Molecular and cellular assays after radiation exposure

Part 1: Influence of temperature during irradiation on the level of DNA damage

Part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors

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Abstract

Part 1: The level of cytogenetic damage induced by ionizing radiation under in vitro conditions in human peripheral blood lymphocytes is analyzed for the purpose of biological dosimetry and for assessing the intrinsic radiosensitivity of the blood donors. A factor that is often not regarded, but may influence the level of DNA damage is blood temperature during exposure. Hence, this part of the thesis was conducted to analyse the impact of temperature during irradiation of lymphocytes at 0°C and 37°C on the level of DNA damage using micronucleus assay and comet assay.

Whole blood cultures were kept at 0°C and 37°C for 15 min before and during exposure to 2 Gy of X-rays. In some experiments isolated PBL were additionally incubated in the presence of 0.5 M DMSO (radical scavenger).

A significantly higher level of micronuclei was found when lymphocytes were kept 15 min before and during exposure at 37°C when compared to 0°C. This effect disappeared in the presence of DMSO, what indicates that the observed temperature effect in micronucleus assay is due to the indirect action of radiation. No temperature effect was observed using alkaline and neutral versions of comet assay.

Part 2: There is some evidence that approximately 10% of the population show an enhanced intrinsic radiosensitivity of normal tissue and hence have a higher risk for developing side-effects during or after radiotherapy. Moreover, higher cellular radiosensitivity may also indicate cancer susceptibility. For that reason there is a need for a fast and robust test to assess individual cellular radiosensitivity. Hence, the aim of the second part of the thesis was to find out whether PBL from prostate cancer patients (PC) with strong clinical side effects following radiotherapy show enhanced rates of in vitro radiation-induced DNA damage when compared to patients without side effects and healthy age–matched donors. The study included 20 prostate cancer patients without and 20 patients with acute side-effects during and after radiotherapy, as well as 20 healthy age-matched donors. From each donor, blood samples were collected, exposed to a radiation dose of 0.5 Gy or 1 Gy of γ–rays and analysed for the following biological endpoints: the initial level of dsb and the repair kinetics (γ–H2AX–assay), apoptosis (Annexin V/PI–assay) and the induction of chromatid–type chromosomal aberrations (G2–assay). Significant higher chromatid aberration yield was found in prostate cancer patients when compared to healthy donors. No differences were observed between both patients groups in any in vitro assay. Clinical radiosensitivity in vivo assessed on the

basis of the EPIC questionnaire correlated with cellular radiosensitivity in vitro assessed on the basis of chromatid aberration 90^{th} cut-off value analysis for 50-62 % of prostate cancer patients.

However, based on the results of all chosen assays 6 prostate cancer patients were identified as cellular sensitive, whereof 4 of them were also clinically sensitive.

I. Introduction

1.1 Short introduction to the history of radiation

Since over 100 years big efforts have been undertaken to understand the influence of ionising radiation (IR) on biological tissue. Under certain circumstances a person or even entire populations might be exposed to radiation and in consequence suffer from the acquired damage. Nuclear bombings of Hiroshima and Nagasaki in 1945 during the 2nd world war are the best known examples for nuclear warfare. Nuclear reactors accidents (e.g. Three Mile Island in 1979, Chernobyl in 1986) affected staff, clean-up workers and the residents of the region close to the place of accident as well as those living many hundred kilometres away. The natural sources of IR such as cosmic radiation or radiation present in the earth's crust (e.g. radon, radium, uranium from the natural radioactive series) contribute to the natural exposure of the population. An approximately equal contribution is due to exposure because of medical diagnostics and therapy. Cancer treatment like radiotherapy enables killing of tumour cells but is also responsible for the damage of the surrounding healthy tissue of patients.

The discovery of ionising radiation by Wilhelm Conrad Röntgen in 1895 and of radioactivity by Henri Becquerel in 1896, further work of Marie Sklodowska-Curie, Pierre Curie, and many other scientists resulted in a huge enthusiasm and hopeful expectations at the beginning of the 20th century. Wearing watches painted using radioactive elements, radium baths, radium bread and candies, drinking of water that contained radioactive elements enjoyed great popularity.

Thereafter, many cases of suspicious illnesses occurred during manufacturing of these watches and when radon bath, radioactive creams etc. were used. Those events brought home to public opinion that ionising radiation might be dangerous when used improperly and that is why there was a need to investigate precisely this phenomenon. The other reason to concentrate the efforts towards understanding the nature of ionising radiation, especially its interaction with tissue is common use of ionising rays in therapy of cancers as well as for the purpose of the biological dosimetry.

1.2 Radiation

The term “radiation” describes a flux of energy in the form of electromagnetic waves (photons or light) or subatomic particles. The energy of the electromagnetic waves and particles determines the effects of radiation on organisms and cells.

The energy deposited in non-ionising radiation is not high enough to overcome the binding energy that keeps electrons in their orbital shells so that no ions are produced. Ionising radiation (IR), on the contrary, contains energy high enough to create electrically charged particles (ions). The rate at which the energy is deposited along the track of radiation classifies the various types of IR into high- or low-LET (LET = linear energy transfer).
High LET radiation, such as protons, neutrons and α-particles produce dense ionisation tracks. These kinds of high-LET ionising radiation possess different amount of energy, mass and speed. As example, α-particles and protons have a larger mass but a lower speed and deposit a large amount of energy over a short distance. In opposite, neutrons, which are uncharged, are highly penetrant (Hall 2000).

Low-LET ionising radiation consisting of X-rays and γ-rays produce sparsely ionisation tracks. Those types of ionising radiation are less effective at creating ions but penetrate deeply into tissues.
Ionising radiation, such as X-rays, electrons and protons are used during radiotherapy of tumours.

The energy deposited by the radiation in a unit mass of matter is called the absorbed dose of IR and measured in Gray (Gy), whereof 1 Gy = 1 Joule/Kg. Due to the difference in the density of ionisation tracks between high- and low-LET the term “equivalent dose” was introduced and measured in Sievert (Sv). In case of low-LET radiation (e.g. X-rays, γ-rays) 1 Gy = 1 Sv, whereas in case of densely (high-LET) radiation the situation is more complex. For example, for α-particles 1 Gy = 20 Sv (Wakeford 2004). In this thesis the peripheral blood lymphocytes were exposed to low-LET radiation (X-rays and γ-rays). The effective dose, also measured in Sievert, is used to compare the stochastic risk of a non-uniform exposure of IR with the risks of a uniform exposure of the whole body.

1.3 The biological effects of ionising radiation

Exposure to ionising radiation of a biological system initiates a cascade of processes that differ in time and therefore, can be divided in three phases: physical, chemical and biological (in accordance with Steel 1993).

The physical phase takes $0\text{-}10^{-18}$ seconds and involves the interactions between charged particles and molecules in the exposed tissue. During this phase ionisations occur followed by excitation and emission of an electron and its ejection from the orbital shell. This electron may excites other atoms and thus starts a chain of ionisations.

During chemical phase free radicals and damaged molecules react with other cellular elements (10^{-12} – 100 seconds). Free radicals have an un-paired electron in the outer shell and thus, they are highly reactive. They result, among others, from the radiolysis of cellular water and are able to induce DNA damage. In this case DNA damage is due to indirect action of ionising radiation (see section 1.3.2).

The biological phase begins approximately 1 second after exposure and may extends to the entire lifespan and subsequent generations. This phase consists of damage recognition and its repair. When DNA damage is substantial the cells may undergo programmed cell death (apoptosis), whereas improper repair of smaller damages may lead to mutations (Steel 1993).

Very high doses of ionising radiation on the whole body are lethal, whereas given as fractionated doses to a tumour during radiotherapy makes this treatment very effective.

Biological effects of radiation to tissues/organs and on the cell/DNA level are described in more details in the sections 1.3.1 and 1.3.2.

1.3.1 Radiation effects in tissues and organs: stochastic versus deterministic effects and acute versus late effects

The effects of IR to normal tissues and organs are usually classified into two categories, depending on the mode of action on the body and the time period considered after exposure. These categories are referred to as stochastic effects and deterministic effects.

Stochastic effects are usually associated with exposure to low level of IR over a long period of time (e.g. years). They are not certain to occur, but the probability of their occurrence increases with the dose. The most important of such stochastic effects are cancer and genetic

defects. For most of stochastic effects, radiation is not the only known cause and the determination whether an effect results from radiation exposure or not is generally not possible (IAEA 2004).

Despite some controversies, the currently accepted radiation protection principles regarding stochastic effects are based on the following assumptions:

- There is no threshold level of radiation dose below which we can certify that cancer or genetic effects will certainly not occur.
- The dose response curve is linear for solid cancers and linear quadratic for leukaemias.
- The severity of stochastic effects is not dose-dependent.

Deterministic effects occur only if the dose is greater than a threshold value, affect all individuals in the exposed group and usually occur after a shorter period of time (seconds to tens of days) than stochastic effects. Deterministic effects have two characteristic features:

- There is a threshold radiation dose, below which the deterministic effects are not observed.
- The severity of deterministic effects are dose-dependent (IAEA 2004).

Depending on the time, an organism or organ/tissue exposed to IR can express a response as an early (acute) or as a late reaction to injury as well as both.
Early side reactions appear during and within 90 days after exposure to IR.
When the body is exposed to IR, the total biological effect of radiation depends on the dose delivered and the proportion of the body exposed to radiation. The changes are mainly seen in highly proliferating hierarchical tissues such as skin, bone marrow and the intestinal track, so that early effects are represented by inflammation, leukopenia, oedema, denudation of epithelia and haemorrhage.
The response of an organism to acute total body irradiation is described as one of the three known specific acute radiation syndromes (reviewed in Hall 2000):

- The bone marrow syndrome, which starts 1 h to 2 days after exposure to a dose between 0.7 – 10 Gy, however the mild effects may occur after a dose of 0.3 Gy. The bone marrow syndrome includes the destruction of the bone marrow stem cells, resulting in infection and haemorrhage. In humans the death caused by haematological damage occurs after about 30 days, but up to 60 days is possible.

For this reason in animal models death due to bone marrow damage described as term of $LD_{50/30}$ (Lethal Dose; a dose causing the death in 50 % of exposed subjects by 30 days). $LD_{50/60}$ in humans (a dose causing the death in 50 % of exposed subjects by 60 days) is between 2.5 – 5 Gy, if no medical treatment is carried out.

- The gastrointestinal syndrome (GI), which starts generally within few hours after exposure to a dose higher than 1 – 2 Gy. In humans the $LD_{100/60}$ (a dose necessary to kill 100 % of the exposed subjects within 60 days) is applied to measure deaths due to damage in gastrointestinal track. $LD_{100/60}$ in humans is about 10 Gy. Medical treatment may postpone somewhat the time of death, but cannot prevent it.

- The cardiovascular/central nervous system syndrome starts within minutes after exposure to a dose higher than about 50 Gy, however some symptoms may develop after 20 Gy. Death follows within 3 days, resulting from collapse of circulatory system and increased pressure in the confining cranial vault.

Late side reactions of radiotherapy occur after latent periods between 3 months and many years, sometimes decades of years (cancer) or in the offspring of exposed humans (genetic/teratogenic effects). They may be caused by the absorption of radiation directly in the target tissue, or as a consequence of acute damage.
These reactions to total body exposure include normal tissue damage such as telangiectasia, atrophy or leukaemia. The induction of a secondary tumour is also observed (Hall 2000).

During radiotherapy of cancer patients usually only a small area of the body is irradiated and the total dose delivered as well as the dose per fraction is well determined. For example, all prostate cancer patients participating in the present study received a total dose in the range of 70.2 - 72 Gy at 1.8 or 2 Gy per fraction.
Many cancer patients receiving radiotherapy will develop very little side effects, but in most patients the normal tissue will show some degree of side effects. This varies in type and amount, depending on the body part, which was treated, the area of normal tissue included in the treatment (Steel 1993), and the individual radiosensitivity. Patients may present different combinations of both early and late effects. In this study only the clinical side effects on the bladder and the rectum were evaluated in reference to individual radiosensitivity, as the

bladder and rectum are the most relevant organs at risk in prostate cancer radiotherapy (for details see Materials and Methods, section 2.2.2.2.1).

Several trials have been undertaken to work out a comprehensive system for grading and reporting of normal-tissue reactions to IR after radiotherapy, such as RTOG/EORTC system, WHO, French/Italian- or European systems. They are either clinically (e.g. RTOG/EORTC) and/or biologically relevant. The ideal system would be relevant for both the clinical aspects and radiobiology (Steel 1993, chapter 12).

For the purpose of the presented study the clinical normal tissue response to IR in prostate cancer patients was assessed on the basis of the EPIC questionnaire (the Expanded Prostate Cancer Index Composite; described in the chapter Materials and Methods, see also Appendix).

1.3.2 Physical and biological effects of IR at the cellular level

When cells are exposed to ionising radiation the first interaction is the excitation and ionisation of atoms or molecules of the tissue. This results, among other types of DNA damage, in the induction of DNA double-strand breaks (dsb), which, if not- or incorrectly repaired, might lead to lethal chromosomal aberrations. This can finally cause the loss of proliferative capacity (Dikomey et al. 2003). A consequence of this is an effect on cell function or cell death.

Physical effects of ionising radiation are based on two effects, the direct and the indirect one mediated by radicals.

When direct action takes place the ionising rays interact with the molecules of the critical target in the cell such as with DNA and induce ionisation or excite it through Coulomb interactions. This may lead to successive events, which produce biological damage. Direct actions dominate in case of exposure of biological material to high Linear Energy Transfer (LET) particles as for example neutrons or heavy ions.

Indirect action of radiation arises from the interaction of DNA with radiation-formed reactive species. Reactive oxygen species (ROS) are mainly formed following radiolysis of water resulting, among others, in the formation of highly reactive hydroxyl radicals ($\bullet$OH).

Following low exposure LET most DNA damage is induced by hydroxyl radicals rather than by direct action of IR (Friedberg et al. 1995).

DNA molecules are considered to be the critical targets for IR in the cell and changes in bases as a response to oxidative stress after irradiation are examined widely (Dizdaroglu and Karakaya 1999, Evans et al. 2004). More than 20 different types of base damage were identified as consequences of oxidative stress caused by ionising radiation (Lindahl 1993). Single strand breaks (ssb) and double strand breaks (dsb) might be induced directly by ionising radiation or result from a conversion of the base damage (Cline and Hanawalt 2003).

Cell response to oxidative damage (ROS) consists of several steps. First line of defence includes enzymes such as catalase, superoxide dismutase, amino acids, vitamins (e.g A, C, E), thiols and polyphenols (Slupphaug et al. 2003). Thereafter, enzymes that hydrolise oxidised dNTPs prevent incorporation of damaged bases into DNA. The third action is the repair of ssb and dsb in DNA by complex mechanisms of DNA repair, e.g. mismatch repair (MMR), homologous recombination (HR) and non-homologous end-joining (NHEJ; Slupphaug et al. 2003), described more detailed in section 1.7. Most of the induced lesions in wild-type mammalian cells can be repaired whereas only a small fraction is irreparable. It is commonly postulated that single-strand breaks (ssb) are accurately repaired while double-strand breaks (dsb) cause often lethal events (Tounekti et al. 1993).

It has also been observed that the difference in accuracy and efficiency of DNA damage repair influences the cellular radiosensitivity (Bishay et al. 2001). Moreover, double-strand breaks are assumed to be the major cause of the lethal effects on cellular level (Cline and Hanawalt 2003). Tounekti et al. (2001) have demonstrated that double strand breaks are intrinsically 300 times more cytotoxic when compared to single strand breaks. Furthermore, un-repaired dsb generate the induction of apoptosis, cause a permanent cell cycle arrest or mitotic cell death. Incorrect repair of double strand breaks can lead to carcinogenesis and finally dsb are precursors for the formation of chromosome aberrations after exposure to IR (Dikomey et al. 1998).

1.3.3 Radiation-induced genomic instability

The term “radiation-induced genomic instability” refers to the elevated rates of different genomic changes such as gene mutations, chromosomal destabilization or an increased level of apoptosis in the unexposed offspring of irradiated cells (Morgan 2003a and 2003b) and organisms. The radiation-induced genomic instability was also observed in un-irradiated cells that had contact with either irradiated cells or factors produced by these irradiated cells. This phenomenon is known as “bystander effect” (reviewed in Lorimore et al. 2003).

Increased ROS production and oxidative stress have been suggested as possible mechanisms responsible for radiation-induced genomic instability (Clutton et al. 1996). Genomic instability was found in cancer cells and different genomic instability syndromes (e.g Ataxia telangiectasia, Fanconi anemia) are associated with specific cancer types. Radiation-induced genomic instability seems to play an important role in radiation-induced carcinogenesis.

In contrast to structural DNA damage, which generally may be repaired, gene mutations result from unrepaired changes in the base sequence of DNA and cannot be recognized after cell division and are, therefore, transmitted to the progeny of the originally affected cell. The biological effect of mutations depends on the place where they occur. For example, a mutation of a gene may lead to a decrease in the normal control mechanisms of cell proliferation and may result in cellular transformation (Steel 1993).

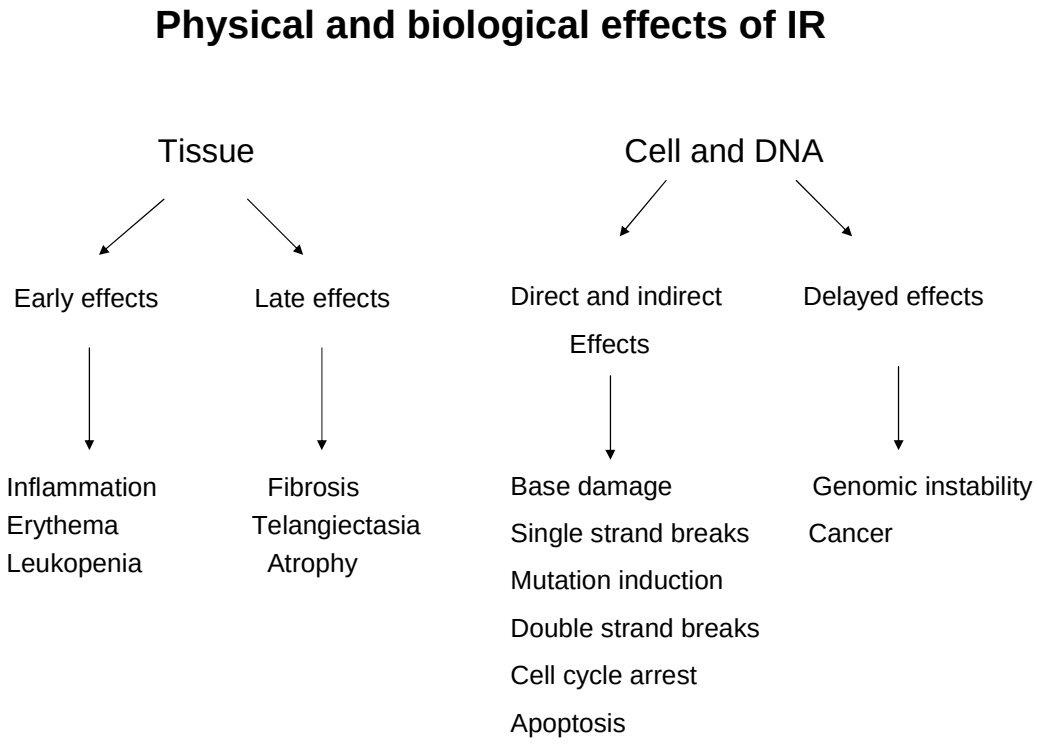


Figure 1.1: The effects of ionising radiation on tissue and cellular level (adapted from Giotopoulos 2008).

1.4 Radiosensitivity related to radiotherapy

The term “radiosensitivity” means the relative susceptibility of cells, tissues, organs or organisms to the damaging effect of ionising radiation (IR). In the cellular response to ionising radiation different processes are involved, such as generation of free radicals, apoptosis, inflammation and DNA repair. Nuclear DNA is the most susceptible target of IR, therefore the repair of DNA damage after irradiation seems to be one major protective mechanism (Gudkov and Komarova 2003). Hence, mutations in proteins that constitute the repair pathways are related to genome instability and radiosensitivity. Defects in these proteins also give rise to many genetic disorders such as cancer predisposition, neurodegeneration, immunodeficiency etc.

Radiotherapy is one of the most effective treatments of cancer. Therefore, there is great interest among clinicians in the in vitro detection of cellular radiosensitivity as an indicator for the risk of side effects in normal tissue after IR treatment (Brock, Tucker 2000). The aim of curative radiotherapy is inactivating cancer cells to reach tumour control with lowest damage of the surrounding normal tissue. This is an important parameter that serves a better quality of life for the patients during and after treatment. However, despite of many therapeutic improvements, efficient cancer therapy is still limited by side effects occurring in the normal tissue due to radiotoxicity (section 1.3.1). A number of patients- and treatment-related factors are known to affect the variability of side effects, but more than 70 % of cases remain unclear (Turesson et al. 1996). Even, when patients are treated using similar radiotherapy schedules, they differ significantly in normal tissue damage (Bentzen and Overgaard 1994, Turesson et al. 1996, Russel and Begg 2002). It is supposed that 5-10 % of all radiotherapy patients suffer from different side effects of normal tissue (Turesson et al. 1996, Andreassen et al. 2002). The identification of those sensitive individuals before the onset of therapeutic treatment has great importance for an optimized and therefore individual radiotherapy and consequently for a patient’s life length and its quality.

Approximately 50 % of patients with malignant tumours will be offered radiotherapy with curative or palliative intention (Bentzen 2006). Inactivation of tumour cells require absorbed doses of tens of Gray (typically in the range 20 – 60 Gy). Too low dose may lead to incomplete treatment, whereas too high dose results in adverse side effects in normal tissue surrounding a tumour, so that the highest priority in radiotherapy is to deliver accurate dose to the tumour (IAEA 2004).

The most common tumours treated with radiotherapy are breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancers, lymphoma, and cancer of the larynx. It is well known, that cells are most susceptible to be damaged by IR during the G2-phase of the cell cycle and mitosis and that most cancer cells proliferate rapidly. Thereby, cancer cells are more likely to be damaged by radiation because there are more cells undergoing division than in the normal cell population (Steel 1993).

Cellular radiosensitivity is directly proportional to the rate of cell division and inversely proportional to the degree of cell differentiation. Hence, as example, lymphoid organs, bone marrow and blood are highly radiosensitive, whereas brain and muscle show low radiosensitivity (Rubin and Casarett 1968).

1.5 Clinical versus cellular radiosensitivity

As mentioned above, efficient radiotherapy of malignant tumours is limited by the adverse normal tissue side effects. That is why during the last few decades great efforts were focused on the development of in vitro assays to predict the normal tissue reactions to IR after tumour treatment.

Several authors have found an enhanced cellular radiosensitivity in the cancer patients that developed severe clinical side reactions after radiotherapy (Borgmann et al. 2007, Hoeller et al. 2003, Widel et al. 2001). Lee et al (2003) have observed such a correlation in prostate cancer patients. In contrast, Lisowska et al. (2006) and Wang et al. (2005) have found no correlation between cellular radiosensitivity and the degree of acute reactions.

The recent advances in both molecular genetics and biology allow the investigation of clinical radiosensitivity by predictive assays based on genotype aspects such as cellular radiosensitivity or subcellular damage endpoints (Peters and McKay 2001; see section 1.8).

This would allow a reduction of the total dose for patients with high cellular radiosensitivity and thus alternative options for therapy may be taken into account. Normal and resistant patients may be exposed to a higher dose of IR. The second option applies especially to tumour entities for which dose escalation is possible, for example in case of prostate cancer. Such an individualization of radiotherapy could improve the whole therapy success rate by more than 20 % (Bentzen 1997, Tucker et al. 1999).

1.6 Cancer

The most important of the stochastic radiation effects is cancer, whose development is a complex and multi-stage process usually taking many years (IAEA 2004). Cancer is one of the leading causes of death in the world (Karim-Kos et al. 2008). It is that in the European Union there will be approximately 1.25 million cancer deaths until 2015, which means almost 130 000 (11 %) more deaths than in 2000. The increase in the predicted number of cancer deaths in 2015 is proportionally greater in males (13 %) than in females (10 %) and also larger in the acceding countries than in the current EU member countries. The highest number of cancer deaths in the current EU member countries will probably occur in 2015 in France and Germany, the Netherlands, Spain and Poland (Quinn et al. 2003).

Over 200 different types of cancer are known, but the most common besides skin cancer are breast, lung, large bowel (colorectal) and prostate cancer. These four cancer types account for over half of all diagnosed cases. Cancer affects mainly older people, whereof about 64 % of cases diagnosed are 65 years old and older (www.cancerresearch.org).

Cancer is a complex disease and carcinogenesis (the conversion of a normal cell into a cancer cell) is a complex multi-step process during which abnormal gene expression due to several mechanisms, such as DNA damage and abnormal gene transcription or translation occurs (Sarasin 2003). The causes of cancer in many cases are not clearly defined, however it is very well known, that both external (radiation and environmental chemicals; Montesano and Hall 2001) and internal factors (immune system defects or genetic predisposition; Peto and Houlston 2001) usually play an important role in cancer initiation. As example, known genes associated with hereditary cancer include the aberrant BRCA1 and BRCA2 genes that increase breast cancer risk and the HNPCC gene that is linked with colon cancer (www.cancerresearch.org).

1.6.1 Prostate cancer

Among cancers, the most commonly diagnosed in men is prostate cancer (PC). It is estimated that one of six men will be diagnosed with this cancer entity over the course of a lifetime. In spite of many risk factors, which have been suggested for prostate cancer, the data are inconsistent (Nomura 1991 and Ross 1996). Over 60 % of cases are diagnosed in men older than 65 years and about 25 % in men older than 75 years, which categorizes PC as old men disease. Prostate cancer ranges from a slow-growing (indolent) to a highly aggressive form

(Fitzpatrick 2008). The risk of PC grows almost linearly with age, from 0.005 % in men younger than 39 years to 2.2 % in men at age ranging between 40-50 years. In man at age 60-79 years the risk of being diagnosed with PC equates almost 14 % (Stangelberger 2008).

The prostate cancer cells may spread from the prostate to other parts of the body, mostly to the bones and lymph nodes. Prostate cancer may cause pain, problems in urinating and during sexual intercourse, or erectile dysfunction (www.cancer.gov).

The common use of prostate-specific antigen (PSA) as a screening test remains controversial. There is no agreement that measurement of PSA level has a predictive value for the risk of prostate cancer (Herman et al. 2009). Its inherent lack of specificity has led to several unnecessary biopsies (Lin et al. 2008). Also the effectiveness of digital rectal examination (DRE) for PC screening is not well established (Wilbur 2008).

All prostate cancer patients chosen for this study suffered from low-stage (T1-3N0M0) prostate carcinoma. Moreover, two further factors such as age and gender are also in this case homogeneous. It is especially important in view of the fact that both factors age and gender are often revealed as affecting the level of DNA damage (Papworth et al. 2001, Mendoza-Nunez et al. 2001).

1.6.2 Susceptibility to cancer

Approximately 80 % of most common cancers are sporadic, 10-15 % of cases are the result of mutations in one or more low penetrance genes, interactions between gene and environment or both and 5-10 % are inherited and arise due to highly penetrant mutations (Nagy et al. 2004). The majority of the discovered genes predisposing to cancer are highly penetrant, but also occur too rarely to be responsible for the induction of more than few percent of most cancer types (Nagy et al. 2004). The second interesting observation is the varying penetrance of the genes. For example in many cases of breast cancer the genes BRCA1 and BRCA2 which are highly predisposing in heterozygotes are involved. Whereas the ATM gene, which causes Ataxia telangiectasia (AT), is only mildly penetrant in obligate heterozygotes. The full gene penetrance appears only in the homozygotes (Swift et al. 1986).

