UNIVERSITY OF PISA

RESEARCH DOCTORATE SCHOOL IN BIOLOGICAL AND BIOMOLECULAR SCIENCES - PhD Course in Biology

"The role of N-acetylcisteine and genetic polymorphisms in XRCC1, XRCC3, GST genes in the modulation of DNA damage and tissue toxicity induced by medical ionizing radiation exposure"

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Academic Year 2010/2013

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Abstract

Ionizing procedures provide essential life-saving information, but great care must be taken regarding their possible long-term health consequences. The biological and clinical burdens of medical radiation represents a worrisome social and medical problem. DNA damage is the main initiating event by which radiation may results in cancer development. Thus considerable efforts should be made to mitigate radiation-induced cell damage. Because radiation induced cellular damage is attributed primarily to the harmful effects of free radicals, the efficacy of non-toxic radioprotectors with radical scavenging properties should be investigated in the clinical setting. These agents may inhibit or reduce free radical toxicity, thus offering protection against radiation. N-acetylcysteine (NAC) is considered a promising radio-protector for its antioxidant and anticarcinogenic properties and could be able to inhibit or reduce free radical toxicity, thus offering protection against radiation.

Moreover, recent evidences have recognized that genetic factors influence the risk of radiation-induced effects and the Seventh National Academy of Science report on Biological Effects of Ionizing Radiation (BEIR VII) included the identification of genes conferring predisposition to radiation-induced health effects as a top research need. Genetic polymorphisms in the detoxification and DNA repair genes are specifically proposed as candidates for genetic predisposition to radiation-induced biological damage. The identification and characterization of genes that enhance prediction of disease risk and improve prevention, treatment, and quality of care remain important goals in the modern imaging practice. Specifically, it is anticipated that the use of genetic markers may serve as the basis for personalized radiotherapy in which cancer management is formulated so that it optimizes the treatment plan for each patient based on their genetic background (radiogenomics).

In order to improve this knowledge, the primary aims of the project were:

- **Aim 1: to evaluate the ability of NAC in conferring protection against radiation induced chromosomal DNA damage.**

- **Aim 2: to assess the value of functional polymorphisms of genes involved in DNA damage repair and oxidative stress response as predictive factors for the occurrence of acute skin reactions.**

To reach aim 1, 65 patients undergoing invasive cardiovascular procedures received the standard hydration protocol consisting of intravenous isotonic saline for 12 h after catheterization (Group I) while 30 patients received a clinically driven double intravenous dose of NAC for 1 hour before and a standard dose for 12 hours following catheterization (Group II). Micronucleus assay (MN) was performed as biomarker of chromosomal damage and intermediate endpoint in carcinogenesis. MN frequency evaluated before, 2 and 24 hours after the radiation exposure showed a significant increase of 24.1% at 2 hours and of 21.4 % at 24 hours in the Group I (p=0.03), while the non-significant increase of MN was 13.1% at 2 hours and 8.7% at 24 hours in Group II (p=0.4). These results suggested that NAC may be an effective promising, well-tolerated antioxidant approach easily usable in the clinical practice to offer protection against DNA damage induced by ionizing radiation exposure during cardiac catheterization procedures.

To reach aim 2, skin toxicity was scored according to Radiation Therapy Oncology Group (RTOG) criteria in 59 breast cancer patients undergoing radiation therapy after conserving surgery. Single nucleotide polymorphisms (SNPs) in XRCC3 (Thr241Met), XRCC1 (Arg399Gln, and Arg194Trp) and in GSTT1 and GSTM1 were determined by PCR-RFPL analysis. According to RTOG criteria, grade 1 and 2 acute skin reactivity was observed in 24 (41%) of the 59 participants. Univariate analysis indicated that XRCC3 241Met variant (OR: 2.5 95% CI: 1.0-7.3, p=0.05) and GSTM1 null genotype (OR: 3.5 95% CI:1.2-10.4 p=0.02) as well as BMI (OR: 3.6 CI: 1.2-11, p=0.02) were associated with the risk of acute skin radiosensitivity. The logistic multivariate analysis confirmed that the two genetic variants increased the individual susceptibility to acute skin reaction. Our findings suggest that the presence of SNPs involved in DNA repair and oxidative stress may in part explain the individual response to acute skin toxicity in patients undergoing partial breast irradiation after conserving surgery. The association analysis between clinical characteristics and genotype with the acute radiation skin toxicity in breast cancer patients suggests that approaches based on (multiple) genetic

markers and clinical characteristics have the potential to predict normal tissue radiosensitivity.

Although our findings are to be carefully assessed with further large, randomized studies, taken together have outmost clinical relevance since add important information to reach a personalized measure of radiation risk in order to implement tailored preventive and chemopreventive strategies.

1 Introduction

1.1 Radiation exposure from medical imaging procedures

Ionizing radiation is daily used in diagnostic radiology and nuclear medicine to better visualize organ and/or vessel anatomy to have physiological information that help the clinicians in the treatment of disease. The diagnostic information can provide a better understanding of a patient disease, prognosis, treatment response, or to guide therapy. Medical imaging is an essential tool in clinical filed as confirmed by its increased use in the United States in the last ten years: from 26 million in 1998 to more than 70 million in 2008. A recent report on medical radiation exposures in the US population shows that the *pro-capite* collective dose of radiation received from clinical imaging has increased by greater than 700% between 1980 and 2006 [Mettler FA, 2008] and cardiac imaging has contributed greatly to this warming [Bedetti G, 2008; Ait-Ali L, 2010; Kaul P, 2010]. Despite many clinical advantages, ionizing radiation is recognized as a proven human carcinogen. The increasing exposure to medical imaging might result in a raising incidence of radiation-related cancer[Brenner DJ, 2007]. Accordingly, medical imaging has been identified as one of the major causes of environmental exposure to carcinogens [President's Cancer Panel, 2010]. Exposure to ionizing radiation carries a carcinogenesis risk that is thought to be linear and cumulative. According to the "linear-no threshold" (LNT) model, no radiation doses - no matter how small - can be considered completely safe [BEIR VII 2006; ICRP 2007, UNSCEAR 2008]. The National Research Council Committee on the Biological Effects of Ionizing Radiation (BEIR VII) of the National Academy of Sciences have confirmed the LNT model in a recent updated report on the health risk of exposure to ionizing radiation [BEIR VII 2006]. This update considers the evidence obtained in epidemiological studies of exposed populations that include atomic bomb survivors, patients exposed to radiation from diagnostic and therapeutic medical studies, as well as studies of occupational and environmental exposures [BEIR VII, 2006].

In the last years, many studies have provided a consistent evidence that several genetic, environmental and dietary factors can affect the variability of damage observed at any given level of radiation. Thus current research is target in shifting epidemiological evidence towards personalized measure of radiation risk in order to implement tailored preventive and chemopreventive strategies.

1.2 Ionizing radiation in cardiac catheterization procedures

Over the last 20 years, the number of interventional cardiovascular procedures has increased rapidly. In Europe, arteriography and interventions were 350,000 in 1993 and > 1 million in 2001 [Togni M, 2004]. On average, a left ventriculography and coronary angiography correspond to a patient radiation exposure of about 300 chest x-rays; and a percutaneous coronary intervention or a cardiac radiofrequency ablation to 750 chest x-rays (range: 350-2350) [Gerber RT, 2009].

The attributable cancer risk of getting cancer from a 15 mSv exposure is 1 in 750 for an adult male and 1 in 500 for a woman Children are especially vulnerable. The same dose confers an extra-risk for a 1-year old infant 10-15 times greater than a 50 year old adult, and female infants have almost double the risk than that of male infants. The typical effective doses for common cardiologic testing are reported in Table 1.

Table 1. Typical effective doses from diagnostic medical exposure

	Diagnostic procedure	Typical effective dose (mSv)	Equivalent number of chest x-rays	Approximative equivalent period of natural background radiation (years)
ADULT	Diagnostic invasive coronary angiogram	7 (2-16)	350	2.9
	Percutaneous coronary intervention	15 (7-57)	750	6.2
	Radiofrequency ablation	15 (7-57)	750	6.2
	Mitral valvuloplasty	29	1450	12.1
	Aortic valvuloplasty	39	1950	16.2
	Head and/or neck angiography	5 (1-20)	250	2.1
	Thoracic angiography of pulmonary artery or aorta	5 (4-9)	250	2.1
	Abdominal angiography or aortography	12 (4-48)	600	5
	Pelvic vein embolization	60 (44-78)	3000	25
	Transjugular intrahepatic portosystemic shunt placement	70 (20-180)	3500	29.1
PEDIATRIC	Diagnostic cardiac cath	6.0 (0.6-23.2)	300	2.5
	ASD	2.8 (1.8-7.4)	140	1.1
	Patent ductus arterovenous occlusion	7.6 (2.1-37)	380	3.1
	Balloon dilation	8.1 (2.9-2.0)	405	3.3

In adult cardiology patient, interventional cardiology procedures account for 12% of examinations, and 48% of the total collective dose [Bedetti G, 2008]. In children with congenital heart disease, invasive cardiology (with diagnostic and interventional catheterization) accounts for 6% of all radiological examinations and 84% of the collective dose [Ait-Ali L, 2010]. Also, the number of professionally exposed subjects in

the catheterization laboratory continues to rise. According to 2008 UNSCEAR estimates, the number of occupationally exposed workers totals 22.8 million (plus military personnel), and 7.35 million of these are medical workers [UNSCEAR, 2008]. Among medical workers, interventional cardiologists have by far the highest exposure, corresponding to the dose equivalent of 200 to 300 chest x-rays per head per year, two-to-three times higher than radiologists or nuclear physicians, with a corresponding levels of long-term lifetime attributable cancer risks in the range of 1 extra-cancer in 50- 1 in 200 [Venneri L, 2009]. These estimates have been also recently corroborated by direct assessment of biological damage resulting from ionizing catheterization procedures in both patients and personnel operating in catheterization laboratory. These studies have shown that interventional cardiology procedures can damage the DNA of the cell to be detectable-acutely and in the long-term as increased chromosomal DNA damage in circulating lymphocytes that represent an intermediate endpoint of cancer [Hagmar L, 1998; Bonassi S, 2007]. Indeed, the lifetime exposure of a young adolescent with congenital heart disease in the range of 20 mSv is associated with a dramatically 200% increased frequency of chromosomal DNA damage when compared to age- and sex-matched control subjects [Andreassi, 2006]. Furthermore, contemporary interventional cardiologists have an increased rate of chromosomal damage when compared to clinical cardiologists [Andreassi, 2005]. Interestingly, radiation-associated chromosomal damage in interventional cardiologists is associated to the presence of specific genetic polymorphisms suggesting that the risk estimates at the population level can be highly inaccurate at the individual level [Andreassi, 2009]

1.3 Radiation therapy for the treatment of breast cancer

Radiation therapy is one of the most important modalities for treating various types of localized cancer, and can be used as a form of palliative therapy for symptoms once a patient develops metastatic disease. Breast cancer is the most common form of cancer in women especially in industrialized areas [IARC, 2000]. In Europe, it represents 30%

of all incident tumors in females with rates ranging from about 40/100000 in Lithuania and Poland, to 75/100000 in Norway, and to over 90/100000 in the Netherlands and some Italian areas [Ferlay J, 2007]. Overall, the annual breast cancer incidence has been increasing worldwide during the last century, but a declining trend in tumor incidence was observed since the year 2000 among women older than 45–50 years in the United States and some European countries. This decreasing trend may be ascribed to the decreased use of hormone replacement therapy [Merlo DF, 2012]. Therefore, the early detection and immediate treatment of breast cancer are two main factors influencing the prognosis of the disease. Surgery, chemotherapy and radiotherapy are the three mainstays in cancer treatment.

