents have been considered.

A) In the first part of this research project, the DNA repair capacity of healthy subjects in response to X-ray irradiation was analysed in relation to different genotypes. The peripheral blood of 50 healthy subjects was irradiated in vitro with 2 Gy of X rays and the induced DNA damage was measured by Comet assay (Tail DNA - TD) immediately after irradiation. DNA repair was been detected by analysing the cells at defined time intervals after the exposure. Furthermore, all subjects were genotyped for XRCC1, OGG1, XPC genes.

Radiation-induced DNA damage and its repair play a critical role for the susceptibility of patients to side effects after RT. Therefore there is much interest among clinicians for in vitro detection of cellular radiosensitivity as an indicator of the extent patient's normal tissue reaction, in order to adjust RT protocol for both sensitive and resistant patients. In order to find a correlation between cellular radiosensitivity and the clinical radiosentitivity of patients, breast cancer patients were enrolled in S.Camillo Hospital (Rome-Italy). Peripheral blood of these patients was irradiated in vitro with X rays (2 Gy) and the induced DNA damage was measured by Comet assay. All subjects were genotyped for XRCC1 and OGG1 genes. Until now 25 breast cancer patients have been enrolled.

B) The second topic discussed in this thesis is the influence of genetic polymorphisms in DNA repair genes and in GST genes after two different exposure conditions.

- Healthy subjects exposed to tobacco smoke: two different cytogenetic assays, the MN and CA assays, were applied to assess persistent damage at the chromatid or chromosome level. Moreover, the correlation between the effects of tobacco smoke on DNA damage induction and some polymorphisms in genes that encode metabolic enzymes (GSTM1, GSTT1, GSTP1 and GSTA1) has been studied.
- Population of nurses handling antineoplastic drugs: the alkaline Comet assay and the micronucleus (MN) test were used to evaluate

DNA and chromosome damage induced by antineoplastic drug exposure in hospital personnel who handle mixtures of chemotherapeutic drugs while following the expected safety precautions. Furthermore all the examined subjects were genotyped for two genes, XRCC1 and XRCC3 in order to analyse the possible influence of the genetically determined variations on individual response to exogenous insults.

C) Finally, to better understand the role of XRCC1, the biological responses induced by X-ray treatment in two hamster cell lines (AA8 and EM9) were analysed. EM9 cells, derived from the parental cell line AA8, are mutant deficient in DNA repair. We studied: -a) the induction of SSBs and the repair kinetics by Comet assay; -b) the induction and the time evolution of DSBs through the analysis of the expression of phosphorylated histone H2AX; -c) the modulation of cell cycle through the flowcytometric analysis of Bromodeoxy Uridine (BrdU) incorporation.

III. RESULTS

PART A
"DNA damage repair and genetic polymorphisms: assessment of individual sensitivity and repair capacity"

DNA repair assessment in healthy subjects

The study involved a total of 50 healthy subjects (Table 4) who were recruited at random regardless of age and gender. Information concerning smoking history, medical history, and occupational exposure was collected from interview-administered questionnaires. Individuals having potential "confounding factors" other than smoking (drug and alcohol consumption, recent radio-diagnostic exposure, major illness) were excluded. A smoker was defined as one who had been smoking up until 1 month before blood collection and who had reported to smoke no fewer than 10 cigarettes per day.

Table 4 - Tail DNA in study subjects before, immediately and 30 and 60 min post irradiation (Cornetta et al , 2006)

Variable		N° of subjects	TD (Mean ± SD)				
			Control	2Gy (min after X ray)			
				0'	30'	60'	P*
All		50	1,06±0,65	7,83±4,16†	4,59±2,67†	3,89±2,91†	<0,0001
Age	≤40	36	1,17±0,69	7,96±4,23†	4,67±2,62†	4,40±3,20‡	<0,0001
	>40	14	0,79±0,48	7,49±4,08†	4,37±2,90‡	2,56±1,27	<0,0001
Gender	M	14	0,93±0,47	7,89±4,70†	5,16±3,88§	3,89±4,03	<0,0001
	F	36	1,12±0,70	7,80±3,99†	4,36±2,06†	3,88±2,41†	<0,0001
Smoke	NS	36	1,11±0,71	7,00±3,22†	4,16±2,41†	3,84±2,97‡	<0,0001
	S	14	0,96±0,45	9,96±5,52†	5,70±3,07‡	4,00±2,86	<0,0001

* Statistical significance calculated with Friedman Test (non-parametric-ANOVA) , obtained when comparing all groups.
† p<0,001 calculated with Post-Test when comparing each group with control one.
‡ p< 0,01 calculated with Post-Test when comparing each group with control one.
§ p<0,05 calculated with Post-Test when comparing each group with control one.

We stratified the subjects for age: younger persons < 40 years (72%) and persons > 40 years (28%); for gender: 14 males and 36 females. The distribution of smokers (S) and non-smokers (NS) was 28% and 72%, respectively. The frequencies of the analyzed polymorphic alleles for XRCC1, OGG1 and XPC in the studied population were, respectively 0.32, 0.28 and

0.56 consistent with literature data (Shen et al, 1998)(Nohmi et al, 2005)(Takezaki et al, 2002).

There were no significant differences in the mean levels of TD values by gender, age, and smoking habits, nor in control condition or after irradiation. However the levels of TD were elevated in exposed situations compared with controls, and a significant difference ($p < 0.0001$ at Friedman test) was always observed when comparing TD values at time 30 min and 60 min after irradiation with 0 time (Table 4).

The contribution of the various genotypes to the in vitro DNA strand-breaks repair was assessed by comparing the in vitro repair capacity of the DNA damage induced by 2 Gy X-rays in subjects with different XRCC1, OGG1 and XPC genotypes.

Figure 11 summarizes the mean TD values obtained from our subjects, in control conditions and after X-ray treatment at successive sampling times, stratified by genotypes. None of the analyzed genotypes was found to modulate TD baseline values. After irradiation, persons with XRCC1 variant Gln399Gln genotype exhibited lower values of TD than those with XRCC1 wild-type Arg399Arg and heterozygote Arg399Gln genotypes. These differences are statistically significant at time 0 ($p < 0.001$) and 30 and 60 min after irradiation ($p < 0.05$).

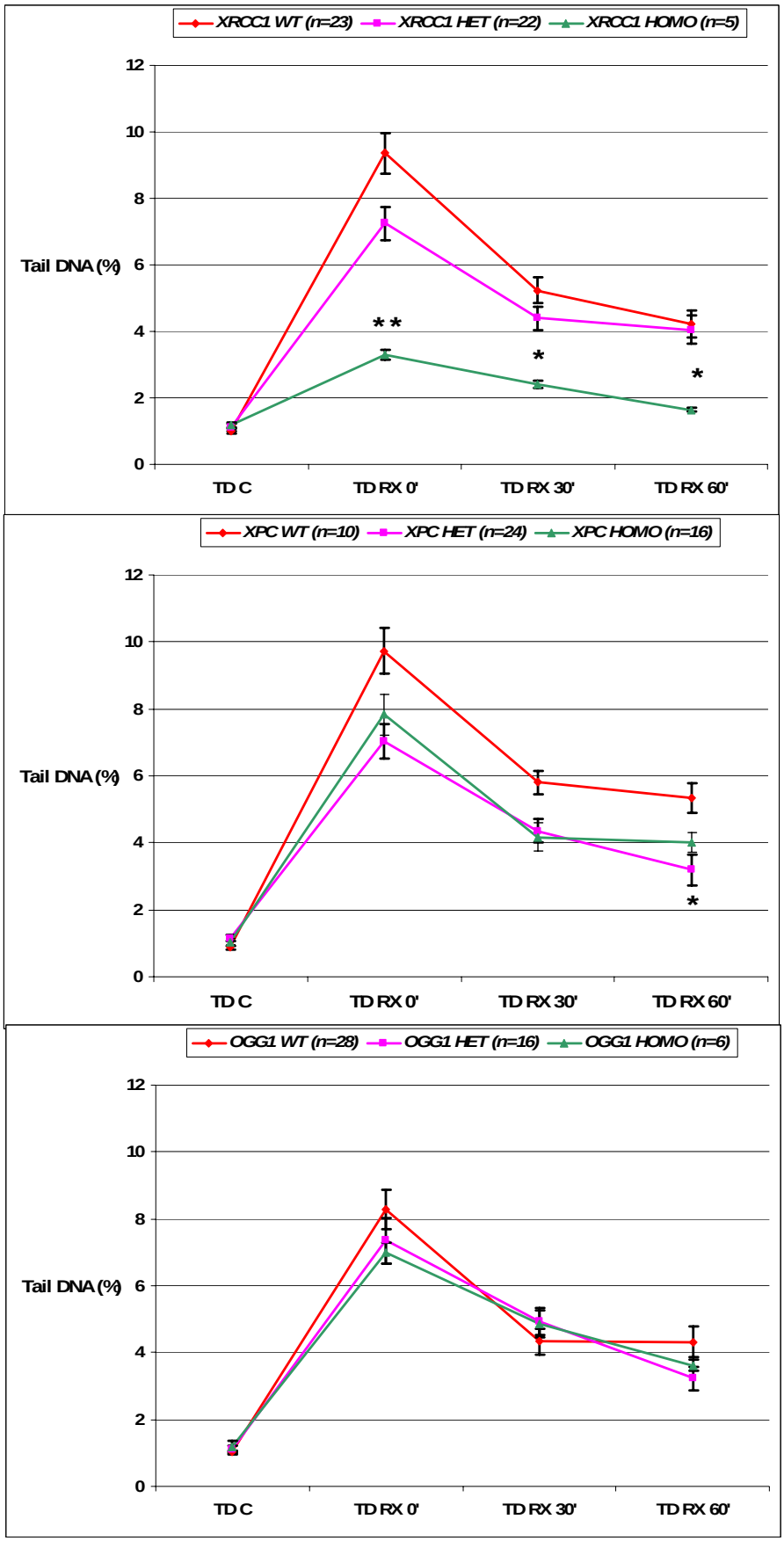


Figure 11 - DNA damage induced by X-ray treatment measured immediately after and 30 and 60 min post irradiation. Data are stratified by XRCC1 codon 399, OGG1 codon 326, and XPC codon 939 genotypes. *p < 0.05; **p < 0.001. TD = Tail DNA (Cornetta et al , 2006)

A significantly lower level of TD ($p < 0.05$) was found only at time 60 min, in persons bearing XPC heterozygote Lys939Gln genotype compared with those with XPC wildtype Lys939Lys and variant Gln939Gln genotypes. No relationship was found between OGG1 codon 326 genotype and TD values.

The distribution pattern of the data is presented as a three-dimensional plot in Figure 12.

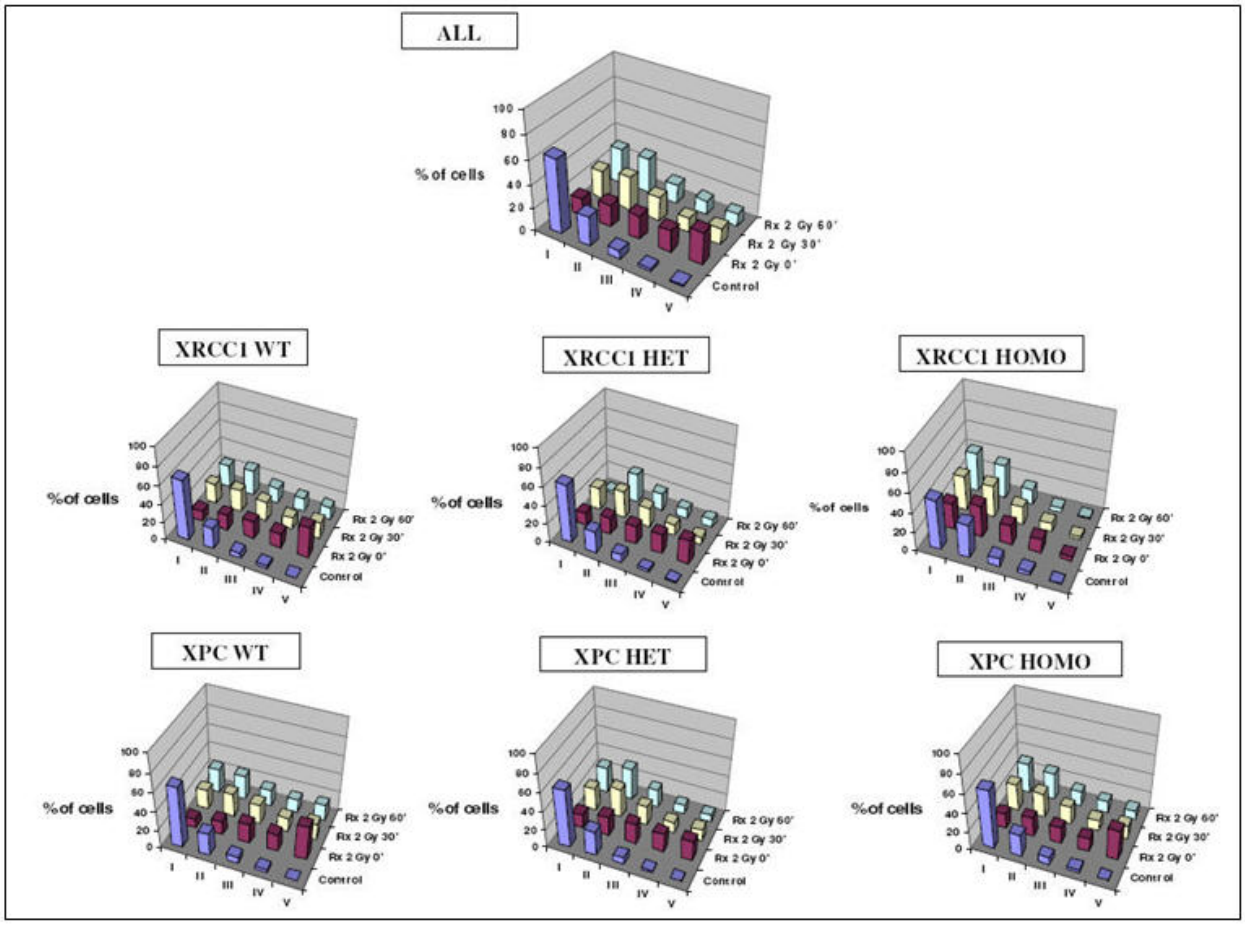


Figure 12 - Distribution of Tail DNA (TD) values in cells from subjects taken all together and stratified for XRCC1 codon 399 and XPC codon 939 genotypes. Values of each class: I = 0; II = 1–3; III = 4–6; IV = 7–10; V ≥ 10. HET = heterozygous; HOMO = homozygous; WT = wild-type (Cornetta et al , 2006)