Now it is well known that a high proportion of cancers arise due to genetic susceptibility. One maintains, however, that the relevant genes are low penetrant and hence, do not cause large multiple families cancer cases.

1.6.3 Detecting cancer susceptibility

Over 30 years ago Taylor et al. (1975) observed an association between cancer predisposition and the in vitro hypersensitivity of human fibroblasts to ionising radiation. Indeed, the elevated chromosomal radiosensitivity has been found further in cancer patients when compared to healthy donors (e.g. Scott et al. 1999, Bonassi et al. 2000, Lisowska et al. 2006).

The identification of individuals with cancer susceptibility (cancer-prone) would allow early detection and effective treatment.
Therefore, this study was undertaken to assess the predictive value of the chosen in vitro assays for cancer predisposition.

Identification of genes responsible for cancer susceptibility causes often trouble because of their low penetrance, gene combination and gene-environment interactions. The only reliable and successful known mechanism for identifying low penetrance genes is the analysis of polymorphisms at candidate loci. The effect of such polymorphisms in combination with each other and also with environmental risk can be only assessed when very large collectives of donors were investigated. Taken this into account it is obvious that alternative methods must be found.
Recently, micro-array technology was developed and gladly used. This technique allows the screening of the expression level of various genes at different times and thus the transcriptional activity of certain regulatory genes as a characteristic of tumours can be assessed. Finally, mutations in potential low penetrance genes can be found. Other analytical tools, based on chromosome aberrations such as micronucleus assay, FISH assay (fluorescence in situ hybridisation), comet assay, apoptosis assay, γ-H2AX assay etc. are investigated as potential tools for prediction of cancer susceptibility.

By use of a selection of the above mentioned assays we wanted to find out, whether the prostate cancer patients in this study show an elevated level of radiation-induced DNA damage in their lymphocytes, when compared to healthy donors.

1.7 Molecular aspects of individual radiosensitivity

There are a number of classification systems characterizing the extent of side reactions in radiotherapy patients (e.g. Wei et al. 2000, Hoeller et al. 2003). The results from a clonogenic assay are presented in values of survival fraction (SF) and parameters α and β describing the survival curve. Measurement of DNA repair kinetics and the amount of repaired damage at certain analysed time points after exposure are supposed to give also some information about the possible development of side effects (Müller et al. 2001, Borgmann et al. 2002).

Mutations in genes, which are involved in DNA repair pathways often lead to an increase in radiosensitivity (Bishay et al. 2001). The first association between a defect in DNA repair, radiosensitivity and cancer was published by Cleaver (1968) who observed that Xeroderma pigmentosum (XP) patients have a genetic defect in the ability to repair DNA damage caused by ultraviolet (UV) light. Lately is has been shown that also several other common hereditary forms of cancer are associated with DNA repair defects and radiosensitivity (Bourguignon et al. 2005). These disorders result mostly from mutations in genes attending in DNA signalling or repair pathways, cell cycle and transcription. The described situations occur in Fanconi anaemia (Duckworth-Rysiecki and Taylor 1985), Bloom's syndrome (Wang et al. 200), Nijmegen breakage syndrome (Demuth and Digweed 2007) etc.

In the repair of dsb, which are regarded as the most critical type of radiation-induced DNA damage (Tounekti et al. 2001), two main repair mechanisms are involved – homologous recombination (HR) and non-homologous end-joining (NHEJ; Smith et al. 2001).

Homologous recombination (HR) uses as a template the homologous sister chromatid and acts post-replication during the late S/G2 – phase, whereas NHEJ "works" in G1/early S – phase (Rothkamm et al. 2003). Both HR and NHEJ play crucial roles in the repair of lesions that arise in certain tissue types. The mutation of either of these pathways can lead to developmental defects and finally embryonic death, increase of cancer induction as well as defects in neurogenesis (Tsuzuki et al. 1996). Nevertheless, there are examples in which the knockout of a certain gene does not lead to a tumour development, as demonstrated by mice with knocked out Rad52 and Rad54 genes coding proteins involved in HR (Smith et al. 2001). Cell lines that are defective in any of the NHEJ genes showed also a high sensitivity to IR and have significant deficiencies in DNA dsb repair (Rothkamm et al. 2003). There are some defects in repair pathways which do not act directly on DNA repair, but cause radiosensitization, such as telomere shortening, signal transduction, transcription or regulation of cell cycle and cell death. For example, higher aberration level and elevated apoptosis rate was found in $mTR^{-/-}$ mice (G5 $mTR^{-/-}$ mice; mice lacking the RNA component of telomerase)

when compared to wild-type controls. In fifth generation $mTR^{-/-}$ mice telomeres are 40 % shorter than in wild-type mice (Goytisolo et al. 2000).

1.8 Measurement of cellular sensitivity in vitro

The concept developed since over 40 years ago that DNA damage is one of the crutial initial events for the development of radiation sensitivity was the reason why great efforts were undertaken to measure DNA damage and repair.

It is well known, that different biological features, such as repair capacity, induction of chromosomal aberrations as well as biological endpoints, such as apoptosis and cell survival are affected by individual radiosensitivity. The existing experimental endpoints include measurement of:

- apoptosis (programmed cell death)
- induction of cellular death (loss of metabolic activity)
- cytogenetic changes such as micronuclei, chromosomal/chromatid aberrations and chromosomal instability
- clonogenic survival
- initial and residual DNA damage and repair capacity using γ-H2AX assay, comet assay or pulsed field gel electrophoresis (PFGE)

(Bourguignon et al. 2005, Joubert et al. 2008)

In the present thesis the G2 assay, apoptosis assay and γ-H2AX foci assay were chosen to analyse the chromatid-type aberration yield, apoptosis rate as well as foci induction and repair in peripheral blood lymphocytes from prostate cancer patients and age-matched healthy donors.

As the G2 assay was used in several studies (e.g. Scott et al. 1994, 1996, 1999, Terzoudi et al. 2000, Lisowska 2006 etc.) and investigated in lymphocytes of many cancer patients and healthy donors, the results of chromatid-type aberrations were regarded in the thesis as point of reference.

1.9 The impact of temperature on radiation-induced cytogenetic damage

The common application of ionising radiation sources for agricultural, military or medical goals in normal life increases the risk for overexposure of people. In case of accidental exposure the estimation of the absorbed dose and its influence on cellular damage is necessary.

The level of cytogenetic damage induced by ionising radiation under in vitro conditions in human peripheral blood lymphocytes (PBL) is analysed for the purpose of establishing calibration curves used in biological dosimetry and for assessing the intrinsic radiosensitivity of the blood donor (International Atomic Energy Agency [IAEA] 2001, Lisowska et al. 2006). It indicates that blood exposure in vitro should be conducted under strictly controlled physical conditions to obtain a high reproducibility of the dose. Temperature control during irradiation in vitro is a factor that often is neglected. However, it has been announced that temperature has influence on radiation-induced chromosome aberrations (e.g Bajerska and Liniecki 1969, Gumrich, Virsik-Peuckert and Harder 1986, Claesson et al. 2007). Mostly a sparing effect of low temperature during exposure on the level of cytogenetic damage was observed (Bajerska and Liniecki 1986, Gumrich et al. 1986). Contrary, there are results showing no influence of temperature on DNA damage (Wojewodzka et al. 1996).

In the first part of this study the influence of temperature on radiation-induced DNA damage was investigated to find out, whether the temperature condition during irradiation is a factor which should be strictly controlled for the purpose of high reproducibility of the results.

1.10 Thesis aims

The goals of this thesis were**:**

In part 1: Influence of temperature during irradiation on the level of DNA damage.

- To find out, whether different temperature conditions during irradiation of peripheral blood lymphocytes have an influence on the radiation-induced level of chromosomal damage.

- To check, whether the cytogenetic temperature effect in peripheral blood lymphocytes is related to the direct or indirect action of radiation.

In part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors.

- To compare individual cellular radiosensitivity between prostate cancer patients with and without clinical side effects after radiotherapy and age-matched male healthy donors to find out, whether the level of DNA damage may be associated with cancer susceptibility.

- To find out whether one or more of the chosen assays might be appropriate to predict the risk for side effects after radiotherapy of prostate cancer patients.

II. Materials and methods

Part 1: Influence of temperature during irradiation on the level of DNA damage.

2.1.1 Materials

2.1.1.1 Equipment

Centrifuge, MPW Med. Instruments, Poland
Cell incubator, NuAire, USA
Electrophoresis chamber, own construction
Eppendorf cups, Eppendorf, Germany
Fluorescence microscope, Nikon Labophot-2, Tokyo, Japan
Irradiation source, ^{137}Cs Gammacell 40, Fa. Atomic Energy, Canada
Irradiation source, X-ray machine, Andreassen, Copenhagen, Denmark
Light microscope, Nikon, Poland
Water bath, MPW Med. Instruments, Poland
Zeiss Fluorescence Microscope Axioplan 2 Imaging, Carl Zeiss, Germany

2.1.1.2 Consumable materials and chemicals

Acetic acid, Sigma, Poland
Agarose Type VII (low melting point), Sigma, Poland
Agarose Type IA (regular agarose), Sigma, Poland
Cytochalasin B, Sigma, Poland
DAPI (4'.6-diamidino-2-phenylindole), Sigma, Poland
DMSO (dimethyl sulphoxide), Gibco, Poland
EDTA, Sigma, Poland
Falcon tubes, Sarstedt, Poland
Gradisol, Sigma, Poland
Sodium acetate, Sigma, Poland
Tris base (Tris(hydroxymethyl)aminomethane), Sigma, Poland

Tris-HCl (Tris Hydrochloride), Sigma, Poland

Trypan Blue, Sigma, Poland

Microscope slides, Marienfeld, Germany

RPMI 1640 medium, Sigma, Poland

Fetal calf serum, Gibco, Poland

Giemsa, Sigma, Poland

Methanol, Sigma, Poland

N-lauroylsarcosine, Fluka, Germany

PBS, Sigma, Poland

Sodium ethylenediaminetetraacetic (Na_2EDTA), Sigma, Germany

Tubes for blood culture, Greiner Bio-One, Germany

Sodium chloride (NaCl), Fluka, Germany

Phytohaemagglutinin (PHA), Gibco, Poland

Penicillin/Streptomycin, Gibco, Poland

Triton X-100, Sigma, Poland

2.1.1.3 Buffers and solutions

Micronucleus assay	Alkaline comet assay	Neutral comet assay
Fix solution I: methanol 0.9 % NaCl acetic acid at a ratio of 12:13:3 Fix solution II: methanol acetic acid at a ratio of 4:1	Lysis buffer, pH 10: 2.5 M NaCl 100 mM Na_2EDTA 10 mM Tris base 1 % Triton X-100 Electrophoresis buffer, pH 13: 1 mM Na_2EDTA 300 mM NaOH Neutralising buffer: 0.4 M Tris, pH 7.5	Lysis buffer, pH 9.5: 2.5 M NaCl 100 mM EDTA 10 mM Tris-HCl 1 % N-lauroylsarcosine 0.5 % Triton X-100 10 % DMSO Electrophoresis buffer: 300 mM sodium acetate 100 mM Tris HCl, pH 8.3 Neutralising buffer: 0.4 M Tris, pH 7.5

2.1.1.4 Software

Comet v.3.0 Kinetic Imaging, Liverpool, UK
Metafer4, version 3.4, MetaSystems, Germany

2.1.2 Methods

2.1.2.1 Collection of blood samples

Peripheral blood lymphocytes were collected by venous puncture from two male non-smokers, age 26 and 46 years and two female occasional smokers of the same age.
For micronucleus assay and alkaline comet assay whole blood or isolated lymphocytes were irradiated and set up for cultures. In all neutral comet assay experiments isolated lymphocytes were used, which resulted in a better quality of comets.

2.1.2.2 Isolation of lymphocytes from whole blood

Peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation using a lymphocyte separation medium (Gradisol). 15 ml whole blood from each donor was diluted with 15 ml of pre-warmed RPMI 1640 medium. 15 ml of cold Gradisol was added to two 50 ml falcon tubes and 15 ml of diluted donors's blood was carefully poured onto the Gradisol solution. The tubes were centrifuged 0.5 h at 400 x g at room temperature (RT) and after that, 4 layers were distinguishable. The lymphocytes were harvested from the thin white layer, mixed with 30 ml warm RPMI 1640 and finally centrifuged 20 min at 300 x g at RT. The supernatant was carefully removed and the pellet was washed 2-3 times by resuspension in RPMI 1640, centrifugation as described above and finally the supernatant was discarded. Immediately after the last washing step the cell pellet was resuspended in 1 ml of RPMI 1640 and the cell number was counted using trypan blue.

2.1.2.3 Exposure conditions

Whole blood or isolated lymphocytes were always aliquoted into Eppendorf cups, which were placed in 150 ml plastic cups, filled with water at 37°C and 0-4°C (crushed, melting ice). Two cups were incubated at 37°C and further two at 0-4°C for 20 min before and during exposure. For each temperature condition one sample was taken as a control and the other exposed to 2

Gy X-rays (dose rate 0.5 Gy/min) for micronucleus assay and alkaline comet assay. For the neutral comet assay, cells were irradiated with a dose of 20 Gy γ-rays at a dose rate of 0.74 Gy/min.

2.1.2.4 Micronucleus assay

Directly after irradiation lymphocyte cultures were set up by adding either 0.5 ml of whole blood (donors 1, 2 and 3) or isolated lymphocytes (about 1.5 x 10^6, donor 1) to 4.5 ml of pre-warmed RPMI 1640 medium, supplemented with 20% fetal calf serum, 10 µg/ml PHA, 100 U/ml penicillin and 100 µg/ml streptomycin. Lymphocytes were then incubated at 37°C in a humidified atmosphere and 5 % CO_2. After 44 h cytochalasin B at a final concentration 5.6 µg/ml was added to block cytokinesis of the cells. Following 72 h of culture time the cells were harvested and fixed according to the protocol of Fenech (2000). The cultures were centrifuged for 15 min at 900 rpm, then the supernatant was carefully removed and after resuspension of the pellet 7 ml of 0.14 M KCl was slowly added to the cells. During addition of the hypotonic solution the tubes were vortexed to get a single cell suspension. The hypotonic treatment for 5 minutes at RT induces the swelling of the lymphocytes which is an important precondition for the bursting of the cell membrane during dropping the cells on slides.

Following hypotonic treatment the samples were centrifuged at 900 rpm for 15 minutes and after removing of supernatant, 7 ml of fresh fix solution (I) was added. The cells were then centrifuged as described above. The pellet was washed 2-3 times with fresh fix solution (II), each time removing the supernatant, vortexing and adding fresh fixative. The cells were kept one or few days at 4°C. Directly before slide preparation the suspension was centrifuged again as above and the cell pellet was resuspended in 1-2 ml of fresh fix solution (II).

2.1.2.4.1 Slide preparation and scoring criteria

The cells were dropped (from a height of approximately 4 cm) on clean slides and allowed to air-dry over night at RT. Slides were stained with 5 % Giemsa for 7-10 minutes and dried over night. Slides were coded and scored at 400x magnification using a light microscope. For each experimental dose point 1000-2000 binucleated cells (BNC) were taken into account with exception of few cultures incubated shortly with toxic DMSO. Micronuclei were scored in agreement to the criteria published by Fenech (2000).

2.1.2.4.2 Treatment with DMSO

For the experiments with DMSO isolated lymphocytes were used. Use of whole blood was unpractical because of the haemolytic properties of DMSO. DMSO was added to the lymphocyte cultures 5 min before exposure (final concentration 0.5 M) and removed by centrifugation for 5 min at 1100 rpm directly after exposure. The set up of the cultures for micronucleus assay was performed as described above.

2.1.2.4.3 Statistical analysis of micronucleus assay

Chi-square test for Poisson-distributed events (Sachs 1984) was used to compare the frequencies of micronuclei exposed at different temperatures. Dispersion index (DI) was calculated for micronuclei by dividing the variance by the mean value. The u test (Edwards et al. 1979) was used to assess a significance of deviation from a Poisson distribution. The paired, two sided Student's t-test was used to compare the mean values of micronuclei per treatment ($P < 0.05$). The proliferation capacity of the lymphocytes was estimated by calculating the replication index (RI) according to the formula:

$$RI = Mono + Bi*2 + Tri*3 + Tetra(+)*4/ N,$$

where: Mono, Bi, Tri and Tetra(+) indicate cells with, respectively 1, 2, 3, 4 or more nuclei, and N is the number of scored cells.

2.1.2.5 Alkaline comet assay

The level of DNA damage was determined using the alkaline version of the comet assay in accordance with Wojewodzka et al. (1998). For each experiment four Eppendorf cups with 0.5 ml blood were prepared. Two of them were incubated at 37°C and two at 0-4°C each for 20 min before and during irradiation. One sample incubated at 37°C and one at 0°C (described as 37°C 2 Gy and 0°C 2 Gy) were irradiated. Not irradiated controls were described as 37°C 0 Gy and 0°C 0 Gy. Following exposure, about 50 µl of whole blood was mixed with 0.5 ml RPMI 1640 (donors 2, 3 and 4). Thereafter, 200 µl of suspension was mixed with the equal volume of warm (~ 37°C) low melting point agarose at a final concentration of 1 %. Finally 100 µl of this suspension was coated carefully on a microscope

slide and covered with a cover slip. Glass slides were prepared in advance by precoating with 0.5 % standard agarose diluted in deionised water and dried over night.

The slides were then left for few minutes on ice. To minimize the influence of DNA repair after irradiation the following order for sample handling was practised: 37°C 2 Gy, 0°C 2 Gy, 37°C 0 Gy and 0°C 0 Gy. After solidification of the agarose, slides were incubated in 50 ml of freshly prepared, cold lysis buffer to release damaged DNA. After 1 h, the slides were washed twice in PBS and placed on a horizontal gel electrophoresis chamber filled with fresh, cold electrophoresis buffer. The slides were incubated in this buffer for 40 minutes at 4°C to allow DNA unwinding. Electrophoresis was performed for 0.5 h at 4°C and a power supply setting of 2 V/cm. Thereafter the slides were washed three times with cold neutralization buffer for 3-5 minutes, stained with 1 µM DAPI and kept in the fridge over night. After staining at least 50 randomly selected comets per slide were captured at 200 x magnification using a fluorescence microscope. Digital images were acquired using the Comet v.3.0 Kinetic Imaging software. The Olive Tail Moment (OTM) was selected for further analysis.

2.1.2.6 Neutral comet assay

The comet assay in neutral version was performed as described by Wojewodzka et al. (2002) with some modification.

Directly after irradiation all samples were put on ice to minimise DNA damage repair and immediately transported to the laboratory (about 10 minutes). The cell suspension (4 x 10^5 cells/ml) was mixed with the equal volume of pre-warmed (37°C) low melting point agarose at a final concentration of 0.75 %. Thereafter, 100 µl of suspension was coated on a microscope slide precoated with a thin layer of 0.5 % standard agarose, covered with a cover slip and kept for 5 min on ice to allow solidification of the agarose. After removing the cover slips, the slides were left at 4°C in the dark for 1-2 h in lysis buffer. After lysis the slides were washed three times for 1-2 min with electrophoresis buffer and left in a horizontal gel electrophoresis chamber filled with cold electrophoresis buffer for 1 h in the dark for DNA relaxing. The electrophoresis was performed for 1 h at 14 V (0.5 V/cm, 4°C). Then, slides were washed three times for 3-5 min with 0.4 M Tris, pH 7.5, stained with DAPI (1 µg/ml) and kept in a fridge over night.

The next day slides were analysed. Images from 50-100 randomly selected comets per slide were automatically captured at 200 x magnification using the fluorescence microscope and the Metafer4 software. The Olive Tail Moment was selected for further analysis.

2.1.2.7 Comet analysis

The Olive Tail Moment is defined as the product of the tail length and the fraction of total DNA in the tail. The Tail Moment allows the measurement of the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces, represented by the fluorescence intensity of the DNA in the tail.

2.1.2.8 Statistical analysis

The average values of the tail moment were estimated for 4 donors in the alkaline version and for 3 donors in the neutral version of the comet assay at each dose and temperature.
The paired, two sided Student's t-test was used to compare the mean values of the Tail Moment per treatment and $p < 0.05$ was considered significant.

Part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors.

2.2.1 Materials

2.2.1.1 Equipment

Casy Counter TTC, Schärfe System, Germany
Centrifuge Multifuge 1s-r, Heraeus, Germany
Centrifuge 5415R, Eppendorf, Germany
Cytospin centrifuge Rotofix 32A, Hettich, Germany
Cell Incubator MCO-20 AIC, Sanyo, Japan
Irradiation source, ^{137} Cs Gammacell 40, Fa. Atomic Energy, Canada
Flow cytometer FACSCanto TM^{II}, BD Biosciences, Germany
Spectrafuge mini C1301, Labnet International, USA
Waterbath SW22, Julabo, Germany
Vortex FB15013 TopMix, Fisher Scientific, Germany
Axioplan 2 Imaging Microscope, Zeiss, Germany

2.2.1.2 Consumables and chemicals

AccuMax, PAA, Austria

Acetic acid, Merck, Germany

Albumin from bovine serum (BSA), Sigma, Germany

Colcemid, PAA, Austria

Culture flasks (25 cm^2), TPP, Switzerland

Culture tubes (4.5 ml), Greiner Bio-One, Germany

Ethanol, Merck, Germany

Falcon tubes (50 ml), Sarstedt, Germany

Fetal calf serum, Biochrom, Germany

Fetal calf serum Gold, PAA, Austria

Filter cards, Hettich Zentrifugen, Germany

Formaldehyde, Fluka, Germany

Giemsa, Merck, Germany

Goat serum, PAA, Austria

Potassium chloride (KCl), VWR, Germany

Lymphocytes Separation Medium, PAA, Austria

Methanol, Merck, Germany

Microscope cover slips, VWR, Germany

Microscope slides, VWR, Germany

Microscope slides Superfrost® Plus, Thermo Scientific, Germany

PBS (phosphate buffered saline without Mg^+ and Ca^+), PAA, Austria

Penicillin/ Streptomycin, PAA, Austria

PHA (phytohaemagglutinin), PAA, Austria

Pipette tips (2.5 µl, 10 µl, 20 µl, 100 µl, 1000 µl), Eppendorf, Germany

Prolong®Gold antifade reagent with DAPI, Invitrogen, USA

RPMI 1640 with 2 mM stable glutamine, PAA, Austria

Triton X-100, Sigma, Germany

Tubes (1.5 ml, 2 ml), Eppendorf, Germany

S-Monovette for blood transport, 10 ml, NH4, Sarstedt, Germany

2.2.1.3 Kits

Annexin V- FITC Apoptosis Detection Kit, BD Biosciences, Germany

2.2.1.4 Buffers and solutions

γ-H2AX assay

1.5 % formaldehyde
2 ml 37.5 % formaldehyde
48 ml PBS

TBP buffer
0.2 ml Triton
1 g BSA
100 ml PBS

0.25 % Triton
0.25 ml Triton X-100
100 ml PBS

FACS buffer
10 ml FCS
90 ml PBS

5 % goat serum
2 ml goat serum
38 ml PBS

2.2.1.5 Antibodies

γ-H2AX assay

Primary antibody: Anti-phospho-histone H2A.X (ser139), clone JBW301, Maus, Upstate, USA
Secondary antibody: Alexa Fluor 488 goat anti- mause IgG, Invitrogen

2.2.1.6 Software

Metafer4, version 3.4, MetaSystem, Germany
FACSDiva, BD Biosciences, Germany

2.2.2 Methods

2.2.2.1 Sample collection

Blood samples from prostate cancer patients, as well as from healthy age-matched donors were collected between September 2007 and May 2009. All blood samples were collected in 9 ml tubes and transported to Forschungszentrum Jülich GmbH in a polystyrene box for ambient temperature as well as to avoid breakage during transport (~1 hour). Then peripheral blood lymphocytes were immediately isolated and for G2 assay blood cultures were prepared.

2.2.2.2 Donors

2.2.2.2.1 Prostate cancer patients

The study was based on patients with localized T1-3N0M0 prostate carcinoma, who were treated with three-dimensional conformal radiotherapy in between the years 2005 and 2006 at the Department of Radiation Oncology, RWTH Aachen University, Germany.
The average patient age was 74 years, ranging from 61 to 84 years. The therapy was based on performing computer tomography (CP) scans in supine position with a slice thickness of 5 mm. Patients were asked to have a full bladder each before making a CT scan and receiving the radiotherapy fraction. In all scans prostate volume, planning target volume (PTV), bladder and rectum were delineated by identifying the external contours. The treatment plans were calculated with the use of a four-field box technique with 15 MeV photons and a multileaf collimator. All patients have received a total dose in the range of 70.2 - 72 Gy at 1.8 or 2.0 Gy daily fractions. All patients have been surveyed prospectively before (time A), at the last day (time B), two months (median, range 6 weeks-6 months) after (time C) and sixteen months (median, range 12-20 months) after (time D) radiotherapy with the use of a validated questionnaire, the Expanded Prostate Cancer Index Composite (EPIC) (Wei et al 2000; Volz-Sidiropoulou et al. 2008). Acute toxicity was assessed at times B and C, whereas late toxicity at time D. The EPIC form consists of 50 questions regarding the urinary, bowel, sexual and hormonal condition. In this study only the clinical side effects on the bladder and the rectum

were evaluated in reference to individual radiosensitivity. The multi-item scale scores were transformed lineary to a 0-100 scale. The higher the scores the worse is health-related quality of life (QoL). In agreement with the literature data mean scores below 5 points was characterised as clinically not significant, 5-10 points was described as “little” changes, 10-20 points corresponded to “moderate” changes and over 20 points represented “very much” changes (Ososba et al. 1998, Pinkawa et al. 2008).

Because the most critical organs at risk in prostate cancer radiotherapy are bladder and rectum, urinary and/or bowel quality of life (QoL) changes were a very important criterion connected with/without side effects after radiotherapy. Patients with severe, side effects were defined as having at least one, but preferably more urinary or bowel bother score changes in comparison to the baseline level: >40 points at time B, >20 points at time C or >10 points at time D – corresponding to those 25% of patients with the most severe reactions at the respective point in time, if the results of all patients are considered. Patients without relevant reactions were defined as patients with urinary or bowel bother score decreases below the mentioned cut-off levels at all times, but preferably always <10 points.

Additionally the patients provided the information concerning smoking, chronic illnesses, taking medicine, diet and allergy (see Table 2.1).

The information about prostate cancer patients were obtained from Professor Michael Eble and Doctor Michael Pinkawa from the Department of Radiation Oncology, RWTH Aachen University in Germany.

Blood samples were obtained from 50 prostate cancer patients after radiotherapy, 25 of them showed strong clinical side effects after radiotherapy and 25 showed no side reactions. By the G2 assay 44 patients were tested, 22 with and 22 without severe clinical reactions after radiotherapy. The apoptosis/necrosis assay was performed for 41 patients, thereof 20 with and 21 without reactions. In the γ-H2AX assay, measuring the mean fluorescence intensity by FACS, 45 patients were analysed, thereof 23 with and 22 without severe side reactions after radiotherapy. The γ-H2AX foci were scored for 44 patients, where 21 showed side reactions and 23 had no reactions after radiotherapy.

2.2.2.2.2 Healthy donors

Additionally blood samples from 23 age-matched male healthy donors were collected. The mean age of all healthy volunteers was 67 years ranging from 47 to 85 years. All healthy volunteers filled a questionnaire comprising questions about smoking, chronic illnesses, taking medicines, diet and allergy (Table 2.1).

Table 2.1: Characteristic of prostate cancer patients and healthy donors.