Surgery is a very effective form of treatment, because solid tumors can be resected in their entirety together with all adjacent tissue into which the tumor may have spread. For a long time, radical mastectomy has been used to treat women with breast cancer. Nowadays, a segmental mastectomy or breast-conserving therapy is used when possible to maintain a normal breast appearance after the surgery [Stephens FO, 2009].

Chemotherapy is the treatment of cancer with cytotoxic drugs affecting cell division. These drugs are generally classified according to their mechanism of action, including antimetabolites (e.g. 5-fluoruracil), DNA damaging agents (e.g. cyclophosphamide), mitosis inhibitors (e.g. taxol) and cancer cell enzyme in-activators (e.g. tyrosine-kinase inhibitors). All these drugs, however, have side effects [Stephens FO, 2009].

The third hallmark of cancer treatment is radiation therapy, which uses X-rays to destroy the tumor. Around 50% of patients are treated with radiation therapy (World Cancer Report [Stewart BW, 2003]), alone or in combination with surgery and chemotherapy. Radiotherapy after breast-conserving surgery is now widely accepted as the standard of care for patients with early breast cancer. This technique reduces the risk of loco-regional recurrence of cancer by approximately 70%, and has been shown to be as effective as radical mastectomy [Fisher B, 2002]. If on the hand radiation therapy destroys the cancer cells remained after surgery, on the other hand causes radiation-induced side-effects in the surrounding normal healthy tissues

[Barnett GC, 2009]. Breast-conserving surgery instead of radical mastectomy represents a good strategy for the reduction of normal tissue toxicity. While today the majority of patients well tolerates standard radiation therapy, clinicians still observe a substantial amount of patients (up to 10%) who suffer from adverse effects arising from the intrinsic sensitivity of healthy tissues [Bentzen SM, 2003]. Normal tissue reactions are of clinical importance since affect the patient quality of life.

2 Early biological effects of ionizing radiation

The biological effects of ionizing radiation may be manifested as clinical symptoms and can be classified into two categories: deterministic and stochastic effects. Deterministic effects, such as erythema or cataracts, are mainly based on extensive cell death. They are most often seen in cases of high doses of radiation delivered over a short period of time (i.e. in the case of acute exposure in radiation accidents or radiation therapy) and they have a threshold dose below which the biological response is not observed. The severity of deterministic effects increases sharply with increasing dose. A stochastic effect is a probabilistic event and there is a known threshold dose. Indeed, damage to the DNA is considered the early event in radiation-induced stochastic effects. Damage to the DNA, which carries the genetic information in the chromosomes of the cell nucleus, is considered to be the main initiating event by which radiation damage to cells results in the development of cancer and hereditary disease in the exposed subjects [Andreassi MG, 2004].

2.1 Radiation induced DNA damage

The damage to DNA can cause single-strand breaks, double-strand breaks (DSBs) and cross-links. A single-strand break (SSB) is formed when the phosphodiester bond between the sugars on the DNA strand is broken. If this occurs on both strands, within a distance of 10–20 base pairs, neither the hydrogen bonds between base pairs nor the chromatin structure will be strong enough to keep the strands together, giving rise to a double-strand breaks [Magnander K, 2012]. This damage can be repaired on a minute time-scale after the damage has occurred by the activation of DNA repair genes. Radiation-induced SSB are usually readily repaired using the opposite DNA strand as a template. Radiation-induced DSBs are the most biologically important DNA lesions, as they are usually accompanied by extensive base damage, a phenomenon termed “locally multiply damaged site(s)“. The capacity of normal cells to repair damage of

DNA is genetically determined and varies between individuals. More than 150 polymorphic genes have been described in DNA repair pathways. This genetic variation is linked with the differing sensitivities of individuals to radiation damage [Feinendegen LE, 2007; Franco N, 2005].

With a delay of several hours, the damaged cells with unrepaired or mis-repaired DNA damage can be removed by apoptosis, necrosis and appropriate immune responses [Martin LM, 2010]. However, some cells may 'escape' from these protective mechanisms and genomic instability and oncogenic transformation with potential cancer development may occur [Sedelnikova OA, 2010; Asaithamby A, 2011].

2.1.1 Direct and indirect DNA damage

Radiation damage to DNA can be ascribed to both direct and indirect mechanism (Fig 1). Direct damage occurs as a result of the interaction of radiation energy with DNA. The indirect DNA damage of ionizing radiation is due to the production of free radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), which are generated during radiolysis of water. These radicals are effective oxidants able to break chemical bonds and initiating DNA damage, within the nano- to microsecond timeframe. If direct and indirect damages following radiation exposure are not repaired, the cell structure and function will be affected [Gaigeot MP, 2010]. Endogenous or exogenous levels of radiation-protective agents (see section 2.1.3) are determinants of the cellular scavenging capacity [Karin M, 2012].

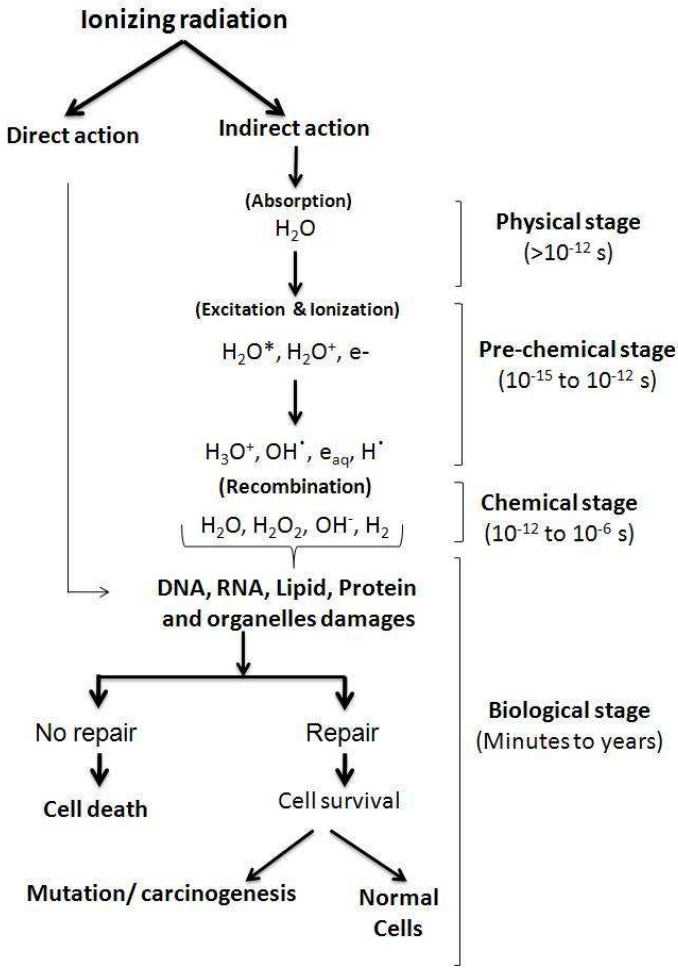

Figure 1. Effect of radiation: chain of the cellular events occurring in the cell/ tissue after ionizing radiation exposure [From Gaigeot MP, 2010]

2.1.2 The micronucleus assay in peripheral lymphocytes as a biomarker of DNA damage

Cytogenetic alterations in cultured peripheral blood lymphocytes, such as chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MN), have been applied as biomarkers of genotoxic exposure and early effects of genotoxic carcinogens for many years [Albertini RJ, 2001; Norppa H, 2004]. The rationale of using these assays derives from the evidence that most established human carcinogens are genotoxic in short-term tests and capable of inducing chromosomal

damage [Norppa H, 2004]. The relevance of the increased frequency of cytogenetic alterations as a cancer risk biomarker is further supported by epidemiological studies suggesting that a high frequency of CAs is predictive of an increased risk of cancer [Hagmar L, 1998; Bonassi S, 2000]. CAs are structural aberrations comprising of chromosome-type and chromatid-type breaks and rearrangements [Norppa H, 2004]. SCEs are interchanges of DNA replication products between sister chromatids at apparently homologous loci, suggested to represent homologous recombination repair of DNA double strand breaks [Johnson RD, 2000]. The two basic phenomena leading to the formation of MN in mitotic cells are chromosome breakage and dysfunction of the mitotic apparatus. MN are small additional nuclei originating from chromosomal fragments or whole chromosomes that lag behind in anaphase (Fig.2) [Falck GC, 2002]. These fragments do not efficiently integrate in any of the daughter nuclei because they are incapable of attaching to the spindle fibers and are left behind during mitosis. During telophase, they are enclosed by the nuclear envelope and arise as micronuclei in the next cell cycle. If two broken chromosome ends are mis-repaired and fuse with each other, a CAs can be formed. Dicentric chromosomes can also be formed by the fusion of two unprotected ends resulting from telomeric dysfunction [Shay JW, 2005]. The two centromeres of the dicentric chromatids can be pulled to opposite poles during the next anaphase, forming a chromosomal bridge that is frequently resolved by breakage. This breakage may result in the formation of acentric fragments that, at the end of mitosis, arise as micronuclei [Hoffelder DR, 2004] or as nuclear blebs, which are micronucleus-like bodies physically connected to the nucleus by a chromatinic filament [Pampalona J_a, 2010; Pampalona J_b, 2010]. The broken bridge often constitutes a source of chromosomal instability, as the resulting unprotected chromosomal ends are susceptible to suffering further reorganization [Terradas M, 2012]. Alternatively, micronuclei can contain a whole chromosome arising from anaphase loss. This loss is a consequence of defects during the mitotic spindle assembly, misregulation of the spindle assembly checkpoint or the presence of supernumerary centrosomes [Fenech M, 2011_b]. It has also been shown that micronuclei can contain whole chromatids derived from the merotelic attachment of chromosomes to the mitotic spindle. In this sense, when a single kinetochore is connected to microtubule bundles coming from both poles, the affected chromatid

lags behind from the bulk of chromosomes and, at the end of mitosis, is enclosed into a micronucleus [Cimini D, 2002].