The figure shows the TD distribution (from class I to class V corresponding with TD values 0, 1–3, 3–6, 6–10, >10) in the cells scored for all subjects taken together (A) and for each single XRCC1 codon 399 (B) and XPC codon 939 (C) genotype. The data are divided into control and 3 sampling times. Immediately after irradiation (time 0) the cells are distributed in the range of TD

values from 0 to > 10, regardless of genotypes, with a prevalence of cells (46%) in the range 6–10 (class IV and V) in all cases apart from XRCC1 variant Gln399Gln cells (60% of cells in class I and II). At 60 min more than 76% of cells from XRCC1 variant Gln399Gln subjects are distributed in the class I–II, corresponding to very low damage and only 7% in classes IV and V; the plots for the other genotypes show the permanence of 20–40% of cells in classes IV and V (heavy damage). In conclusion, these data show that cells from XRCC1 variant Gln399Gln genotypes never bear heavy DNA damage, nor immediately or after DNA repair occurs.

Table 5 – Influence of XRCC1 codon 399, XPC codon 939 and OGG1 codon 326 genotypes on Residual % DNA Damage at 30 and 60 min post irradiation (Cornetta et al , 2006)

Genotypes	RD 30' (±SD)	P*	RD 60' (±SD)	P*
XRCC1				
Arg399Arg	53,11±27,78		40,17±30,10	
Arg399Gln	47,46±33,74		41,06±27,89	
Gln399Gln	53,44±31,59		**17,74±16,26**	**<0,05**
XPC				
Lys939Lys	60,71±19,60		49,33±24,58	
Lys939Gln	49,38±30,39		**30,53±28,32**	**<0,05**
Gln939Gln	46,28±35,80		43,11±29,16	
OGG1				
Ser326Ser	48,77±29,28		38,43±27,12	
Ser326Cys	52,53±30,01		37,16±34,01	
Cys326Cys	54,47±40,95		40,86±21,98	

*Significantly different biomarker levels are shown in bold, including P values (Mann - Whitney U-test), obtained when comparing polymorphic genotypes with wild type ones.

Table 5 shows the contribution of XRCC1, XPC and OGG1 genotypes to the in vitro repair capacity of DNA damage induced by X rays, measured by RD30 and RD60 (RD – Residual % DNA damage) values. At 30 min from treatment, no significant difference in RD was found between different genotypes. At 60 min, XRCC1 variant Gln399Gln genotypes show a significantly lower RD ($p < 0.05$) compared with XRCC1 wild-type Arg399Arg and Arg399Gln genotypes. The XPC genotypes do not show any different

significant contribution to RD at 30 min. At 60 min after irradiation, XPC Lys939Gln genotypes show a significant ($p < 0.05$) lower RD value compared with XPC wild-type Lys939Lys and variant Gln939Gln genotypes. No differences were found in RD30 and 60 values for OGG1 codon 326 genotype.

To analyze the specificity of the genotype/phenotype response to X-ray–induced damage at the individual level, we evaluated the individual repair capacity with respect to XRCC1, XPC and OGG1 genotypes, taking into account the RD60 values of each subject.

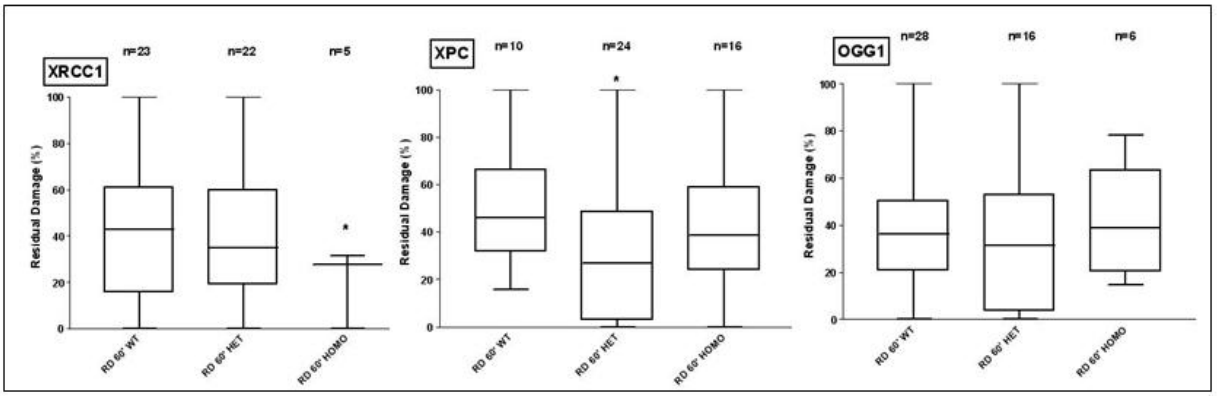


Figure 13 - The distribution of residual DNA (RD) damage 60 min post irradiation in XRCC1 codon 399, OGG1 codon 326 and XPC codon 939 genotypes. The upper hinge defines the 75 percentile and the lower the 25 percentile; the line represents the median value; the vertical bar represents max and min value. *$p < 0.05$. HET = heterozygous; HOMO = homozygous; WT = wild-type (Cornetta et al , 2006)

Results in Figure 13 show that the RD 60 min after irradiation was lower in persons with the XRCC1 variant Gln399Gln genotype compared with those with XRCC1 Arg399Arg and Arg399Gln genotypes. XPC Lys939Gln persons also show significantly lower mean RD 60 values compared with XPC Lys939Lys and Gln939Gln genotypes. There was no significant contribution of OGG1 codon 326 polymorphism on RD values.

Table 6. Mean Tail DNA values in relation to combination of different genetic polymorphisms (Cornetta et al , 2006)

Genotypes		N° of subjects	Mean Tail DNA ± SD			
			TD C	TD RX0'	TD RX30'	TD RX60'
XRCC1 399 Arg→Gln	**OGG1 326 Ser→Cys**					
Wt	Wt	11	0,92±0,46	10,33±4,21	4,93±2,61	5,15±3,18
Var	Wt	17	1,10±0,36	6,95±3,60	3,93±2,65	3,78±3,37
				***p=0,0369**		
Wt	Var	12	1,07±0,71	8,47±4,45	5,52±3,17	3,36±2,50
Var	Var	10	1,17±1,07	5,79±3,57	4,19±2,05	3,29±2,02
				***p=0,0295**		
XRCC1 399 Arg→Gln	**XPC 939 Lys→Gln**					
Wt	Wt	6	0,90±0,47	10,86±5,63	5,91±2,99	6,11±3,34
Var	Wt	4	0,81±0,13	8,02±3,12	5,64±1,70	4,21±2,90
Wt	Var	17	1,03±0,64	8,8±3,86	5,00±2,88	3,55±2,55
Var	Var	23	1,17±0,73	6,25±3,63	3,75±2,43	3,49±2,96
						***p=0,0307**
XPC 939 Lys→Gln	**OGG1 326 Ser→Cys**					
Wt	Wt	3	1,10±0,16	11,20±6,07	4,73±1,33	7,50±3,80
Var	Wt	25	1,01±0,42	7,92±3,86	4,27±2,76	3,94±3,11
Wt	Var	7	0,76±,38	9,09±4,55	6,26±2,74	4,42±2,61
Var	Var	15	1,28±0,99	6,39±3,90	4,28±2,60	2,81±1,93

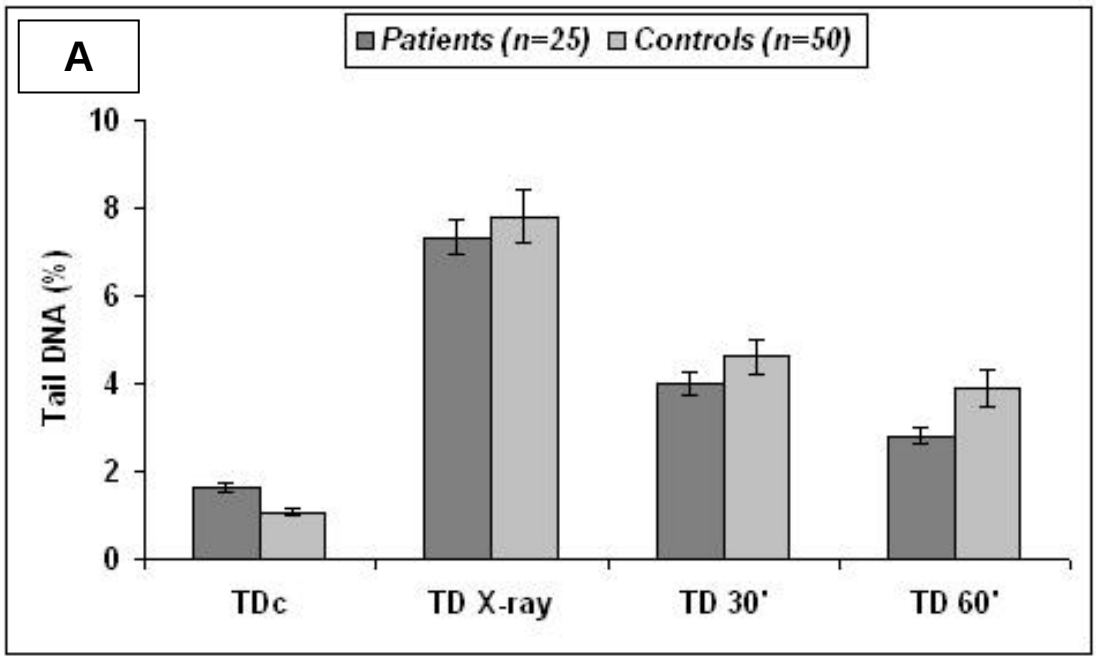
Abbreviations: Var, homozygous + heterozygous variant genotypes; Wt, wild-type genotypes
*p-Value based on Mann - Whitney U-test obtained when comparing combinations bearing at least one polymorphic allele (Var-Wt, Wt-Var and Var-Var) with Wt-Wt.

In Table 6, we show the data on genotype combinations and their association with TD. The data are presented as a combination between genotypes bearing at least 1 polymorphic allele (homozygous plus heterozygous), indicated as variant and wild-type genotypes. The combination of XRCC1 variant genotypes with OGG1 wild-type and variant genotypes shows significantly lower TD values at time 0' ($p = 0.03$) compared with wild-type genotype combination. The combination of XRCC1 variant genotypes with XPC variant genotypes also shows lower TD values at time 60 min ($p < 0.03$) compared with wild-type genotype combination. Binary combinations of XPC and OGG1 genotypes did not significantly affect DNA damage, even if TD at time 60 min was lower in subjects bearing the combination of XPC variant with OGG1 variant genotypes.

DNA repair assessment in breast cancer patients

To continue on the analysis of the association between DNA damage induced by X-ray treatment in vitro and the genetic background, a new investigation was started in order to find a correlation between cellular radiosensitivity and clinical radiosentitivity in cancer patients.