	Number of donors		
	Patients with side effects (S)	**Patients without side effects (0)**	**Healthy donors (HD)**
No smokers	20	23	18
Passive smokers	-	-	1
Smokers	5	2	4
Previous illness (-es):			
Yes	3	3	16
No	22	22	6
No information	-	-	1
Taking of drugs:			
Yes	23	24	18
No	2	1	4
No information	-	-	1
Allergy:			
Yes			3
No	-	-	19
No information			1
Weight:			
Normal weight			13
Underweight	-	-	2
Overweight			8

All patients and healthy volunteers agreed with the test personal information sheet about the experiments and with the regulations concerning the data privacy protection. Ethical permission was obtained for the study (EK 130/08) by the ethics committee of the medical faculty, university clinics of RWTH Aachen, Germany.

2.2.2.3 The G2 assay

The G2 assay detects chromatid-type chromosome aberrations, mainly chromatid breaks and gaps occurring after irradiation of the cells during the G2 phase of the cell cycle. The G2 assay was performed according to a modified version of the method of Scott et al. (1999).

The samples were cultured within 3 hours after venous puncture. 1 ml heparinised whole blood was added to 9 ml of complete, pre-warmed (37°C) culture medium consisting of RPMI 1640 with 2 % stable glutamine, 10 % donors own plasma, 2.5 % PHA

(phytohaemagglutinin) and 1 % antibiotics. PHA is a mitogen stimulating lymphocytes to become mitotically active cells (Chandler and Yunis, 1978) by making the plasma membrane permeable for growth factors. The medium is supplemented with donors own plasma and stable glutamine to provide the cell culture with factors identified as essential for growth such as adhesion factors, hormones, mineral elements, vitamins and binding proteins.

Twenty four hours after stimulation by PHA the RNA synthesis in the cells increases, subsequently the nucleus enlarges and the DNA synthesis begins. Hence, the first mitoses are seen at about 48 hours after stimulation.

The cultures were grown in 25 cm^2 tissue culture flasks in a CO_2 incubator at 37°C for 72 hours. For each donor 2 cultures were set up, one for irradiation and one as a control to enable the determination of the spontaneous chromatid aberration yield.

After 69 hours, the cultures were irradiated with a dose of 0.5 Gy γ-rays (dose rate 0.74 Gy/min.) and placed immediately on ice during transport. Cultures were further incubated at 37°C. Colcemid (final concentration 0.1 µg/ml) was added 1.5 h before harvest. Colcemid prevents the spindle formation, which causes an arrest between metaphase and anaphase and thus allows the preparation of metaphase chromosomes, which are highly condensed and therefore visible under the microscope.

Thereafter, cultures were centrifuged at 1100 rpm for 10 minutes. Supernatants were removed and prewarmed 75 mM KCl was added to the pellets and vortexed to resuspend the cells. The suspension was incubated for 15 minutes at 37°C, which induces the swelling of the lymphocytes due to hypotonic treatment. Cells were then fixed 3 to 4 times in cold methanol: acetic acid (3:1), each time the supernatant above the cell layer was removed and the remaining pellet vortexed with fresh methanol: acetic acid solution. Tubes containing fixed lymphocytes were stored at -80°C overnight or longer. Incubation of lymphocytes at -80°C results in higher quality of metaphases on slides.

2.2.2.3.1 Slides preparation

Cell suspension was taken out from the freezer and centrifuged at 1100 rpm for 10 minutes. The supernatant was removed and the cell pellet was dissolved in 1 ml of fresh fixative, 100 µl of cell suspension was dropped from a height of approximately 40-50 cm on a clean fat-free slide. The slide was dried at room temperature over night.

Because irradiation results in a reduced mitotic index, it was necessary to make 2-6 slides to obtain enough metaphases to score for each irradiated samples. For not irradiated controls 1-2

slides were sufficient. Staining of the slides was performed with 10 % Giemsa for a few minutes. The slides were dried at room temperature over night.

2.2.2.3.2 Scoring criteria

Slides were coded for anonymity, placed on the microscope stage and scanned at 10x magnification until a metaphase was found. The magnification of the microscope was changed to 100x objective using immersion oil and the quality of the metaphase was assessed before scoring for any chromosome damage. Only well spread metaphases were selected for analysis. Thereafter, chromosomes were counted and only if there were 46, they were taken into account. For analysis, 100 cells were scored for aberrations, which were mainly chromatid breaks with a few exchanges and chromatid gaps.

Chromatid aberrations were classified according to Sanford et al. (1989) and Scott et al. (1999), who defined breaks as chromatid discontinuities with relocation of the broken segment and gaps as showing no displacement of the segment distal to the lesion. Gaps were included in the final G2 score only in case, if they were wider than the chromatid width. Chromatid breaks and gaps were added together to give the total G2 score for both irradiated and control samples. The spontaneous as well as radiation-induced chromatid yield was determined.

2.2.2.3.3 Statistical analysis of chromatid aberrations

2.2.2.3.3.1 Mean spontaneous and radiation-induced aberration yields

Mean spontaneous as well as induced chromatid aberration frequencies were calculated by dividing the total number of aberrations observed by 100 (the total number of scored cells).

The unpaired t test was used to compare G2 scores of the healthy donors with the prostate cancer patients with side effects after radiotherapy, as well as with the patients without effects.

The unpaired t test was also used to compare G2 scores between both groups of patients.

2.2.2.3.3.2 Analysis of aberration distribution in accordance with the 50th percentile

The analysis of average values of chromatid aberration is very sensitive on values lying in the ends of Gauss graph of chromatid distribution. In practise, it means that extreme high or low average chromatid aberration yields have a very strong influence on the mean value of the entire group of donors. To assess scope, where the aberration scores for 50 % patients are assembled, the aberration yields for each group of donors were separately divided by three quartile values into four equal parts. The middle quartil is also known as a median value and cuts data set (in this case aberrations yield) in half. Lower quartile (known also as 25th percentile) cuts off lowest 25% of data and upper quartile (or 75th percentile) cuts off highest 25% of data. The area between lower and upper quartil is assembled around the median value and contains 50 % of all the scores in a group.

2.2.2.3.3.3 Analysis of aberrations dispersion

The u-test according to Edwards et al. (1979) was used to study the distribution of chromatid-type aberrations amongst metaphase cells in each donor. The chance of developing an aberration in a cell is randomly distributed, what means that each cell has an equal chance, if the observed distribution follows Poisson statistics. A positive u-value indicates over-dispersion, whereas negative values indicate under-dispersion.

If the u-value is greater than the value of 1.96 or -1.96 then the over-/under-dispersion is significant.

Analysis of aberration dispersion was performed for samples exposed to 0.5 Gy of γ-rays. Not irradiated samples were excluded in this analysis because of very low aberration yields counted for 100 control cells.

2.2.2.3.3.4 Determining radiosensitivity

The proportion of radiosensitive individuals was determined by using a cut-off value of the 90th percentile of spontaneous and radiation-induced chromatid aberration frequencies per 100 cells for the control population, as proposed by Roberts et al. 1999 and Scott et al. 1999.

2.2.2.4 Isolation of lymphocytes from whole blood

Peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation on Lymphocytes Separation Medium (LSM). 30 ml whole blood per donor was diluted with pre-warmed 30 ml RPMI 1640, supplemented with 2% stable glutamine. 15 ml of cold LSM were added to a 50 ml falcon tube each and 15 ml of diluted blood were carefully poured onto the LSM solution. The next steps were performed in accordance with the protocol above (2.1.2.2). Immediately after the last centrifugation the cell pellet was resuspended in 1 ml of RPMI 1640 and the cell number was counted with a CASY Counter. Information about total cells number per sample, number of vital lymphocytes and cell viability were documented. Thereafter, 10 % own donor's plasma and 1 % antibiotics were added, as well as additional amount of RPMI 1640 medium to get a final concentration of 10^6 cells/ ml. The culture was incubated at 37°C, 5 % CO_2 over night to give the cells time for the repair of DNA damage and to recover from shearing forces due to isolation procedure.

2.2.2.5 Sample irradiation for γ-H2AX assay and apoptosis/ necrosis assay

After over night incubation each sample was divided in two culture flasks, one for irradiation and one as a control. The flasks were then put on ice, transported to the γ-ray machine and then irradiated with 1 Gy γ-rays at a dose rate of 0.74 Gy/min. The irradiation and the return to the laboratory were conducted also on ice to inhibit DNA repair.

The irradiated sample and the control were divided each into three volumes (for 0.5 h, 5 h and 24 h cultures) and placed in a 5 % CO_2 gassed incubator at 37°C. The experiments for both γ-H2AX assay and apoptosis/necrosis assay were performed 0.5 h, 5 h and 24 h starting from the incubation after irradiation.

2.2.2.6 The γ-H2AX assay

Phosphorylation of the histone H2A occurs within minutes after irradiation as response to the presence of DNA double strand breaks and is thought to recruit repair enzymes to these sites (Rogakou, Boon 1999; Paull, Rogakou 2000).

When double strand breaks (dsb) are induced in the DNA, a complex cellular response is triggered. At sites flanking dsb the histone H2A becomes phosphorylated at the serine 139 residue (Rogakou et al., 1999; Sedelnikova et al., 2002). After phosphorylation the histone is called γ-H2AX (Ragakou et al., 1998). Using an anti-γ-H2AX antibody labeled with a

fluorescent dye, the quantity and the pattern of γ-H2AX foci can be detected and visualised. The induction of γ-H2AX foci and the repair kinetics after irradiation with 1 Gy and different incubation times (0.5 h, 5 h and 24 h) at 37°C, 5 % CO_2 was analysed.

After the time points 0.5 h, 5 h and 24 h each irradiated and not irradiated sample (control) was treated as follows. 10^6 lymphocytes per sample were centrifuged in Eppendorf tubes for 5 min at 3000 rpm (RT), resuspended and pretreated for 10 min with AccuMax at 37°C in order to detach cell aggregates. After centrifugation supernatants were discarded and pellets were resuspended in 0.5 ml of 1.5 % formaldehyde in PBS and fixed for 10 min, RT. Following centrifugation pellets were resuspended first in 0.3 ml of PBS and 0.7 ml of ice-cold 100 % ethanol afterwards and kept at -20°C over night or longer (max. 2 weeks), for thorough fixation. Thereafter, the samples were centrifuged for 5 min with 3000 rpm at RT, then the supernatant was removed and the cells suspended in 1 ml of 0.25 % Triton X-100 and incubated 0.5 h on ice to fix the lymphocytes. The presence of nonionic detergent such as Triton X-100 increases the permeability of the plasma membrane for the primary and secondary antibody. Afterwards cells were centrifuged as above and the pellet was incubated with 0.5 ml of 5 % goat serum, which was used as blocking agent reducing non-specific binding of proteins. After 1 h incubation at RT, samples were centrifuged, washed with 1 ml of PBS to reduce background by removing unbound antibody, and finally incubated under gentle shaking for 2 h at RT with the primary antibody, diluted 1:500 in TBP buffer. Thereafter, 0.5 ml of TBP buffer was added and the samples were centrifuged for 5 min with 3000 rpm at RT.

The supernatant was removed and the FITC labeled secondary antibody was added to the pellet for detection of γ-H2AX foci.

After 1 h incubation in the dark the pellet was resuspended in 1 ml of FACS buffer.

2.2.2.6.1 Slide preparation and foci scoring

100 µl aliquots of the above described lymphocyte cell suspension prepared for FACS analysis was taken for cytospin centrifugation. Samples were centrifuged for 5 min at 400 x g, RT. Centrifugation using cytospin centrifugation allows to deposit a single layer of cells on a defined area of a glass slide, keeping the cellular integrity intact. The residual fluid was absorbed into the sample chamber's filter card. Because lymphocytes adhere better to a positive charged surface Superfrost Plus glass slides were used for this procedure. For each sample 100-120 cells, stained with DAPI were scored. Images were taken using a Zeiss

microscope equipped with the Metafer4 software, version 3.4. (Metasystem GmbH), which was used for automatic analysis of foci.

During the analysis microscope slides were scanned field by field at 10-fold magnification. Images were taken by scanning stained cells using the 40x objective. The desired objects were automatically identified and image galleries and objects features like foci number or distribution, as well as its positions were recorded.

2.2.2.6.2 Fluorescence intensity analysis (FACS)

900 µl of lymphocytes, suspended in FACS buffer were analysed by flow cytometry using the FACSDiva software (BD Biosciences). For each sample 10 000 events were recorded. Debris and cell aggregates were excluded from the analysis by a standard approach using the gate for lymphocytes in the forward (FSC) and side scatter characteristic (SSC). The γ-H2AX assay, described in this thesis based on the measurement of the median green Alexa Fluor 488 dye fluorescence signal. The excitation wavelength of Alexa Fluor 488 is 488 nm, the emission maximum at 519 nm. A greater value of the fluorescence signal corresponds to the number of phosphorylated histones H2AX, according to the higher level of DNA damage. In contrast, a low fluorescence signal was interpreted as low level of γ-H2AX foci.

2.2.2.6.3 Statistical analysis of γ-H2AX data

The unpaired t test was used to compare fluorescence intensity or foci number of the treatment groups for the different time points. Statistical significance was designated if $p< 0.05$.

2.2.2.6.4 Determining radiosensitivity

The proportion of radiosensitive individuals was determined by using a cut-off value of the 90^{th} percentile of both spontaneous and radiation-induced relative fluorescence intensity per 10.000 events and foci number per 100 cells for the control population, as proposed by Roberts et al. 1999 and Scott et al. 1999 for chromosome aberrations.

2.2.2.7 The apoptosis/necrosis assay

In normal vital cells phospholipid phosphatidylserine (PS) is located on the inner membrane surface. During early apoptosis PS is translocated from the inner to the outer leaflet of the plasma membrane. The protein Annexin V binds because of its high affinity to PS. Annexin V conjugated with the fluorochrome FITC (fluorescein isothiocyanate) allows to detect and to visualise early apoptotic cells by FACS analysis and fluorescence microscopy. The loss of membrane integrity is a feature of late apoptosis. Uptake of Propidium Iodide (PI), as an intercalating DNA binding dye, allows discrimination between early and late apoptotic/necrotic cells. In a combined staining protocol using Annexin V as well as Propidium Iodide, vital (non-apoptotic) cells (Annexin V-FITC and PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative) and late apoptotic/necrotic cells (Annexin V-FITC and PI positive) can be visualised separately.

This assay can not distinguish between cells that have undergone apoptosis and those that have died because of the necrotic pathway, because in both cases dead cells are stained with FITC but also with PI.

The Annexin V-FITC Apoptosis Detection Kit I was used. Briefly, lymphocytes (10^5 cells/ sample) were washed twice with cold PBS and centrifuged for 5 min at 300 x g. Thereafter, the pellet was resuspended in 100 µl of 1x binding buffer, then 5 µl of Annexin V- FITC and 5 µl of PI were added to the sample. The cells were then incubated for 15 minutes at RT in the dark. Finally, 400 µl of 1x binding buffer was added and samples were immediately analysed by flow cytometry.

2.2.2.7.1 Flow cytometry

By flow cytometry the properties of individual particles can be measured. The cell suspension injected to a flow cytometer is directed into a stream of single particles that can be detected and analysed by the machine's detection system, managed by a fluidics system. Each particle passes through one or more beam lights. The most commonly lights sources nowadays are the laser and the arc lamp. Light scattered in the forward direction corresponds with the forward scatter channel (FSC) and its intensity provides information about the particle size. Light measured approximately at a 90° angle to the excitation line is called side scatter channel (SSC) and provides information about the granularity of a cell.

The excitation wavelength of FITC is in the range of 400-550 nm (blue light) with the maximum peak at 490 nm. The fluorochrome emits green fluorescence light above a range of

475-700 nm peaking at 525 nm. Propidium iodide is excited by green light at 490 nm and emits light with the maximum peak at 630 nm (red).

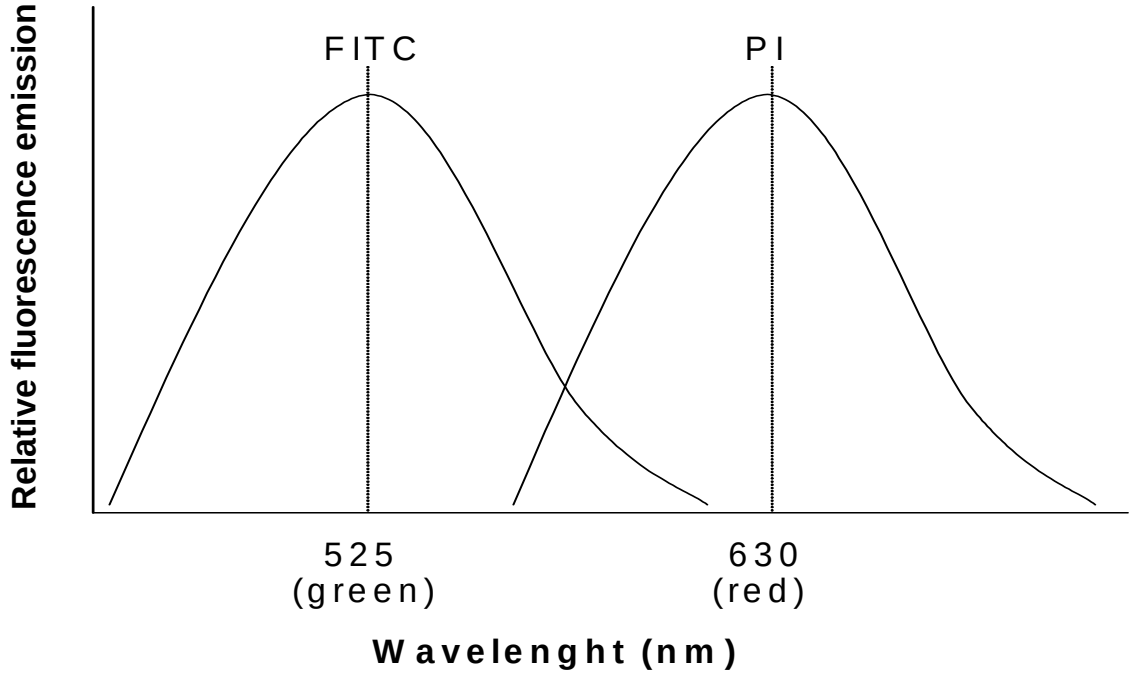


Figure 2.2: Emission spectra of FITC and PI excited by blue light (490 nm).

2.2.2.7.2 Apoptosis/ Necrosis measurement

Apoptosis/necrosis was measured within 1 h after finish of sample preparation. The FSC and SSC values, as well as the compensation values were chosen using an unstained sample, a sample stained with Annexin V-FITC dye alone, and a sample stained with PI dye alone. The lymphocyte populations were gated according to physical characteristics on a dot plot diagram and monocytes as well as debris were excluded from the analysis. For each sample 10 000 events were analysed. The histograms below (Figure 2.3) show typical scatters distinguishing between vital cells, early apoptotic cells and late apoptotic/ necrotic cells.

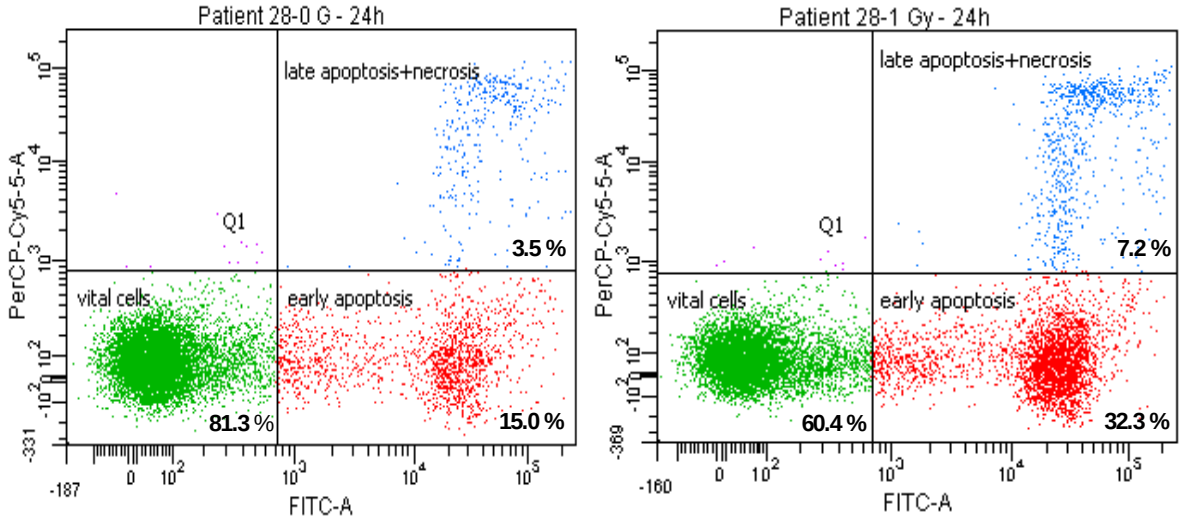


Figure 2.3: Typical two-parameter histograms for apoptosis/necrosis results. Lymphocytes were stained with FITC (x-axis, FITC-A channel) and PI (y-axis, PerCP-CY5-5-A channel). Non-irradiated sample (left) and a sample irradiated with 1 Gy, both incubated 24 h at 37°C after exposure are presented.

2.2.2.7.3 Statistical analysis of apoptosis/necrosis data

The unpaired t test was used to compare percent of apoptotic/necrotic cells of the healthy donors with those of the prostate cancer patients with effects after radiotherapy, as well as with the patients without effects at each time point. The unpaired t test was also used to compare percent of apoptotic/necrotic cells between both groups of patients.

2.2.2.7.4 Determining radiosensitivity

The proportion of radiosensitive individuals was determined by using a cut-off value of the 90^{th} percentile of spontaneous and radiation-induced early apoptotic as well as late apoptotic/necrotic lymphocytes for healthy donors (based on Roberts et al. 1999 and Scott et al. 1999).

III. Results

Part 1: Influence of temperature during irradiation on the level of DNA damage.

3.1.1 Micronucleus assay

The micronucleus assay results for 3 donors analysed in 6 independent experiments are presented in Table 3.1 and Figure 3.1. No difference was detected between not irradiated peripheral blood lymphocytes incubated at 0°C and 37°C. After exposure of PBL to X-rays a significantly higher frequency of micronuclei ($p < 0.05$) was observed in the samples incubated at 37°C in comparison with the samples kept at 0°C. This is true for both experiments with whole blood and isolated lymphocytes (see Table 3.1, Figure 3.1). The values for the dispersion index (DI, ranging from 0.94 to 1.29) calculated for all irradiated samples, indicate that this difference is not due to the presence of a few cells with a high number of micronuclei, which would occur in case of high DI values. The replication indices (RI) calculated for the 3 experiments with whole blood (donors: 1, 2 and 3) showed that the proliferation capacity of lymphocytes is similar for both temperatures in exposed samples, as well as in not irradiated samples. The RI obtained in the cultures of lymphocytes exposed to X-rays is graphically shown in Figure 3.2.

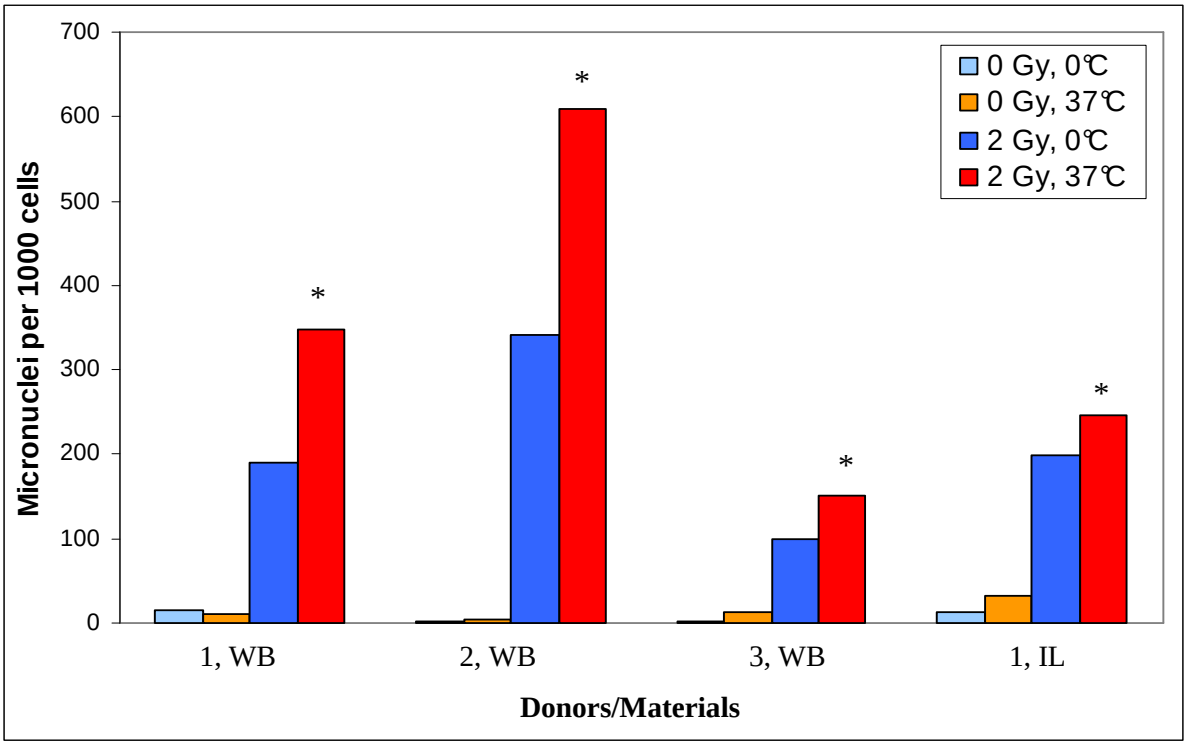
Two independent experiments were performed with DMSO added to the isolated lymphocytes of donor 1. For the samples irradiated with 2 Gy X-rays at 0°C and 37°C in the presence of DMSO similar frequencies of micronuclei were observed (Table 3.1). The average values for the frequency of micronuclei in the presence and absence of DMSO are presented in Figure 3.3.

Table 3.1: Frequency of micronuclei in peripheral blood lymphocytes exposed to X-ray.

Donor	Dose (Gy), Rad. Type	Temp. (℃)	State at exposure	DMSO	Number of BNC with Mn					DI	Mn per 1000 BNC	Cells scored
					0	1	2	3	≥4			
1	0 X	0	WB	no	1975	22	2	1	0		14.5	2000
	0 X	37	WB	no	1979	21	0	0	0		10.5	2000
	2 X	0	WB	no	1666	289	42	3	0	1,24	191	2000
	2 X	37	WB	no	1438	447	99	14	2	1,09	347.5*	2000
2	0 X	0	WB	no	998	2	0	0	0		2	1000
	0 X	37	WB	no	996	4	0	0	0		4	1000
	2 X	0	WB	no	729	216	41	12	2	1,18	342	1000
	2 X	37	WB	no	598	249	108	35	10	1,29	610*	1000
3	0 X	0	WB	no	998	2	0	0	0		2	1000
	0 X	37	WB	no	989	10	1	0	0		12	1000
	2 X	0	WB	no	906	89	5	0	0	1	99	1000
	2 X	37	WB	no	886	119	12	4	0	1,16	151.8*	1021
1	0 X	0	IL	no	989	9	1	1	0		14	1000
	0 X	37	IL	no	484	16	0	0	0		32	500
	2 X	0	IL	no	830	142	27	1	0	1,1	199	1000
	2 X	37	IL	no	549	127	21	1	0	1,03	246.4*	698
1	0 X	0	IL	yes	995	5	0	0	0		5	1000
	0 X	37	IL	yes	607	7	0	0	0		11.4	614
	2 X	0	IL	yes	342	33	6	0	0	1,15	118.1	381
	2 X	37	IL	yes	663	68	5	0	0	1,02	106	736
1	0 X	0	IL	yes	389	3	0	0	0		7.7	392
	0 X	37	IL	yes	308	5	1	0	0		22.3	314
	2 X	0	IL	yes	544	62	6	1	0	1,11	125.6	613
	2 X	37	IL	yes	182	26	1	0	0	0,94	134	209

WB, whole blood; IL, isolated lymphocytes; BNC, binucleated cells; DI, dispersion index

*Difference between treatment groups significant with $p < 0.05$



WB, whole blood; IL, isolated lymphocytes

*Difference between 2 Gy, 37°C and 2 Gy, 0°C significant with $p < 0.05$

Figure 3.1: The frequency of micronuclei for experiments with whole blood and isolated lymphocytes.