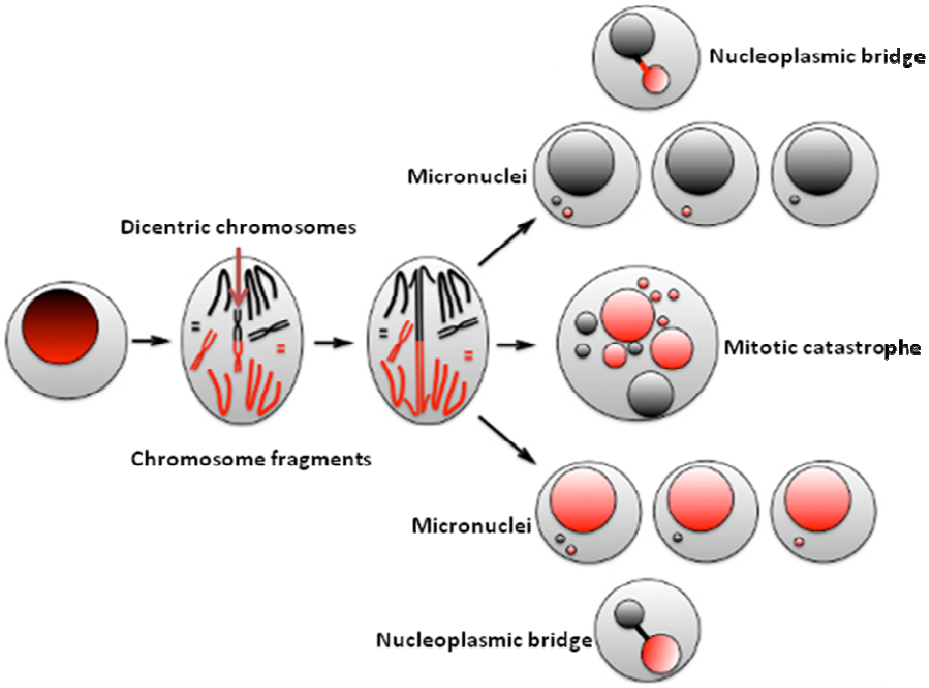

Figure 2. Micronuclei and nucleoplasmic bridge formation in cells undergoing nuclear division

In comparison with chromosomal aberrations (CA), the scoring of MN is simpler, requires shorter training and is less time consuming. In principle, the MN assay can be expected to be more sensitive than the CA assay, because of the increased statistical power brought about by the fact that the number of cells analyzed can easily be increased to thousands when only a hundred or a few hundred cells are usually scored for CA.

MNs, usually detected in lymphocytes by the cytokinesis-block MN assay [Fenech M, 1985] are the most frequently used chromosomal biomarker in human lymphocytes in order to study genotoxicity and cytotoxicity *in vitro* [Fenech J, 2007]. The use of the MN assay in peripheral lymphocytes is also employed as a biological dosimeter in order to evaluate *in vivo* ionizing radiation exposure [Vral A, 2011]. According to the different origin of micronuclei, their presence in cells is not only an indicator of mutagenic agent exposures but also an indicator of ongoing chromosome instability. Most importantly,

Bonassi et al. provided evidence that high micronucleus frequencies in peripheral blood lymphocytes of healthy individuals are predictive of higher cancer risks, suggesting that increased micronuclei formation is associated with early events in carcinogenesis in non exposed humans [Bonassi S, 2011; Bonassi S, 2007]. Recently, Ait-Ali et al. have shown an increase of micronucleus frequencies in peripheral blood lymphocytes of exposed children few hours after the end of the catheterization procedure [Ait-Ali L, 2010]. Significant somatic DNA damage, measured by an acute increase in micronuclei in circulating lymphocytes has been also demonstrated in patients undergoing invasive cardiovascular procedures and medical staff [Andreassi MG, 2005; Andreassi MG, 2007].

2.1.3 Radiation-protective agents in the reduction of radiation damage

The term 'radiation-protective agent' refers to any agent that protects against radiation-induced damage, whether administered before, during, or after irradiation [Stone HB, 2004]. Radioprotectants, radiation mitigators (or mitigants), and therapeutic agents are the three main classes of 'radiation-protective agent' as defined in Table 1. Radioprotectants are administered before radiation exposure to prevent damage; mitigators are administered during or after radiation exposure with the aim of preventing or reducing the action of radiation on tissues before the appearance of symptoms. Finally, therapeutic agents are administered after radiation exposure to treat or facilitate recovery from various aspects of the acute radiation syndrome and delayed effects of radiation exposure [Dumont F, 2010].

Table 2. Main classes of radiation countermeasure agents [From Dumont F et al, 2010]

Category	Properties	Timing of administration
Radioprotectant	Prevent injury from IR-induced free radicals and other reactive species. Enhance radiation tolerance of critical tissues by increasing their cellularity and the production of protective mediators	Given before irradiation
Radiation mitigator	Reduce the potential severity of IR injury. Minimize the risk of clonogenic death of normal cells. Accelerate tissue recovery and repair processes	Given after irradiation, but before phenotypic expression of tissue damage and appearance of overt symptoms of IR injury
Therapeutic	Palliative and supportive in nature. Diminish the pathophysiology of IR injury by reducing chronic oxidative stress and inflammation. Facilitate tissue recovery and regeneration. Prevent or reverse tissue fibrosis and other late effects	Given after irradiation and the appearance of overt symptoms of IR injury

IR: Ionizing radiation.

Thousands of compounds have been tested for radio-protective effects but most of them have only been tested on cell cultures and rodents using lethality as an end point [Liu Y, 2007]. An extensive review has been conducted by Weiss and Landauer [Weiss JF, 2009]. The main types of antioxidants are sulfhydryl compounds, polyphenols, superoxide dismutase, and vitamin E analogs [Coleman CN, 2003]. The best known class of them are the sulfhydryl compounds. The radioprotective properties of such compounds, including cysteine and cysteamine, were investigated as early as the late 1940s. These compounds rely on a potential sulfhydryl group at the end of the molecule for free-radical scavenging and also hydrogen atom donation to facilitate direct chemical repair at sites of DNA damage.

N-Acetylcysteine (NAC) is a natural compound found in several vegetables that is considered to be the most “natural” of the thiol protectors and is approved for human use for various purposes [Weiss JF, 2003]. NAC, an aminothiol and synthetic precursor of intracellular cysteine and GSH, has been used as a mucolytic agent. In addition, it has been shown to prevent radiation-induced DNA damage [Mansour HH, 2008]. Kuefner et al. have shown that the administration of a radiation-protective oral agent containing a mixture of antioxidants such as α–tocopheryl succinate, N-acetylcysteine, glutathione elevating compounds, can reduce DNA damage by 23% after CT examinations [Kuefner MA, 2012]. Recently radioprotective effects of NAC have been

demonstrated on radiation toxicity in intestinal [Sridharan S, 2002] and hepatic tissue [Mansour H, 2008]. Several clinical studies also demonstrated the efficacy of NAC in the prevention of contrast-induced nephropathy in unselected populations undergoing procedures involving intravascular contrast administration [Isenbarger W, 2003; Birck R, 2003; IAEA, 2001; Wu MY, 2013]. The evidence from in vivo studies suggests that NAC is capable of replenishing intracellular GSH by reducing extracellular cystine to cysteine [Issels RD, 1988], or by supplying sulfhydryl groups that can stimulate GSH synthesis and enhance glutathione-S-transferase activity [De Flora S, 1985]. NAC is a potent free radical scavenger as a consequence of its nucleophilic reactions with reactive oxygen species (ROS) [De Flora S, 2001]. Thus, NAC treatment may be beneficial for conditions of free radical formations during oxidative stress [Reliene R, 2004; Mansour HH, 2008].

2.2 Radiation induced tissue toxicity

A substantial amount of cell killing, sufficient to result in detectable tissue reactions, may be the direct consequence of high dose radiations. This cellular response may occur early (days) or late (months to years) after irradiation, depending on the tissue. The manifestations of tissue injury vary from one tissue to another depending on cellular composition, proliferation rate and mechanisms of response to radiation. Examples include cataracts of the lens, cell depletion in the bone marrow causing hematological deficiencies, non-malignant damage to the skin (i.e. skin-toxicity during radiation therapy) [Annals of the ICRP, 2011].

Radiation-induced skin toxicity is a prominent clinical problem affecting the majority of breast cancer patients receiving breast radiation therapy (RT) after conservative surgery. Acute and chronic toxicities have been assessed in patients treated with adjuvant breast RT, including skin (30-40%) and heart toxicity (1.5%) [Clarke M, 2005; Darby SC, 2005]. Today most patients tolerate well standard RT but RT-induced skin

toxicity is still a relevant clinical problem affecting a substantial amount of breast cancer patients receiving adjuvant RT and can lead to temporary or permanent cessation of treatment [Chen MF, 2010].

2.2.1 Non-neoplastic-tissue reaction after Radiation Therapy (RT)

Non-neoplastic or normal tissue toxicity to RT is commonly classified according to the time taken to exhibit clinical injury into early and late effects. Early tissue reactions (hours to a few weeks after irradiation) may be of an inflammatory nature, occurring as a result of cell permeability changes and release of inflammatory mediators. Subsequent reactions are often a consequence of cell loss e.g. mucositis and desquamation in epithelial tissues, although non-cytotoxic effects on tissues also contribute to these early reactions. Early reactions include erythema, epilation and desquamation.

Late tissue reactions (months to years after irradiation) are called “generic” if they occur as a result of injury directly in the target tissue e.g. vascular occlusions leading to deep tissue necrosis after protracted irradiations, or “consequential” if they occur as a result of severe early reactions, e.g. dermal necrosis as a result of extensive epidermal denudation or chronic infection, and intestinal strictures caused by severe mucosal ulceration [Dorr W, 2001]. Late reactions include dermal erythematous reactions, atrophy, induration, telangiectasia, necrosis and fibrosis. However, it is important to realize that early and late tissue reactions are not mutually exclusive and may often coexist. Skin reaction during RT is the most frequent normal tissue side-effects and can affect the therapeutic program and worsen the quality of life of patients [Bentzen SM, 2003].

2.2.2 Inter-individual variations in the development of tissue toxicity

There has been intense interest in the phenomenon of inter-individual variation in normal tissue toxicity in response to RT treatment. Next to the issue of normal tissue toxicity in the RT treatment of cancer patients, large inter-individual variations in the rate and severity of the development of these reactions is seen. This accounts for acute reactions as well as for late reactions. The parameters influencing the individual radiosensitivity can be subdivided into:

• **Therapy-related factors** (total dose, dose per fraction and volume irradiated, irradiation site, dose inhomogeneity and use of concomitant chemotherapy).

• **Patient characteristics** (age, smoke status, hemoglobin level and co-morbid conditions such as diabetes, hypertension, vascular diseases).

• **Genetic background** (genetic variants, most often, single nucleotide polymorphism in DNA repair and detoxification genes may influence inter-individual variation in normal tissue toxicity).

Therapy related factors such as the total tumor dose, the dose per fraction, and the dose-volume on the healthy tissues have an impact on the incidence and severity of acute as well as chronic normal tissue toxicities [Stone HB, 2003]. Additional treatment modalities like chemotherapy and surgery cause further traumas that can significantly aggravate the radiation responses [Joiner M, 2009; Stone HB, 2003; Azria D, 2008].