Breast cancer patients had been admitted in S. Camillo Hospital (Rome-Italy). Peripheral blood of cancer patients was irradiated in vitro with X rays (2 Gy) and the induced DNA damage was measured by Comet assay. All subjects were genotyped for XRCC1 and OGG1 SNPs. Until now 25 breast cancer patients have been registered. Preliminary results did not show significative differences in DNA repair capacity and in DNA repair genes polymorphism frequencies between healthy subjects and cancer patients (Figure 14-A e B).



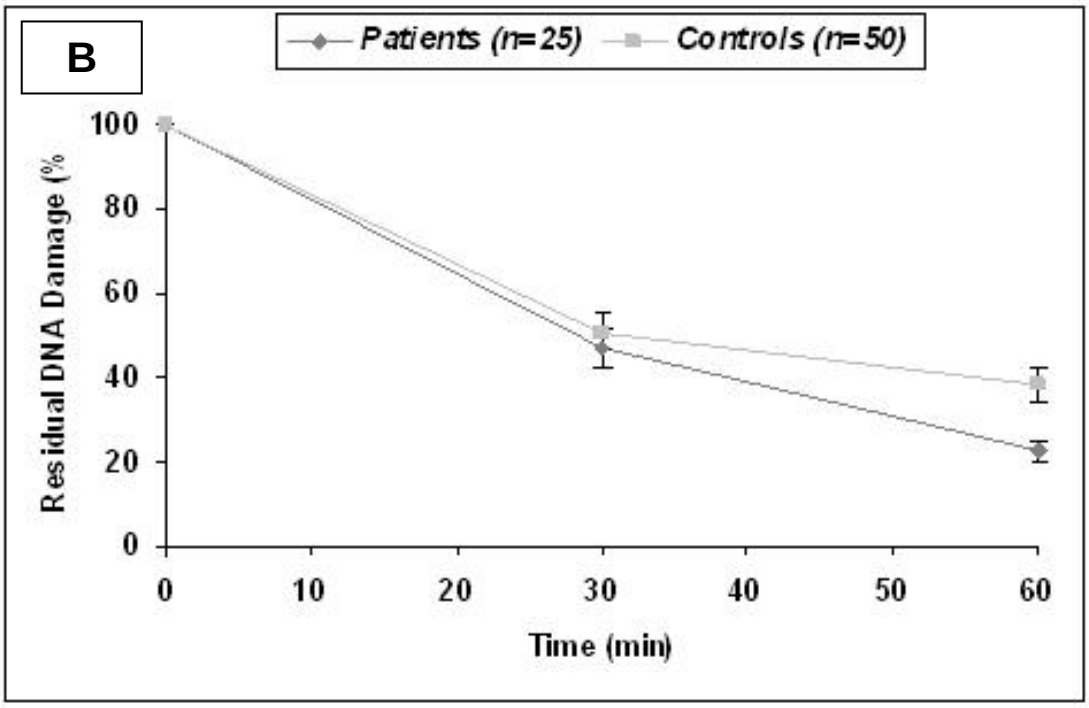


Figure 14 – DNA damage induced by X-ray treatment measured immediately after and 30 and 60 min post irradiation (A) and repair kinetics of radiation-induced DNA damage as a function of time after treatments (B), in breast cancer patients and in healthy controls.

The analysis of side effects and general clinical radiosensitivity is in progress.

Until now, cryopreserved blood samples from breast cancer patients, previously treated with radiotherapy in S. Camillo hospital, have been collected. These patients will be genotyped for several DNA repair gene polymorphisms. Data on RT adverse side effect will be provided from medical personnel.

PART B

"Influence of genetic polymorphisms in DNA repair genes and in xenobiotic metabolism genes after two different exposure conditions"

Healthy subjects exposed to tobacco smoke

Seventy-two healthy volunteers (25 smokers and 47 non-smokers, mean age 38.9±8.7 and 34.3±8.1 years, respectively) of Caucasian ethnicity were selected for the study. Among the smokers, 13 were females and 12 males; among the non-smokers, there were 30 females and 17 males. All subjects were individually interviewed and a "personal health questionnaire" was completed (Carrano and Natarajan, 1988) for the evaluation of "lifestyle confounding factors". Individuals having potential confounding factors such as drug or alcohol consumption, recent radiodiagnostic exposure and major illnesses were excluded. All smokers had reported to smoke more than 20 cigarettes per day.

Table 7 shows the distribution and the related frequencies of GSTs genotypes in the entire population and in different subgroups. The frequencies of the analysed polymorphic alleles were in Hardy–Weinberg equilibrium (P > 0.1 with Chi-squared test for each genotype) and consistent with literature data (Ye et al, 2006)(Ahn et al, 2006).

Table 7 - Distribution of GSTM1, GSTT1, GSTP1 and GSTA1 genotypes (Palma et al, 2007)

	GSTM1			GSTT1			GSTP1				GSTA1			
	Null	Present	Null Frequency	Null	Present	Null Frequency	I105I	I105V	V105V	V105V Frequency	C-69C	C-69T	T-69T	T-69T Frequency
All (n=72)	39	33	0,54	20	52	0,27	37	31	4	0,27	30	33	9	0,35
Non-Smokers (n=47)	24	23	0,51	13	34	0,27	23	22	2	0,27	20	21	6	0,35
Smokers (n=25)	15	10	0,60	7	18	0,28	14	9	2	0,26	10	12	3	0,36
Females (n=43)	24	19	0,56	11	32	0,25	24	17	2	0,24	18	19	6	0,36
Males (n=29)	15	14	0,52	9	20	0,31	13	14	2	0,31	12	14	3	0,34

Table 8 summarizes the mean values of chromosomal damage in the entire population, subdivided according to smoking habit and gender. Smokers showed a significant increase in MN compared with non-smokers (P < 0.05, Mann–Whitney U-test). Comparing female smokers with male smokers, we found significantly higher values in females for all CA parameters. In contrast,

no difference between females and males was observed among non-smokers. Furthermore, we found that female smokers showed significantly higher values of all CA types compared with females who did not smoke.

Within the smoker population, we found a significant difference in MN frequency between female and male subjects, with females having the highest value (Table 8). Moreover, female smokers showed an increased MN frequency compared with non-smokers.

Table 8 - Mean values of CA and MN according to gender and smoking habit (Palma et al, 2007)

	Gender	CsA (%)	CtA (%)	CA tot (%)	BNMN (‰)
Smokers (Mean Age = 36,9 ± 8,7)	All	1,1 (1,1)	3,0 (3,5)	4,1 (3,9)	**8,3*** (4,4)
	Female (n=13)	1,7$^{a\Omega}$ (1,3)	5$^{b\ \Phi}$ (3,9)	6,7$^{c\Psi}$ (3,9)	10,7$^{a\Omega}$ (4,1)
	Male (n=12)	0,5 (0,5)	0,9 (0,6)	1,3 (0,5)	5,7 (3,1)
Non- Smokers (Mean Age = 34,4 ± 8,1)	All	1,0 (1,1)	1,5 (1,2)	2,5 (1,6)	5,9 (3,8)
	Female (n=30)	0,9 (0,9)	1,7 (1,3)	2,5 (1,7)	7,4 (3,9)
	Male (n=17)	1,2 (1,3)	1,0 (0,7)	2,2 (1,5)	3,9 (2,7)

CsA, chromosome-type aberration; CtA, chromatid-type aberration; CA tot, total chromosome aberration; BNMN, binucleated cells with micronucleus. Values in parentheses are SD values.
*for direct comparison between smokers and non-smokers. *P< 0,05
Latin letters for direct comparison of female smokers versus male smokers : ^{a}P< 0,01; ^{b}P< 0,001; ^{c}P< 0,0001
Greek letters for direct comparison between female smokers versus female non-smokers
$^{\Omega}$P< 0,05; $^{\Phi}$P< 0,01; $^{\Psi}$P< 0,001. p-value based on Mann-Whitney U-test

Multivariate analysis (Table 9) on the whole population showed that among the biomarkers considered in this study, the CtAs, the CAtot and the frequency in micronucleated binucleate cells (BNMN) were affected by smoke and gender, while a significant influence of age was noted only for the BNMN frequency.

As far as the influence of the genotype on chromosomal damage is concerned, a significant correlation was found only between the BNMN frequency, in smokers, and GSTM1 and GSTP1 genotypes (Table 9).

Table 9 - Multivariate analysis of confounding factors and gene polymorphisms in CAs and BNMN frequencies in non-smoker and smoker subjects (Palma et al, 2007)

Population	Biomarker[a]	Indipendent Variables[b]	Partial R^2	R^2	P-Value
All (n=72)	CtA	Gender	0,0012		**0,0048**
		Smoking Habit	0,0381		**0,0003**
				0,30	0,0002*
	CAtot	Gender	0,0012		**0,0054**
		Smoking Habit	0,0381		**0,0001**
				0,32	0,0001*
	BNMN Age		0,0391		**0,0063**
		Gender	0,0012		**<0,0001**
		Smoking Habit	0,0381		**0,0198**
				0,40	<0,0001*
Non Smokers (n=47)	BNMN Age		0,1248		**<0,0001**
	Gender		0,1550		**0,0057**
				0,55	<0,0001*
Smokers (n=25)	BNMN Gender		0,2635		**0,0287**
		$GSTM1^{null}$	0,2944		**0,0469**
		$GSTP1^{I105V}$	0,1531		**0,0383**
				0,63	0,0338*

[a] Biomarkers used as dependent variables: CtA; CsA; CAtot, BNMN.
[b] Indipendent variables used in the model: Age, Gender, Smoking Habit, $GSTM1^{null}$, $GSTT1^{null}$, $GSTP1^{I105V}$, $GSTA1^{C-69T}$.
*Significance level in the model; Values in bold are P-values for each of the independent variables

Figure 15 shows the correlation found between the mean BNMN value and GSTM1 and GSTP1 polymorphism in smokers and non-smokers: smokers carrying the GSTM1-null genotype showed a significantly higher frequency of MN compared with GSTM1-positive subjects ($P < 0.05$); no association was found in non-smokers. As far as GSTP1 genotype is concerned, a slight but not significant increase was found comparing carriers of the wild-type genotype with carriers of the variant genotypes.

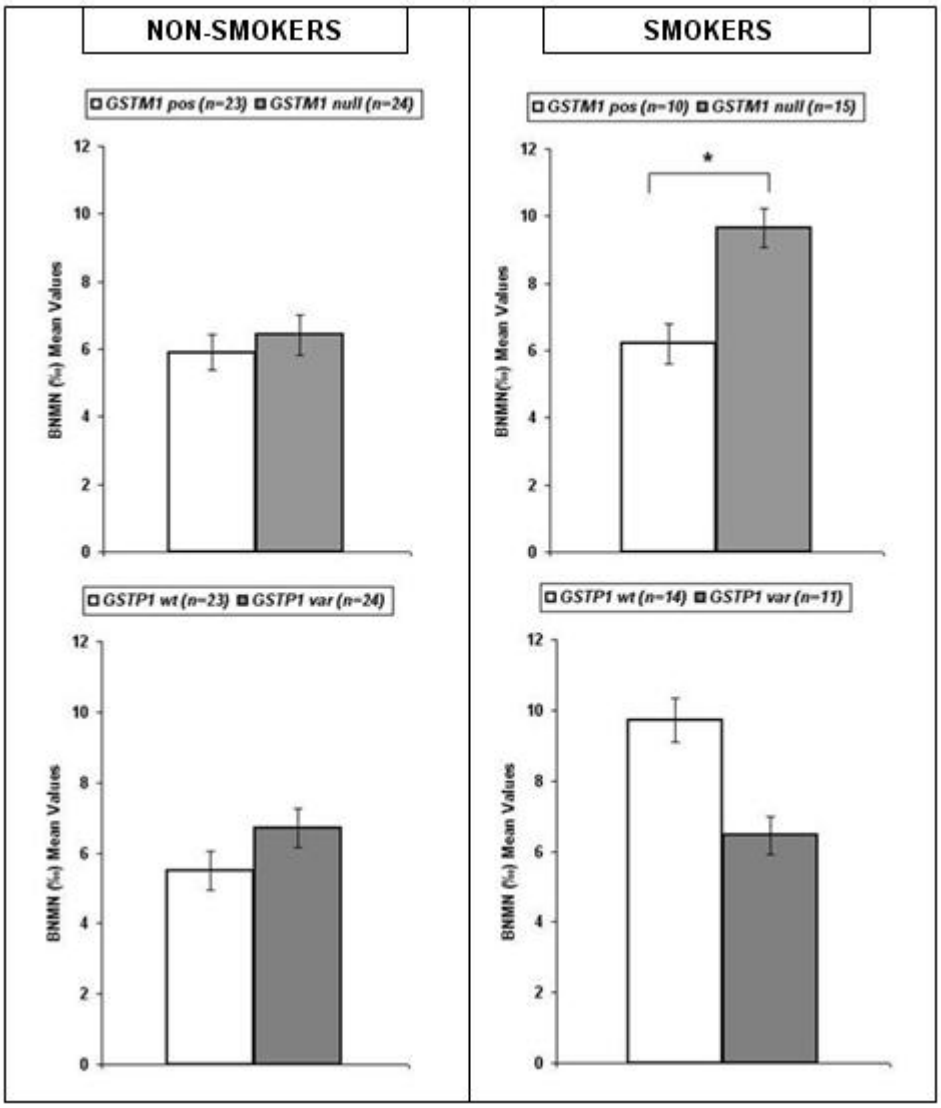


Figure 15 - Effect of BNMN values and GSTM1 gene polymorphisms; BNMN, binucleated cells with micronuclei ; pos, positive; wt, wild type; var, variant allele.* P< 0,05 based on Mann-Whitney U-test (Palma et al, 2007)

In Table 10 we show that a particular gene combination seems to influence BNMN frequencies in smokers, but not in non-smokers. Subjects bearing GSTM1-null/GSTP1-wild-type genotype have the highest BNMN value compared with the other three genotype combinations, showing a significant difference between GSTM1-null/GSTP1-wild-type subjects and GSTM1pos/GSTP1variant.