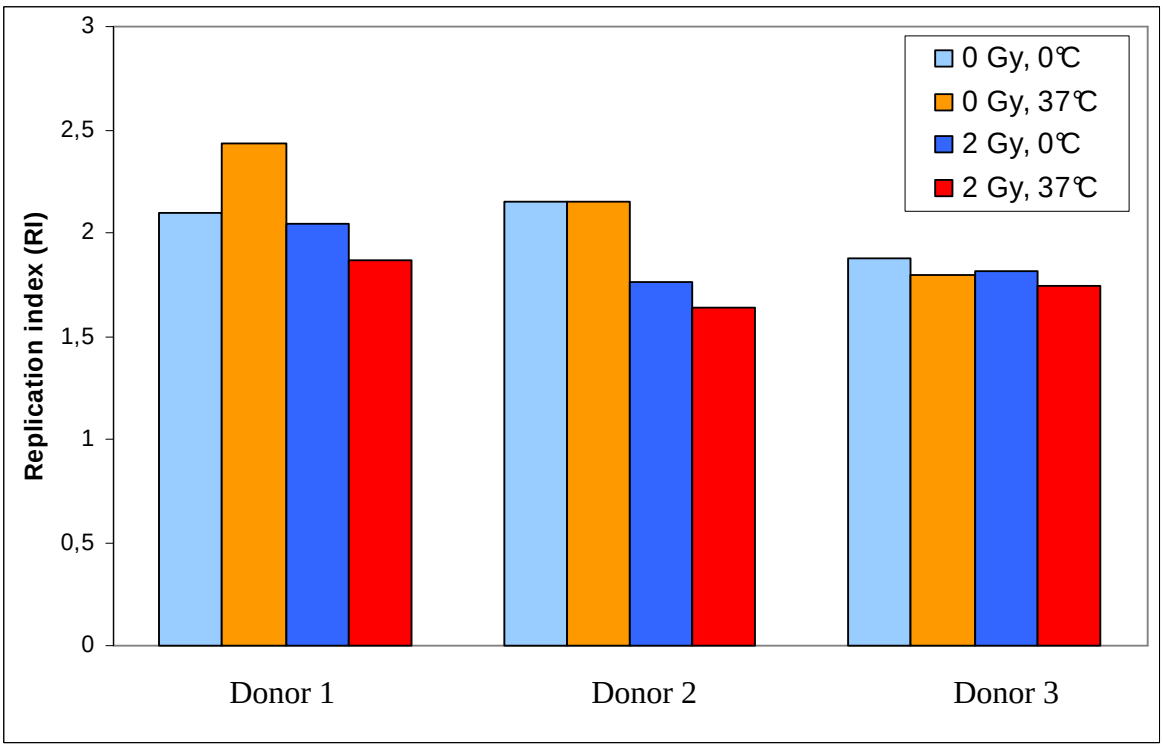


Figure 3.2: Replication index obtained in PBL from donors 1, 2 and 3 exposed to 2 Gy X- rays at 0°C and 37°C.

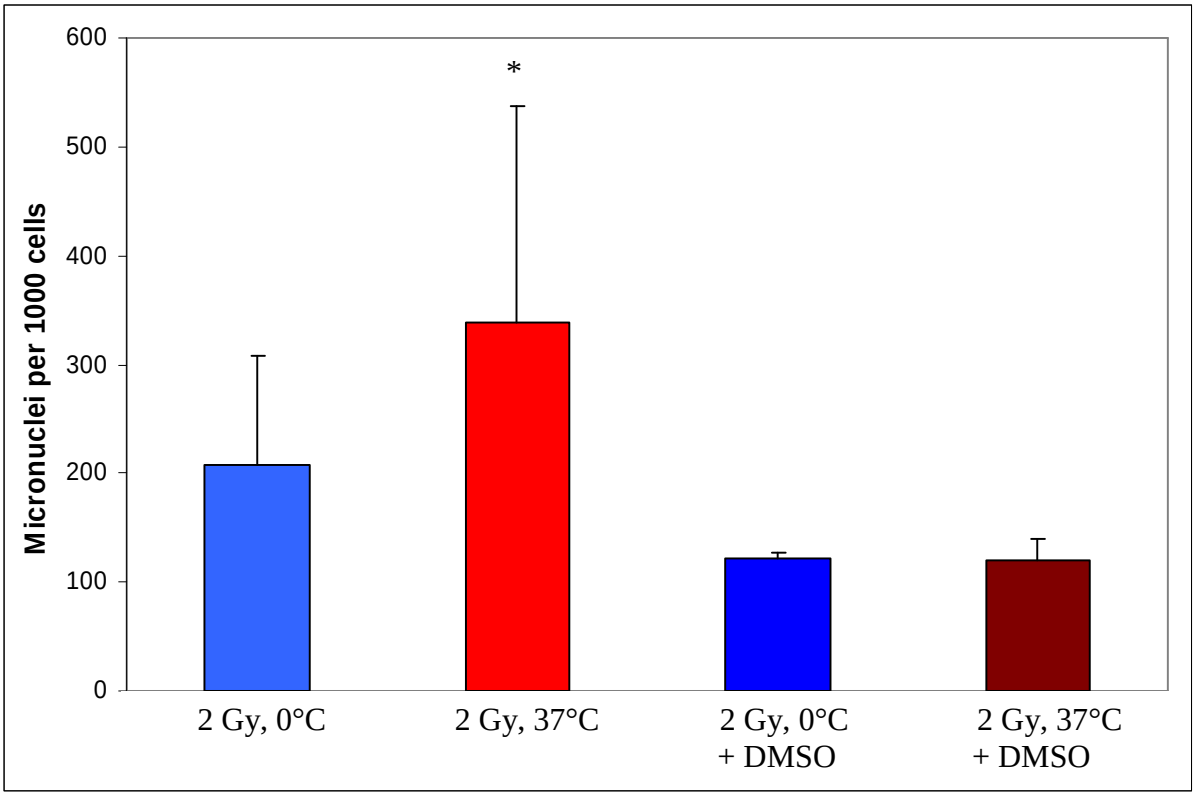


Figure 3.3: Average values of micronuclei frequencies after exposure of lymphocytes to X-rays in the absence or presence of DMSO at different temperatures. Error bars indicate standard deviations.

3.1.2 Comet assay

Results of the comet assay in the alkaline and neutral version are presented in Table 3.2 and graphically illustrated in Figure 3.4 (alkaline) and for the neutral version in Figure 3.5.

The alkaline version of the comet assay was performed with peripheral blood lymphocytes from donors 1 (2 experiments), 2, 3 and 4. No difference was found between samples incubated at 0°C or 37°C shortly before and during exposure. The mean radiation-induced TM ± SD was 51.7 ± 24.5 and 43.1 ± 15.8 for the samples incubated at 0°C and 37°C, respectively. The values of Tail Moment for not irradiated samples are similar for both temperatures.

Results of the neutral comet assay were obtained on donors 1, 2 and 3. There was no significant difference between cells incubated at different temperatures for both, controls and exposed samples.

Furthermore, a somewhat higher level of damage was observed with both assays in samples exposed at 0°C. This tendency was not evident in not irradiated cells.

Table 3.2: Results of the comet assay showing the average values of the tail moment per experiment and the mean value from 5 (alkaline version) and 3 (neutral version) independent experiments. Standard deviations are shown in brackets.

			Donor number					
Dose (Gy)	**Temp. (°C)**	**Comet assay version**	**1**	**1**	**2**	**3**	**4**	**Mean**
0	0	A	8.1 (11.2)	9.4 (7.8)	11.0 (8.2)	54.3 (24.2)	37.1 (20.5)	23.9 (20.8)
0	37	A	11.3 (13.2)	11.8 (9.8)	11.3 (6.6)	52.9 (21.5)	23.5 (14.9)	22.2 (18.0)
2	0	A	54.5 (26.2)	25.4 (15.1)	33.7 (18.7)	88.9 (27.8)	56.2 (20.5)	51.7 (24.6)
2	37	A	42.7 (24.2)	26.9 (16.9)	39.5 (23.3)	69.3 (28.6)	37.1 (20.5)	43.1 (15.8)

			Donor number			
			1	**2**	**3**	**Mean**
0	0	N	0.68 (0.29)	0.16 (0.27)	0.28 (0.41)	0.37 (0.28)
0	37	N	0.34 (0.15)	0.75 (0.54)	0.20 (0.27)	0.43 (0.29)
20	0	N	2.62 (1.04)	3.96 (1.39)	1.14 (1.47)	2.57 (1.40)
20	37	N	1.79 (0.66)	2.87 (0.87)	1.22 (1.56)	1.96 (0.84)

A, alkaline version; N, neutral version of the comet assay

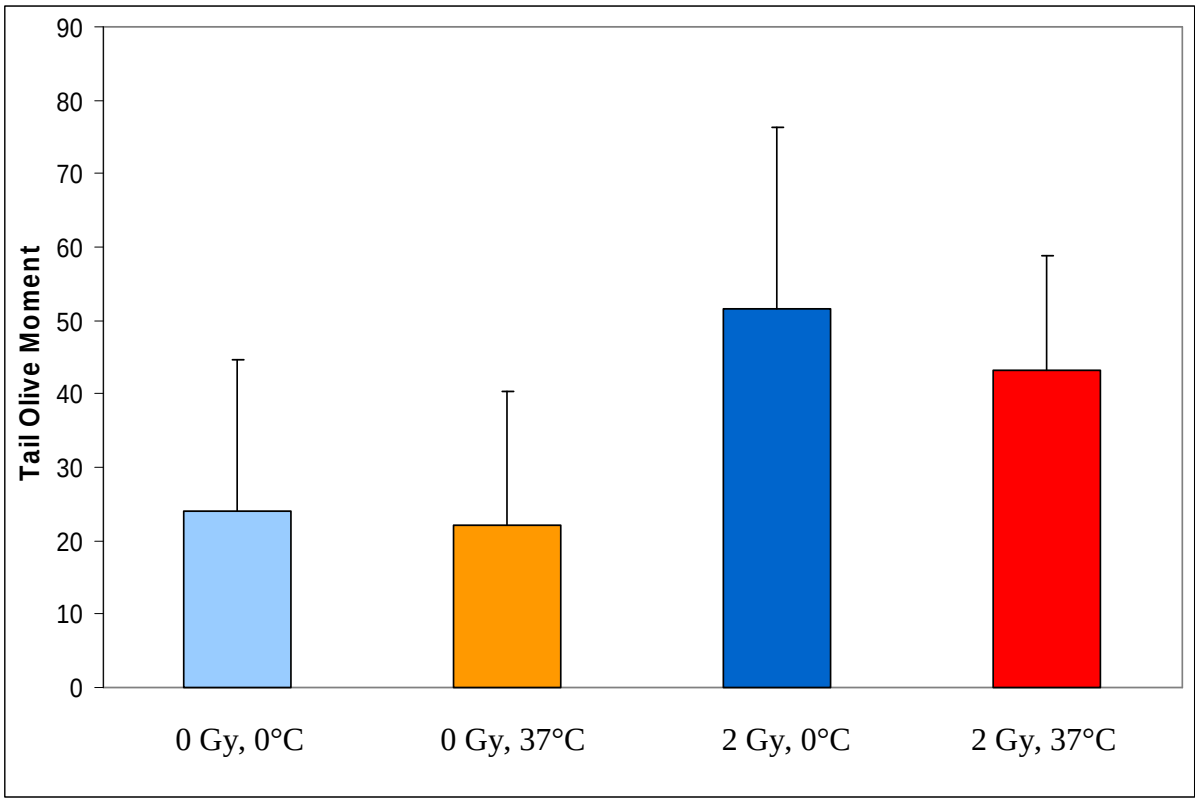


Figure 3.4: Results of the alkaline comet assay. Error bars indicate standard deviations.

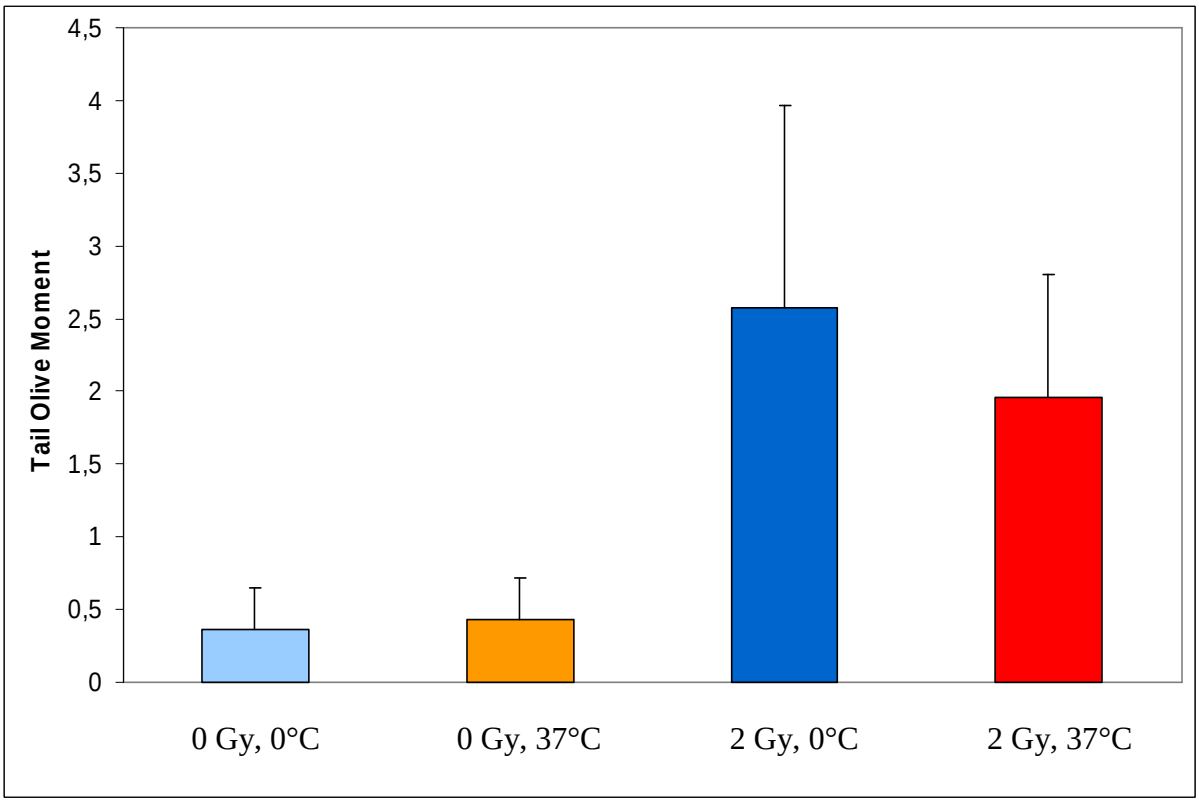


Figure 3.5: Results of the neutral comet assay. Error bars indicate standard deviations.

Part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors.

3.2.1 The G2 assay

3.2.1.1 Mean spontaneous and radiation-induced aberration yield

Results were obtained for 44 prostate cancer patients after radiotherapy who donated blood for the study. Out of these, 22 patients showed strong in vivo side effects after the treatment whereas no in vivo (clinical) effects were observed in the other 22 patients. Chromatid aberration yields (number of gaps, breaks and other aberrations per 100 metaphases), standard deviations and standard errors for all groups of blood donors are given in Table 3.3. There was a significantly higher aberration yield in both spontaneous ($p = 0.029$) and radiation-induced ($p = 0.004$) aberrations in patients with severe reactions when compared to healthy donors. In addition, a weak but significant difference was found in radiation-induced aberration yields in patients without severe side effects in comparison with healthy donors ($p = 0.021$), see Table 3.3, also Figure 3.6. No difference was observed in the aberration yields between patients with and without side effects.

Table 3.3: Mean spontaneous and radiation-induced aberration yields for prostate cancer patients with (S) and without (0) side effects after radiotherapy, as well as for healthy donors (HD).

Donors; Dosis	N	Mean	Stand. Deviat.	SEM
S; 0 Gy	22	2.0[a]	2.4	0.5
S; 0.5 Gy	22	73.6[b]	45.3	9.7
0; 0 Gy	22	1.2	1.5	0.3
0; 0.5 Gy	22	67.1[c]	44.8	9.5
HD; 0 Gy	21	0.8[a]	1.0	0.2
HD; 0.5 Gy	21	42.1[b,c]	17.6	3.8

S, patients with side effects; 0, patients without side effects; HD, healthy donors;

N, number of donors; SEM, standard error of the mean,

Difference significant with $p < 0.05$ between:

a - patients S, 0 Gy and HD; 0 Gy; b - between patients S; 0.5 Gy and HD; 0.5 Gy;

c – between patients 0; 0.5 Gy and HD; 0.5 Gy

3.2.1.2 Distribution of donors based on radiation-induced chromatid aberrations

The number of chromatid aberrations in lymphocytes is very variable in different individuals. In order to exclude donors with extremely high and low aberration yields from the analysis, donors with aberrations frequency between the 25^{th} and 75^{th} percentiles were selected for comparison.

Data used for this analysis were obtained by subtraction of the aberration yield for not irradiated cells (controls) from radiation-induced aberration yield for each donor separately. The median value was not included in the halves when calculating the quartiles. The analysis allowed determination of regions containing 50 % of scores for the group S (aberration frequency < 40; 99 >), the group 0 (aberration frequency < 32; 87 >) and healthy donors (aberration frequency < 28; 54.5 >), separately.

Even if the extremely low and high aberration scores were excluded, the differences between the groups of patients and healthy donors are observable (Figure 3.6). Additionally, statistical analysis (Student's t test) of data for the middle regions (between the 25^{th} and 75^{th} percentiles) for both patient groups and healthy donors was performed. There is a statistically significant difference between patients with side effects (group S) and healthy donors ($p = 0.014$), as well as between patients without side effects (group 0) and healthy donors ($p = 0.012$). The mean ± SD values for the aberration yields are 57 ± 19 and 57.5 ± 18.9 aberrations per 100 cells in patients S and 0, respectively. The mean ± SD magnitude within healthy donors having aberration frequencies between lower and upper quartiles was 40.64 ± 8.17. Notable, the ranges between 25^{th} and 75^{th} quartiles for both patient groups are similar, whereas for healthy donors the range is much smaller. The results are graphically shown in Figure 3.6.

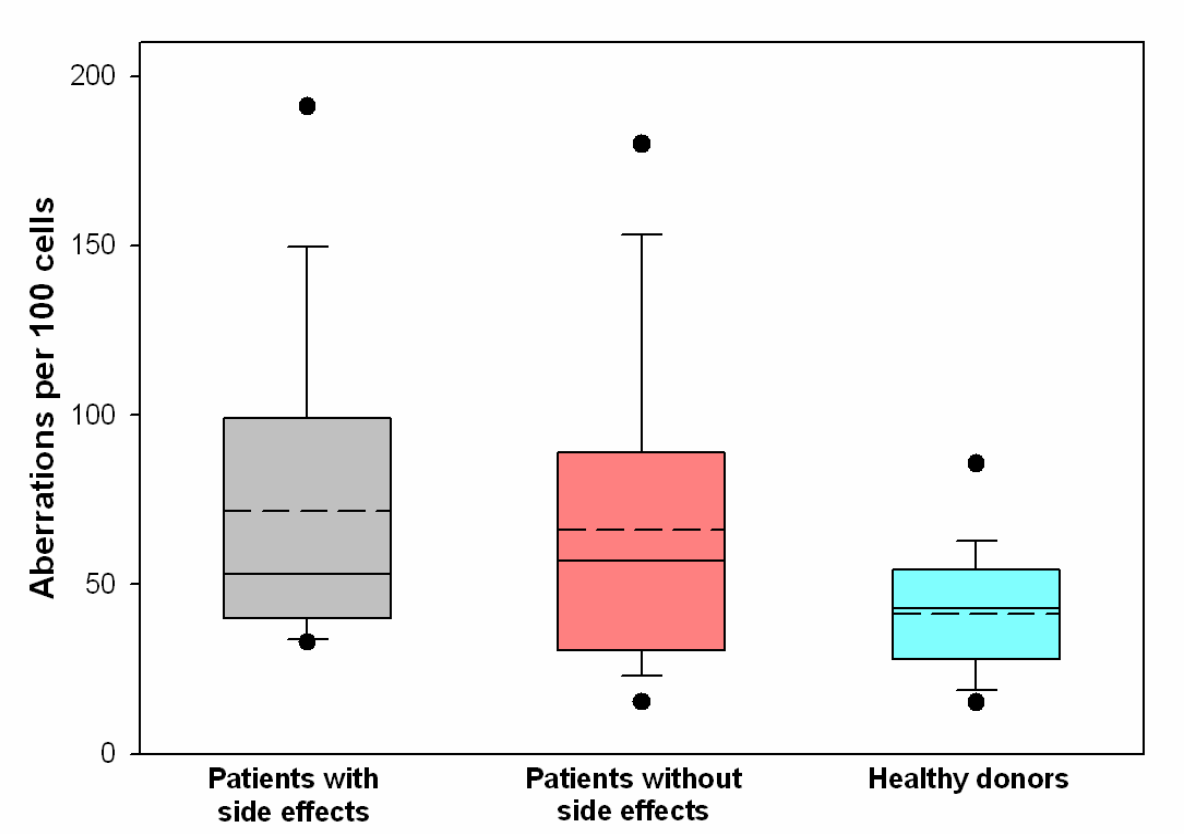


Figure 3.6: Results of chromatid aberration frequency in cells exposed to γ-rays for 3 groups of donors. Data are presented as box plots. The boxes extend from the 25th percentile to the 75th percentile with a horizontal line at a median (50th percentile) and a dotted line at the mean value. Error bars above and below the boxes indicate the 90th and 10th percentiles. The points above (●) the boxes mean top extreme values, the points below (●) mean bottom extreme values of aberrations.

3.2.1.3 Determining the degree of cancer proneness, based on the frequency of spontaneous and radiation-induced aberrations.

The 90th percentile of the chromatid aberration frequencies for the control group (age-matched healthy donors) was used to differentiate cancer-prone from non cancer-prone individuals. For this reason the number of healthy donors with a high level of aberrations was assessed on the basis of a 90th cut-off point, with respect to spontaneous and radiation-induced aberration yields. Thereafter, using the cut-off value computed for healthy donors, the number of cancer-prone individuals among the cancer patients (both patients S and 0) was determined.

The cut-off value in the group of healthy donors estimated in this study was 2 chromatid aberrations for spontaneous aberration yield. Individuals were classified as having a high G2 aberrations yield (indicative of cancer proneness) if they exceeded the cut-off value of 2 aberrations per 100 cells. This resulted in 9.5 % healthy donors (2/21) and 18 % (8/44) patients revealing enhanced spontaneous aberration yield (Figure 3.8, Table 3.4).

The same procedure was applied to define individuals with an increased radiation-induced aberration frequency. The cut-off point in the group of healthy donors was assessed to be 57 aberrations per 100 cells. This value allowed to identify 2 healthy donors (9.5 %) and 22 patients (50 %) as sensitive (Figure 3.9; Table 3.4, ** and ***). The amount of 22 (50 %) radiosensitive patients in vitro (according to the frequency of radiation-induced aberrations, Fig. 3.9; Table 3.4, ** and ***) is in agreement with the number of patients, which showed side effects after radiotherapy (22 patients sensitive in vivo, representing 50 % of all patients). However, among 22 sensitive in vivo patients, only 11 were sensitive in vitro too (see Figure 3.7, Table 3.4). The results of radiosensitivity in vitro (on the basis of chromatid aberrations yield) were compared with the radiosensitivity in vivo (on the basis of EPIC questionnaire), see Table 3.4.

According to the spontaneous aberrations yield 1 patient in the group S and 2 patients in the group 0 are sensitive (Table 3.4, *).

In agreement with radiation-induced aberration rate there are 7 sensitive patients in the group S and 10 patients in the group 0 (Table 3.4, **).

According to both spontaneous and radiation-induced aberration yield 4 patients in the group S and 1 in the group 0 were assessed as sensitive (Table 3.4, ** and ***).

Eleven patients have been assessed to be sensitive both in vivo and vitro (Figure 3.7).

Moreover, ~ 11 % of the patients (P 2, P 8, P 18, P 27 and P 19) have been found with enhanced spontaneous as well as radiation-induced aberration yields (see Table 3.4, ***). An interesting observation is that 4 of them were classified as also clinical sensitive (P 2, P 8, P 18, P 27, see Table 3.4).

Amongst 8 sensitive patients in accordance to spontaneous aberration yield, 5 of them (P 2, P 8, P 18, P 26 and P 27) showed clinical side effects in vivo, whereas 3 of them (P 12, P 19, P 22) were not sensitive in vivo.

No correlation was observed between elevated spontaneous and radiation-induced aberrations in any healthy donor.

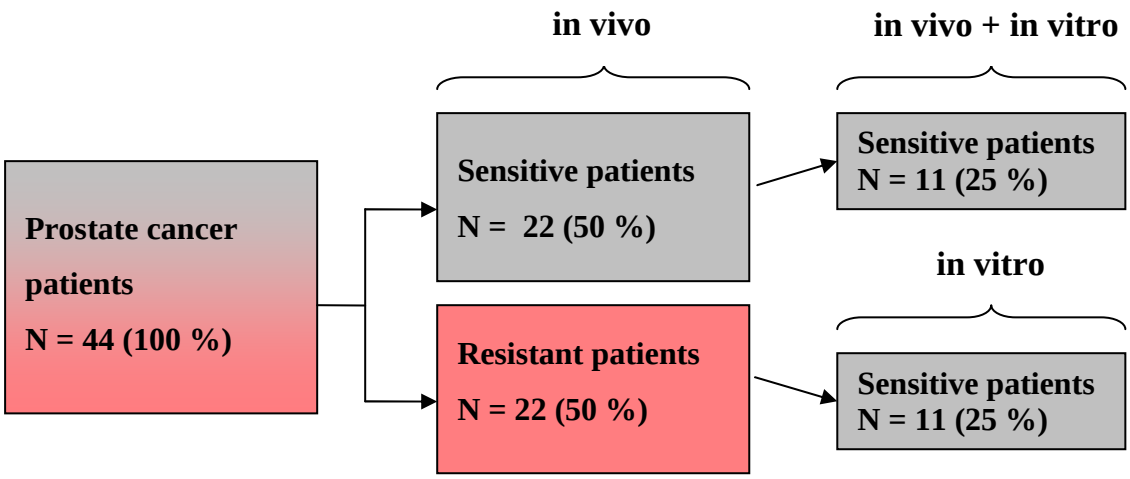


Figure 3.7: In vivo and in vitro (according to radiation-induced aberrations) sensitivity of patients.