The dose-effect relationship is stronger for the late responsive tissues compared to the acute responding tissues. Treatment regimes that result in a decrease in the total tumor dose therefore could reduce the incidence and severity of late adverse effects [Azria D, 2008]. The dose per fraction generally influences the severity of both the acute and late effects, although late responding tissues are the most sensitive to changes in the dose per fraction [Stone HB, 2003]. Late responding tissues therefore show a greater ability to recover during fractionated exposure than early responding tissues. Hyperfractionation regimes (lower dose per fraction, multiple fractions a day)

could lower the incidence of late toxicities without a decrease in total tumor dose and therefore without affecting the cure rates. This is because the shoulder (large for late responding tissues) of the cell survival curve at low radiation doses is repeated when the dose is split in multiple fractions [Willers H, 2006]. The difference in fractionation sensitivity of early- and late responding tissues is described using the α/β ratio. The α–component is responsible for the linear component of the survival curve (unrepairable damage). The downward bending component is caused by the □-component ('shoulder': repair of sublethal damage). The α/β ratio represents the dose for which α and β equally contribute to the cell damage [Bomford CK, 1993].

The risk of adverse effects after RT is also influenced by general condition and habits of the patients. Age, nutritional status, medications, recent surgery in an irradiated site and co-morbidities, especially those affecting normal vascular function like diabetes, connective tissue disease and arterial hypertension are widely considered to affect the expression of radiation-induced morbidity [Hölscher T, 2006]. Lifestyle behaviors such as smoking tobacco, consuming alcohol, eating spicy can intensify the response to RT as well [Joiner M, 2009]. Patients who continue to smoke during their therapy for head and neck cancer show a significant increase in acute skin reactions [Porock D, 2004]. Treatment- and patient-related parameters can explain only a part of variability existing among individuals. The hypothesis that patient radiosensitivity can be affected by the genetic alterations in some genes originates from the studies of patients with certain rare genetic syndromes such as ataxia telangiectasia, Nijmegen breakage syndrome, Fanconi's anaemia and Bloom's syndrome. Although these syndromes are rare and not representative for the general unselected cancer patients, they do act as a proof of principle that genetic factors can influence an individuals' sensitivity to ionizing radiation. Acute side effects (erythema and desquamation of skin) occurring during or shortly after radio-treatment are normally checked by clinicians since they are particularly interested in predicting the normal tissue reactions of patients before radiotherapy starting in order to personalize the therapy and optimize the results. During last decade mutations in repair genes have been detected in extremely radiosensitive cancer patients, not suffering from any known syndrome [Rogers PB, 2000]. Normal-tissue toxicity in breast cancer patients following RT after conserving

surgery correlate with several different genetic alterations [Suga T, 2007; Moullan N, 2003]. Abnormal DNA repair and cell death regulation in such individuals may result in higher vulnerability to irradiation. Some of them also manifest chromosome instability that is associated with higher incidence of cancer [Bourguignon MH, 2005]. I*n vitro* assays for the radiosensitivity of peripheral blood lymphocytes have suggested that breast cancer patients are more radiosensitive than healthy controls [Burrill W, 2000; Scott D, 1998].

3. Individual genotype and ionizing radiation susceptibility

Mammalian cells have evolved distinct mechanisms to repair different types of DNA damage to maintain genomic integrity. Humans showing severely compromised repair capacity have increased mutation rates, genomic instability and an increased risk of cancer [Berwick M, 2000]. Healthy subjects can also differ in intrinsic capacity in repairing DNA damage [Setlow RB, 1983]. Single nucleotide polymorphisms (SNPs) in DNA repair gene can affect the individual susceptibility to radiation exposures. These SNPs significantly contribute to the increased amount of unrepaired DNA damage that in turn, results in a raised mutation frequency, genetic instability and acute skin toxicity [Chang-Claude J, 2005; Andreassen CN, 2005]. Nowadays, radio-genomic, the research focused on the study of genetic variations to explain the inter-individual differences in response to therapeutic radiation exposure, is of increasing interest. The modulation of repair capacity by SNPs in genes responsible for DNA damage repair and detoxification enzymes might affect the individual sensitivity to radiation damage [Parliament MB, 2010]. As a consequence of radiation-induced DNA damage, the cells activate highly conserved mechanisms for the maintenance of genomic integrity and reparation of DNA lesions:

- Base excision repair (BER) removes and corrects damaged bases and single strand breaks;
- Nucleotide excision repair (NER) removes pyrimidine dimers and large chemical adducts;
- Homologous Recombination Repair (HRR) and Non-Homologous End Joining (NHEJ) are employed to repair double strand breaks.

Nuclear excision repair (NER) and base excision repair (BER) are activated after DNA single-strand breaks. In addition, to repair DNA double strand breaks (DSB) the cells can use two distinct pathways: non-homologous end joining (NHEJ) or homologous recombination (HR) repair systems. To minimize the harmful effects of radiation-increased oxidative stress levels the cells have also evolved a variety of antioxidant enzymes, such as glutathione S-transferases, that play an important role in the

detoxification of ROS generated by radiation-induced oxidative stress. [Ambrosone CB, 2001]

3.1 DNA single-strand break repair genes

Single-strand breaks (SSBs) in DNA are considered as transient promutagenic lesions, representing direct effects of damaging agents. They may also be related to apurinic/apyrimidinic sites (alkali-labile sites appearing as breaks) and also represent intermediates in cellular repair, since both Nucleotide Excision Repair (NER) and Base Excision Repair (BER) cut out the damage and replace it with undamaged nucleotides [Vodicka P, 2004].

NER and BER are two major cellular responses that correct DNA damage in mammalian cells. NER is one of the most versatile and important pathways by which mammalian cells remove a variety of DNA lesions, such as bulky chemical adducts, pyrimidine dimers and interstrand cross-links that distort the DNA helix. BER, however, is critically involved in the repair of single-strand breaks induced by reactive oxygen species, alkylation or ionizing radiation [Almeida KH, 2007]. Similar to BER, NER has four steps involving damage recognition and enzymatic denaturation, stabilization of damage, elimination of damaged nucleotides, gap-filling through DNA sysntesis and ligation (sealing) (Fig. 3). Defects in NER are associated with the inherited condition xeroderma pigmentosum, which is characterized by photosensitivity and a predisposition to cancer and neurological degeneration [Scriver CR, 1989]

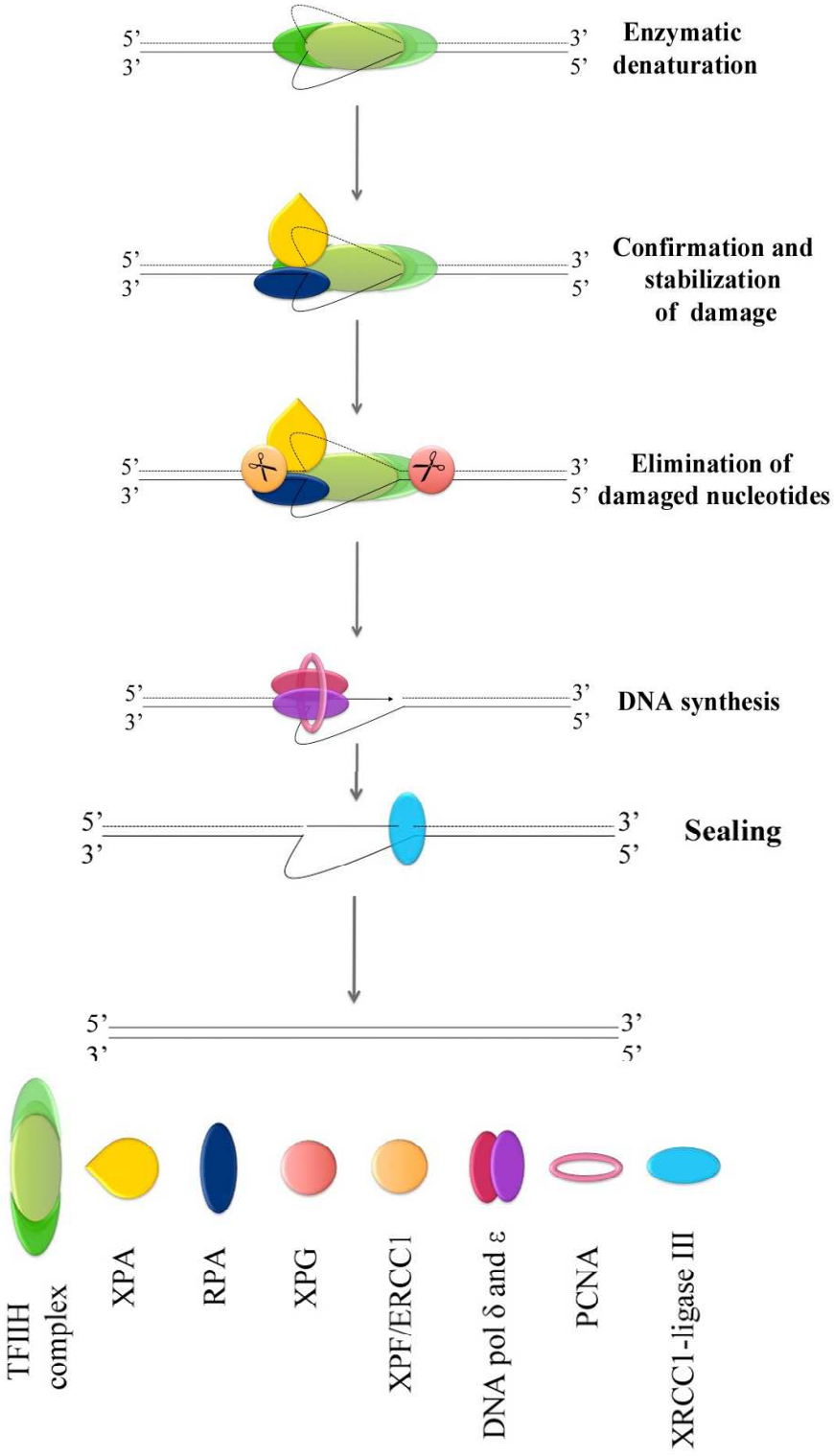

Figure 3. Model for nucleotide excision repair pathway

BER is composed of three main steps. (Fig 4) The first step is lesion recognition/strand scission by a lesion-specific glycosylase which catalyzes the hydrolysis of the N-

glycosidic bond of the damaged deoxynucleoside. The damaged base is converted into an abasic site, which is a substrate for enzymes with endonucleolytic activity in a second step called Gap Tailoring. A poly (ADP-ribose) polymerase (PARP1) and a complex formed by DNA Ligase III with XRCC1, ligates the nucleotide to the DNA strand. The final step of repair is DNA synthesis and ligation [Schötz U, 2011]. Depending on the extent of replaced nucleotides, BER can be distinguished as a short patch (1 nucleotide) or a long patch (2–13 nucleotides) [Hegde ML, 2008].