Table 10 - Mean values ± S.D. for BNMN frequencies stratified for a combination of GSTM1 and GSTP1 polymorphisms in Non-Smoker and Smoker subjects (Palma et al, 2007)

Genotypes		Non-Smokers		Smokers	
		N° of subjects	BNMN Mean values ± SD	N° of subjects	BNMN Mean values ± SD
GSTM1	GSTP1 105 Ile→Val				
Pos	Wt	11	5,3±3,7	6	7,2 ± 4,5
Null	Wt	12	5,7±4,3	8	**11,6 ± 3,4***
Pos	Var	12	6,2±4,1	4	4,7 ± 3,9
Null	Var	12	7,2±3,9	7	7.4 ± 3.8

Pos, positive; var, variant (A/B or B/B) genotypes; wt, wild-type genotypes
* P≤ 0,05 for direct comparison among subjects with GSTM1 null/GSTP1 wt versus GSTM1 positive/GSTP1variant genotypes. p-value based on Mann-Whitney U-test.

Population of nurses handling antineoplastic drugs

Eighty-three probands, employed in oncology units of Italian hospitals (S. Martino Hospital, Genova; Umberto I Hospital, Roma) and regularly handling antineoplastic drugs, and 73 unexposed controls, selected from volunteers employed in administrative offices, were involved in this study. All subjects gave their informed consent for participating in this study and were individually interviewed by filling out a modification of the “personal health questionnaire” (Carrano and Natarajan, 1988). This questionnaire included information on age, gender, life habits (dietary, smoking, alcohol consumption, use of medicines), employment, cytostatic drugs handled, safety prevention measures adopted, time of exposure. Alcohol drinkers (more than 200 ml daily, equal to 24 g of alcohol), subjects with previous treatment by chemotherapy and radiotherapy or with recent viral or bacterial infections, subjects recently (over a period of 3 months) taking drugs or having vaccinations, individuals with known genetic defects in the family or major illnesses were not included. Exposed and controls were matched by gender, age and smoking status (smoker and non-smoker). All subjects contributed to the study with a single blood donation. Blood (approximately 10 ml) was taken at the beginning of work-shift of the third working day, by venipuncture into heparinized tubes.

During the work hours, all the exposed subjects used adequate protective precaution, consisting of both individual (gloves, overalls, goggles, masks) and environmental equipment (vertical air-flow cabinet). These safe handling practices are recommended in the guidelines promulgated by the

Italian Government (Italian Government “Gazzetta Ufficiale”, 1999). Overall mean length of service was 12.2±7.3 years and the standardworking time was 36 h/week. The hospital personnel reported handling a diversity of antineoplastic drugs, often in a mixture including more than five agents while preparing solutions for infusion and administering them. Data obtained by questionnaire made it possible to know which drugs had been handled by exposed subjects over a period of 6 months before blood collection (Table 11). Data are reported as the relative percentage of each drug being 100% the totality of the handled drugs.

Table 11 - Relative mean frequencies of antineoplastic drug handling (Cornetta et al, 2008)

Type of drug	Frequency (%)
Azathioprine	3.4
Busulfan	10.2
Cyclophosphamide	7.2
Citarabine	9.6
Doxorubicin	5.9
Etoposide	6.5
Fluorouracil	9.8
Hydroxyurea	3.0
Methotrexate	5.4
Thiotepa	4.6
Tretinoin	4.1
Vincristine	2.5
Others[a]	27.8
Total	**100**

[a] Includes antineoplastic drugs with an individual handling frequency less than 2.5%. Data were obtained from questionnaire information over a period of six months, of 83 exposed subjects. Data are reported as the relative percentage of each drug being 100% the totality of the handled drugs

The characteristics of the population considered in this study are indicated in Table 12 . Most of subjects were females and non-smokers, both in control and exposed groups. The two populations, controls and exposed, were age-matched, the range being 26–58 and 23–56, respectively. The distribution of the analysed genotypes was in Hardy–Weinberg equilibrium. The frequencies obtained for XRCC1 399Gln and XRCC3 241Met variant alleles were 0.37–0.40 and 0.36–0.41, consistent with literature data obtained for Caucasian population (Shen et al, 1998).

Table 12 - Baseline characteristics of study populations (Cornetta et al, 2008)

Variables	Controls	Exposed
	N (%)	N (%)
Gender		
Female	53 (73)	67 (81)
Male	20 (27)	16 (19)
Current Smoking		
No	51 (70)	64 (77)
Yes	22 (30)	19 (23)
Mean Age (years)±SD [range]	37.0±10.0 [23-56]	37.6±6.7 [26-58]
Mean Time Exposure (years)±SD [range]		12.2±7.3 [3-28]
*XRCC1*codon399		
Arg/Arg	32 (44)	37 (45)
Arg/Gln	27 (37)	38 (46)
Gln/Gln	14 (19)	8 (9)
*XRCC3*codon241		
Thr/Thr	30 (41)	37 (45)
Thr/Met	33 (45)	36 (43)
Met/Met	10 (14)	10 (12)

Table 13 shows the results obtained with the comet assay, expressed in mean TD, and MN test expressed as mean of micronuclei on 1000 binucleated cells. The exposed subjects show a very significant increase both in DNA damage and in chromosome damage in respect to controls. Moreover, when gender is taken into account, females show a significant higher MN value when compared to males, both in control and in exposed groups. As far as the smoking habit is concerned, control smokers show a significantly higher MN value compared with control non-smokers. Within the exposed group, the difference in MN values between smokers and non-smokers does not reach any statistical significance.

Table 13 - Average values of biomarkers of genetic damage in study subjects grouped by DNA repair genotypes (Cornetta et al, 2008)

	Controls			Exposed		
	N°	TD (%)±SD	BNMN (‰)±SD	N°	TD (%)±SD	BNMN (‰)±SD
All	73	0.77±0.47	6.70±4.01	83	1.16±0.82*	13.11±6.70**
Gender						
Female	53	0.73±0.48	8.12±4.10	67	1.16±0.78	13.81±6.94
Male	20	0.88±0.45	4.62±3.03[a]	16	1.13±0.98	9.62±4.74[b]
Current Smoking						
No	51	0.75±0.46	5.91±3.83	64	1.20±0.90	13.51±6.93
Yes	22	0.80±0.52	8.10±4.20[b]	19	1.00±0.39	11.71±6.14
*XRCC1*codon399						
Arg/Arg	32	0.81±0.50	6.63±3.62	37	1.12±0.79	11.12±5.50
Arg/Gln+Gln/Gln	41	0.74±0.45	6.68±4.46	46	1.19±0.84	14.53±7.21[c]
*XRCC3*codon241						
Thr/Thr	30	0.71±0.46	5.96±4.09	37	1.15±0.84	12.88±6.96
Thr/Met+Met/Met	43	0.81±0.48	7.08±3.94	46	1.16±0.81	13.19±6.57

*P<0.001 - **P<0.0001 significantly different from controls at Mann-Whitney U-Test (TD) and Unpaired T-Test (BNMN)
[a]P<0.001 when comparing Female Controls to Male Controls
[b]P<0.05 when comparing Females Exposed to Males Exposed and Smoker Controls to Non-Smoker Controls
[c]P<0.05 significantly different from Exposed with wild type genotypes

Results from Comet assay and MN test with regard to the different genetic polymorphisms analysed are also gathered in Table 13. Regarding the XRCC1 gene, a significant increase ($P < 0.05$ unpaired t-test) in MN value was obtained for exposed individuals bearing the Gln variant allele.

Multiple regression analysis, including age, exposure time, gender, smoking habit and gene polymorphisms was performed considering both biomarkers (DNA and chromosome damage). As shown in Table 14, MN values tended to significantly rise with age and gender, in total population, in controls and in exposed subjects. Smoking exerts its influence on BNMN in controls but not in the exposed. Furthermore, XRCC1 gene, but not XRCC3, influences the BNMN in the exposed subjects. No significant association was found between Comet assay parameters and any variables analysed.

Table 14 - Regression analysis of confounding factors and gene polymorphisms on BNMN frequencies in control and exposed subjects (Cornetta et al, 2008)

Population	Biomarker	Independent Variables	Regression Coefficients		R^2	P-Value*
			Unstandardized B (95% CI)	Standardized β		
All (n=156)	BNMN	Age	0.287 (0.177÷0.397)	0.338		<0.0001
		Gender	4.771 (2.838÷6.703)	0.322		<0.0001
		Smoking Habit	-0.116 (-2.003÷1.771)	-0,008		0.903
		Exposure	4.944 (3.233÷6.656)	0.381		<0.0001
		XRCC1^{399}	1.897 (0.194÷3.601)	0.145		0.029
		XRCC3^{241}	-0.105 (-1.831÷1.620)	-0,008		0.904
					0,45	
Controls (n=73)	BNMN	Age	0.152 (0.052÷0.252)	0.318		0.0035
		Gender	4.389 (2.598÷6.180)	0.502		<0.0001
		Smoking Habit	1.951 (0.077÷3.825)	0.215		0.0416
		XRCC1^{399}	0.510 (-1.260÷2.279)	0.061		0.5666
		XRCC3^{241}	1.120 (-0.704÷2.944)	0.130		0.224
					0.40	<0.0001**
Exposed (n=83)	BNMN	Age	0.479 (0.239÷0.720)	0.462		0.0002
		Exposure Time	0.026 (-0.177÷0.228)	0.029		0.8015
		Gender	5.010 (1.621÷8.399)	0.292		0.0043
		Smoking Habit	-1.201 (-4.317÷1.915)	-0,077		0.4445
		XRCC1^{399}	2.988 (0.235÷5.742)	0.222		0.0339
		XRCC3^{241}	-1.514 (-4.253÷1.226)	-0,113		0.2742
					0.35	<0.0001**

* P-values for each of the independent variables; ** Significance level in the model

PART C

"XRCC1 involvement in cell cycle control and DNA strand break repair: characterization of Chinese hamster cell lines AA8 and EM9"

a) Induction of SSBs and the repair kinetics analysed by the Comet assay:

In figure 16 radiation dose-response of TD in EM9 and AA8 cells is shown. Values of TD are significatively higher in EM9 than AA8 cells at all used X ray doses. In figure 17 repair kinetics of radiation-induced DNA damage as a function of time after treatments, is shown. EM9 cells have significatively higher values of RD at 30 and 60 min after X ray treatment, than AA8 cells.