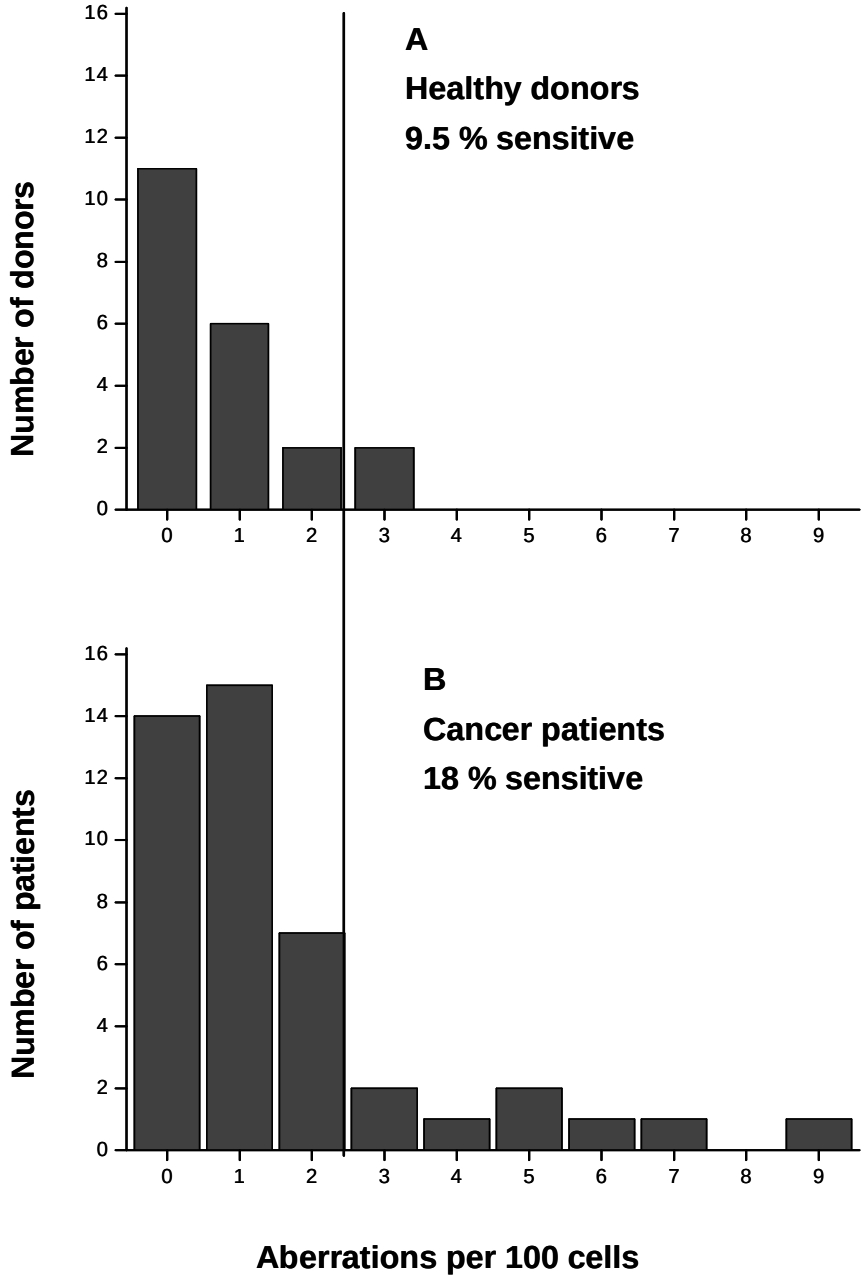


Figure 3.8: Distribution of healthy donors (A) and prostate cancer patients (B) according to the frequency of spontaneous chromatid aberrations. The vertical line marks the 90th percentile cut-off value.

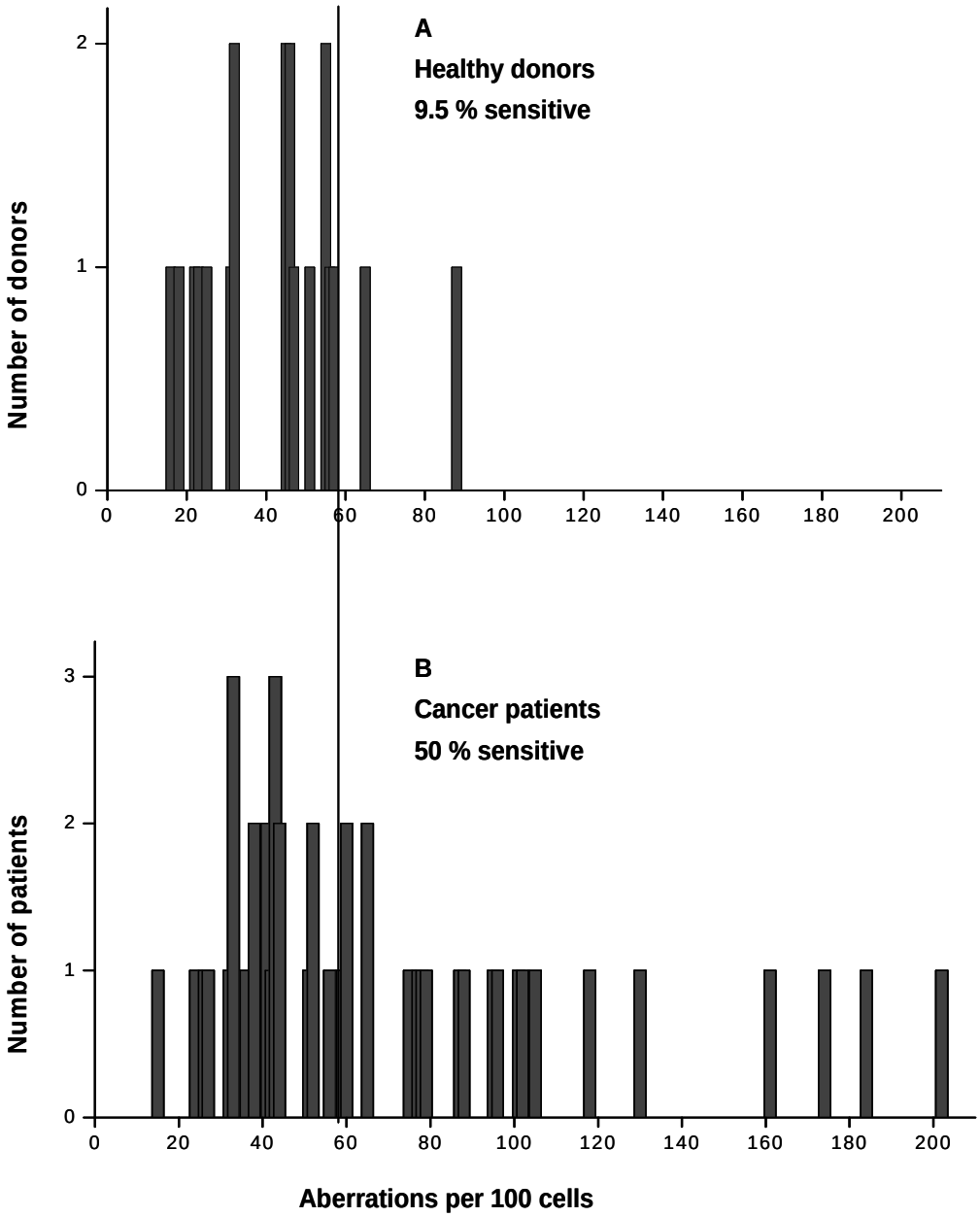


Figure 3.9: Distributions of healthy donors (A) and prostate cancer patients (B) according to the frequency of radiation-induced (0.5 Gy) aberrations. The vertical line marks the 90th percentile cut-off point.

Table 3.4: In vivo/in vitro individual radiosensitivity of patients.

Aberrations yield for patients sensitive in vivo (with effects, S)			**Aberrations yield for patients not sensitive in vivo (without effects, 0)**		
Patients	**Spontaneous**	**0.5 Gy**	**Patients**	**Spontaneous**	**0.5 Gy**
P 2***	3	161	P 9**	2	79
P 4**	2	102	P 10	1	15
P 8***	9	118	P 12*	6	32
P 11	0	33	P 15	1	33
P 13**	2	75	P 17**	0	174
P 14	1	44	P 19***	3	184
P 16	1	43	P 22*	4	27
P 18***	5	202	P 23	1	26
P 20	1	52	P 24	1	24
P 25	1	44	P 29**	0	65
P 26*	7	51	P 30**	1	60
P 27***	5	60	P 31**	0	95
P 28	2	38	P 35**	0	105
P 33**	2	101	P 37	1	43
P 34**	2	88	P 39	0	43
P 36**	0	66	P 41**	1	96
P 38	1	41	P 42**	0	77
P 40**	0	130	P 43	0	36
P 44	0	38	P 46**	1	78
P 45	0	33	P 47	1	56
P 48	1	41	P 52**	0	87
P 53**	0	59	P54	2	42

* patients sensitive **in vitro** according to the spontaneous aberration yield

** patients sensitive **in vitro** according to the radiation-induced (0.5Gy) aberration yield

*** patients sensitive **in vitro** according to both spontaneous and radiation-induced aberrations yield

3.2.1.4 Analysis of aberration distribution

Analysis of radiation-induced (0.5 Gy) chromatid aberration distribution amongst the cells in all 3 groups of blood donors revealed an over-dispersion compared to Poisson distribution. There are, however, differences between the groups. The u-values for the patients in group S range between -1.88 and 9.24, with 5 negative values, 17 positive, and 11 of them having a magnitude exceeding 1.96, which is explicitly correlated with significant over-dispersion.

In the group 0 the u value ranges from -1.034 to 4.88, for 3 patients u values are negative, whereas for 19 it is positive. For 9 patients chromatid aberrations are significantly over-dispersed because the u value is greater that 1.96.

For healthy donors the range of u values is the smallest one (-1.67 to 2.74). In 17 healthy donors the u value indicates an over-dispersion, but only in 4 cases over-dispersion is significant ($u > 1.96$).

3.2.2 The γ-H2AX results

3.2.2.1 Fluorescence intensity data

Formation and loss of γ-H2AX foci in prostate cancer patients with (S) and without (0) side effects after radiotherapy and in age-matched healthy male donors (HD) was investigated. For statistical data analysis the background FACS signal in each sample was subtracted from the signal obtained in irradiated sample to get the induced yield of DNA damage measured as mean fluorescence intensity.

As expected, in all groups of donors the maximum expression of γ-H2AX foci was detected 0.5 h after irradiation. Interestingly, the highest fluorescence signal 0.5 h after exposure was observed in healthy donors (4632 ± 2783). The lowest FITC signal was detected in patients with severe side reactions (2717 ± 1822). The relative fluorescence intensity in patients without side effects was 3636 ± 1304. A decrease of the fluorescence signal was observed 5 h after exposure in all groups. The lowest fluorescence signal was detected in patients 0 (1448 ± 689) followed by patients S (1493 ± 1012), and healthy donors (2201 ± 1273). In agreement with DNA damage repair a further decrease of the FITC signal was observed until 24 h after irradiation. The lowest fluorescence signal was detected in patients S (100 ± 454), followed by patients 0 (161 ± 677) and healthy donors (189 ± 428). The fluorescence intensity of the initial (0.5 h after exposure) DNA damage in patients S was significantly lower than in

healthy donors (*, p = 0.014, Figure 3.10). A significant difference was found 5 h after irradiation between patients 0 and healthy donors (**, p = 0.02, Figure 3.10).

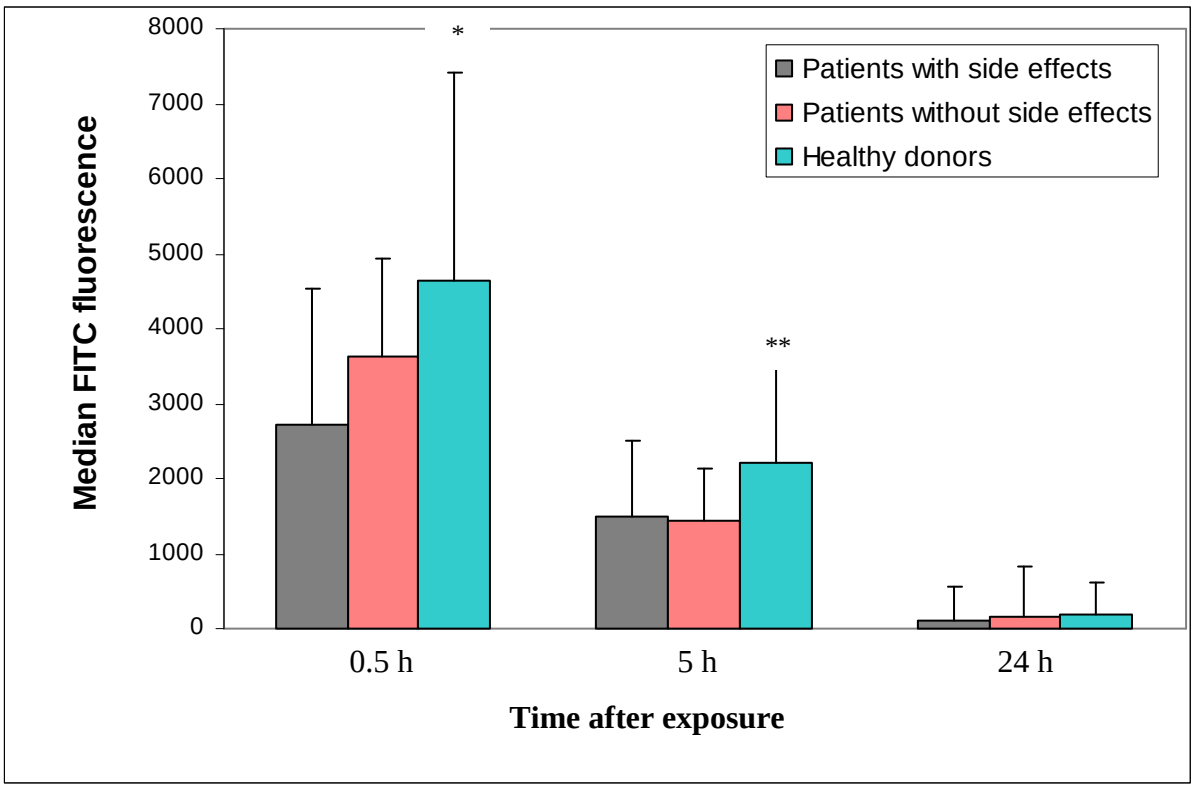
The values of FITC intensity in not irradiated lymphocytes showed no differences between all groups of donors for all analysed incubation times (Table 3.5). This indicates that the observed elevated level of DNA double strand breaks in HD lymphocytes is due to ionising radiation.

3.2.2.2 Determining the degree of cancer proneness, based on the relative FITC fluorescence values measured in not irradiated and irradiated lymphocytes.

The 90^{th} percentile of the relative fluorescence values for the control group (age-matched healthy donors) was used to differentiate cancer-prone from non cancer-prone individuals.

The analysis was performed for time points 0.5 h and 24 h after irradiation in accordance with spontaneous and radiation-induced DNA damage. The cut-off point in the healthy donors was 3037 in not irradiated lymphocytes as measured at time point 0.5 h, and 2875 after 24 h. In case of the irradiated lymphocytes, the cut-off points were 108 and 3315 for the time 0.5 h and 24 h, respectively. This procedure identified one healthy donor (HD 5) according to spontaneous as well as radiation-induced DNA damage, 14 patients (31 %) exceeding cut-off point for spontaneous damage as well as 9 patients (20 %) being sensitive according to radiation-induced DNA damage. Nevertheless, only 4 (~ 9 %) patients (P 20, P 26, P 41 and P 45) showed an enhanced DNA damage level (measured as fluorescence intensity of γ-H2AX foci) for both spontaneous and radiation-induced. However, in case of radiation-induced damage the obtained results concern only for the measurement of time point 24 h.

In summary, using 90^{th} cut-off value the above mentioned 4 patients (9 %) were identified to have an enhanced DNA damage level in agreement with both spontaneous and radiation-induced DNA damage 0.5 h as well as 24 h after exposure. Thus, one can postulate that these patients are cancer-prone.



* Difference between healthy donors and patients with side effects significant with $p < 0.05$

** Difference between healthy donors and patients without side effects significant with $p < 0.05$

Figure 3.10: Radiation-induced FITC fluorescence 0.5 h, 5 h and 24 h after exposure to γ-rays in prostate cancer patients and healthy donors. Error bars indicate standard deviations.

Table 3.5: The mean fluorescence intensity in not irradiated lymphocytes of patients S, patients 0 and healthy donors.

	Mean ± SD 0.5 h	**Mean ± SD 5 h**	**Mean ± SD 24 h**
Patients S	2485 ± 715	2470 ± 639	2454 ± 728
Patients 0	2555 ± 591	2.51 ± 466	2474 ± 459
HD	2442 ± 574	2474 ± 474	2514 ± 440

S, patients with side effects; 0, patients without side effects; HD, healthy donors;
SD; standard deviation

3.2.2.3 γ-H2AX foci number

The number of the background γ-H2AX foci in a control lymphocyte sample was subtracted from the number of an exposed sample. In accordance with FACS results, the highest level of DNA damage, expressed as foci number per cell, was observed 0.5 h after exposure to 1 Gy. In contrast to FACS data, there was no significant difference in the average foci number between patients S and 0, as well as in healthy donors. There are, however, differences in foci number analysed at different time points. The mean ± SD for the foci number measured 0.5 h after exposure was 7.33 ± 4.02; 8.46 ± 2.97 and 7.31 ± 2.72 in patients with side effects, patients without effects and healthy donors, respectively. After 5 h a decrease of the foci number in all donor groups was measured with an average value of 2.9 ± 1.36 per cell in patients with side effects, 3.27 ± 1.38 in patients without side effects and 3.68 ± 2.04 in healthy donors. The lowest mean foci number was observed 24 h after exposure (1.15 ± 1.21; 1.49 ± 1.67 and 1.87 ± 1.71 in patients S, patients 0 and healthy donors, respectively). In case of spontaneous foci number a slightly decreasing tendency with the incubation time was observed in all donors groups (Table 3.6).

No significant difference could be detected in all analysed groups (Figure 3.11). However, a slightly higher average foci number was counted at the time points 5 h and 24 h in healthy donors, when compared with patients S and patients 0.

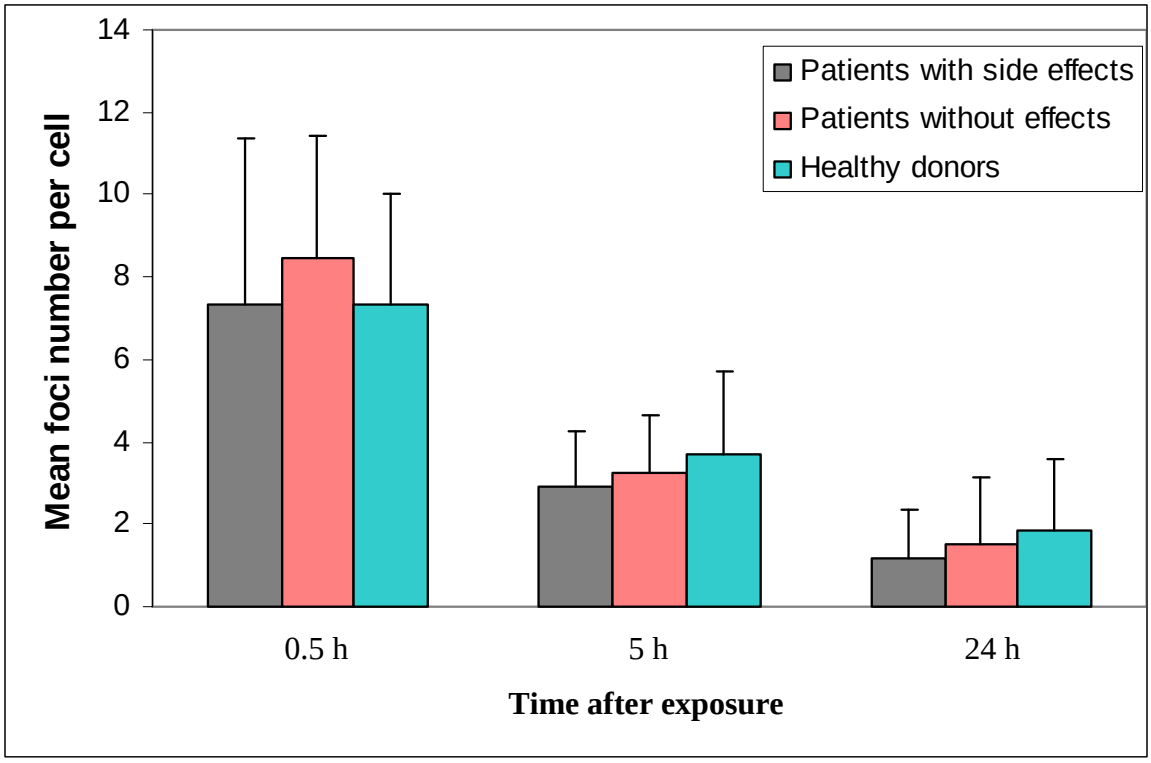


Figure 3.11: Mean radiation-induced foci number 0.5 h, 5 h and 24 after exposure to γ-rays in prostate cancer patients and healthy donors. Error bars indicate standard deviations.

Table 3.6: Mean foci number in not irradiated lymphocytes of the patients S, patients 0 and HD.

	Mean ± SD 0.5 h	**Mean ± SD 5 h**	**Mean ± SD 24 h**
Patients S	2.03 ± 1.5	1.66 ± 0.87	1.52 ± 0.77
Patients 0	2.00 ± 1.08	1.75 ± 0.95	1.60 ± 0.93
HD	2.08 ± 1.21	1.86 ± 0.84	1.2 ± 1.03

S, patients with side effects; 0, patients without side effects; HD, healthy donors;
SD; standard deviation

3.2.3 Apoptosis/ Necrosis results

The analyses were conducted with peripheral blood lymphocytes using the Annexin V-FITC flow cytometry assay. Cells were analysed for early apoptosis and late apoptosis/necrosis 0.5 h, 5 h and 24 h post-exposure. This assay does not recognize differences between late apoptotic and necrotic cells, because of their morphological similarity. Statistical analysis was done on the means of the data obtained from patients with side effects after radiotherapy (S), patients without side effects (0) and healthy donors (HD). For each donor, the score for a not irradiated sample was subtracted from the yield in exposed cells to give the radiation-induced yield of early apoptotic and late apoptotic/necrotic cells. In addition, the results obtained in not irradiated samples are presented to show whether any changes in percent of spontaneous apoptosis/necrosis were or were not observed. Generally, in both cases, spontaneous and radiation-induced apoptosis and necrosis varied between individuals in all donor groups, representing an intrinsic feature of each donor.

3.2.3.1 Early apoptosis results

An increase of radiation-induced early apoptosis was observed in all donor collectives 5 h and 24 h post-exposure. The increase in percentage of apoptotic cells between 0.5 h and 5 h was ~0.6 %; ~0.5 % and ~0.8 % in cells of the S patients, patients 0 and healthy donors, respectively. Between 5 h and 24 h the observed increases of early apoptosis were much higher; ~8 %; ~7 % and ~13 % in cells of the S patients, patients 0 and healthy donors, respectively. The average percent of apoptotic cells in healthy donor cells at the time point 24 h is higher (but not significantly) when compared to both patients groups ($p = 0.054$, see Table 3.7, Figure 3.12). Nevertheless, there is no statistically significant difference between patients and healthy donors, or between both patient collectives neither 0.5 h, nor 5 h nor 24 h after exposure. In not irradiated lymphocytes a stable amount of early apoptotic lymphocytes was detected. The lowest level of spontaneous, early apoptotic cells was found after 5 h in all donor groups (~10-11 %). After incubation times of 0.5 h and 24 h a value of about 13-14 % of early apoptosis was detected. The data concerning spontaneous early apoptosis are presented in the Table 3.8.

Table 3.7: Percent of radiation-induced early apoptosis in PBL of prostate cancer patients with and without side effects after radiotherapy and healthy donors 0.5 h, 5 h and 24 h after exposure.

Donors	Time after exposure (h)	N	Mean	SD	SEM
S	0.5	20	0.43	1.87	0.42
0		21	0.26	2.23	0.49
HD		21	-0.04	2.98	0.65
S	5	20	1.06	1.87	0.42
0		21	0.73	3.15	0.69
HD		20	0.79	2.63	0.59
S	24	20	9.25	5.98	1.33
0		21	7.99	7.90	1.72
HD		17	13.94	10.57	2.56

S, patients with side effects; 0, patients without side effects; HD, healthy donors;
N, number of donors; SD, standard deviation; SEM, standard error of the mean

Table 3.8: Mean percent of spontaneous early apoptotic lymphocytes in the patients S, patients 0 and HD.

	Mean ± SD 0.5 h	**Mean ± SD 5 h**	**Mean ± SD 24 h**
Patients S	14.16 ± 6.48	11.89 ± 5.70	13.84 ± 5.50
Patients 0	13.55 ± 4.86	10.73 ± 4.09	14.06 ± 4.64
HD	13.36 ± 5.45	11.21 ± 5.36	13.09 ± 7.73

S, patients with side effects; 0, patients without side effects; HD, healthy donors;
SD; standard deviation

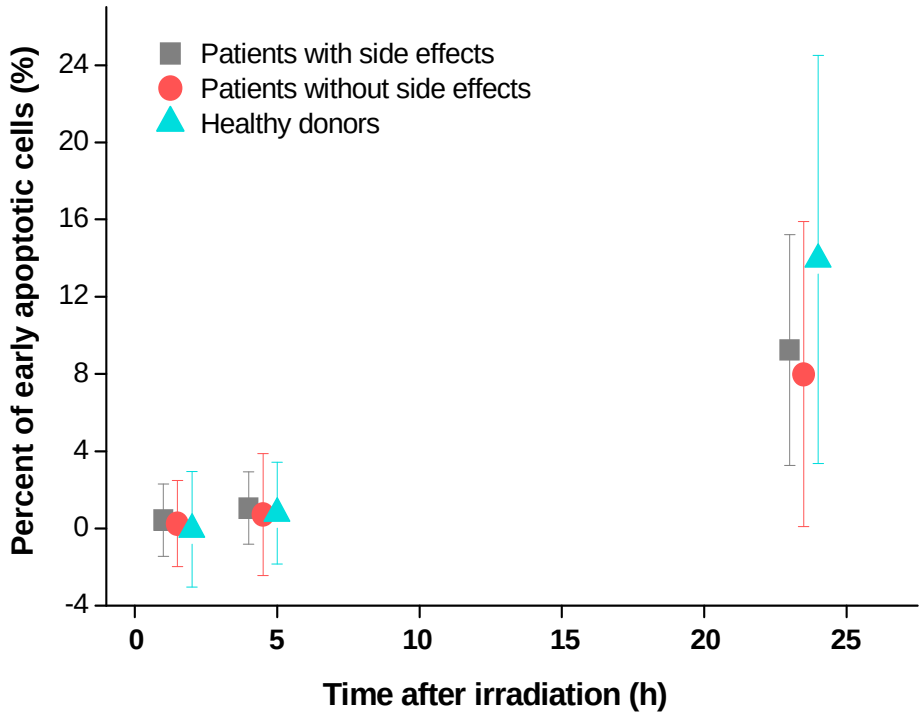


Figure 3.12: Percent of radiation-induced early apoptotic PBL in prostate cancer patients and healthy donors 0.5 h, 5 h and 24 h after exposure. Error bars indicate standard deviations.

3.2.3.2 Late apoptosis/necrosis results

The data obtained for radiation-induced late apoptosis/necrosis show an increase with time until 24 h after exposure (Table 3.9, Figure 3.13). Generally, the results measured in lymphocytes of all donor groups 0.5 h and 24 h after irradiation do not differ in each of these time points between patients and healthy donors or between patients S and patients 0 (Table 3.9, Figure 3.13). However, the results for healthy donors at time point 5 h are slightly but significantly higher when compared to patients S ($p = 0.04$) and also patients 0 ($p = 0.02$), see Table 3.9, Figure 3.14.