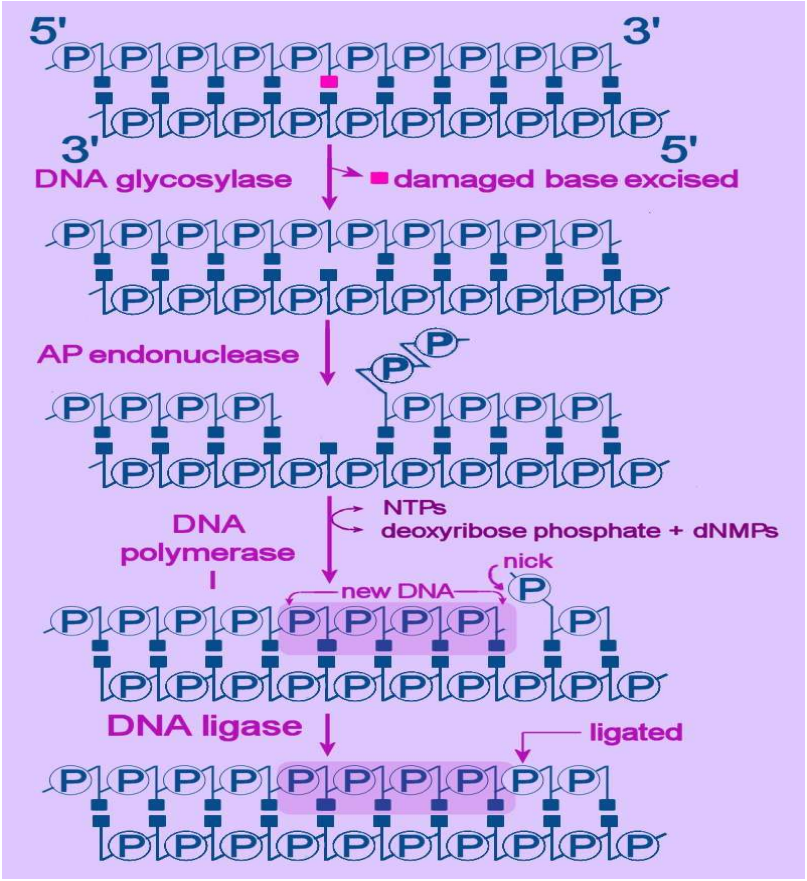

Figure 4. Base Excision Repair mechanism

A number of proteins are involved in the BER process, of which poly (ADP-ribose) polymerase, X-ray repair cross-complementing group 1 (XRCC1) and apurinic/apyrimidinic endonuclease/redox effector-1 play important roles. XRCC1 is a major player in the base excision repair pathway. Cells defective in XRCC1 have been

shown to have an increased sensitivity to mitomycin, UV, and ionizing radiation [Thompson LH, 2000]. Common SNPs in XRCC1 gene, have been extensively studied for their influences in individual sensitivity to radiation exposure [Aka P, 2004; Vodicka P, 2004; Angelini S, 2005; Au WW, 2006; Cornetta T, 2006]. The XRCC1 gene is located on chromosome 19q13.2, and 33 kb in length. It consists of 17 exons, and encodes a protein of 633 amino acids which acts as a scaffold to coordinate BER proteins at the repair site. In order to correct DNA damage this protein interacts with poly (ADP-ribose) polymerase (PARP1) at the site of damage. XRCC1 protein has two BRCA1 carboxyl-terminal domains (BRCT1 and BRCT2), the BRCT1 domain binds protein PARP1 to repair the damage. More than 300 SNPs in the XRCC1 gene have been validated in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), including Arg399Gln and Arg194Trp affected amino acid sequence and correlate with susceptibility to ionizing radiation damage. The polymorphism Arg399Gln is located close to BRCT1's C-terminal boundary and codon 194 resides in a linker region connecting the domains BRCT1 and DNA polymerase. The functional consequence of both polymorphisms on the overall function of the protein is not clear yet. It was suggested that the mutation in codon 399 and also the threonine (Thr) to Met polymorphism at codon 241 are non-conservative changes in protein structure. These polymorphisms act by modifying the interactions between XRCC1 and other BER proteins involved in the repair pathway. Therefore, the reduced capacity in DNA damage restoration may result in an increased sensitivity to ionizing radiation damage [Chang-Claude J, 2005].

3.2 DNA double-strand break repair pathways

DNA double-strand breaks (DSBs) are considered to be the most relevant lesion for the deleterious effects of ionizing radiation exposure [Rothkamm K, 2003]. DSBs Repair involves two main mechanisms: DNA non-homologous end joining (NHEJ) and homologous recombination (HR).

In NHEJ, the DSB ends are blocked from 5'end resection and held in close proximity by the double-stranded DNA (dsDNA) end-binding protein complex. NHEJ promotes direct ligation of the DSB ends, but in an error-prone manner, frequently resulting in small insertions, deletions, substitutions at the break site, and translocations if DSBs from different parts of the genome are joined [Lieber MR, 2010]. In contrast to NHEJ, HR is largely error free and is initiated when the DSB is resected by nucleases and helicases, generating 3'single-stranded DNA (ssDNA) overhangs onto which the RAD51 recombinase assembles as a nucleoprotein filament. This structure can invade homologous duplex DNA, which is used as a template for repair DNA synthesis. Although NHEJ is active throughout the cell cycle, HR is more prevalent after DNA replication, since an identical sister chromatid is available as a template for repair. HR requires a nucleolytic reaction that leads to formation of a single-stranded DNA (ssDNA) overhang at the break. A central role is played by the RAD51 complex, a small monomeric molecule which assembles into long helical polymers that wrap around the ssDNA tail at the break site [Johnson RD, 2001]. In addition to RAD51, a family of proteins known as the RAD51 paralogs and consisting of five proteins (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3), play an essential role in the DNA repair reactions through HR. The RAD51 paralogs act to transduce the DNA damage signal to effector kinases and to promote break repair [Suwaki N, 2011]. The XRCC3 gene is localized on chromosome 14q32.3, he encodes for a protein that directly interacts with and stabilizes Rad51 and is required for efficient repair of chromosome breaks [Brenneman MA, 2000]. The Thr241Met substitution is the most investigated polymorphism in XRCC3 due to a C18067T transition at exon 7. The functional consequence of XRCC3Thr241Met polymorphism on the overall function of the protein is yet not clear. It was suggested that conversion from one with a neutral hydrophilic hydroxyl group (Thr) to a hydrophobic one with a methyl sulphur group (Met) could represent a substantial change in protein characteristics which could affect protein structure and integrity. A large number of molecular epidemiologic studies have been preformed to evaluate the role of XRCC3 polymorphisms on the risk of various cancers [Manugueira M, 2006]. In addition, it has shown that XRCC3 Met241 variant influence radio sensitivity of human fibroblasts and that more risk allele of susceptible genes have a combined effect on cellular radiation response, suggesting that individuals with

multiple risk alleles could be more susceptible to radiation effects than those with fewer risk alleles [Alsbeih G, 2007].

3.3 Glutathione-S-transferase genes

Glutathione S-transferases (GSTs) are members of a multigene family of isoenzymes expressed in almost all living organisms. In both eukaryotes and prokaryotes they can be regarded as cellular housekeeping proteins, detoxifying many endogenous compounds as well as xenobiotics [Habig WH, 1974; Hayes JD, 2005]. GSTs are located in cytosol, mitochondria and microsomes; in some mammalian organs, they represent as much as 10% of the cytosolic protein [Boyer TD, 1989]. They comprise a large, functionally diverse family of enzymes, assigned in humans to 6 distinct classes: alpha, mu, theta, pi, zeta and omega (GSTA, GSTM, GSTT, GSTP, GSTZ and GSTO) *GSTs* carry out a wide range of functions in cells. They catalyses the conjugation of reduced glutathione (GSH) to electrophilic centers on a wide range of substrates. The conjugation between environmental pollutants and oxidized biomolecules to GSH, catalyzed by GSTs, is the major detoxification pathway in humans. GSTs also remove reactive oxygen species also formed by ionizing radiation exposure as confirmed by the activation of GST enzymes in response to radiation therapy [Helland A, 2007]. GSTs have also been shown to have a non-enzymic role. GSTs of the A, P and M classes modulate signalling pathways that control cell proliferation, cell differentiation and so on [Laborde E, 2010]. The genes encoding some GST enzyme loci are too polymorphic in humans; several polymorphisms in the GSTs may alter protein expression and/or function and can modify the risk in individuals exposed to toxic substrates (such as polycyclic aromatic hydrocarbons from smoke) or environmental pollutants [Manfredi S, 2007]. Deletion polymorphisms in the *GSTM1* and *GSTT1* genes, important members of the GST family, may result in complete function lack of GSTM1 and GSTT1 proteins [Pemble S, 1994]. During last year many studies have shown the association between DNA repair pathway and GST polymorphisms, mostly for GSTM1 and GSTT1, and

increased radiosensitivity in breast cancer patients [Mangoni M, 2011]. Polymorphisms resulting in reduced or absent activity in the GSTs activity have been associate with reduced hazard of death and risk of recurrence following treatment for breast cancer [Ambrosone CB, 2001]. Moreover GSTM1 and GSTT1 polymorphism may be significant in determining the level of adverse effects after radiotherapy. Variations in the level, activity, and ability to induce antioxidant enzymes may therefore be an indicator of radiosensitivity [Edvardsen H, 2007].

4. Aim of the thesis

Ionizing procedures provide essential life-saving information, but great care must be taken regarding the possible long-term health consequences. The biological and clinical burdens of medical radiation represents a worrisome social and medical problem.

Considerable efforts should be made to mitigate radiation-induced cell damage. Because radiation induced cellular damage is attributed primarily to the harmful effects of free radicals, the efficacy of non-toxic radioprotectors with radical scavenging properties should be investigated in the clinical setting. These agents may inhibit or reduce free radical toxicity, thus offering protection against radiation.

The identification and characterization of genes that enhance prediction of disease risk and improve prevention, treatment, and quality of care remain important goals in the modern imaging practice. Specifically, it is anticipated that through the use of genetic markers may serve as the basis for personalized radiotherapy in which cancer management is formulated so that it optimizes the treatment plan for each patient based on their genetic background (radiogenomics). In order to improve this knowledge, the primary aims of the project were:

- **Aim 1: to evaluate the ability of NAC in conferring protection against radiation induced chromosomal DNA damage.**
- **Aim 2: to assess the value of functional polymorphisms of genes involved in DNA damage repair and oxidative stress response as predictive factors for the occurrence of acute skin reactions.**

5. Methods

5.1. Study populations

To evaluate the ability of NAC in conferring protection against radiation induced chromosomal DNA damage, we studied a population of 65 patients (52 males, age 64.4 ±11.9 years) who underwent to invasive cardiovascular procedures, including peripheral trans-luminal angioplasty (PTA; n =45), cardiac resynchronization therapy (CRT; n =15) and ablation therapy (AT; n =5). Exclusion criteria included the inability to obtain consent for participation in the study and the presence of acute or chronic inflammatory disease, immunological disease, and neoplastic disease. Eligible patients were classified into two groups: 35 patients (26 males, age 63.4±11.1 years) receiving the standard hydration protocol consisting of intravenous isotonic saline for 12 h after catheterization (Group I) and 30 patients (26 males, age 65.5±12.9 years) at risk for radiocontrast nephropathy with preexisting renal insufficiency (Group II). Group II patients received a double intravenous dose of NAC (6 mg/kg/h diluted in 250 mL of NaCl 0.9%) for 1 hour before and a standard dose (6 mg/kg/h diluted in 500 mL of NaCl 0.9%) for 12 hours following catheterization.