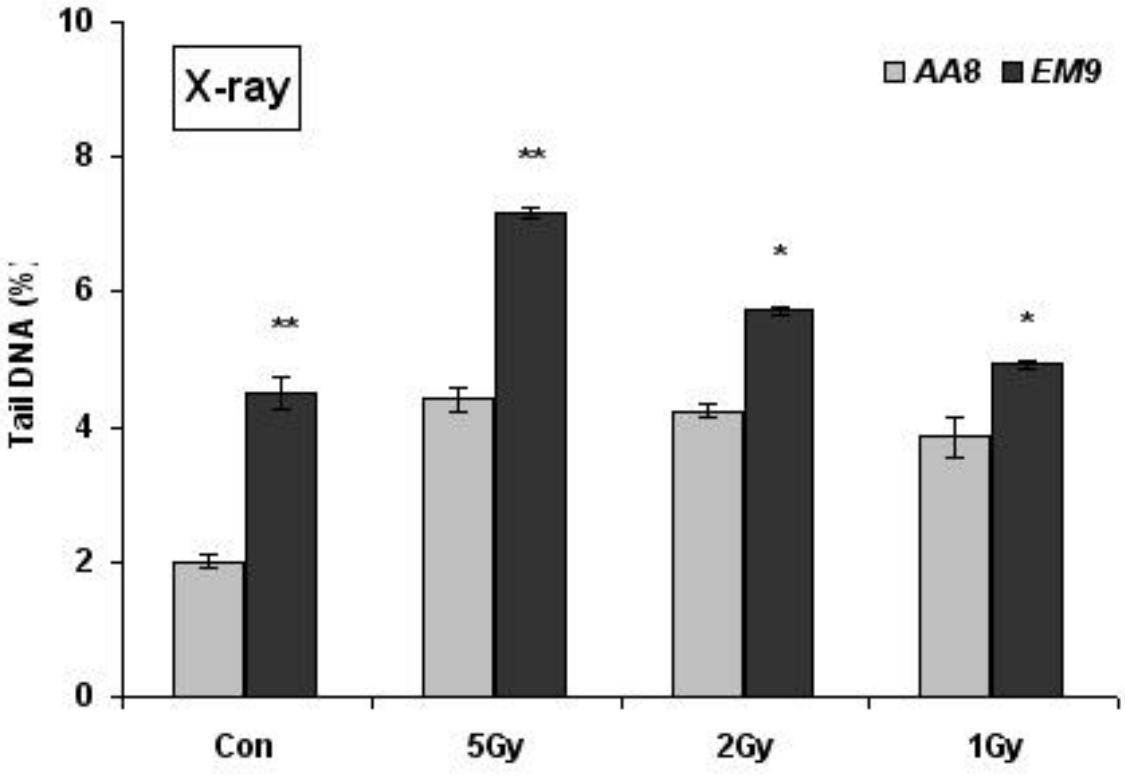


Figure 16 - radiation-dose response of % Tail DNA in EM9 and AA8 cells (*P<0.05; **P<0.001)

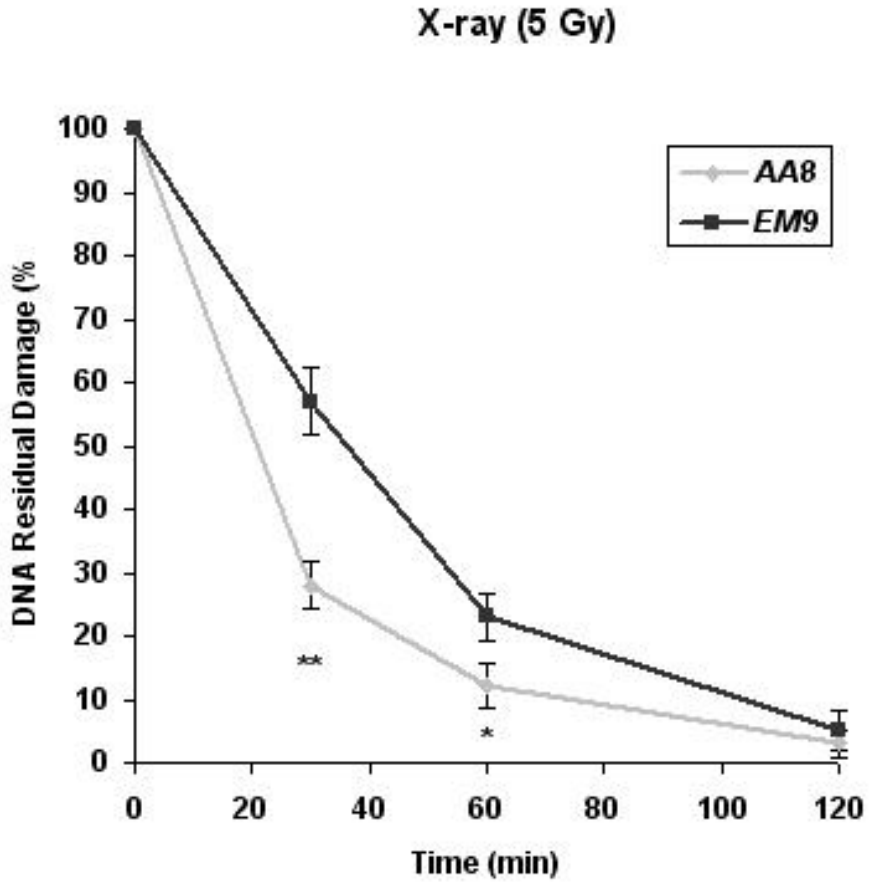


Figure 17 - Repair kinetics of radiation-induced DNA damage as a function of time after treatments (*P<0.05; **P<0.001).

b) the induction and the time evolution of DSBs through the analysis of the expression of phosphorylated histone H2AX:

In figure 18 the radiation dose-response of γH2AX expression in EM9 and AA8 cells, 1 hour after irradiation, is shown. The EM9 cells show higher levels of γH2AX expression than AA8 1 hour after irradiation, at all used doses. However both cell lines show a lower level of γH2AX after the highest dose (15 Gy), probably due to an extensive mortality induced by this dose. In EM9 cells the maximum γH2AX expression is observed 1 hour after treatment; then a time-dependent decrease in γH2AX levels is observed at 2, 4 an 24 hours following 5 and 10 Gy treatment (Figure 19-EM9). In AA8 cells the maximum γH2AX expression is observed 2 hours after irradiation with a time-dependent decrease at 4 and 24 hours later (Figure 19-AA8).

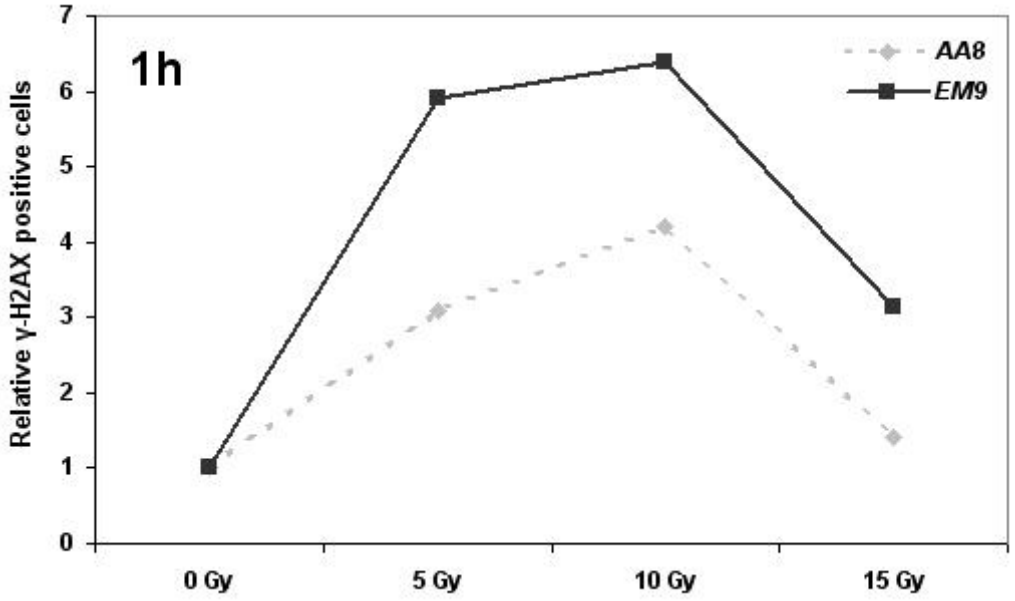


Figure 18 - Radiation dose-response of γH2AX expression in EM9 and AA8 cells, 1 hour after irradiation

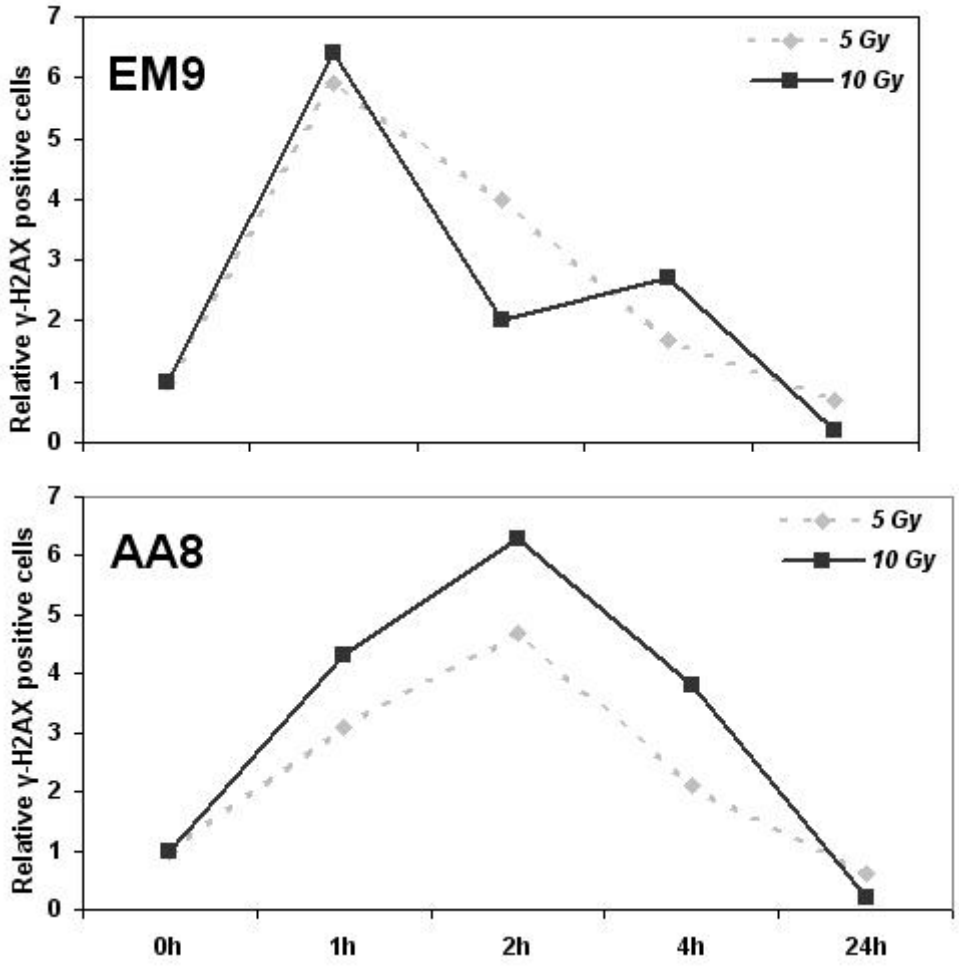


Figure 19 - Kinetics of γH2AX decrease as a function of time after irradiation (immediately after or 1, 2, 4, 24 hours later), measured by bivariant flow citometry analysis in both cell lines.

c) Modulation of cell cycle through the flowcytometric analysis of Bromodeoxy Uridine (BrdU) incorporation:

As XRCC1–deficiency has been linked to the perturbation of DNA replication, we assessed whether EM9 cells displayed an impaired cell cycle progression in control condition and after X-ray treatment. The biparametric analysis of the BrdU labelling vs DNA content allowed to record the cell progression through G1/S/G2 phases and to distinguish the early-median S from G1 and late S/G2 (Figure 20 and Table 15).

Both cell lines show an accumulation in G2 phase 8 hours after irradiation as expected. No differencies were observed in cell cycle progression when comparing EM9 to AA8 cells both in control and X-ray treated samples.