The percentage increase of late apoptotic/necrotic cells between 0.5 h and 5 h was below 0.4 % in all donor groups, whereas the observed increase of early apoptosis was much higher for the same time points: ~ 9 %; ~ 6 % and 6.6 % in cells of patients S, patients 0 and healthy donors, respectively.

Nevertheless, no significant difference between the in vivo sensitive patients (group S) and patients without any side reactions (group 0) was found.

Interestingly, in contrast to the results of spontaneous early apoptosis (Table 3.8), an increase of spontaneous late apoptotic and necrotic lymphocytes was observed after 5 h of culture time and a further increase (about 2-fold when compared to 0.5 h) after 24 h in all donor groups (Table 3.10).

Table 3.9: Percent of radiation-induced late apoptosis/necrosis in PBL of prostate cancer patients with and without side effects after radiotherapy and healthy donors 0.5 h, 5 h and 24 h after exposure.

Donors	Time after exposure (h)	N	Mean	SD	SEM
S	0.5	20	0.13	0.45	0.10
0		21	0	0.72	0.16
HD		21	0.023	0.40	0.09
S	5	20	0.01*	0.51	0.11
0		21	0**	0.56	0.12
HD		20	0.41*,**	0.65	0.15
S	24	20	9.11	12.63	2.83
0		21	5.80	3.41	0.74
HD		17	7.03	12.84	3.11

S, patients with side effects; 0, patients without side effects; HD, healthy donors;

N, number of donors; SD, standard deviation; SEM, standard error of the mean

Difference significant with $p < 0.05$: * – between patients S and HD; ** – between patients 0 and HD

Table 3.10: Mean percent of spontaneous late apoptotic/necrotic lymphocytes in the patients S, patients 0 and HD.

	Mean ± SD 0.5 h	**Mean ± SD 5 h**	**Mean ± SD 24 h**
Patients S	2.89 ± 2.02	3.25 ± 2.46	5.54 ± 3.43
Patients 0	2.31 ± 2.09	2.70 ± 1.87	4.85 ± 2.10
HD	3.14 ± 2.76	2.69 ± 2.0	4.24 ± 2.5

S, patients with side effects; 0, patients without side effects; HD, healthy donors;
SD; standard deviation

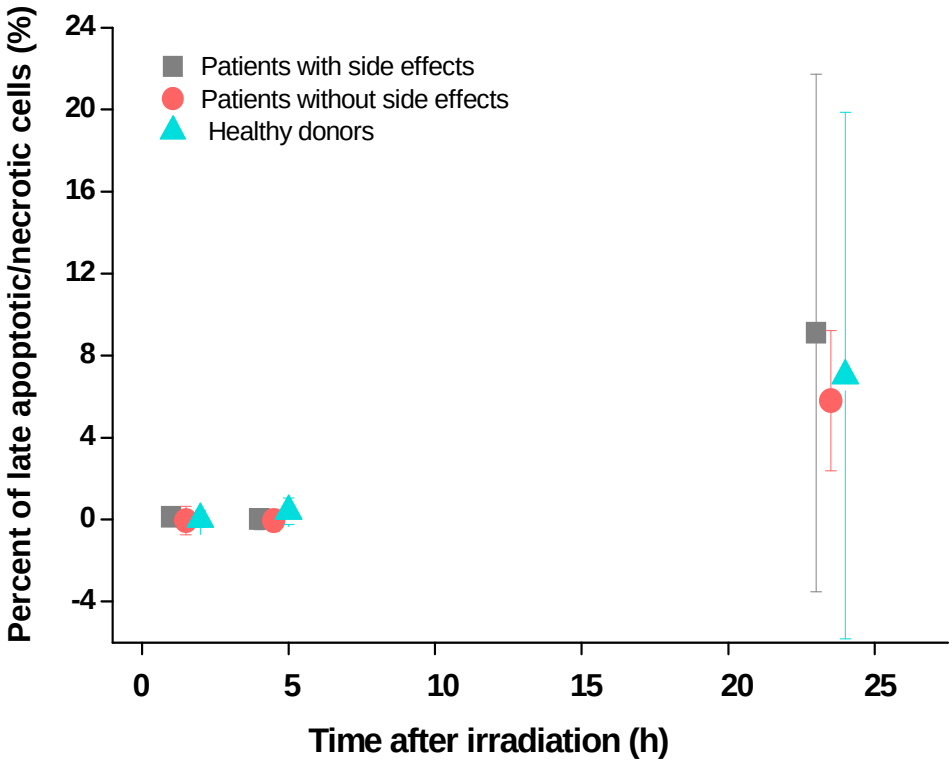
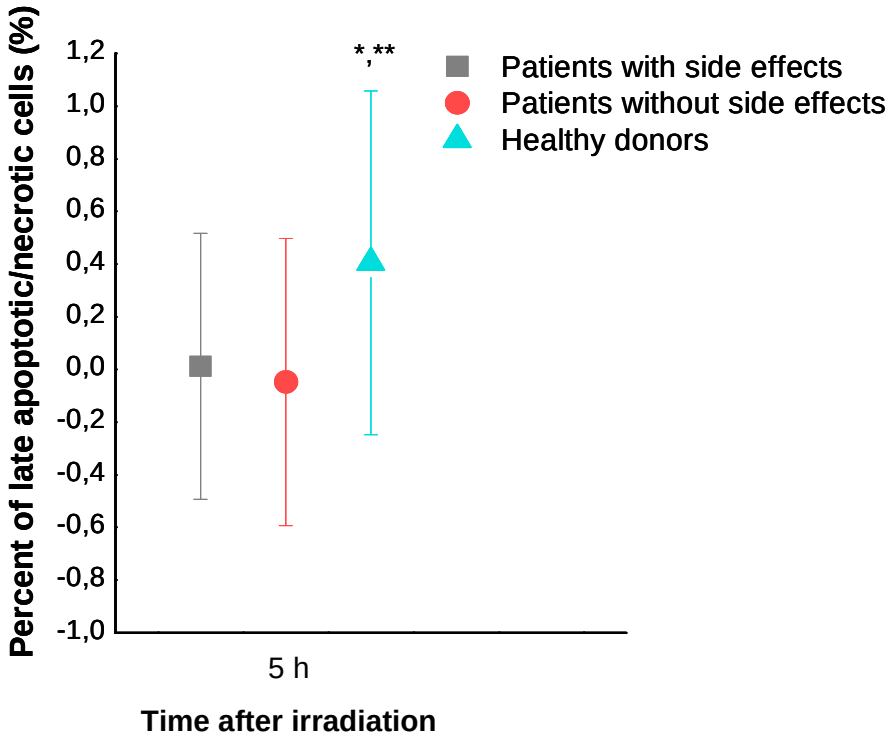


Figure 3.13: Percent of radiation-induced late apoptotic/necrotic PBL in prostate cancer patients and healthy donors 0.5 h, 5 h and 24 h after exposure to 1 Gy. Error bars indicate standard deviations.



Difference significant with $p < 0.05$: * – between patients S and HD; ** – between patients 0 and HD.

Figure 3.14: Percent of radiation-induced late apoptotic/necrotic PBL in prostate cancer patients and healthy donors 5 h after exposure to 1 Gy. Error bars indicate standard deviations.

3.2.4 Determining the degree of cancer proneness in accordance to the results of the 90thpercentile cut-off analysis as measured by G2-, γ-H2AX- and apoptosis/necrosis-tests. Potential application of the used tests as predictive assays.

The 90th percentile analysis was performed for chromatid-type aberrations and FACS analysis (results described above), as well as for γ-H2AX foci and apoptosis/necrosis assay. The aim of the analysis was to find out whether these assays are able to determine an enhanced spontaneous and/or radiation-induced DNA damage in the same donors. Or expressed as question: Is it possible to predict the cancer proneness of donor lymphocytes by using one or more of these assays?

The cut-off points were estimated in the group of healthy donors for spontaneous and radiation-induced DNA damage for each assay separately.

Generally, no relationship between the assays was found when sensitive healthy donors (according to both spontaneous and radiation-induced) were analysed; in each assay, different healthy donors were assessed as sensitive (Table 3.11). However, in late apoptosis/necrosis assay and FACS analysis of γ-H2AX assay, one healthy donor (HD 5) showed enhanced (over cut off point) fluorescence intensity in both not irradiated and irradiated cells at the time points 0.5 h and 24 h (Table 3.11).

Thereafter, 6 patients (P 17, P 18, P 20, P 34, P 41 and P 42) were found to have radiation-induced DNA damage above the cut-off value when the results for spontaneous and radiation-induced DNA damage of few of the assays were taken into account (see Table 3.12), what could indicate these patients as cancer-prone.

Table 3.11: Sensitive healthy donors assessed on the basis of 90th cut off point for spontaneous and radiation-induced DNA damage measured using G2-, γ-H2AX- and apoptosis/necrosis assays.

Assay Time after exposure	Spontaneous	Radiation-induced
	Sensitive healthy donors	
G2	HD 7; HD 23	HD 1; HD 2
γ-H2AX; FACS		
0.5 h	HD 5; HD 18	HD 4; HD 5
24 h	HD 5; HD 20	HD 5; HD 16
γ-H2AX; Foci		
0.5 h	HD 15; HD 21	HD 7; HD 12
24 h	HD 17; HD 24	HD 1; HD 2; HD 16
Early apoptosis		
0.5 h	HD 3; HD 13	HD 9; HD 13
24 h	HD 9; HD 19	HD 19; HD 22
Late apoptosis/necrosis		
0.5 h	HD 3; HD 23	HD 3; HD 23
24 h	HD 23; HD 24	HD 5; HD 22

HD 5 –healthy donor number 5, etc.

Table 3.12: Sensitive prostate cancer patients assessed on the basis of 90th cut off point for spontaneous and radiation-induced DNA damage measured using G2-, γ-H2AX- and apoptosis/necrosis assays. The cut off points were estimated for each assay for spontaneous and radiation-induced DNA damage in healthy donors, respectively.

Assay Time after exposure	Spontaneous	Radiation-induced
	Sensitive Patients	
G2	P 2; **P 8**; P 12; **P 18**; P 19; P 22; P 26; P 27	P 2; P 4; **P 8**; P 9; P 13; **P 17**; **P 18**; P 19; P 27; P 29; P 30; P 31; P 33; **P 34**; P 35; P 36; P 40; **P 41**; P 42; P 46; P 52; P 53
γ-H2AX; FACS		
0.5 h	**P 8**; P 9; **P 20**; P 23; P 25; P 26; P 36; **P 41**; P 45	no sensitive patients
24 h	P 8; P 9; P 19; **P 20**; P 26; P 27; P 28; **P 34**; P 40; **P 41**; P 45	**P 17**; **P 18**; **P 20**; P 26; **P 34**; P 40; **P 41**; P 42; P 45
γ-H2AX; Foci		
0.5 h	P16; P 25; P 29; P 31; P 33; **P 34**; P 36	P 6; **P 8**; P 12; P 13; P 14; **P 17**; P 29; P 33; **P 34**; P 35; P 39; P 49
24 h	P 30; P 31; **P 34**; (lack of results for P 16; P 25; P 36)	P 6; **P 8**; P 12; **P 17**
Early apoptosis		
0.5 h	P 15; **P 18**; **P 20**; P 44; P 50	P 15; P 16; **P 17**; **P 18**; **P 20**; P 21; P 44; P 50
24 h	**P 17**	P 6; P 8; P 43
Late apoptosis/necrosis		
0.5 h	P 6; P 14; P 16	P 6; P 14; P 16
24 h	P 6; **P 8**; P 14; **P 20**; P 30	**P 8**; P 10; P 14; P 29

P 2 – patient 2 ; P 8 – patient 8, etc.

IV. Discussion

Part 1: Influence of temperature during irradiation on the level of DNA damage.

The influence of the temperature during irradiation on the level of cytogenetic damage in peripheral blood lymphocytes is known since a long time (Bajerska and Liniecki 1969, Gumrich et al. 1986, Virsik-Peuckert and Harder 1986). In contrast, Claesson et. al (2007) observed no temperature effect for the DNA dsb induction by alpha irradiation.
The goal of this part of the thesis was to find out whether the temperature has an influence on the level of DNA damage and if yes, what is the reason for it, direct or indirect action of radiation?

4.1.1 Comparison of micronucleus and comet assay results

In present study a significantly reduced level of micronuclei in cells exposed to 2 Gy X-rays at 0°C as compared to 37°C was observed. This was true for both experiments with whole blood and isolated lymphocytes. This temperature effect disappeared when the lymphocytes were exposed in presence of DMSO, which is known as a radical scavenger. Thus, it seems that the observed temperature effect is due to the indirect action of radiation mediated by radicals.
Surprisingly, the results of the comet assay did not confirm the results of the micronucleus assay. There was no difference between cultures exposed to X-rays at 0°C and 37°C, neither in alkaline nor in neutral version of the comet assay.
The alkaline version of comet assay allows detection of single and double strand breaks, also alkali-labile sites (Singh et al. 1991). However, it is well known, that 1 Gy of X-rays induces about 1000 ssb and only about 40 dsb per cell (Ward 1988; Whitaker 1992). Thus, combining low radiation dose and alkaline comet assay, gives generally the information about ssb.
The neutral comet assay, generally detects DNA dsb (Olive and Banath, 1993, Wojewodzka et al. 2002).
Altogether, single and double breaks have a potential to generate chromosomal damage (Nowak and Obe, 1984). Hence, the temperature effects observed at the level of micronuclei should also be seen in both alkaline and neutral comet assay. However, many literature data

do not confirm such a correlation. For example Wojcik et al. (1996) observed an adaptive response in lymphocytes, characterized as less initial damage and an increased repair capacity measured with the comet assay. Intriguing, the results of chromosomal aberrations analysed in the lymphocytes of the same donors, did not reflect the comet assay data. On the contrary, Wojewodzka et al. (1996) have found an adaptive response to ionising radiation in peripheral blood lymphocytes for the induction of micronuclei but not for DNA damage measured by the alkaline comet assay.

The reason for this disagreement is not clear. It is known, however, that DNA damage can be measured by the comet assay regardless of the proliferative status of the analysed cells. In contrast, chromosome aberrations can only be analysed in dividing cells which undergo mitosis. Hence, the results of the comet assay can be biased by early apoptosis (Choucroun et al. 2001, Lankoff et al. 2004). The results of dispersion index values of micronucleus test for cultures exposed to the different temperatures indicate however, that no selective cell elimination influenced the discrepancy between both methods. In addition, the replication indices (RI) obtained in cultures of donors 1, 2 and 3 in micronucleus test exposed to 0 Gy and 2 Gy at 0°C and 37°C showed no impairment in replication of lymphocytes kept at 4°C when compared to 37°C.

Another possible explanation deals with the chromatin structure. Long time ago it has been proposed that the variation in the chromatin structure could be responsible for differences in the induction of DNA damage between cell lines (Olive et al. 1986, Jorgensen et al. 1990, Ward 1990, Ljungman 1991, Schwartz et al.1993) and also in cellular radiosensitivity (Gordon at al. 1990, Lynch et al. 1991). Moreover, Woudstra et al. (1996) have showed that a modified chromatin structure was able to modify the cellular radiosensitivity.

4.1.2 Comet assay results at 0°C and 37°C

A further interesting observation was somewhat higher Tail Moment values in samples exposed to X-rays at 0°C as compared to 37°C. This tendency was true for both alkaline and neutral comet assay. In not irradiated samples this trend was not observed. It is well known, that repair of DNA damage starts very effectively within minutes after irradiation (Dikomey and Franzke 1986). In our experiments the irradiation for the alkaline version of comet assay took 4 min and for neutral version 27 min, so that the cells had enough time to start repair of ssb and dsb.

DNA repair at 37°C is much more effective than at 0°C, so that the difference in the cellular ability to repair DNA damage during and within minutes after irradiation may be a factor preventing the detection of temperature effects by comet assay.

4.1.3. The possible explanation of sparing effect of low temperature

The lack of agreement between micronucleus assay and comet assay results does not change our conclusions drawn from experiments with micronucleus test in presence and absence of radical scavenger (DMSO).

There is still one question left to be answered: why are lymphocytes more sensitive to irradiation performed at 37°C when compared to 0°C?

Densely ionising radiation acts on DNA mainly through an indirect effect (Roots et al. 1985), so if our assumption concerning indirect action of radiation is true, there should be no temperature effect or minimal effect after exposure of the cells at 0°C and 37°C to high LET radiation. For this reason, the blood from donors 1 and 3, used in the experiments described in this thesis were also exposed to 6 MeV neutrons at 0°C and 37°C and about 1000 cells per dose point were scored. The exposure to high LET and Mn scoring were conducted by Christian Johannes (Essen, Germany) and the results were published (Brzozowska et al. 2009). Surprisingly, a significantly reduced level of micronuclei was found in cells exposed to neutrons at 0°C, when compared to 37°C. No differences were found between not irradiated samples, incubated at 0°C or 37°C (Brzozowska et al. 2009).

On the contrary, Claesson et al. (2007), who analysed a DNA damage induction after gamma and alpha irradiation, observed a temperature effect in cells exposed to gamma but not to alpha particles. However, Claesson et al. used different radiation types, also different cells (fibroblasts) and endpoints, which could cause the discrepancy between our results. In spite of this, we should remember that even at very high LET radiation the indirect effect exists and may contribute as much as 30 % of the whole biological effect of radiation (Ito et al. 2006). Additionally, exposure to neutrons is also connected with the emission of gamma rays, which corresponds to 10 % in case of exposure to 6 MeV neutrons (Wuttke et al. 1998).

In summary, the comet assay results, as well as the lack of agreement between data of Claesson et al. (2007) and Brzozowska et al. (2009) do not exclude the hypothesis about a temperature effect in association with the indirect effect of radiation.

There are a number of publications revealing a sparing effect of low temperature on radiation-induced DNA damage. In 1959 Deschner and Gray found a 13 % reduction of the aberration

frequency when the temperature was lowered from 37°C to 3°C during irradiation. Belli and Bonte (1963) observed a 25 % higher mean lethal dose in HeLa cells exposed at 5°C in comparison to 37°C. Bajerska and Liniecki (1969) have observed a reduction in the yield of dicentrics and acentric fragments at 20°C as compared to 37°C. However, the frequency of rings and minutes was similar at 20°C and 37°C. Also Gumrich et al. (1986) found a reduced dicentrics yield in lymphocytes kept at 4°C for 30-50 min before, during and 10 min after irradiation when compared to 37°C. Moreover, Gumrich et al. observed characteristic S-shaped temperature dependency, where the dicentric chromosome aberration yield was low in a temperature range between 4°C and 10°C, achieving maximum aberration frequency at 20°C. During last 10 years series of experiments concerning the temperature effect have been conducted by Elmroth and co-workers. They found a protective effect of low temperature (2°C) using halo assay before and during exposure to X-rays on inhibition of DNA supercoils rewinding in MCF-7 cells when compared to 37°C (Elmroth et al. 1999a). The same results were obtained using diploid fibroblasts (Elmroth et al. 1999b). Also the experiments from the colony forming assay confirmed a protective effect of low temperature during exposure (Elmroth et al. 2000a).

However, in presence of radical scavenger DMSO during exposure at 37°C the radiation-induced damage in diploid fibroblast cells was reduced. No effects were observed, when the cells were irradiated in presence of DMSO at 0°C, indicating the temperature dependence of the indirect effect (Elmroth et al. 1999b and 2000b).

Somewhat confusing, no temperature effect assessed on the level of micronuclei was detected in human fibroblasts (Larsson et al. 2007). These results remain unexplained.

Even, if the reasons of the discrepancy between results examined with the use of different methods are still not clear, there are enough data presenting that irradiation in low temperature makes cells less sensitive to ionising radiation when compared to the physiological temperature of 37°C. There are many possible explanations for this effect.

The first hypothesis is the assumption, that hypothermia leads to condensation of the chromatin, what makes the chromatin less accessible for free radicals and thus protects the DNA from damage. In this context it is interesting to recall the results of Vergani et al. (2004). They confirmed that internal and external cellular factors, such as for example temperature, induce changes in the cell shape and are able to remodel the chromatin structure and gene expression. Lowered temperature causes depolymerisation of microtubules (Cassimeras et al. 1986), what causes histone deacetylation and finally condensation of the chromatin (Le Beyec et al. 2007).

The role of chromatin conformation in the cell response to radiation has been investigated already in the 70s. A higher frequency of DNA damage was observed in euchromatin when compared to heterochromatin (Holmberg and Jonasson 1973; Bauchinger and Götz 1979). Lately, Falk et al. (2008) observed less susceptibility to dsb induction by γ-rays in condensed chromatin in comparison to decondensed (open) chromatin.

Altogether, a reduced level of micronuclei after exposure to low LET radiation in cells kept at 0°C when compared to 37°C can be expected due to the chromatin condensation at lowered temperature in association with the indirect effect of radiation.

The second possible explanation of the temperature effect in cells during irradiation could be inhibition or elimination of the bystander effect at lowered temperature. Although the occurrence of the bystander effect has been found in a variety of biological systems, no mechanism responsible for this effect was identified. It is supposed, that multiple pathways are involved in this phenomenon, moreover, different cell lines respond in different ways (Hei et al. 2007). The bystander effect appears as a consequence of a signalling cascade in the cell (Hei et al. 2007) and thus, should be inhibited at low temperatures. Ryan and co-workers assessed bystander effect to be responsible for 20-50 % of the observed damage induced by radiation (Ryan et al. 2007). This agrees with the results of present thesis, where the sparing effect in cells exposed to X-rays at 0°C was in the range 20 - 45 %. Additionally, our results with DMSO also support the assumption, since it is known that the bystander effect is mediated by reactive oxygen species (Hei et al. 2008). Nevertheless, there are no evidences indicating that the bystander effect does not occur at 0°C, so that the assumption that the bystander effect could be responsible for the temperature effect during irradiation remains hypothetical.

A last factor, which was taken into consideration could be a possibly decreasing of the ability to proliferate for cells exposed at 0°C. In consequence, the cells with a high level of DNA damage would divide slower and remain undetected in the micronucleus assay, as it has been presented by Hoffmann et al. (2002). Nonetheless, no differences were found in replication indices in PBL irradiated at 0°C or 37°C, what indicates that the temperature effect is not due to decreased proliferation capacity of cells kept at 0°C.

4.1.4 Summary of the first part the thesis

Taken together, the results obtained in this part of the study confirmed the previous reports about the sparing effects of low temperature during irradiation to the level of cytogenetic damage. This effect disappeared in the presence of the radical scavenger DMSO, which indicates the indirect action of radiation being responsible for the result observed.
The mechanisms, possibly responsible for the effect of temperature during irradiation and measured with micronuclei, could be a condensation of chromatin at lowered temperature or inhibition/abolition of the bystander effects.
The lack the temperature effect as measured with the e comet assay remains unexplained and needs further investigation.

The conclusion derived from this part of the study is that control of temperature during irradiation is critical for reliable and reproducible results. Keeping cells at 37°C during transport to irradiation source and during irradiation allows the maintenance of physiological conditions. On the other hand, when transport to irradiation source and back takes 20 minutes, like in this study, the temperature could decrease when the container with samples was opened, especially during the winter. In this case the conditions during transport and irradiation cannot be permanently controlled. For this reason it has been decided to transport and irradiate the lymphocytes and blood cultures from prostate cancer patients and healthy donors in the second part of this study on ice.

Part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors.

Over 30 years ago Taylor et al. (1975) observed an association between cancer predisposition and the hypersensitivity of cells to ionising radiation in vitro. This finding was confirmed by many researches in the following years. Some of them found an enhanced level of chromosome aberrations in lymphocytes of cancer patients when compared to healthy individuals (Scott et al. 1999, Roberts et al. 1999, Lisowska et al. 2006). It was, therefore, postulated that a higher cellular sensitivity to radiation in vitro might indicate a susceptibility to cancer development. The present study objective was to find out whether, indeed, prostate

cancer patients show an enhanced cellular sensitivity when compared to age-matched healthy donors.

The second aspect of this part of the thesis deals with the dependence between clinical side effects and individual cellular radiosensitivity. The association between individual radiosensitivity and the risk of acute side effects after radiotherapy appears to be not obvious; some reports showed a clear correlation (e.g. Barber et al. 2000a, Widel et al. 2003), whereas other authors revealed no evident relationship (e.g. Begg et al. 1993, Rudat et al. 1997 and 1998). If the individual risk of side effects would be known before radiotherapy, a cancer treatment could be conformed to each patient individually. For this reason it was highly interesting to find out whether the assays chosen for this study might be used as reliable tools for prediction of a risk for side effects development on the basis of individual cellular radiosensitivity. Hence, all prostate cancer patients participated in this study, have donated blood samples minimum 12 months after the completion of radiotherapy treatment This allowed clinical identification of those patients who developed early and/or late severe side effects (patients S) and patients who showed no side effects (patients 0) during this time.

Exposure to IR during tumour treatment induces several chromosomal aberrations, which might be found in peripheral blood lymphocytes months and years after radiotherapy. In the present study, however, chromatid aberrations such as breaks and gaps were analysed. As chromatid aberrations are induced followed by irradiation of lymphocytes that are in G2-phase of cell cycle and not in G0-phase, the potential influence of radiotherapy on the level of chromatid aberrations should be considered as marginal.

4.2.1 Chromosomal aberrations. Cancer susceptibility

Enhanced G2 chromosomal radiosensitivity as measured with the G2 assay was observed in various cancer patients compared to controls. Lisowska et al. (2006) analysed 38 larynx cancer patients (mean age 57) after radiotherapy and 40 healthy donors (mean age 47). Thirty four patients and 36 healthy donors were male. The mean frequency of aberrations in patients was significantly higher compared to healthy donors. Additionally, 39.5 % of patients showed an enhanced chromosomal radiosensitivity. A weak but significant correlation between aberration frequency and age was observed. Elevated G2 chromatid radiosensitivity was also observed in fibroblasts from individuals with dyskeratosis congenita, when compared to healthy donors (DeBauche et al. 1990). These authors supposed that increased

susceptibility of chromatid breakage induction by X-rays could be a cellular marker of diagnostic value.

A number of published data deal with chromosomal radiosensitivity in breast cancer patients (Scott et al. 1994 and 1999, Terzoudi et al. 2000, Mozdarani et al. 2005). The majority of the data has been collected for breast cancer patients whose lymphocytes were exposed to radiation in the G2 phase of cell cycle. Scott et al. (1994) analysed DNA damage in PBL of 50 breast cancer patients and 74 healthy donors. They found out that breast cancer patients are significantly more cellular sensitive than controls. These data were later confirmed with a larger number of cases (130 breast cancer patients and 105 normal donors, Scott et al. 1999). Using a cut-off point of the 90th percentile of healthy donors they found about 40 % sensitive patients. Howe et al. (2005) observed an elevated level of radiation-induced chromatid aberrations in patients with benign prostatic hyperplasia (BPH) or prostate cancer in comparison with healthy donors. The study was performed with blood samples drawn from 15 patients with BPH, 17 prostate cancer patients and 14 healthy donors. Using the 90th percentile cut-off value they detected 7 % of healthy donors, 40 % of the BPH patients and ~ 88 % (15 patients) of the prostate cancer group to be radiosensitive.

In this thesis a significantly higher level of radiation-induced chromatid aberrations was observed in PC patients when compared to healthy donors.