The X-ray equipments used in this study were Philips Integris H5000C Monoplane and Integris Allura Monoplane with the X- ray tube for both Systems: MRC 200 0508 ROT GS 1001. The DAP (Gy cm2) has been used for the estimation of the radiation dose received by the patient [Efstathopoulos EP, 2004; Kocinaj D, 2006] and is considered a valid indicator of a patient's dose and consequent risk for radiation induced effects. Samples were collected from each subject and the laboratory analyses were performed in a random order.

Conversely, to assess the value of functional polymorphisms of genes involved in DNA damage repair and oxidative stress response as predictive factors for the occurrence of acute skin reactions we studied female breast cancer patients receiving breast radiation therapy after conservative surgery (Stage I-III) at unit of Radiation Oncology of Brindisi Hospital "A. Perrino". Fifty-nine patients were included in this prospective

single-arm study. The median age of patients was 58 years (range, 35-80 years). A detailed anamnestic history was collected from all patients, and blood sampling was performed for each of them, with written informed consent.

All the patients received a typical breast-irradiation treatment. The planning target volume (PTV) dose prescription ranged from 40 Gy (54) and 50 Gy (2). All patients were given whole breast radiotherapy with conventional fractionation. Breast conserved patients received an additional boost (10 Gy) to the tumoral bed in 48/59 cases. Conventional fractionations (200 cGy/day) were used. Clinical radiation skin reaction within the radiation field of the breast was documented during the course of treatment and at the end of RT. The skin tissue reactions were graded according to the Radiation Therapy Oncology Group (RTOG) acute radiation morbidity scoring criteria [Cox J, 1995]. Skin tissue reactions after treatment for the 60 patients studied are shown in Table 3. Early acute toxicity (grade>1) was the end-point analyzed and was defined as early when occurred within few days after radiotherapy.

Table 3. Acute skin tissue reaction at the end of radiotherapy course

Acute skin toxicity, RTOG grade	
• Grade 0	No change
• Grade 1	Follicular, faint or dull erythema, epilation, dry desquamation
• Grade 2	Moderate erythema
• Grade 3	Several desquamation
• Grade 4	Ulceration, hemorrhage, necrosis

5.2 Cytokinesis-block micronucleus test

Blood samples were collected at baseline, 2 hours following the procedure and 24 hours after end of the catheterization procedures for MN assay as previously described [15-17]. Briefly, two separate cultures from each sample were set up by mixing 0.3 mL of whole blood with 4.7 RPMI 1640 medium: the cultures were incubated at 37°C for 72 hrs. Cytochalasin B (6 μg/ml) was added 44 h after culture initiation. Cells were then harvested and fixed according to the standard methods. For each sample, 1000 binucleated cells were scored under optical microscope (final magnification 400x) for micronucleus analysis, following the criteria for micronucleus acceptance. We evaluated the MN frequency as the number of micronucleated cells per 1000 cells (‰). For each sample, 1000 binucleated cells were scored under optical microscope (final magnification 400x) for micronucleus analysis, following the criteria for micronucleus acceptance. We evaluated the MN frequency as the number of micronucleated cells per 1000 cells (‰).

5.3 PCR-RFLP Genotyping Assays

All subjects enrolled in the study provided a blood sample (~3 mL) collected using standard venipuncture techniques before the start of radiotherapy. Whole blood samples for DNA analyses were immediately frozen at -80°C until processing.

Genomic DNA was extracted from peripheral blood leukocytes. Genetic polymorphisms were analyzed by PCR combined with restriction fragment length polymorphism (RFLP) [Ambrosone CB, 2006; Veronesi U, 2001; Cancer Therapy Evaluation Program, 1998; Brenneman MA, 2000]. The primer pairs used were: (a) XRCC3: *Thr241Met*, F5'-GGTCGAGTGACAGTCCAAAC-3' and R5'-TGCAACGGCTGAGGGTCTT-3' (b) XRCC1: *Arg399Gln*, F5'-AGTAGTCTGCTGGCTCTGG-3' and R5'-TCTCCCTTGGTCTCCAACCT-3' (c) XRCC1: *Arg194Trp*, F5'-GCCCCGTCCCAGTA-3' and R5'-AGCCCCAAGACCCTTTCACT-3'

Genomic DNA was isolated from cells in the venous blood using QIAmp kit (QIAmp DNA blood Mini Kit, Qiagen), following the manufacturer's instructions, and the DNA quality was evaluated by the spectrophotometer analysis (NanoDrop, Thermo Scientific instrument). Details of annealing temperature, restriction enzymes and fragment sizes (pb) used to assess *XRCC3* (Thr241Met), *XRCC1* (Arg399Gln) and *XRCC1* (Arg194Trp) genetic polymorphisms are listed in Table 4.

Genotyping of *GSTM1* and *GSTT1* deletions was carried out using a duplex PCR (in a volume of 50 µl) with the Albumin gene (*ALB*) serving as an internal positive control to prove the successful PCR amplification [Chen CL, 1997; Naveen AT, 2004]. In Table 4 details of annealing temperature and fragment size.

Table 4. Condition for amplification, restriction enzyme and restriction patterns

Primers	Annealing Temperature (°C)	Restriction Enzyme	Fragment Sizes (pb)
XRCC3 (Thr241Met) **Forward primer** GGTCGAGTGACAGTCCAAAC **Reverse primer** TGCAACGGCTGAGGGTCTT	60	*Nla III*	315+140 (*Thr/Thr*) 210+140+105 (*Met/Met*)
XRCC1 (Arg399Gln) **Forward primer** GTTGGGCTCAAATATACGGTGG **Reverse primer** TCTCCCTTGGTCTCCAACCT	56	*MspI*	269 + 133 (Arg/Arg) 402 (Gln/Gln)
XRCC1 (Arg194Trp) **Forward primer** GCCCCGTCCCAGTA **Reverse primer** AGCCCCAAGACCCTTTCACT	58	*Pvu II*	490 (Arg/Arg) 294 + 196 (Trp/Trp)
GSTM1 **Forward primer** GCCCCGTCCCAGTA **Reverse primer** GAACTCCCTGAAAAGCTAAAGC	64		215
GSTT1 **Forward primer** TTCCTTACTGGTCCTCACATCTC **Reverse primer** TCACCGGATCATGGCCAGCA	64		480
ALB **Forward primer** GCCCTCTGCTAACAAGTCCTAC **Reverse primer** GCCCTAAAAAGAAAATCGCCAATC	64		380

5.4 Statistical analysis

Statistical analyses of the data were conducted with the Stat view statistical package, version 5.0.1 [Abacus Concepts, Berkeley, CA, USA]. Data are expressed as mean (+ SD). The sample size was projected to be 60 patients, with 30 to the treatment group and 30 to the control group, based on an increase of MN of 15%, a two-tailed alpha of 0.05, and a power of 0.80. Qualitative and quantitative comparisons in demographic characteristics between Groups I and II were evaluated by $\chi 2$ analysis and the Student's t-test, respectively. Statistical differences in MN data between the two paired samples were determined with the non parametric Wilcoxon matched pairs test. Variations of MN with time in either group were assessed by using repeated-measures analysis of variance (ANOVA). Comparisons among groups at each time point were made by means of the Kruskal–Wallis. A two-tailed p-value < 0.05 was chosen as the level of significance. In the logistic regression analysis, the homozygote of the most frequent allele was used as a reference. For odds ratio and 95% confidence interval, logistic regression analysis was used. The values of p-value < 0.05 were considered statistically significant.

6. Results

6.1 NAC reduces chromosomal DNA damage

The Clinical and demographic characteristics of study population are summarized in Table 5. Two groups were similar at baseline on demographic and clinical characteristics, including smoking status, dyslipidemia, hypertension, and diabetes mellitus. In particular, prior to the catheterization procedures there was no significant difference between frequencies (p=0.5).

Table 5. Clinical and demographic characteristics of study patients

Group	I° (n=35)	II ° (n=30)	p-value
Mean age (±SD)	63.4±11.1	65.6±12.9	0.5
Male sex, n (%)	26 (74.3)	26 (86.7)	0.2
Hypertension, n (%)	19 (54.3)	12 (40)	0.2
Diabetes, n (%)	5 (15.6)	4 (14.8)	0.9
Dyslipidemia, n (%)	14 (40)	13 (43.3)	0.8
Smoking, n (%)			0.9
Never smokers	11(31.4)	8 (26.7)	
Former smokers	18 (51.4)	16 (53.3)	
Smokers	6(17.2)	6 (20)	
Baseline MN frequency (%)	14.5±4.7	13.7±7.0	0.5

Median effective DAP values were found to be significantly higher (p = 0.0001) in NAC-treated patients (median 126.2 Gy cm2 range 15.9–260 Gy cm2) as compared to control group median 58.2 Gy cm2 range 7.5–114 Gy cm2), as shown in Fig 5.

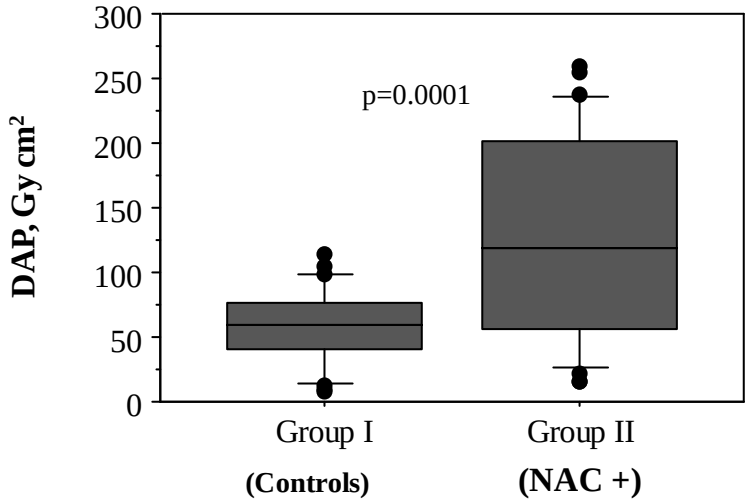

Figure 5. DAP values for Group I (controls) and Group II (NAC+).