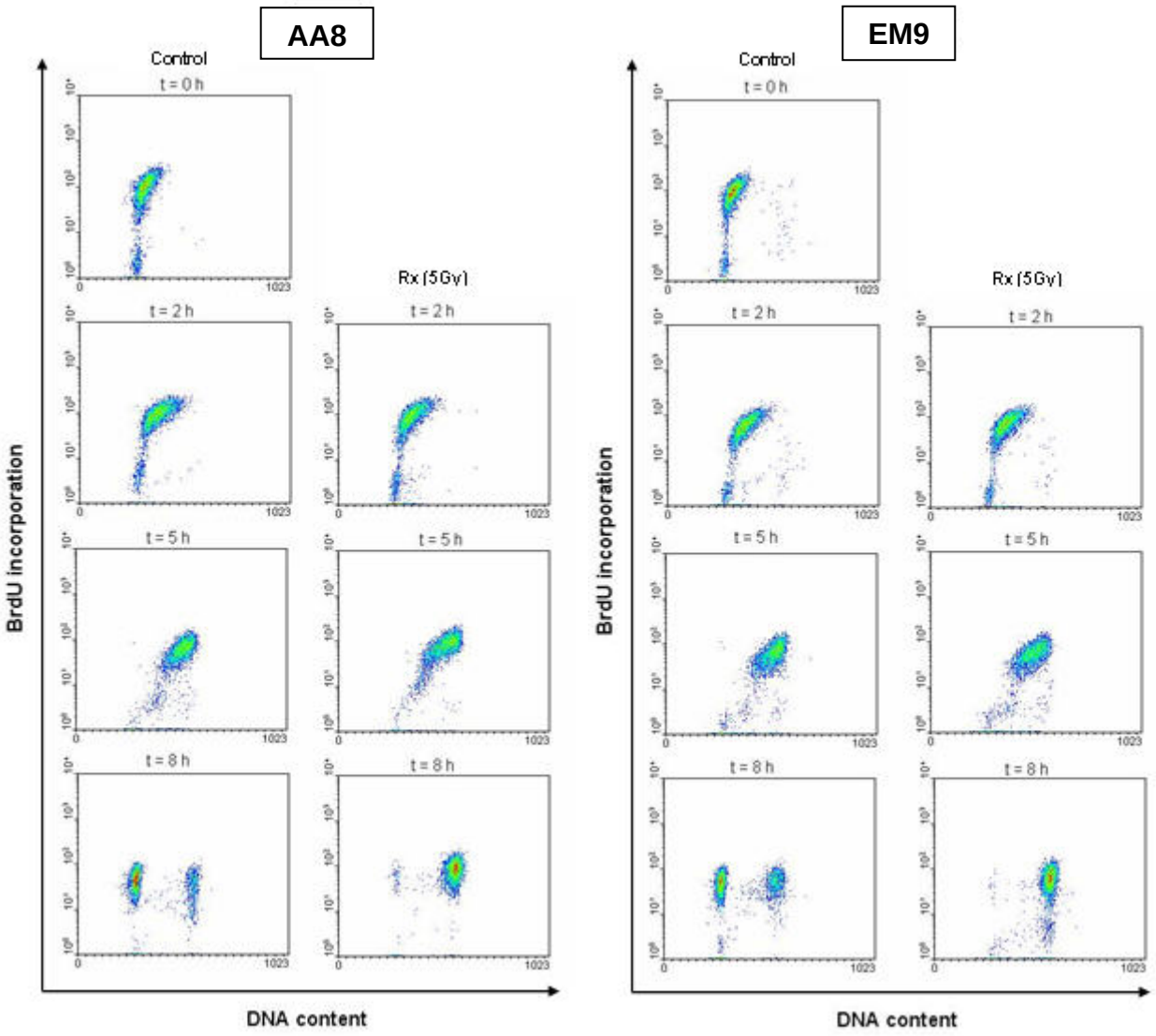


Figure 20 - Biparametric analysis of the distribution of AA8 and EM9 cells in G1 S-G2 phases. Cells irradiated with 5 Gy X rays were pulsed with BrdU 30 min before sampling in order to check for S phase progression.

Table 15 – Percent of cells in cell cycle phases at different recovery times after X-eray treatment

AA8	Recovery Time	Cell Cycle Phases (%)			
		G1	Early S	Medium S	Late S/G2
Control	0 h	15,5	72	9,7	4
Control	2 h	11	23	60,3	7,5
Control	5 h	5,3	1	20,4	65,3
Control	8 h	4,2	62,9	4,2	26,9
Rx 5 Gy	2 h	15,6	34	47	3,6
Rx 5 Gy	5 h	4,8	0,9	27,7	62
Rx 5 Gy	8 h	1,6	5	4,5	88

EM9	Recovery Time	Cell Cycle Phases (%)			
		G1	Early S	Medium S	Late S/G2
Control	0 h	10	69	17,6	5,6
Control	2 h	13,6	22,8	56,4	7,6
Control	5 h	7	0,7	16	69,5
Control	8 h	5,8	49	5	38,2
Rx 5 Gy	2 h	12,6	33,6	49,7	6,4
Rx 5 Gy	5 h	6,8	1	27,3	57,7
Rx 5 Gy	8 h	4	2	2,2	87,6

I V. DISCUSSION

The major topic of this research project has been the investigation of the genetic basis of individual response to DNA damage due to exogenous exposures.

The relationship between polymorphisms in the BER genes XRCC1 and OGG1, and the NER gene XPC and the repair of DNA damage induced by in vitro X-ray irradiation in peripheral blood cells of healthy subjects has been assessed. To analyse induced DNA damage, we used the alkaline version of Comet assay because it is able to detect the major part of these radiation induced lesions, in contrast to the neutral Comet assay which detects, specifically, double-strand breaks. The Comet assay was used in DNA repair capacity assessment and proved to be a very sensitive and reliable method (Mayer et al, 2002) because it allowed the determination of both the initial DNA damage and the residual DNA damage remaining after repair, as a measure of individual radiosensitivity (Plappert et al, 1997).

The ionizing radiation-induced DNA damage is considered to be responsible for clinical radiation sensitivity, and mutations/variations in DNA repair genes must affect late radiation reactions, including secondary malignancies (Popanda et al, 2003).

Most of these studies employed an experimental design that included patients previously submitted to RT and for whom the severity of side effects was already known. The biological end point of all the assays was an estimation of cellular effects in terms of “cell lethality” and a correlation with radiation-induced toxicity. Comment could be made to the fact that acute and late effects on normal tissues are now better understood and clearly different in their pathogenesis. For acute effects, the role of cellular response evaluated by *in vitro* tests appear more clear (even with contrasting experimental findings), whereas late effects are probably not entirely dependent on cell lethality and modulated by multiple factors (extracellular matrix response, inflammatory cytokines, vascular damage), making a correlation with cellular response of irradiated cells more difficult to define.

So, studying individual repair capacity in vitro in relation to genetic background should contribute to a preventive evaluation of late effect risks.

Our data suggest that XRCC1 and XPC polymorphisms, but not OGG1, have an effect on DNA damage manifestation. XRCC1 is a molecular scaffold

protein that coordinates the assembly of repair complexes at damaged sites (Brem and Hall, 2005). In the current study we have focused our investigation on the characterization of the role of one of the most common polymorphism, a G to A substitution at position 28152 (codon 399, exon 10) (Shen et al, 1998). XRCC1 codon 399 is located within the BRCTI motif, and the exon 10 variant could have an altered repair activity.

The presence of the 399Gln variant has been correlated with the persistence of DNA damage (Matullo et al, 2001), elevated formation of SCE (Hu et al, 2002), and radiation-induced cell cycle delay (Duell et al, 2000).

Our data show that cells from persons bearing XRCC1 Gln399Gln genotype had fewer DNA breaks than wild-type subjects, after X-ray treatment at time 0 and after 30 and 60 min of repair.

These data, apparently contrasting with previously cited reports, are consistent with a different, faster ability in repairing strand breaks induced by X rays when 399Gln alleles are present.

In vitro radiation experiments have showed that the majority of this DNA damage is repaired very quickly and is almost completely repaired within 2 hours (Plappert et al, 1997). The rejoining double strand breaks is completed 12 to 16 hours after irradiation (Dikomey et al, 2003). The faster repair of SSB is attributable to BER (Vodicka et al, 2004) and this may account for the observed significant variation in repairing between XRCC1 codon 399 variant and wild-type genotypes.

Au and collaborators (Au et al, 2003) found that a combination of variant XRCC1 and XRCC3 genotypes showed an increase in chromosome aberrations induced by X rays. These results do not agree with ours but we believe that the discrepancy is based on the use of different biomarkers. In fact, the Comet assay does not provide information about the fidelity with which DNA lesions are repaired, and misrepaired lesions can originate chromosome-type damage.

A number of polymorphic genes have been shown to be associated with the onset of therapy-related acute myeloblastic leukemia (t-AML). In particular Seedhouse and co-workers (Seedhouse et al, 2002) found that the presence of the XRCC1 399Gln allele is protective against the development of t-AML. The

same protective effect was found in studies on bladder cancer (Stern et al, 2001) and non-melanoma skin cancer (Nelson et al, 2002).

The authors suggested that XRCC1 399Gln allele might reduce the efficiency of repair, and damaged cells are more likely to be driven toward apoptosis. In wild-type cells, the damage might be repaired originating cells harboring mutations. Consequently, in cells with polymorphic codon 399 genotypes, the result is protection through the elimination of potentially transformed cells; in wild-type cells, mutated cells can originate clonal disease. We believe that this hypothesis could also explain our findings. In fact, we have found that the XRCC1 polymorphic genotype is associated with lesser TD values after X-ray irradiation and lower residual DNA damage after 60 min of repair. This finding suggests the subsequent interpretations: XRCC1 399Gln allele promotes a faster resolution of open breaks induced by X rays; these possibly misrepaired lesions might in part originate chromosome damage; these cells with genetic instability could be driven toward apoptotic death.

In the present study, individuals bearing XPC heterozygous Lys939Gln genotype show significantly lower TD values and lower residual damage 60 min after irradiation. This finding is quite difficult to explain even if one could hypothesize that in these subjects the XPC codon 939 polymorphic allele exhibits linkage disequilibrium with other XPC SNPs that cause a modified repair activity (Khan et al, 2000).

In order to find a correlation between cellular radiosensitivity and clinical radiosentitivity, the analysis of the association between DNA damage induced by X-ray treatment in vitro and the genetic background in breast cancer patients has been started.

Preliminary results did not show significative differences in DNA repair capacity and in DNA repair genes polymorphism frequencies between healthy subjects and cancer patients.

Breast Cancer is the common type of malignancy in females, accounting for approximately 21% of all cancer cases in women worldwide (Parkin et al, 1999). Out of all breast cancer patients, 2% have a strong genetic predisposition, caused by the highly penetrant BRCA1 and BRCA2 genes (Peto et al, 1999). Because these genes cannot account for the overall increased risk in the relatives of BC cases (Baeyens et al, 2002), it was suggested that a

substantial proportion of breast cancer patients may be predisposed to cancer through mutations in low penetrance genes (Scott, 2004), which may be genes involved in DNA damage processing and repair.

The deficient DNA repair capacity has been proposed as a predisposing factor in familial and in some sporadic breast cancer cases (Parshad et al, 1996). Genomic instability has also been described for various hereditary cancers including hereditary breast cancer (Baeyens et al, 2002).

A convenient test to evaluate both genetic instability and DNA repair capacity is the single-cell gel electrophoresis or Comet assay (Olive et al, 1990). Djuzenova and collaborators (Djuzenova et al, 1999) have found the background and induced DNA damage in the peripheral blood lymphocytes from breast cancer patients to be similar to that in control individuals. Consistent with these data, non-irradiated lymphocytes from patients with multiple tumours (Muller-Vogt et al, 2003), lung cancer (Rajaee-Behbahani et al, 2001) and breast cancer (Alapetite et al, 1999) have also been reported to exhibit the same range of DNA damage as control cells. Similarly, no differences has been revealed by the Comet assay between cells from control subjects and patients with BRCA1 mutation, after irradiation with 2 Gy *in vitro* (Rothfuss et al, 2000).

Our preliminary data are in agreement with previous literature data, showing no significative differemces in DNA repair capacity between healthy subjects and breast cancer patients

The influence of genetic polymorphisms in DNA repair genes and in GST genes after two different exposure conditions was assessed.

We have found a significant increase of DNA damage in smokers compared with non-smokers only when considering BNMN values. This increase seems to be influenced not only by age and gender as previously reported (Fenech et al, 1999), but also by the genetic constitution. In particular, our results indicate that GSTM1-null smokers have a higher BNMN frequency than GSTM1-positive smokers. An analogous effect was previously described, when an increase was reported in CA and SCE frequencies in peripheral blood lymphocytes of smokers deficient in GSTM1 (Tuimala et al, 2004), but these data were not confirmed by others (Leopardi et al, 2003).

However, the correlation between BNMN, tobacco smoke and genotype is still controversial (Kirsch-Volders et al, 2006)(Norppa, 2004), probably due to

the difficulty in interpreting these relationships. Moreover, the amount of DNA damage seems to be highly influenced by a particular combination of GST genotypes. In fact, a lower frequency of BNMN has been found in subjects carrying the GSTP1 variant allele in combination with the GSTM1-positive genotype. The subjects with the highest level of DNA damage carried a combination of GSTM1-null and GSTP1-wild type, suggesting that the GSTP1-wild-type isoform is less active in conjugating PAH diol epoxides than the variant enzyme.

Understanding the role of different genotype combinations on the expression of DNA damage is relevant in particular when the complex network of metabolic pathways is considered. Regarding the influence of GSTM1 and GSTP1 genotype combinations on the DNA damage induced by tobacco-smoke exposure, Butkiewicz and co-authors found that the combined GSTM1 and GSTP1 genetic polymorphisms could modulate PAH-DNA adduct levels (Butkiewicz, 2000).

There is an inherent interest in monitoring genotoxicity independently of cancer as an endpoint, especially as far as exposure to known carcinogens/mutagens is concerned (Albertini et al, 2000). This is the case for the exposure to antineoplastic drugs in healthcare personnel. It is well known that these substances are carcinogens/mutagens to humans and so nurses handling them normally adopt individual and environmental protective measures, as recommended by government guidelines. Nevertheless contamination in the work environment is still possible and the safety precautions adopted are not always enough to prevent health hazards related to antineoplastic drug management.

Our study combines genotype analysis of DNA repair genes with DNA and chromosome damage measured in blood cells of hospital personnel, occupationally exposed to antineoplastic drugs.

We restricted our analysis to two genes, XRCC1 and XRCC3, involved in base excision repair and homologous recombination, respectively.

The genotoxicity of antineoplastic drugs is controversial: an increase in comet assay parameters has been shown (Maluf et al, 2000)(Laffon et al, 2005) while the data on chromosome damage are in less agreement (Pilger et al 2000}{Cavallo et al, 2005}. These differences could probably be explained by

the multitude of factors influencing the outcome of the biomonitoring studies (exposure conditions, doses, drugs, etc.).