Moreover, using the 90th percentile cut-off method, 50 % (22/44) of prostate cancer patients were identified to be radiosensitive in vitro on the basis of radiation-induced aberration yield. Our results are in agreement with the data of Scott et al. (1994, 1999), Howe et al. (2005) and also Lisowska et al. (2006) which indicates that the procedure used in these experiments was prepared correctly. Although the case numbers do not exceed 22 in the patients and healthy donors groups, on the basis of the data published by Scott et al. (1994, 1999), the results obtained in this study should be accredited as reliable.

Additionally, in both groups of prostate cancer patients also a higher spontaneous aberration yield was observed. This finding supports the suggestion of Bonassi et al. (2000, 2004), that an enhanced level of spontaneous aberrations in lymphocytes may be a marker for cancer predisposition.

The difference in average spontaneous aberrations yield between patients with side effects after radiotherapy (S) and healthy donors was significant. Eight patients (18 %) were found to have spontaneous aberration frequencies above the 90th percentile of the cut-off value. Out of these 5 showed also side effects (clinically sensitive in vivo).

For only 5 patients the aberration yield for both spontaneous and radiation-induced aberrations was above the cut-off thresholds. The missing correlation between spontaneous and radiation-induced aberrations above the 90th cut-off point suggests that different cellular mechanisms may be responsible for the G2-sensitivity after irradiation and the spontaneous DNA damage.

Further, there are also known data revealing no difference in G2 radiosensitivity between cancer patients and healthy donors. Hence, Baria et al. (2001) observed no elevated lymphocyte sensitivity in patients with cervix and lung cancer. A somewhat confusing result was published by Papworth et al. (2001). These authors found an enhanced radiosensitivity only in those larynx cancer patients that were younger than 45 years old.

The reason for the lack of agreement between the results obtained in this study with the data of Baria et al (2001) and Papworth et al. (2001) is not clear. A possible explanation could be different temperatures during exposure and transport of the samples. Papworth et al. (2001) performed all steps at 37°C, whereas in these experiments the transport to the irradiation source in another building and back to the laboratory, as well as the exposure were conducted on ice. Furthermore, some differences in the protocols have to be mentioned. Papworth et al. (2001) added colcemid 0.5 h after irradiation and the cells were harvested after 1 h, whereas in these experiments colcemid was added 1.5 h after exposure followed by cell harvest after further 1.5 h. The experiments described by Baria et al. (2001) have been performed in agreement with Papworth et al. (2001). Finally, a factor, that should be taken into account is the individual differences between persons in manual aberrations analysis.

In view of the potential importance of the chromatid analysis for detecting of individual radiosensitivity and predisposition of cancer there is a need to find out which mechanisms underlie the hypersensitivity. Scott et al. (1994) have suggested that G2 chromosomal radiosensitivity is a marker for low penetrance predisposing genes in a substantial number of breast cancer patients. This observation was later confirmed by Parshad et al. (1996) and Patel et al. (1997). Howe et al. (2005) have found that mitotic inhibition values were lower in benign prostatic hyperplasmia patients (BPH) than in prostate cancer patients (PC). Thus, mitotic inhibition indicates different mitotic delay times in lymphocytes from BPH and PC patients. It has been, therefore, suggested that different cellular and molecular processes occur in response to exposure to IR in these both cancer patients groups (Howe et. al 2005).

4.2.2 Clinical versus cellular radiosensitivity measured by G2 assay

The goal of curative radiotherapy is inactivation of cancer cells one the one hand and greatest achievable protection of the normal tissue on the other hand. Nevertheless, the success of radiotherapy is limited by the risk of side effects. The reduction of possible side effects after radiotherapy offer the patients a chance for a better life quality. It is well known, that the occurrence of side effects depends on many factors such as total dose, tumour volume, comorbidity of patients and many others (Bentzen and Overgaard 1994, Turesson et al. 1996). It should be also kept in mind that even after similar radiotherapy treatment, patients develop a broad range of radiation injury in the normal tissue (Turesson 1990, Borger et al. 1994, Burnet et al. 1998, Raaphorst et al. 2002). This is due to the variation in individual intrinsic radiosensitivity, largely determinated by genetic factors (Turesson et al. 1996, Borgmann et al. 2007).

Taken together, the main question is whether the chromosomal radiosensitivity of in vitro irradiated lymphocytes can be used to predict the risk of side reactions in cancer patients before radiotherapy?

A number of scientists have undertaken trials to find a correlation between the reactions of healthy tissue to radiotherapy and the chromosomal radiosensitivity in peripheral blood lymphocytes. A positive correlation was reported for breast cancer (Hoeller et al. 2003, Borgmann et al. 2008), cervix cancer (De Ruyck et al. 2005, Widel et al. 2001) as well as for prostate cancer (Lee et al. 2003). In contrary, no relationship between cellular and clinical sensitivity was found by Lisowska et al. (2006), Papworth et al. (2001), Wang et al. (2005), Slonina et, al (2000) and Barber et al. (2000).

Hoeller et al. (2003) conducted a study with 86 patients after breast conserving surgery and irradiation with a median dose of 55 Gy. Thereafter, the stage of fibrosis (grades: 0, 1, 2 and 3) was compared with radiosensitivity in vitro, as measured with radiation-induced chromosomal damage (G0 assay). They observed a 2-3-fold higher annual rate for fibrosis in patients with high cellular radiosensitivity in comparison with patients with intermediate and low radiosensitivity.

Borgmann et al. (2008) observed an enhanced individual radiosensitivity in PBL of breast cancer patients and in patients with different tumour sites showing acute reactions after radiotherapy of grade 2-3. Intriguing, these results were true for the in vitro exposure dose of 6 Gy and not so clear after irradiation with 3 Gy. The authors concluded that determining of individual radiosensitivity after irradiation with 6 Gy seems to be a good tool for prediction of acute effects after curative radiotherapy.

Continuing, Lee et al. (2003) irradiated ex vivo lymphocytes from prostate cancer patients before the onset of radiotherapy to find out whether the level of micronuclei correlates with the clinical reactions after treatment. They found a significant greater level of micronuclei in PBL of over-reacting patients when compared to average-reacting patients.

In contrast, Lisowska et al. (2006) observed no relationship between radiation-induced aberration frequencies and the degree of acute reactions. Additionally, Papworth et al. (2001) observed no significantly greater DNA damage as measured with G2 and G0 assays in young head and neck tumour patients with enhanced toxicity to radiotherapy. Wang et al (2005) analysed DNA damage and repair using comet assay in 100 nasopharyngeal cancer patients after radiotherapy (total radiation dose of 70 Gy). Twenty-one patients showed an enhanced initial radiation damage and 19 patients showed a reduced DNA repair capacity 15 and 30 min after exposure. These patients were supposed to be radiosensitive in vivo. However, the obtained data indicate no apparent relationship between the acute skin reactions and in vitro radiation effects in lymphocytes. Only 3 of the patients suffered from enhanced acute skin reactions after the treatment.

In present study 22 from 44 prostate cancer patients showed an increased clinical radiosensitivity (side effects) as assessed on the basis of validated EPIC questionnaire (the Expanded Prostate Cancer Index Composite). Twenty two of the patients showed also elevated radiation-induced chromosomal radiosensitivity as assessed on the basis of 90^{th} percentile cut-off point. However, an intriguing observation was that only 11 from 22 radiosensitive in vitro patients were also clinically sensitive. Moreover, the other 11 radiosensitive in vitro patients showed no clinical side effects.
Hence, the correlation between clinical sensitivity in vivo and cellular radiosensitivity in vitro observed in this thesis on the basis of radiation-induced aberration yield was true for 50 % of the analysed prostate cancer patients.

In agreement with the results for spontaneous aberration yield and 90^{th} percentile cut-off value 8 prostate cancer patients have been assessed as sensitive in vitro, whereof 5 of them showed also clinical side effects. So that the correlation between clinical sensitivity in vivo and cellular sensitivity in vitro observed in spontaneous aberration yield was true for 62 % of the analysed PC patients.

Taken together, using G2 assay, we found 22 (50 %) of the prostate cancer patients, participated in the study, which showed an elevated radiation-induced chromatid aberration yields, whereof 11 of the patients were also clinically sensitive. Elevated yields of spontaneous aberrations were found in 8 prostate cancer patients (18 %), whereof 5 of the patients were also clinically sensitive.

Hence, the clinical individual radiosensitivity was reflected in this study at the level of cellular sensitivity in 50-62 % of prostate cancer patients.

In conclusion, the results for clinical versus cellular sensitivity seems to be promising, however further studies are suggested.

Concerning the discrepancy in results revealed by authors above named, there were several different factors in the experiments such as age of the blood donors, tumour volume, total dose of radiation, daily fraction of radiation and the most important, the different tumour entities, that could influence the results. For example the patients examined by Papworth et al. (2001) were at the moment of diagnosis younger than 50 years, the average age of patients examined in this study was 72.5 years and all donors were older than 58 years. A significant influence of age on chromosome aberration levels was observed by many authors (e.g. Papworth et al. 2001, Lisowska et al. 2006).

However, it does not explain existing (Hoeller 2003, Borgmann 2008) or lack of correlation (Scott et al. 1994, 1999, Lisowska 2006, Howe 2005) between clinical sensitivity in vivo and cellular sensitivity in vitro.

Andreassen et al. (2002) suggested that “clinical normal tissue radiosensitivity should be regarded as so-called complex trait dependent on the aggregate effect of many ‘minor genetic determinats’ and that single nucleotide polymorphisms (SNPs) could account for a proportion of such genetic component”. Moreover, the authors supposed that some genetic variations affect mostly normal tissue response to IR, whereas others behaved differently. At present, a great attention is focused on SNPs analysis. It is supposed that there is a correlation between SNPs and clinical response to ionising radiation (Andreassen et al. 2002 and 2003). As SNPs represent a very numerous type of genetic variations and tens or hundreds of genes could participate in the response to IR, it seems to be understandable that the potential correlation between radiosensitivity in vivo and in vitro could be very complex. The present state of knowledge and assumptions concerning SNPs should be still, however, regarded as preliminary.

In conclusion of G2 assay, a significantly enhanced level of chromatid aberrations was found in PBL of prostate cancer patients in accordance to both spontaneous and radiation-induced aberration yield when compared to healthy donors. No difference was observed between patients with and without side effects.

The present results show that the chromosomal radiosensitivity of prostate cancer lymphocytes may be a marker of cancer predisposition. A predictive value for the risk of developing side effects to radiotherapy was true in this study for 50-62 % of prostate cancer patients, when the clinical sensitivity in vivo on the basis of the EPIC questionnaire and cellular sensitivity on the basis of spontaneous and radiation-induced chromatid aberrations level were analysed.

4.2.3 The γ-H2AX assay

"The DNA damage induced directly after irradiation is considered by several authors to be responsible for clinical radiation sensitivity, because a large amount of DNA damage will be harmful to the cell" (Wang et al. 2005).

Moreover, the current theory suggests that the phosphorylation of the histone H2A is a marker of the induction of DNA dsb as well as of radiosensitivity (Rothkamm and Löbrich 2003).

The literature gives information revealing the maximum number of phosphorylated γ-H2AX histones in human lymphocytes in the range between 10 min – 1.5 h after irradiation (Takahashi and Ohnishi 2005, Löbrich et al. 2005, Andrievski and Wilkins 2009). In this study the maximum γ-H2AX foci expression was assessed after 0.5 h culture time at 37°C, 5 % $C0_2$. This is in agreement with Löbrich at al. (2004) who assessed a maximum foci number at 0.5 h after exposure of lymphocytes to 1 Gy. However, they found a higher foci level (20 foci/cell/ 1 Gy) in comparison to the results presented in this thesis (~ 7-8 foci/cell/ 1 Gy). One basic difference in the mode of evaluation is the manual foci counting performed by Löbrich et al., whereas in this study the foci were counted automatically. The fact that the automatically working system cuts-off the fluorescence intensity of a focus underneath a certain user defined value, might be one possible explanation for these differences in foci numbers.

The FACS data in the presented thesis showed a 1.7-fold higher initial fluorescence signal 0.5 h after exposure in healthy donors compared to patients S. The fluorescence intensity in patients S corresponds to about 58.5 % of the value measured in healthy donors. The higher level of the fluorescence signal was also observed in healthy donors 1 h after exposure when

compared to prostate cancer patients (n = 10) and cervical cancer patients (n = 20; Wegierek-Ciuk, personal communication).

In this study the results obtained for fluorescence intensity of dsb measured by FACS 0.5 h after exposure were not confirmed when γ-H2AX foci were counted by fluorescence microscopy. The discrepancy between these assays could be due to various parameters which were measured.

A significant reduction (40 % - 50 %) of both fluorescence signal and foci number was observed in all groups of donors 5 h after exposure when compared to the initial level of damage. This correlates very well with the observation of Banath et al. (2004). They found 6 h after exposure to 2 Gy about half the amount of γ-H2AX foci they measured 1 h after irradiation in cervical cancer lines.

A further decrease of the fluorescence signal was detected until time point 24 h after exposure which represented about 4 % of the initial DNA damage. This was true for both groups of patients and for healthy donors.

Interestingly, Wegierek-Ciuk observed a decrease of the fluorescence signal in lymphocytes of cervical cancer patients, but not in prostate cancer patients. Moreover, prostate cancer patients showed 24 h after exposure a higher level of γ-H2AX fluorescence signal than after 1 h post-exposure.

The reduction of foci number was not so clearly after 24 h and corresponded to ~16 % of the initial foci number in patients S, ~18 % in patients 0 and ~26 % in healthy donors. A decrease of DNA dsb during incubation of lymphocytes at 37°C is in agreement with the results of Olive and Banath (2004) indicating that the decline of γ-H2AX foci correlates with DNA dsb repair processes.

It does not explain, however, the differences between the patients and the healthy donors. A possible explanation of this observation could be that the decreased foci number in prostate cancer patients was due to modulated DNA damage recognition. An adjustment process could be develop due to fractionated radiotherapy of tumours. It might results in no recognition of DNA damage under a certain level (Löbrich et al. 2005).

It existed no information concerning in vivo radiosensitivity for the healthy donors examined in this thesis. That means that they were not pre-separated in sensitive or normal responders like this was done for prostate cancer patients.

In brief, no significant differences have been found between patients with and without side effects after radiotherapy in the spontaneous and radiation-induced initial level of DNA dsb.

The enhanced level of DNA damage in HD could be due to a slower activation of appropriate molecular mechanisms or the heterogeneous population of healthy donors which were unselected for radiosensitivity.

4.2.4 Apoptosis/ necrosis assay

"Lymphocytes were described to be the most radiosensitive immunocompetent cells, showing a dose dependent increase in apoptosis" (Crompton and Ozsahin 1997). Based on this assumption, the measurement of apoptosis rate in lymphocytes might give more information about in vitro radiosensitivity of prostate cancer patients as well as healthy donors.

A correlation between cellular radiosensitivity and radiation-induced apoptosis is not certain. High apoptosis is correlated with elevated radiosensitivity in several cell lines as published by Dewey et al. (1995), Barber et al (2000b), whereas Crompton et al. (1999 and 2001) and Ozsahin et al. (2005) found decreased apoptosis in lymphocytes of radiosensitive individuals.

The rate of radiation-induced early apoptosis and late apoptosis/necrosis showed in the current study increases with culture time. The same increasing tendency appeared also in spontaneous late apoptosis/ necrosis. An increase of spontaneous and radiation-induced apoptosis in lymphocytes with incubation time was also observed by many authors (Hertveld et al, 1997, Kern et al. 1999, Bordon et al. 2009). Hertveld et al. observed a lower level of spontaneous and a slightly higher level (between 15-18 %) of early apoptosis (Annexin V positive cells) than described in this thesis (~8-14 %). This difference could be due to irradiation time. A common practise is to irradiate lymphocytes directly after isolation, whereas in this study the isolated lymphocytes were incubated over night to allow the cells to repair DNA damage caused by shearing forces during isolation steps.

Additionally, interesting results about a negative correlation concerning the age of blood donors and the level of radiation-induced apoptosis in CD4 T-lymphoctyes have been published by Crompton et al. (1999). They postulated that with each 10 years of life, a dose of 9 Gy X-rays induces 6.5 % less apoptosis. A lot of published results were obtained using lymphocytes of younger donors, when compared to the age of prostate cancer patients and healthy donors from this study. It could therefore, explain why the level of radiation-induced early apoptosis as well as late apoptosis and necrosis measured in this study 24 h after exposure was not higher than 14 %.

Big efforts were undertaken to improve the suitability of the apoptosis assay as predictive test for cancer proneness. Bordon et al. (2009) maintain that estimating the cellular radiosensitivity of PBL is possible using the radiation-induced apoptosis rate measured after Annexin V/PI staining.

Discrepancy between the results of Bordon et al. and the data described in this thesis could be due to many reasons. First of all, Bordon et al. (2009) have performed a more complex study about the relationship between individual radiosensitivity and programmed cell death. They analysed apoptosis in PBL 24 h, 48 h and 72 h after exposure to different radiation doses. The cervical patients were divided into groups due to clinical toxicity (sexual, bowel, rectal and urinary). Only 4 healthy donors were included in this study. There was no separation into early apoptotic and late apoptotic/necrotic cells. Apart from that, sample irradiation and preparation was performed under different conditions. However, Bordon and co-workers found in lymphocytes of cervical carcinoma patients with late toxicity after radiotherapy a lower apoptotic response when compared to patients who had not developed late toxicity. This finding agrees with previous studies (Crompton et al. 2001, 1999), in which no significant differences between healthy donors and cancer patients with normal as well as with hypersensitivity was found for apoptosis in CD4 and CD8 lymphocytes measured 48 h after exposure to 2 Gy and 9 Gy of X-rays. A clearly reduced level of radiation-induced apoptosis in donors with elevated toxicity to radiation was observed. The authors can not explain which mechanism is responsible for the relationship between elevated radiation toxicity and reduced apoptosis rate. They assumed that the increased late toxicity could be due to a delay in mobilizing the physiological response to radiation injury. Thus, they conclude that individuals expressing high levels of apoptosis activate the physiological response to radiation rapidly whereas individuals with a low apoptosis level mobilize this response slowly.

Altogether, Crompton et al. (1999, 2001) and Ozsahin et al. (2005) believed the leukocytes apoptosis assay to be a useful predictor of an increased late toxicity to radiation therapy.

In contrary, Barber et al. (2000b) observed no correlation between apoptosis level measured by TUNEL assay and toxicity in healthy donors, breast cancer patients and individuals with ataxia telangiectasia (AT). Apart from that, the authors found a somewhat reduced level of apoptosis in breast cancer patients and AT individuals when compared to healthy donors. The same tendency was observed in this thesis for radiation-induced early apoptosis 24 h after exposure. Moreover, 5 h after exposure the difference between radiation-induced late apoptosis/necrosis in healthy donors was significantly higher when compared to patients S

and patients 0. Barber et al. (2000b) supposed that the reduced apoptosis level in breast cancer patients may be connected with a genetic predisposition to this cancer entity. At least, the authors did not recommend application of apoptosis assay as a reliable tool for the prediction of normal tissue response to radiotherapy.

Generally, the apoptosis/necrosis data obtained in this study do not differ between patients S, patients 0 and healthy donors (the exception described above) what makes this assay not a good candidate for a reliable predictive test.

4.2.5 Prediction of cancer proneness using G2-, γ-H2AX- and apoptosis/necrosis assays

The comparison of the G2 assay and apoptosis/necrosis assay is somewhat baffling. For apoptosis/necrosis assay, as well as for the γ-H2AX assay not stimulated lymphocytes were used, whereas in G2 test, PHA was added. In most proliferating cells apoptosis is induced by residual DNA damage and occurs either in the late interphase or after one or more mitoses (Dewey et al. 1995, Hendry and West 1997). Nevertheless, in the thesis the efforts were focused to find out whether assessed cancer susceptibility can be confirmed in the same patients when different assays were used.

As was mentioned earlier, an enhanced level of chromosomal damage is supposed to be a marker of cancer predisposition. Big trials were made to find a correlation between cellular individual radiosensitivity measured as chromosomal aberrations (G2, G0 assays) and cancer susceptibility and thus a lot of results were published (Scott et al. 1996, Barber et al. 2000a, Papworth et al. 2001, etc.). For this reason in the present thesis the results of the G2 assay were used as reference data for the discrimination between cancer-prone and non-prone patients with reference to the data obtained with the other assays.

The results obtained in this study are presented in chapter 3 Results, subsection 3.2.4 and in Table 3.11. Generally, 6 patients (P 8, P 17, P 18, P 20, P 34, P 41) were found to be sensitive according to spontaneous and radiation-induced DNA damage analysed with various assays.

Four of them were assessed as also clinically sensitive (P 8, P 18, P 20, P 34), whereas P 17 and P 41 were classified on the basis of the EPIC questionnaire as patients without side effects after radiotherapy. With the exception of P 20, the rest of them showed high induced chromosome aberration yield as assessed with the G2 assay. Despite P 41 was not sensitive in vivo (without clinical side effects after radiotherapy), in this study he has been classified as sensitive in vitro when G2 (0.5 Gy) and FACS results for 90^{th} cut-off point were taken into consideration. Moreover, P 41 showed also an enhanced level of chromosomal aberrations

when lymphocytes were exposed to γ-rays in G0 phase of cell cycle (FISH assay, Schmitz S, personal communication). In P 41, 18 metaphases with aberrations have been found in not irradiated lymphocytes and 46 metaphases with 96 various aberrations after exposure to 2 Gy, when 100 metaphases were analysed. Blood of P 41 was drawn once and the cultures for both assays (G2, G0) were set up within 3 h after venous puncture.

Taken together, a partial agreement was found with respect to identification of cancer-prone patients when the results of the chosen assays were taken into consideration. The highest correlation was observed for G2- and FACS (γ-H2AX assays) data.

Four patients out of 6 were assessed as radiosensitive in vitro on the basis of nearly all chosen assays and showed also clinical side effects. So far, however, it is not proved that clinical radiosensitivity must be reflected on cellular level.

V. Summary and final conclusions

The goal of this thesis was:

Part 1

- To find out, whether different temperature conditions during irradiation of peripheral blood lymphocytes have an influence on the radiation-induced level of chromosomal damage
- To check, whether the cytogenetic temperature effect in peripheral blood lymphocytes is related to the direct or indirect action of radiation

Part 2

- To find out whether one or more chosen assays might be appropriate to predict cancer susceptibility
- To compare individual radiosensitivity between prostate cancer patients with and without clinical side effects after radiotherapy and age-matched male healthy donors – prediction of the risk of development any side effects after radiotherapy.

Results:

Part 1

- A significantly higher level of micronuclei was found when lymphocytes were kept 15 min before and during exposure at 37°C when compared to 0°C. This effect disappeared in the presence of DMSO (radical scavenger)
- The observed temperature effect in micronucleus assay is supposed to be due to the indirect action of radiation
- No temperature effect was observed using alkaline and neutral versions of comet assay

Part 2

- Significant differences between prostate cancer patients and healthy donors in G2-, γ-H2AX (FACS) and late apoptosis/necrosis assays were observed.

- No significant differences between prostate cancer patients with side effects (S) and without side effects (0) after radiotherapy were found.

- Clinical radiosensitivity in vivo assessed on the basis of the EPIC questionnaire correlated with cellular radiosensitivity in vitro assessed on the basis of chromatid aberration 90^{th} cut-off value analysis for 50-62 % of prostate cancer patients.
- Six prostate cancer patients were identified as notably sensitive in vitro when spontaneous and/or radiation-induced DNA damage were analysed. Four of them were also sensitive in vivo on the basis of EPIC questionnaire.

Final conclusions:

Part 1

- The observed sparing effect of low temperature could be due to:
  - The condensation of the chromatin in lowered temperature, what makes the chromatin less accessible for free radicals and thus protects the DNA from damage.
  - The inhibition or elimination of the bystander effect in lowered temperature.
- This effect disappeared in the presence of the radical scavenger DMSO, an observation which favours the indirect action of radiation as being responsible.
- The lack of the temperature effect as measured with the comet assay remains unexplained and needs further investigation.
- The conclusion derived from this part of the study is that control of temperature during irradiation is critical for reliable and reproducible results.

Part 2

- The chromosomal radiosensitivity in lymphocytes of prostate cancer patients:
  - May be a marker of cancer predisposition.
  - Seems to have a predictive value for the risk of developing side effects to radiotherapy for about 50-62 % of prostate cancer patients analysed in the study.
- The enhanced level of DNA damage as measured with the γ-H2AX assay (FACS) in HD could be due to a slower activation of appropriate molecular mechanisms or the lack of a pre-selection of sensitive/resistant individuals.
- The apoptosis/necrosis data do not differ between patients S, patients 0 and healthy donors, which indicates that this assay alone is not a reliable predictive tool.

- Further investigations using G2-, γ-H2AX- and apoptosis/necrosis assays are necessary to find out whether the likeliness to identify in vivo and in vitro sensitive individuals might be increased by using 2 or more of these methods in combination in order to get a good agreement concerning correlation of clinical and cellular sensitivity of cancer patients.

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Abbreviations

AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
BNC	binucleated cell
CP	computer tomography
DMSO	dimethyl sulphoxide
DI	dispersion index
DAPI	4’.6-diamidino-2-phenylindole
dsb	double strand breaks
EPIC	Expanded Prostate Cancer Index Composite
FACS	fluorescence activated cell sorting
FISH	fluorescence in situ hybrydisation
FITC	fluorescein isothiocyanate
FSC	forward scatter channel
γ-H2AX	phosphorylated histone H2AX
Gy	gray
G5 $mTR^{-/-}$	mice lacking a RNA component of telomerase
HNPCC	hereditary non-polyposis colorectal cancer
HR	homologous recombination
KCL	potassium chloride
Kg	kilogram
LMS	lymphocytes separation medium
MeV	mega-electron volt
M	mol
ml	mililiter
Mn	micronuclei
UV	ultraviolet light
PC	prostate cancer
PBL	peripheral blood lymphocytes
PFGE	pulsed field gel electrophoresis
PHA	phytohaemagglutinin
PI	propidium iodide
PSA	prostate specific sntigen

PS	phospholipid phosphatidylserine
PTV	planning target volume
RI	replication index
RT	room temperature
ROS	rective oxygen species
SF	survival fraction
SSC	side scatter channel
Sv	sievert
TM	Tail Moment
T1-3N0M0	TNM grading system to describe prostate tumour
T	describes the tumour and uses different numbers to explain how large it is
N	stands for nodes and tells whether the cancer has spread to the lymph nodes
M	means metastatic and tells whether the cancer has spread throughout the body
WB	whole blood
XP	xeroderma pigmentosum

Appendix

List of Tables

Table 3.12: Sensitive prostate cancer patients assessed on the basis of 90^{th} cut off point for spontaneous and radiation-induced DNA damage measured using G2-, γ-H2AX- and apoptosis/necrosis assays. The cut off points were estimated for each assay for spontaneous and radiation-induced DNA damage in healthy donors, respectively.