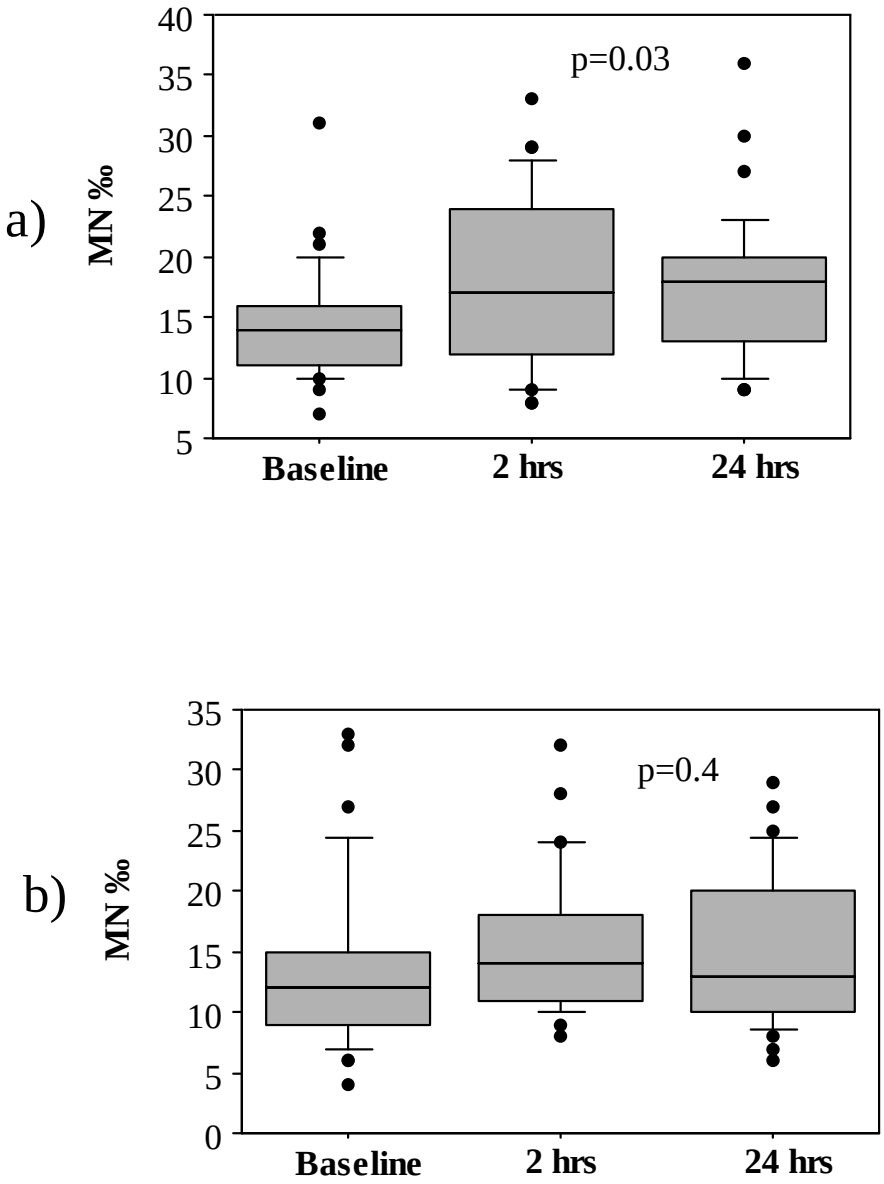

Figure 6. Frequency of MN at baseline, 2 and 24 h after cardiac catheterization procedures in the a) control patients (Group I) and b) NAC-treated patients (Group II). The results are expressed as boxes with 5 horizontal lines, displaying the 10th, 25th, 50th

MN frequency was 13.7 ± 4.7‰ at baseline and showed a significant rise at 2 h (18.0 ± 6.8 p=0.01) and 24 h (17.6 ± 5.9, p=0.03) in the Group I (*Fig 6*). On the contrary, there was no significant increase of MN in the Group II (13.7 ± 7.0, 15.5 ± 6.0 and 14.9 ± 6.3 for baseline, 2 h and 24 h respectively, p = 0.4).

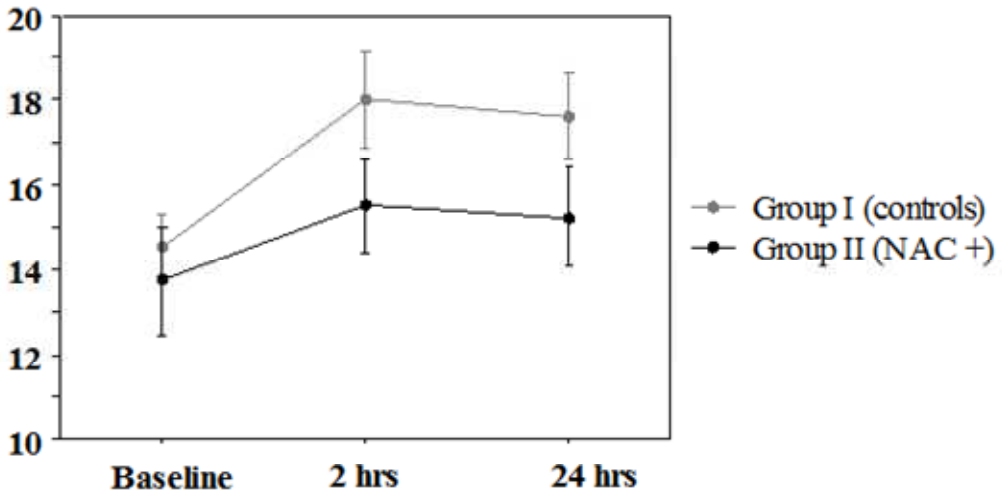

Figure 7. MN levels at baseline, 2 h and 24 h after cardiac catheterization procedures

A significant difference in MN frequency was also found between the treatment group and the control group at 24 h after procedures (Figure 7). We did not observe any relationship between DAP and % MN increase at 24 h for both Group I ($r = 0.1$, $p=0.54$) and Group II ($r = 0.1$, $p=0.71$).

6.2 Association between genetic polymorphisms and acute skin toxicity in breast cancer

The clinical and demographic characteristics of the whole set of breast cancer patients are shown in Table 6. Twenty-four (41%) of the 59 participants experienced faint to moderate acute skin toxicity (RTOG grade 1–2, grade ≥1) while 35 (59%) patients had no acute skin reactions (RTOG grade 0). A significant difference was recorded for the BMI value ($p<0.01$). Conversely, the two study population were similar for age, smoke status and RT treatment (boost therapy to the surgical bed).

Genotype distributions for *XRCC3* Thr241 →Met and *XRCC1* Arg399 →Gln satisfied Hardy-Weinberg equilibrium. Conversely, the *XRCC1* Asp194Trp violated the equilibrium at the $p < 0.05$ level thus these polymorphisms were excluded from further analysis. The distributions of variants alleles in the whole set of breast cancer patients are shown in Table 5.

Table 4. Clinical and demographic characteristics in the whole set of breast cancer patients (n = 59) after stratification according to skin toxicity criteria (Grade 0: no change; Grade ≥ 1: faint-moderate erythema)

Clinical variable	**Grade 0 n = 35**	**Grade≥ 1 n = 24**	**p-value**
Mean (SD) age, years	57 (11.6)	59.3 (11.7)	0.4
BMI, mean (SD)	24.8 (4.3)	27.6 (4.7)	0.01
Smoke, n (%)	4 (7)	3 (5)	0.9
Tumor characteristics			
Histology, n (%)			
• Ductal-inasive (D)	24 (41)	15 (25)	
• Lobular-invasive (L)	3 (5)	3 (5)	
• Ductal in situ (DCIS)	2 (3)	2 (3)	
• Mixed (D + L)		2 (3)	
• Other	6 (10)	2 (3)	
Tumor stage, n (%)			
I	21 (37)	13 (22)	
IIA	6 (10)	3 (5)	
IIB	1 (2)	3 (5)	
III		1 (2)	
IIIA	1 (2)	3 (5)	
IIIB	3 (5)	1 (2)	
IIIC	1 (2)	1 (2)	
Systemic therapy			0.6
Hormonal therapy (H), n (%)			
• None, n (%)	6 (10)	5 (8)	
• Tamoxifen n (%)	14 (23)	5 (8)	
• Aromatase inhibitors n (%)	18 (32)	11 (18)	
Chemotherapy (C) n (%)			
• None, n (%)	12 (20)	12 (20)	
• Antracycline n (%)	11 (18)	7 (12)	
• Taxane n (%)	13 (23)	4 (6)	
Tumor bed boost therapy, n (%)			0.09
• Yes	26 (74)	22 (92)	

Table 5. Genotype and allele frequencies of Thr241 →Met polymorphism in XRCC3 gene, Arg399 →Gln polymorphism in XRCC1 gene and GSTM1null and GSTT1null genotype in the study population

	Grade 0 ***n = 35***	**Grade ≥ 1** ***n = 24***	**p-value**
|*XRCC3*| Thr^{241} →Met polymorphism			0.1
Thr/Thr, n (%)	21 (60)	9 (37.5)	
Thr/Met, n (%)	11 (31.4)	9 (37.5)	
Met/Met, n (%)	3 (8.6)	6 (25)	
TT vs TM + MM			0.08
Allele			
Thr, n (%)	53 (75.7)	27 (56)	
Met, n (%)	17 (24.3)	21 (44)	
|*XRCC1*| Arg^{399} →Gln polymorphism			0.1
Arg/Arg, n (%)	22 (63)	11 (46)	
Arg/Gln, n (%)	9 (26)	12 (50)	
Gln/Gln, n (%)	4 (11)	1 (4)	
AA vs AG + GG			0.2
Allele			
Arg, n (%)	53 (76)	34 (71)	
Gln, n (%)	17 (24)	10 (29)	
GSTM1			
Wild-type, n (%)	25 (71)	10 (42)	
Null, n (%)	10 (29)	14 (59)	0.02
GSTT1			
Wild-type, n (%)	25 (71)	16 (67)	
Null, n (%)	10 (29)	8 (33)	0.7

At the univariate analysis the association between clinical variables and acute skin toxicity showed that a higher BMI (defined as BMI>24 kg/cm^2) had a significant

adverse effects on acute skin toxicity. Patients with higher BMI have 3.6-fold risk to develop side reactions (CI: 1.2-11, p=0.02). Other factors evaluated, including age, smoking status and tumor bed boost therapy were not associated with an increased risk of acute skin toxicity. Regarding to patient genotype, subjects with Met/Met genotype in *XRCC3* gene had a 2.5-fold increase risk of acute radiation skin toxicity as compared to patients with wild-type genotype (95% CI: 1.0-7.3, p=0.05) while the Arg399→Gln polymorphism in *XRCC1* gene had no effect on acute skin toxicity. Similarly, the presence of GSTM1 null genotype increased almost 3-folds the risk of acute skin toxicity (OR: 3.5 CI: 1.2-10.4 p=0.02). No effect seemed to have the GSTT1 null genotype in our population. ORs for genetic polymorphisms at univariate analysis are shown in Table 8.

Table 6. Association between genetic polymorphisms and radiation-induced acute toxicity in breast cancer patients.