In this study we have shown that nurses handling antineoplastic drugs have a very significative increase in DNA damage, particularly chromosome damage as evidenced by the MN data, in spite of the use of safety precautions. Since the mean time of exposure to antineoplastic drugs is more than 10 years, the cumulative effects of these agents on the human genome must be considered. However, as the MN level is influenced by the age of the nurses, a relationship between age and time of exposure is conceivable. Also gender exerted an influence on MN level, both in control and in exposed groups, being higher in females than in males. This is in agreement with current knowledge on the effect of gender on genetic damage which determines a 1.5 time greater MN frequency in females than in males (Fenech, 1998). A significant difference has been shown when considering MN mean value in smoking control versus non-smoking groups. This difference was not observed in exposed groups: the additional occupational exposure could have a more pronounced effect on non-smokers than on smokers in consequence of an adaptive response in smokers (Testa et al, 2005).

Furthermore we found that exposed subjects bearing at least one XRCC1 variant allele (399Gln) show an higher value of MN. In our opinion this result is in agreement with those found by Qu and collaborators (Qu et al, 2005) who showed that the XRCC1 cDNA containing Arg399Gln polymorphism did not fully correct the DNA repair defect in XRCC1- knockout cells, measured by the MN test.

Recently, Angelini et co-authors (Angelini et al, 2005) have shown that MN frequency was higher in subjects exposed to low levels of ionizing radiation and bearing XRCC1 variant genotypes.

The cytostatic drugs used by exposed subjects induce both DNA single and double strand breaks that are potentially repaired by BER and HR. Recently the involvement of XRCC1 protein also in DSB repair has been well established (Audebert et al, 2004)(Levy et al, 2006).

On the whole our results provide evidence that occupational exposure to antineoplastic drugs, even if in safety controlled conditions, represents a serious health risk since – as it has been reported by Bonassi and collegues

(Bonassi et al, 2007) – the increased MN formation is associated with early events in carcinogenesis. Furthermore we have shown that the presence of XRCC1 genetic polymorphism could contribute to increase the genetic damage in susceptible individuals who have been occupationally exposed to dangerous substances.

Finally, to better understand the role of XRCC1, the biological responses induced by X-ray treatment in two hamster cell lines (AA8 and EM9) was analysed.

The EM9 cells appear to be more sensitive to DNA damage induction by X rays. As far as the repair kinetic, EM9 shows a slower DNA repair kinetic following exposure to radiation.

The high radiosensitivity of EM9 cells is confirmed by the high levels of γH2AX and by its fast expression 1 hour after treatment. Apparently the short clearance kinetic is not in agreement. Because the expression level of H2AX phosphorylated correlates with the number of DSBs induced by X rays and its loss reflects the repaired lesions (Taneja et al, 2004), one could hypothesize that these cells are able to rapidly repair DSBs with reduced efficiency. This hypothesis is confirmed by our preliminary results on Chromosomal Aberrations: the % ChAb after 2 and 5 Gy irradiation are higher in EM9 cells than in AA8 cells (72 and 256% vs 24% in untreated cells; 60 and 154% vs 16% in untreated cells respectively).

In addition to operating in BER, recent evidence also suggests that XRCC1 acts as a part of a complex to foster DSBs repair, and defects in XRCC1 specifically lead to impaired NHEJ (Audebert et al, 2004). However, the contribution of XRCC1 in DSBs repair is largely in a back-up capacity, and thus, is not the primary function of this protein (Wilson and Thompson, 2007). Moreover, unrepaired SSBs encountered by the progressing replication machinery could be converted to DSBs that are potential substrates for HR repair (Wilson and Thompson, 2007).

As far as cell cycle progression, the hypersensitivity of XRCC1-deficient CHO cells to DNA damaging agents has been linked to perturbation of DNA replication, leading to a delay of S phase progression (Brem and Hall, 2005).

In our experimental conditions EM9and AA8 cells did not show any difference in cell cycle progression as expressed by the distribution of cells in

the different phases after DMSO (dimethyl sulfoxide) synchronization in G1 phase. Furthermore EM9 cells efficiently responded to X-ray treatment with an arrest in G2 phase, such as AA8 cells, without perturbating S phase progression.

Conclusion

Our data confirm the role of genetic polymorphisms in DNA repair and xenobiotic metabolizing genes in DNA damage response and in individual susceptibility to exogenous exposure. In particular, the role of XRCC1 polymorphism (exon 10 codon 399, Arg to Gln) in SSBs (data from Comet Assay) and in DSBs (data from Micronuclei) due to IR and antineoplastic drugs has been assessed. Moreover, data from in vitro studies on EM9 and AA8 cell lines failed to show any involvement of XRCC1 gene in cell cycle modulation.

Due to the considerable number of subjects anlysed in these studies, and results from in vitro investigations, our conclusions can be considered as a part of a new experimental approach in biomedical sciences: pharmacogenomics.

Pharmacogenomics, is a new science born with the publication of human genome in 2001 (International Human Genome Consortium, 2001). Pharmacogenomics is the branch of pharmacology which deals with the influence of genetic variation on drug response in patients by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy or toxicity (Kalow W, 2002). By doing so, pharmacogenomics aims to develop rational means to optimise drug therapy, with respect to the patients genotype, while ensuring maximum efficacy with minimal adverse effects.

In addition, a new term has been coined in analogy to pharmacogenomics: radiogenomics (Andreassen et al, 2002). Radiogenomics is the study of genetic differences in the response to radiation and this is an emerging field of research in which attempts at investigating a possible genetic background for variations in clinical radio-responsiveness have concentrated on SNPs in selected candidate genes and the screening of multiple genes using gene-expression arrays (Bentzen, 2006).

Such approaches promise the advent of "personalized medicine", in which therapies (chemical- and radio-therapy) are optimised for each individual's unique genetic makeup.

V. MATERIALS AND METHODS

In vivo studies

In vitro irradiation and repair capacity assessment

Fresh blood samples, kept in plastic tubes, were exposed to 2 Gy of X-rays generated by a Gilardoni apparatus (250 kV, 6 mA, 0.2 mm copper; Gilardoni, Lecco, Italy) at a dose rate of 53 cGy/min. Exposure was carried out on ice. After irradiation, 200 µl of whole blood were used to assess cell viability by propidium iodide (PI) exclusion assay, while an aliquot (20 µl) of the blood was immediately subjected to the Comet assay. For the repair studies, the remaining blood was incubated at 37 °C to allow time for DNA repair, and aliquots taken after 30 and 60 min were analyzed by the Comet assay to measure residual DNA damage.

Nonviable cells were microscopically identified as red cells and they never exceeded 20% of total cells.

Alkaline Comet assay

The alkaline Comet assay was performed as described by Singh et al. (Singh et al, 1988), with minor modifications as previously described (Festa et al, 2003). Twenty microliters of whole blood embedded in 180 µl of 0.7% low melting point agarose in phosphate-buffered saline (PBS) (Ca and Mg free) at 37 °C, and immediately pipetted onto a frosted glass microscope slide precoated with a layer of 1% normal melting point agarose, similarly prepared in PBS. Two slides were prepared for each experimental point. The agarose was allowed to set at + 4 °C for the necessary time and the slides incubated in a lysis solution (2.5 M NaCl, 10 mM TRIS-HCl, 100 mM Na_2EDTA, NaOH to pH = 10, 1% Triton, 10% DMSO) for 50 min. After lysis, slides were placed onto horizontal electrophoresis unit containing fresh buffer (1 mM Na_2EDTA, 300 mM NaOH, pH = 13) for 20 min to allow DNA unwinding. Electrophoresis was conducted for 15 min (25 V, 300 mA) at + 4 °C. Subsequently, slides were gently washed with in neutralization buffer solution for 5 min (0.4 M TRIS-HCl, pH = 7.5), fixed in 100% freshly methanol for 3 min, and stained with ethidium bromide (2 µg/mL). Slides were analyzed using a fluorescence microscope (Leica, Milan, Italy) equipped with a camera. Fifty comets on each slide, coded

and bindly scored, were acquired using “I.A.S.” software automatic image analysis system purchased from Delta Sistemi (Rome, Italy) (Figure 21)

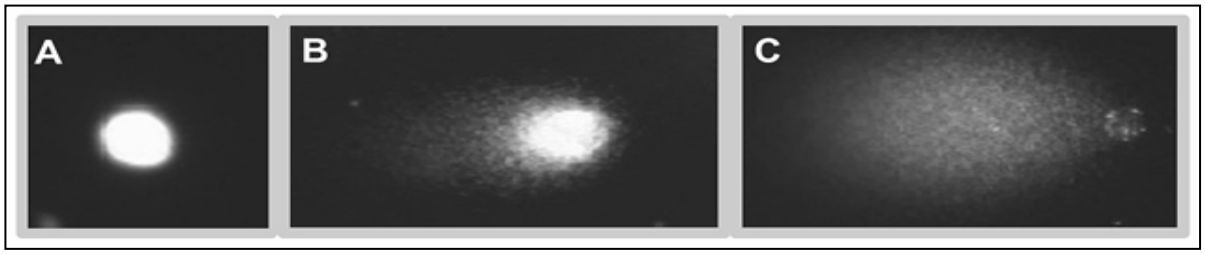


Figure 21- Images of comets (from lymphocytes), stained with ethidium bromide. A) Control cell; B) X-ray induced DNA damage; C) Highly damaged cell

To quantify the induced DNA damage, Tail DNA (TD), which is a measure of the percentage of migrated DNA in the tail (Collins, 2004) was used.

The percentage of damage remaining after 30 and 60 min repair time (residual DNA damage [RD]) was calculated as follows:

$$\frac{[TDt30(or\ 60)Xr - TDcont]}{[TDt0Xr - TDcont]} X\ 100$$

TDt30 o 60Xr = Tail DNA 30 or 60 min after irradiation

TDcont = Tail DNA of non-irradiated sample (control)

TDt0Xr = Tail DNA immediately after irradiation

Lymphocyte cultures

For lymphocyte cultures, 0.5 mL of whole blood was added to 4.5 mL of RPMI 1640 medium (MP Biomedicals), supplemented with 10% heat-inactivated fetal calf serum (Gibco-Invitrogen), 2% phytoemoagglutinin (Glaxo, Wellcome, HA15) from a stock solution of 2.25 mg/mL, 1.5% penicillin-streptomycin (5000 IU/mL and 5000 mg/mL) (Sigma Chemical Co.) and 1% L-glutamine (Sigma Chemical Co.). Cultures were grown at 37 °C.

Micronucleus assay

The MN test was performed as described by Fenech (Fenech, 2000). Lymphocytes cultures were grown at 37 °C for 72 h; 44 h after incubation, cythochalasin B (Cyt-B; Sigma Chemical Co.) was added at a final concentraton

of 6 mg/mL to arrest cytokinesis. Air-dried preparations were stained by the conventional Giemsa method. The presence of micronuclei was evaluated by scoring a total of 1000 binucleated (BN) cells with well-preserved cytoplasm.

For each subject, all slides were scored in double-blind-coded fashion by two observers to mitigate technician variability.

Genotyping of DNA repair and GSTs gene polymorphisms

DNA was isolated from the blood samples of the study participants using the Gentra Puregene extraction kit (Gentra Systems), following the manufacturer's instruction.

In Table 16 are summarized primers, annealing temperatures and restriction enzymes used for DNA repair and GSTs gene polymorphisms genotyping.

Genetic polymorphisms analysis for the GSTM1 and GSTT1 genes was conducted with PCR (Polymerase Chain Reaction)-based assays, according to published methods (Zhong et al, 1993)(Pemble et al, 1994). In Figure 22 are shown PCR products obtained for GSTM1 and GSTT1 polymorphisms analysis.

Polymerase chain reaction followed by restriction enzyme digestion was used for the genotyping of the other genes.

The genetic analysis of the DNA repair and GSTs gene polymorphisms was performed according to published methods (Tuimala et al, 2002)(Festa et al, 2005)(Ito et al, 2002)(Harries et al, 1997)(Coles et al, 2001)

In Figure 22 are shown PCR products obtained for DNA repair genes (i.e. XRCC1 and XPC) after enzymatic digestion.

All genotype analysis were performed on at least two separate occasions with appropriate positive controls, and only genotypes that showed consistent results were accepted.