	OR (95% CI)	**p-value**
XRCC3 Thr^{241}→Met		
Thr/Thr	1.00 (ref)	
Thr/Met + Met/Met	2.5 (1.0-7.3)	**0.05**
XRCC1 Arg^{399}→Gln		
Arg/Arg	1.00 (ref)	
Arg/Gln + Gln/Gln	2 (0.7-5.7)	0.2
GSTM1^{null}		
Wild-type	1.00 (ref)	
Null	3.5 (1.2-10.4)	**0.02**
GSTT1^{null}		
Wild-type	1.00 (ref)	
Null	1.2 (0.4-3.8)	0.7

At logistic regression analysis, a BMI>24kg/cm^2 was confirmed as an independent predictors of acute radiation-induced skin toxicity in breast cancer patients. Indeed, a higher BMI 3.6-fold increased the risk. Referring to genetic factors, also the Met/Met

genotype in *XRCC3* gene and the *GSTM1* null genotype were independent associated with the risk of acute breast skin toxicity following radiotherapy. The predictive efficiency of the logistic multivariate model according to the three independent predictors is summarized in the classification table reported in Table 9.

Table 7. Predictive factors of acute skin toxicity by multivariate logistic regression analysis

	OR (95% CI)	**p-value**
XRCC3 Thr^{241}→Met		
Thr/Thr	1.00 (ref)	
Thr/Met + Met/Met	3.12 (1.0 – 10.8)	0.05
GSTM1^{null}		
Wild-type	1.00 (ref)	
Null	3.1 (1.0 – 10.0)	0.05
BMI kg/cm^2		
BMI≤24 kg/cm^2	1.00 (ref)	
BMI>24 kg/cm^2	4.5 (1.3 – 15.9)	0.02

7 Discussion

The study proposal has been designed to evaluate the efficacy of NAC as new radioprotector in order to inhibit or reduce the free radical toxicity and, thus, offering protection against radiation during interventional cardiovascular procedures. In addition, the project assessed the role of genetic inter-individual differences in radiation-induced response.

Regarding the efficacy of NAC treatment, this antioxidant resulted able to reduce radiation-induced chromosomal DNA damage in human lymphocytes after invasive cardiovascular procedures, possibly through its known action against free radicals.

Indeed, it is known that invasive cardiovascular procedures can induce both DSBs (i.e. γ-H2AX) and chromosomal DNA damage [Geisel D, 2008; Bonassi S, 2008] largely by the generation of free radicals and ROS [Cadet J, 2012]. The ability to scavenge free radicals and reduce ROS is a critical function of radiation-protective agents [Weiss JF, 2000].

The first *in vivo* studies on protection by chemicals against ionizing radiation were conducted almost 50 years ago. Patt et al., reported in 1949 that cysteine, a sulfur-containing amino acid, could protect rat from a lethal dose of X-rays [Patt HM, 1949]. More than 4000 thiol-containing compounds have been screened in mice but the majority of these compounds are too toxic. Conversely NAC is a no toxic, safe drug without major side-effects. It has been used in clinical setting for more than four decades [Miller LF, 1983; Kelly GS, 1998] and multiple clinically relevant effects have been described. Indeed, in addition to its direct ROS scavenging activity, NAC enhance the synthesis of GSH, and reduces inflammation [De Flora S, 2001]. NAC has been examined for its potential against radiation-induced injury, predominantly in vitro assays and, to some extent, in animals. NAC seems reduce ionizing radiation-induced DSB formation in human microvascular endothelial cells *in vitro* [Kataoka Y, 2007] and oxidative DNA damage in the liver of mice [Liu Y, 2007]. NAC treatment prior to radiation was found to decrease the lipid peroxidation, total nitrate/nitrite (NOx), DNA fragmentation and significantly increase the antioxidant status [Mansour H, 2008]. Finally, NAC showed protective effect against MN frequency in human blood

lymphocytes exposed in vitro to γ radiation [Tiwari P, 2009]. Our findings strongly support these evidences, suggesting the efficacy of NAC as radioprotector able to inhibit or reduce the free radical toxicity in human.

This finding may have major clinical relevance since the use of radiation in medical diagnosis in western societies is increasing, especially for the growing use of computed tomography and interventional cardiology. In particular, in the United States, the dose from medical exposures has increased by a factor of six in the last 25 years [Mettler FA, 2008].

The attributable cancer risk of getting cancer from medical radiation is estimated around 5 to 10% [Picano E, 2004], with approximately 29,000 future cancers (2% of all cancers) related to computed tomography scans in US [Berrington de González A, 2009]. To date, in adult cardiology patients, interventional cardiology procedures account for roughly 12% of examinations, and 48% of the total collective dose [Bedetti G, 2008]. They contribute substantially to the high cumulative radiation doses of contemporary patients [Chen J, 2010].

The problem of medical ionizing radiation is more marked in children with congenital heart disease where the invasive radiology (with diagnostic and interventional catheterization) accounts for 6% of all radiological examinations and 84% of the collective dose [Ait Ali L, 2010]. With cumulative radiation exposure, the patient acquires increasing risks of developing cancer during their lifetime.

As recommended in April 2010 by US President's Cancer Panel, any possible action should be taken by health care provides to minimize radiation exposure by medical sources, recognized as one of the 6 major causes of environmental cancer [President’s Cancer Pannel, 2010].

Dose optimization and is, therefore, of crucial importance for limiting radiation dose in cardiac catheterization procedures, especially for pediatric cardiac testing. For that, the use of specific radiation protector agents which acts as scavengers of reactive oxygen species is a crucial mean to reduce cell toxicity. NAC is a safe, inexpensive, and well-tolerated antioxidant with a well-defined mechanism of action [Millea PJ, 2009].

The findings of this thesis support the notion that the use of NAC may be a promising approach in order to offer protection against DNA damage in patients undergone to interventional catheterization procedures.

In the last years, many studies have provided a consistent evidence that several genetic, environmental and dietary factors can affect the variability of damage observed at any given level of radiation. In particular a major role of genetic polymorphism of genes involved in DNA damage and repair in modulating the vulnerability to radiation exposure in the very low dose range has been evidenced.

Accordingly, the seventh National Academy of Science report on Biological Effects of Ionizing Radiation (BEIR VII) recommended the identification of genes conferring predisposition to radiation-induced health effects as a top research need. So that, the characterization of genes that enhance prediction of disease risk and improve prevention, treatment, and quality of care remain important goals. In particular, the use of genetic markers may serve as the basis for personalized radiotherapy in which cancer management is formulated to optimize the treatment plan for each patient based on their genetic background (radiogenomics). Skin reaction during radiotherapy, though reversible in the large majority of cases, is the most common side effect in breast cancer patients. Acute effects such as erythema (redness, warmth, rash-like appearance), dry desquamation (dryness, itching, peeling), or moist desquamation (moist, oozing, tender, redness and exposure of the dermis) occur during or shortly after therapy. However cancer patients exhibit large patient-to-patient variability in acute skin reactions when the same treatment regimen is applied. Several observations support the hypothesis that radiosensitivity of clinical normal tissue is influenced by several polymorphic genes in DNA repair mechanisms and also oxidative stress [Zhou L, 2010, Ambrosone CB, 2006]. Some genetic variants of *XRCC1* and *XRCC3* genes have been shown to correlate with hypersensitivity to radiotherapy [Moullan N, 2003]. In addition, Alsbeih G et al. have showed that XRCC3 Met241 variant influence radiosensitivity of human fibroblasts and that more risk allele of susceptible genes have a combined effect on cellular radiation response, suggesting that individuals with multiple risk alleles could be more predisposed to radiation effects than those with fewer risk alleles [Alsbeih G, 2007]. Recently, Mangoni M et al. showed the protective

role of XRCC3 wild type genotype towards acute skin side effects [Mangoni M, 2011]. In this study we confirm the association of *XRCC3* Thr241→Met polymorphism as independent predictor of individual susceptibility to acute skin toxicity. Indeed, the presence of this polymorphism 3-fold increased the risk (95% CI 1.0 – 10.8; $p<0.05$).

Similarly, the genetic background of detoxification enzyme may confer an individual susceptibility to ionizing radiation. Indeed, it is known that radiotherapy leads to the induction of antioxidant enzymes, such as glutathione S-transferases. The GSTs are important members of the cellular phase II detoxification system, and they catalyze the reactive oxygen species scavenging process by conjugation of the tripeptide glutathione to a variety of endogenous and exogenous electrophilic compounds. A lack of GSTM1 and GSTT1 enzyme activity is caused by homozygous deletion of the corresponding genes and is observed in 50% and 20% of the Caucasian population, respectively [Sharma A, 2012].

In our study, the evaluation of the potential effects of null GST genotypes has showed that breast cancer patients carrying null GSTM1 genotype was associated with a more than three-fold increase in risk for experiencing acute skin toxicities (OR: 3.5 CI: 1.2-10.4 p=0.02). This suggests that a reduced or absent activity in the GSTM1 enzyme may result in a greater risk of radiation-associated toxicity likely by influencing the cellular redox state. Thus, polymorphisms in genes associated with higher generation of ROS appear to increase susceptibility to development of normal tissue complications, whereas gene variants associated with lower ROS production may decrease risk of these effects [Chang-Claude J, 2009]. The identification of risk factors, related to radiation therapy, among clinical characteristics and genetic background of cancer patients are important feature to predict the probability of undesirable effects and also to allow optimization of radiotherapy treatment. The genetic polymorphisms evaluated may be promising candidates for predicting acute radiosensitivity, but further studies will need to be carefully confirm these results in largest population.

Moreover, our results also showed that a higher BMI increased the risk of early acute skin reaction. The finding of BMI as a risk factor is compatible with previous observations which underline breast size as an important prognostic factor for acute

toxicity since BMI and breast size are likely to be highly correlated [Gray JR, 1991]. A greater dose in-homogeneity across the breast and a greater field separation in patients with larger breasts could be reasons for this association.

8. Conclusions

In conclusions, the results reported in this thesis provide evidence that:

- NAC may be an effective promising, safe, inexpensive, and well-tolerated antioxidant approach easily usable in the clinical practice to offer protection against DNA damage induced by ionizing radiation exposure during cardiac catheterization procedures. This observation may have potential application in clinic practice in order to improve the protection of the patients from the adverse health effects of ionizing radiation, especially for pediatric patients.

- SNPs in DNA repair and detoxification genes can modify the susceptibility to inter-individual differences in response to therapeutic ionizing radiation exposure in breast cancer patients following radiation therapy. The association analysis between clinical characteristics and genotype with the acute radiation skin toxicity in breast cancer patients suggests that approaches based on (multiple) genetic markers and clinical characteristics have the potential to predict normal tissue radiosensitivity.

Although our findings are to be carefully assessed with further large, randomized studies, taken together have outmost clinical relevance since add important information to provide new insights to reach a personalized measure of radiation risk. All this in order to implement tailored preventive and chemopreventive strategies. Indeed, in the era where the use of ionizing radiation is exponentially increasing, a parallel advance in the knowledge of new strategy to protect cellular DNA damage as well as in the knowledge of individual susceptibility is strongly need for a better therapeutic program able to improve the life quality of patients.

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