Table 16 - Primers and restriction enzymes used for gene polymorphisms analysis

Gene Polymrphism	Primer sequence	Annealing Temperature (°C)	Restriction enzyme
XRCC1 399 (G>A)	**F** GCC CCT CAG ATC ACA CCT AAC	61	*HpaII*
	R CAT TGC CCA GCA CAG GAT AA		
OGG1 326 (C>G)	**R** CCCAACCCCAGTGGATTCTCATTGC	60	*Fnu4HI*
	F GGTGCCCCATCTAGCCTTGCGGCCCTT		
XPC 939 (A>C)	**F** GAT GCA GGA GGT GGA CTC TCT	61	*PvuII*
	R GTA GTG GGG CAG CAG CAA CT		
XRCC3 241 (C>T)	**F** GCT CGC CTG GTG GTC ATC	60	*Hsp92II*
	R CTT CCG CAT CCT GGC TAA AAA		
GSTM1 null	**P1** CGC CAT CTT GTG CTA CAT TGC CCG		
	P2 ATCTTC TCC TCT TCT GTC TC		
	P3 TTC TGG ATT GTAGCA GAT CA		
GSTT1 null	**F** TTC CTT ACTGGT CCT CAC ATC TC		
	R TCA CCG GAT CAT GGC CAG CA		
GSTP1 105 (A>G)	**F** ACC CCA GGG CTC TAT GGG AA	55	*BsmaI*
	R TGA GGG CAC AAG AAG CCC CT		
GSTA1 -69 (C>T)	**F** TGT TGA TTG TTT GCC TGA AAT T	54	*EarI*
	R GTT AAA CGC TGT CAC CGT CCT		

Statistical analysis

Statistical analysis of the data was carried out using GraphPad software Instat and GraphPad software Prism (version 3.02) (Graph Pad Inc.) and SPSS version 10.0 (SPSS Inc.).

The distribution of all parameters was evaluated by the Kolmogoroff-Smirnoff test. Chi-squared test was used to verify the Hardy-Weinberg equilibrium of the polymorphic alleles analyzed. Mann-Whitney U-test, unpaired t-test and Friedman test (non-parametric analysis of variance) were applied to assess statistical significance.

Finally, multivariate regression analysis was performed to examine the possible influence of confounding factors.

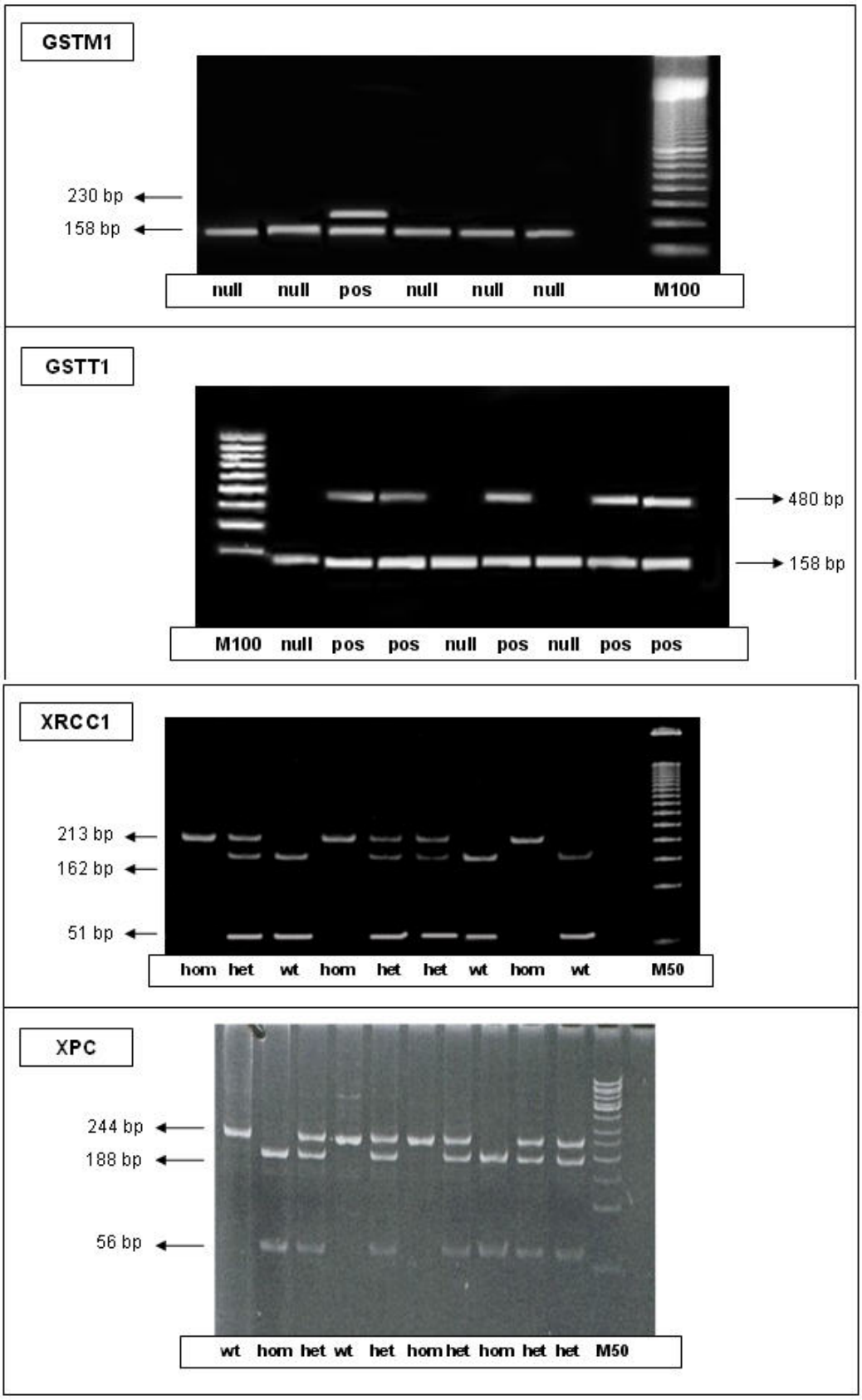


Figure 22 - Example of gel images obtained after PCR (GSTM1 and GSTT1 polymorphisms) and after RFLP-PCR (XRCC1 and XPC SNPs). Wt>wild-type respect to SNP; het>heterozygous respect to SNP; hom>homozygous respect to SNP; null>presence of gene deletion; present>no deletion (wild-type condition)

In vitro studies

Cell lines

Parental CHO (Chinese Hamster Ovary) cells, AA8 and EM9 (mutant deficient in DNA repair) were originally obtained from Dr L. H. Thompson (University of California, Lawrence Livermore National Laboratory, USA), and kindly provided by professor F. Palitti (University of "Tuscia", Viterbo, Italy).

The cells were maintained as monolayer cultures in Hams F10 (Euroiclone) medium supplemented with 10% foetal bovine serum (Euroclone), 4 mM L-glutamine (Euroclone) and the antibiotics penicillin (50 IU/mL; Euroclone) and streptomycine (50 µg/mL; Euroclone). Cells were cultured at 37 °C in 5% CO_2 atmosphere.

In vitro irradiation and determination of the repair capacity

AA8 and EM9 cells were exposed to X-rays generated by a Gilardoni apparatus (250 kV, 6 mA, 0.2 mm copper; Gilardoni, Lecco, Italy) at a dose rate of 53 cGy/min. Exposure was carried out on ice. Different doses were used to assess radiation dose-response and repair kinetics with Comet assay and γ-H2AX expression.

For assessment of X-ray induced DNA damage and DNA repair capacity, Comet assay was used as previously described **(see MATERIALS and METHODS, page 84)**. Residual DNA damage was measured after 30, 60 and 120 min after irradiation.

Flow cytometric analysis of DNA content and BrdU incorporation

To distinguish between early and late S phase cells from G1 and G2, respectively, AA8 and EM9 cells harvested at 2, 5 and 8 hours after irradiation were simultaneously analysed for PI and FITC (fluoresceinisothiocyanate)-BrdU (Bromodeoxyuridine) fluorescence to detect DNA content and BrdU incorporation. Both cell lines were synchronized in G1 phase before X-ray treatment, as previously described (Fiore et al, 2002). Cells were exposed to 45 µM BrdU during the last 25 min prior to harvesting, collected and washed with PBS and fixed in 1:1 absolute methanol:PBS mixture. Immunostaining with BrdU monoclonal antibody consisted of DNA denaturation by incubating $1X10^6$

cells in 1 mL of 3 N HCl for 45 min at room temperature and buffered with 1 mL 0.1 M sodium tetraborate (Sigma) to neutralize HCl. BrdU detection was carried out by incubating cells with primary mouse monoclonal antibody anti-BrdU (Dako) plus 5 µl goat serum in 100 µl PBS for 45 min at room temperature in the dark.

Then cells were washed twice with PBT (0.5% Tween-20 in PBS) and incubated with the anti-mouse FITC-conjugated anti-Ig G antibody (Vector Laboratories) plus 5 µl goat serum in 100 µl PBS for 30 min. Samples were washed twice with PBT and finally stained in 20 µg/mL PI for 15 min at room temperature. Flow cytometric analysis was carried out using a Galaxy flow cytometer (Dakocytomation) equipped with laser (488nm-25mV) and UV lamp (HBO-100W). Red fluorescence (DNA content) was detected with a 600 nm wavelength long pass filter and green fluorescence with a 514 nm bandpass filter. Ten thousand events were collected for each sample and biparametric analysis of total DNA content and BrdU content was performed using WinDMI 2.7 software.

Flow cytometric analysis of phosphorylated histone H2AX

Cells were fixed for 10 min in 2% ice-cold paraformaldehyde solution (Sigma) at 37 °C, and permeabilized with 90% methanol at -20 °C for 30 min on ice. After twice rinsing in PBS, cells were incubated overnight at 4 °C, in 50 µLof 0.5% BSA/PBS containing 1:100 diluition of phosphor-specific (Ser-139) histone H2AX (γ-H2AX) rabbit polyclonal antibody (mAb) (Cell Signaling Technology). After incubation cells were washed twice with PBS and then incubated in 50 µL of 1:300 diluition of Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) for 30 min at room temperature in the dark, washed twice with PBS, counterstained for 30 min with 50 µg/mL PI solution (Sigma), and the analysed by flow cytometry (Galaxy, Dakocytomation). A biparametric analysis, γ-H2AX vs DNA content, was performed by FloMax software (Partec). Cell percentage of positive γ-H2AX cell population was calculated by electronic gate (Figure 23).

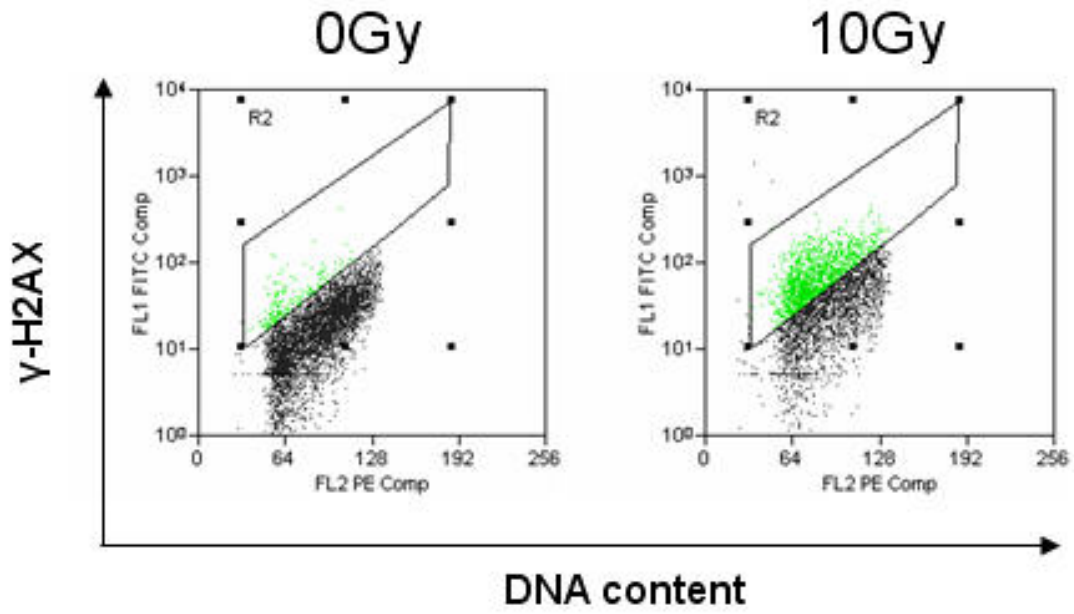


Figure 23 - Bivariate distribution of γH2AX versus DNA content in untreated and 10 Gy-treated cells. The rectangular gates show the position of γH2AX positive cells.

Statistical analysis

Statistical analysis of the data was carried out using GraphPad software Instat and GraphPad software Prism (version 3.02) (Graph Pad Inc.) and SPSS version 10.0 (SPSS Inc.).

The distribution of all parameters was evaluated by the Kolmogoroff-Smirnoff test.

Mann-Whitney U-test, unpaired t-test and Friedman test (non-parametric analysis of variance) were applied to assess statistical significance.

Statistical analysis

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VII. APPENDIX

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AKNOWLEDGMENTS

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Dr. Stefano Leone
Dr. Silvia Sterpone
Valeria Mastellone

ENEA – Casaccia Research Center, Rome, Italy

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