List of Figures

Raw data

Appendix A: The numbers of scored spontaneous and radiation-induced chromatid aberrations

	Aberrations per 100 cells			Aberrations per 100 cells			Aberrations per 100 cells		
Group S	**Control**	**0.5 Gy**	**Group 0**	**Control**	**0.5 Gy**	**Group HD**	**Control**	**0.5 Gy**
P 2	3	161	**P 9**	2	79	**HD 1**	0	88
P 4	2	102	**P 10**	1	15	**HD 2**	0	65
P 8	9	118	**P 12**	6	32	**HD 3**	1	55
P 11	0	33	**P 15**	1	33	**HD 4**	0	45
P 13	2	75	**P 17**	0	174	**HD 5**	1	56
P 14	1	44	**P 19**	3	184	**HD 7**	3	46
P 16	1	43	**P 22**	4	27	**HD 8**	0	55
P 18	5	202	**P 23**	1	26	**HD 9**	1	37
P 20	1	52	**P 24**	1	24	**HD 10**	0	31
P 25	1	44	**P 29**	0	65	**HD 12**	0	25
P 26	7	51	**P 30**	1	60	**HD 13**	0	22
P 27	5	60	**P 31**	0	95	**HD14**	0	18
P 28	2	38	**P 35**	0	105	**HD 15**	1	16
P 33	2	101	**P 37**	1	43	**HD 16**	0	47
P 34	2	88	**P 39**	0	43	**HD 17**	1	32
P 36	0	66	**P 41**	1	96	**HD 18**	2	46
P 38	1	41	**P 42**	0	77	**HD 20**	0	32
P 40	0	130	**P 43**	0	36	**HD 21**	0	23
P 44	0	38	**P 46**	1	78	**HD 22**	1	51
P 45	0	33	**P 47**	1	56	**HD 23**	3	37
P 48	1	41	**P 52**	0	87	**HD 24**	2	57
P 53	0	59	**P 54**	2	42			

Appendix B: Mean foci number per 100-120 cells

Patients with effects (S)						Patients without effects (0)						Healthy donors (HD)					
Patients	**Control 0.5 h**	**1 Gy 0.5 h**	**1 Gy 5 h**	**1 Gy 24h**	**Control 24 h**	**Patients**	**Control 0.5 h**	**1 Gy 0.5 h**	**1 Gy 5 h**	**1 Gy 24h**	**Control 24 h**	**Donors**	**Control 0.5 h**	**1 Gy 0.5 h**	**1 Gy 5 h**	**1 Gy 24h**	**Control 24 h**
P 8	1,48	14,27	6,55	5,86	2,04	**P 6**	0,88	12,84	6,9	6,06	1,1	**HD 1**	1,38	11,24	8,2	6,22	1,72
P 11	0,663	6,4	1,655	1,555	0,773	**P 7**	1,58	12,5	6,62	4,68	1,18	**HD 2**	0,58	9,44	6,56	4,76	0,98
P 13	1,01	15,73	6,05	2,61	0,86	**P 9**	1,26	11,59	4,97	1,3	0,84	**HD 3**	0,74	8,3	5,96	3,24	1,7
P 14	0,79	17,63	5,03	2,99	1,05	**P 10**	1,36	10,79	6,02	3,39	1,22	**HD 4**	1,59	12,66	7,76	1,5	1,47
P 16	5,083	5,901	3,659	3,016		**P 12**	1,44	13,22	4,94	6,02	1,91	**HD 5**	0,77	11,66	6,8	2,81	0,67
P 20	0,8	10,24	4,09	2,67	0,75	**P 15**	0,96	11,7	4,71	3,11	0,72	**HD 7**	0,52	13,32	3,45	2,02	0,3
P 25	3,516	7,033	4			**P 17**	1,42	17,14	7,14	5,36	1,17	**HD 8**	0,5	4,783	1,258	1,325	0,975
P 26	1,232	5,617	1,795	0,808	1,285	**P 22**	1,402	6,839	4,045	3,327	0,991	**HD 9**	1,9	6,708	2,825	3,492	2,317
P 27	0,864	7,536	3,964	3,064	2,018	**P 23**	0,214	8,556	1,207	0,685	0,225	**HD 10**	1,793	9,125	6,55	3,678	3,433
P 28	1,017	7,15	4,508	2,3	0,967	**P 24**	2,636	9,318	4,396	2,082	1,432	**HD 11**	3,017	8,851	4,57	4,15	1,132
P 32	1,456	2,442	2,603	4,425	2,275	**P 29**	3,488	12,983	7,367	2,183	1,902	**HD 12**	3,3	12,207	7,1	2,967	1,728
P 33	3,76	13,208	7,339	2,754	2,292	**P 30**	2,942	11,4567	5,554	2,942	4,117	**HD 13**	1,633	2,767	3,385	4,437	2,767
P 34	3,883	14,342	7,182	4,292	3,593	**P 31**	4,892	11,417	6,785	4,042	3,683	**HD 14**	2,678	9,675	6,117	2,918	
P 36	4,659	10,669	6,157			**P 35**	2,817	12,309	6,408	3,289	2,43	**HD 15**	4,261	9,094	7,775	3,933	
P 38	0,167	7,44	2,55	0,777	0,992	**P 37**	2,025	8,694	4,408	2,192	1,5	**HD 16**	2,508	8,692	4,383	7,032	1,793
P 40	1,125	7,017	2,767	2,133	1,388	**P 39**	2,358	12,851	6,075	3,612	1,75	**HD 17**	3,367	10,642		3,215	3,672
P 45	2,342	9,808	4,653	3,194	0,943	**P 41**	1,225	4,592	2,308	2,587	0,458	**HD 18**	2,975	10,623	5,75	4,421	1,025
P 48	2,612	9,388	4,992	0,727	1,831	**P 42**	2,372	9,433	4,15	3,333	1,675	**HD 20**	2,253	9,439	6,653	4,458	1,517
P 50	2,6	11,388	6,355	3,617	2,375	**P 43**		3,867	4,325	2,6	2,333	**HD 21**	4,405	12,092	6,205	3,81	1,521
P 51	0,022	6,167	3,442	0,869	0,717	**P 47**	0,856	6,175	3,55	1,475	0,525	**HD 22**		9,467	6,608	3,518	2,721
P 53	3,3	7,033	6,57	1,74	1,355	**P 49**	1,785	12,458	5,603		2,066	**HD 23**	0,742	3,717	1,583	1,542	2,925
						P 52	3,129	8,208	3,861	2,636	1,708	**HD 24**	2,892	10,328	5,508	4,117	4,052
						P 54	3,008	9,744	4,358	4,342	1,959						

Appendix C: FACS results

Patients with effects (S)						Patients without effects (0)						Healthy Donors (HD)					
Patients	**Control 0,5 h**	**1 Gy 0,5 h**	**1 Gy 5 h**	**1 Gy 24 h**	**Control 24 h**	**Patients**	**Control 0,5 h**	**1 Gy 0,5 h**	**1 Gy 5 h**	**1 Gy 24 h**	**Control 24 h**	**Donors**	**Control 0,5 h**	**1 Gy 0,5 h**	**1 Gy 5 h**	**1 Gy 24 h**	**Control 24 h**
P 8	3,164	9,875	6,307	2,391	2,881	**P 9**	3,172	8,928	4,744	2,489	2,879	**HD 1**	1,121	6,079	4,661	2,851	1,794
P 11	1,704	5,378	3,838	1,965	1,8	**P 10**	1,925	5,743	3,932	1,845	1,828	**HD 2**	2,028	9,816	5,653	2,277	2,338
P 13	1,73	6,119	3,508	1,855	1,536	**P 12**	1,852	5,211	3,621	1,953	1,569	**HD 3**	2,093	9,493	6,523	2,864	2,805
P 14	1,823	5,603	3,296	2,084	1,665	**P 15**	1,933	5,915	2,998	2,318	2,148	**HD 4**	2,671	14,127	8,691	3,312	2,628
P 16	1,276	1,535	1,107	1,083	1,455	**P 17**	1,131	5,774	4,296	4,343	1,402	**HD 5**	3,049	12,79	6,545	3,347	3,386
P 18	1,678	4,448	4,171	4,041	2,661	**P 19**	1,609	7,043	3,591	3,022	3,208	**HD 6**	2,359	10,572	5,149	2,433	2,648
P 20	3,218	6,476	5,696	3,562	3,144	**P 21**	2,86	7,684	5,145	3,104	2,832	**HD 7**	1,524	5,312	2,597	1,531	1,606
P 25	3,384	2,839	1,691	1,054	0,725	**P 22**	2,849	7,572	4,938	2,66	2,832	**HD 8**	1,383	6,587	3,138	1,604	1,656
P 26	3,795	9,529	6,103	3,216	3,542	**P 23**	3,606	9,303	3,004	2,295	2,362	**HD 9**	2,772	7,172	4,91	2,981	2,634
P 27	2,91	5,989	4,551	2,846	3,224	**P 24**	3,002	9,094	4,1	2,471	2,434	**HD 10**	2,294	4,168	4,361	2,812	2,704
P 28	2,66	7,641	5,719	3,095	2,992	**P 29**	2,849	5,263	5,053	2,147	2,442	**HD 11**	2,624	5,348	4,397	2,941	2,183
P 32	2,435	3,972	2,915	2,194	2,68	**P 30**	2,669	4,944	3,65	2,232	2,746	**HD 12**	2,866	5,953	4,437	2,5	2,868
P 33	2,094	5,388	4,096	2,326	2,243	**P 31**	2,508	5,529	4,106	2,4	2,149	**HD 13**	2,732	5,41	3,93	2,755	2,308
P 34	2,988	6,703	5,406	3,405	3,294	**P 35**	2,909	5,452	4,691	2,96	2,753	**HD 14**	2,666	5,869	3,913	2,486	2,714
P 36	3,092	4,62	4,381	2,464	2,716	**P 37**	2,523	5,282	3,977	2,78	2,584	**HD 15**	2,513	4,733	3,837	2,731	2,703
P 38	1,538	3,702	2,423	2,483	2,159	**P 39**	2,451	4,456	3,267	2,496	2,311	**HD 16**	2,887	5,302	4,105	3,487	2,481
P 40	2,733	5,184	5,547	3,947	3,124	**P 41**	3,345	5,585	4,54	3,692	3,092	**HD 17**	2,634	5,657	3,734	2,47	2,855
P 44	2,659	6,878	4,58	2,772	2,85	**P 42**	2,738	6,045	4	3,389	2,869	**HD 18**	3,302	5,818	4,236	2,992	2,475
P 45	3,345	5,476	4,07	3,333	2,922	**P 43**	2,82	5,679	3,643	2,452	2,507	**HD 20**	3,036	6,131	5,052	2,983	2,942
P 48	1,542	2,342	2,401	1,514	1,392	**P 49**	2,925	6,168	3,564	2,932	2,603	**HD 21**	2,295	5,142	3,711	2,715	2,56
P 50	2,94	5,688	4,667	3,088	2,551	**P 52**	2,389	5,099	3,406	2,514	2,695						
P 51	2,063	3,57	3,064	2,06	2,227	**P 54**	2,163	4,449	2,934	2,175	2,199						
P 53	2,397	3,968	3,464	2,568	2,671												

Appenix D: Early apoptosis results

	Patients with effects (S)							Patients without effects (0)							Haelthy donors (HD)					
	Controls			Irradiated samples				Controls			Irradiated samples				Controls			Irradiated samples		
Patients	0.5 h	5 h	24 h	0.5 h	5 h	24 h	Patients	0.5 h	5 h	24 h	0.5 h	5 h	24 h	Donors	0.5 h	5 h	24 h	0.5 h	5 h	24 h
P 8	15,1	17,2	19	15,7	22,7	41,4	**P 6**	9,4	7,6	7,9	5	10,4	39,55	**HD 3**	25,4	4,9		15,1	6,1	13,6
P 11	5,3	4,1	5	5,85	5,3	17,4	**P 10**	9,5	8,5	15,4	7,65	11,75	19,95	**HD 4**	2		7	1,3	3	20,9
P 13	17,6	15,1	7,1	21	16,8	22,6	**P 12**	8,6	12,8	9,7	12	8,85	11	**HD 5**	14	11,7	8,1	14,9	14	15,9
P 14	18,1	16,2	21,1	17,35	19,1	25,35	**P 15**	26,8	20,5	17,7	23,45	20,05	22,2	**HD 7**	6,6	5,1	4,1	6,5	8,8	13,8
P 16	19,3	16,7	13,1	21,65	14,05	14,8	**P 17**	19,3	15	22,7	19,55	19,5	27,35	**HD 8**	12	8,8	6,3	11,6	16	23,5
P 18	23,6	20	15,6	23,95	18,6	22,35	**P 19**	15,6	10,8	15,2	16,7	12,7	19,75	**HD 9**	18,2	24,3	22,6	24,8	18,7	33,4
P 20	21,6	21,1	27	27,1	24,1	27	**P 21**	20	15,5	21,8	19,9	16,5	31,2	**HD 10**	14,1	10,1	13,4	15,1	11,2	26,6
P 25	13,7	11,7	13,2	14,9	12,1	24,5	**P 22**	15	16,8	21,6	19	15,1	18,7	**HD 11**	14,4	10,1	14,3	14,3	10,5	26,1
P 27	7,7	6,7	10	7,7	7,7	26	**P 23**	11,9	9,8	16,4	10,8	10,8	19,7	**HD 12**	10,6	8,3	10,4	11,5	10,6	23
P 28	13,7	12,7	14,9	13,7	11	32,3	**P 29**	11,8	8,7	11,6	15,9	10,9	24,6	**HD 13**	22,4	21,2		23,1	19,4	
P 32	5,7	4,8	7,1	6	5,7	21,7	**P 30**	13,7	11,9	15,2	14,2	14,5	25,6	**HD 14**	10,1	10,2		10,6	9,1	
P 33	9,3	6,2	12	8,6	7,1	20,6	**P 31**	13,7	10,1	8,2	14,6		23,9	**HD 15**	13,1	9,2		13,5	8,8	
P 34	9,9	10,3	14,8	10,7	10,8	20,1	**P 35**	11,3	9,4	14	11,2	9,9	18,8	**HD 16**	9,7	5,7	6,2	10	6,8	12,3
P 36	8,8	7	12,7	9,3	8,3	16,7	**P 37**	13,1	8	12,9	11,5	9,3	14,9	**HD 17**	14,9	13,6	16,1	14,6	12,3	27,6
P 38	19	15,5	20,4	18,2	18,1	29,9	**P 39**	12	8,3	12,1	11,6	8,8	17,9	**HD 18**	8,2	6,8	7,8	7,9	8,4	22,6
P 40	5,7	5,4	8,7	3,8	7,2	22,3	**P 41**	14,9	9,7	14,1	16,5	11,9	22,7	**HD 19**	14,7	15,3	33,9	18,4	18,4	37,3
P 44	22,8	11,4	15,1	22,4	14,3	19,8	**P 42**	10,9	7,3	12,8	14,3	10,7	21,8	**HD 20**	13,7	10,1	15,5	10,8	8,8	21
P 45	11,5	7,3	11,9	11	7,9	16,5	**P 43**	18,2	14,3	16	17,2	18	39,9	**HD 21**	8,2	5,9	7,5	7,6	6,4	12,4
P 48	9,1	7,1	8,9	10,7	7,9	12,4	**P 46**	10,8	7	10,4	10,5	6,4	13,9	**HD 22**	15	15,4	17	14,7	15,6	44,6
P 50	25,7	21,4	19,3	22,3	20,4	28,3	**P 47**	4,5	2,7	5,5	4,1	3,2	7,2	**HD 23**	20	16,4	19,3	19,3	15,6	21,4
							P 54	8,9	6,4	10,6	9,7	7,2	19,1	**HD 24**	14,3	14,1	22	15,2	17,5	28

Appendix E: Late apoptosis/necrosis results

	Patients with effects (S)							Patients without effects (0)							Haelthy donors (HD)						
	Controls			Irradiated samples				Controls			Irradiated samples				Controls			Irradiated samples			
Patients	0.5 h	5 h	24 h	0.5 h	5 h	24 h	Patients	0.5 h	5 h	24 h	0.5 h	5 h	24 h	Donors	0.5 h	5 h	24 h	0.5 h	5 h	24 h	
P8	5,9	8,8	15,8	5,6	7,9	32,9	P6	9,6	7,3	7,8	6,75	7,6	16,4	HD3	11,4	4		11,3	6,3	9,7	
P11	1,7	4,2	3,5	1,95	2,85	16,85	P10	3,2	6,2	8	3,4	5,25	17,4	HD4	4,4		5,2	3,5	5,4	12,5	
P13	1,7	1,9	6,6	2,05	2,35		P12	0,9	0,9	1,9	1,1	0,9	4,5	HD5	1,2	1,9	3,2	1,8	1,7	18,6	
P14	7,2	9,1	9,5	7,6	9,25	28,25	P15	4,7	4,2	4	4,9	4,7	11,45	HD7	2,8	2,6	2,3	2,7	2,7	4,9	
P16	7,7	7,2	4,6	7,9	6,55	9,25	P17	1,4	1,8	4,1	1,4	2,25	13,05	HD8	3,5	4,1	4,6	3	5,3	10,3	
P18	1,4	2,9	5,6	1,85	2,85	14,25	P19	1,2	1,9	3,4	1,35	2,2	9,9	HD9	0,1	0,1	0	0	0	0	
P20	4,5	4,7	12,2	5,4	5,7	12,2	P21	1,7	2,1	5,5	2,3	2,5	13	HD10	2,5	2,1	2,9	2,5	3,3	8,2	
P25	1,6	2	3	1,5	2,2	8,7	P22	2	2,7	5,8	2,4	2	15,3	HD11	4,7	4,4	5,4	4,6	4,6	8,3	
P27	3,9	1,9	3,4	4,1	2	5,8	P23	3,4	3,4	4,5	3,1	3,6	8,6	HD12	1,4	1,6	3,8	1,5	1,8	8,4	
P28	4,1	1,8	3,7	4,1	2	7,2	P29	1,8	3,4	6,6	2	3,9	20,6	HD13	4,3	3,5		4,4	4,1		
P32	1,3	1,8	3,6	1,8	1,9	13,9	P30	2,9	4,9	8,2	3,1	5,5	15,7	HD14	0,6	0,6		0,6	0,9		
P33	1,7	2,2	5,1	1,8	2,5	12	P31	1	1,2	6,2	0,9		14,8	HD15	0,7	1		1	1		
P34	1,2	1,4	2,4	1,5	1,1	7,6	P35	1,6	1,4	4,2	1,4	1,4	7,5	HD16	1	0,9	1	1,2	0,9	2,2	
P36	1,9	1,3	3,7	1,5	1,3	5,6	P37	0,8	1,1	3,8	1	1,1	6,7	HD 17	1,9	2,3	6,3	1,6	2,2	9,9	
P38	1	1,1	3,2	1,2	1,3	8,3	P39	0,9	1	2,3	1	0,7	5	HD18	1,7	1,8	2,5	1,8	2,1	4,2	
P40	2,5	2,2	4,8	1,6	2,4	6,6	P41	1,1	0,9	2,6	1	1,4	7,3	HD19	2,6	3	5,7	2,7	2,6	7,1	
P44	2,5	2,8	5,9	2,7	2,9	9,6	P42	1,4	2,2	5,9	1,9	2,4	12,2	HD20	2,5	2,3	4,9	2,7	3,2	7,2	
P45	2,7	3	5,7	1,9	2,9	8	P43	2,1	3,6	6,9	1,5	2,7	9,2	HD21	3,4	2,1	2,8	3,2	2,8	4,3	
P48	1,1	0,6	1,8	1,5	0,8	5,6	P46	4,3	3,5	4,6	4	2,6	6,3	HD22	3,3	3,6	7,1	3,4	3,4	19,4	
P50	2,2	4,2	6,7	2,8	4,6	15,4	P47	0,3	0,4	0,7	1	0,5	2,2	HD23	8,9	9,2	10,2	10,1	10,2	13,5	
							P54	1,7	2,3	4,1	1,5	2,2	5,7	HD24	5,9	5,9	8,4	5,7	6	16,8	

EPIC questionnaire

Appendix F: EPIC questionnaire (The Expanded Prostate Cancer Index Composite)

Beschwerden beim Wasserlassen ***in den letzten vier Wochen***
(Zutreffendes bitte ankreuzen /Angaben freiwillig: Fragen, die Sie nicht beantworten möchten, können Sie überspringen)

1. Wie oft hatten Sie ungewollten Urinabgang?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie
🕓	🕓	🕓	🕓	🕓

2. Wie oft hatten Sie Blut im Urin?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie
🕓	🕓	🕓	🕓	🕓

3. Wie oft hatten Sie Schmerzen oder Brennen beim Wasserlassen?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie
🕓	🕓	🕓	🕓	🕓

4. Was beschreibt Ihre Fähigkeit, das Wasser zu halten, am besten?

keine Urinkontrolle	häufiges Träufeln	gelegentliches Träufeln	komplette Kontrolle
○	○	○	○

5. Wie viele Vorlagen brauchen Sie täglich, um den unwillkürlichen Abgang zu beherrschen?

drei oder mehr täglich	zwei täglich	eine täglich	keine
○	○	○	○

Wie belastend waren die folgenden Beschwerden für Sie?

	großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
6. ungewollter Harnabgang					
7. Schmerzen oder Brennen beim Wasserlassen					
8. Blut im Urin					
9. Schwacher Harnstrahl oder inkomplette Entleerung					
10. Aufwachen,um Wasser zu lassen					
11. häufigesWasserlassenwährend des Tages					

12. Wie belastend war insgesamt das Wasserlassen für Sie?

großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem

Beschwerden beim Stuhlgang ***in den letzten vier Wochen***
(Zutreffendes bitte ankreuzen /Angaben freiwillig: Fragen, die Sie nicht beantworten möchten, können Sie überspringen)

13. Wie oft hatten Sie Stuhldrang (Gefühl, zur Toilette gehen zu müssen, ohne Stuhlgang)?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie

14. Wie oft hatten Sie unkontrollierten Stuhlabgang?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie

15. Wie oft hatten Sie wässrige Stühle (keine feste Form)?

immer	üblicherweise	ungefähr die Hälfte der Zeit	selten	nie

16. Wie oft hatten Sie blutige Stühle?

immer	üblicherweise	ungefähr die Hälfte der Zeit	selten	nie
☐	☐	☐	☐	☐

17. Wie oft hatten Sie Schmerzen beim Stuhlgang?

immer	üblicherweise	ungefähr die Hälfte der Zeit	selten	nie
☐	☐	☐	☐	☐

18. Wie oft hatten Sie Stuhlgang an einem normalen Tag?

fünfmal oder häufiger	drei- oder viermal	zweimal oder seltener
☐	☐	☐

19. Wie oft hatten Sie krampfhafte Schmerzen im Bauch-, Becken- oder Enddarmbereich?

mehrmals täglich	ungefähr einmal täglich	mehrmals wöchentlich	ungefähr einmal wöchentlich	selten oder nie
☐	☐	☐	☐	☐

Wie belastend waren die folgenden Beschwerden für Sie?

	großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
20. Stuhldrang	☐	☐	☐	☐	☐
21. vermehrte Häufigkeit des Stuhlgangs	☐	☐	☐	☐	☐
22. wässrige Stühle	☐	☐	☐	☐	☐
23. unkontrollierte Stuhlabgang	☐	☐	☐	☐	☐
24. blutige Stühle	☐	☐	☐	☐	☐
25. krampfhafte Bauch-/Becken-/Enddarmschmerzen	☐	☐	☐	☐	☐

26. Wie belastend war insgesamt der Stuhlgang für Sie?

großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
☐	☐	☐	☐	☐

Sexuelle Beschwerden ***in den letzten vier Wochen***
(Zutreffendes bitte ankreuzen /Angaben freiwillig: Fragen, die Sie nicht beantworten möchten, können Sie überspringen)

Wie würden Sie folgende Punkte einschätzen?

	sehr gering oder nicht vorhanden	gering	zufrieden-stellend	gut	sehr gut
27. Ihr sexuelles Verlangen	☐	☐	☐	☐	☐
28. Ihre Fähigkeit, eine Erektion zu haben	☐	☐	☐	☐	☐
29. Ihre Fähigkeit, einen Höhepunkt zu erreichen	☐	☐	☐	☐	☐

30. Wie würden Sie die übliche QUALITÄT Ihrer Erektionen einschätzen

keine	nicht ausreichende Festigkeit für sexuelle Aktivität	ausreichende Festigkeit nur für das Vorspiel	ausreichende Festigkeit für Geschlechtsverkehr
☐	☐	☐	☐

31. Wie würden Sie die übliche HÄUFIGKEIT Ihrer Erektionen einschätzen (wenn Sie eine Erektion wollten)?

nie	weniger als die Hälfte der Fälle	mehr als die Hälfte der Fälle	immer
☐	☐	☐	☐

32. Wie oft sind Sie mit einer Erektion morgens oder in der Nacht aufgewacht?

nie	seltener als einmal wöchentlich	ungefähr einmal wöchentlich	mehrmals wöchentlich	täglich
☐	☐	☐	☐	☐

33. Wie oft hatten Sie <u>irgendeine</u> sexuelle Aktivität?

nie	seltener als einmal wöchentlich	ungefähr einmal wöchentlich	mehrmals wöchentlich	täglich
☐	☐	☐	☐	☐

34. Wie oft hatten Sie Geschlechtsverkehr?

nie	seltener als einmal wöchentlich	ungefähr einmal wöchentlich	mehrmals wöchentlich	täglich
☐	☐	☐	☐	☐

35. Wie würden Sie Ihre Fähigkeit zur Sexualität insgesamt einschätzen?

sehr schlecht	schlecht	zufriedenstellend	gut	sehr gut
☐	☐	☐	☐	☐

Wie belastend waren die folgenden Punkte für Sie?

	großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
36. Ihr sexuelles Verlangen	☐	☐	☐	☐	☐
37. Ihre Fähigkeit, eine Erektion zu haben	☐	☐	☐	☐	☐
38. Ihre Fähigkeit, einen Höhepunkt zu erreichen	☐	☐	☐	☐	☐

39. Wie belastend war insgesamt Ihre Sexualität bzw. Mangel an Sexualität für Sie?

großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
☐	☐	☐	☐	☐

Hormonelle Beschwerden ***in den letzten vier Wochen***

(Zutreffendes bitte ankreuzen /Angaben freiwillig: Fragen, die Sie nicht beantworten möchten, können Sie überspringen)

40. Wie oft hatten Sie Hitzewallungen?

häufiger als einmal täglich	ungefähr einmal täglich	häufiger als einmal wöchentlich	ungefähr einmal wöchentlich	selten oder nie
☐	☐	☐	☐	☐

41. Wie oft hatten Sie Schmerzen in der Brustdrüse?

häufiger als einmal täglich	ungefähr einmal täglich	häufiger als einmal wöchentlich	ungefähr einmal wöchentlich	selten oder nie
☐	☐	☐	☐	☐

42. Wie oft waren Sie depressiv verstimmt?

häufiger als einmal täglich	ungefähr einmal täglich	häufiger als einmal wöchentlich	ungefähr einmal wöchentlich	selten oder nie
☐	☐	☐	☐	☐

43. Wie oft fühlten Sie einen Energiemangel?

häufiger als einmal täglich	ungefähr einmal täglich	häufiger als einmal wöchentlich	ungefähr einmal wöchentlich	selten oder nie
☐	☐	☐	☐	☐

44. Welche Gewichtsänderung trat bei Ihnen in den letzten vier Wochen auf?

5kg oder mehr zugenommen	weniger als 5kg zugenommen	keine Gewichtsänderung	weniger als 5kg abgenommen	5kg oder mehr abgenommen
☐	☐	☐	☐	☐

Wie belastend waren die folgenden Beschwerden für Sie?

	großes Problem	mäßiges Problem	kleines Problem	sehr kleines Problem	kein Problem
45. Hitzewallungen	🕙	🕙	🕙	🕙	🕙
46. Brustsdrüsenschmerzen/ -vergrößerung	🕙	🕙	🕙	🕙	🕙
47. Verlust der Körperbehaarung	🕙	🕙	🕙	🕙	🕙
48. Depressive Verstimmung	🕙	🕙	🕙	🕙	🕙
49. Energiemangel	🕙	🕙	🕙	🕙	🕙
50. Gewichtsänderung	🕙	🕙	🕙	🕙	🕙

Publications

1. Brzozowska K, Johannes K, Obe G, Hentschel R, Morand J, Moss R, Wittig A, Sauerwein W, Liniecki J, Szumiel I, Wojcik A. Effect of temperature during irradiation on the level of micronuclei in human peripheral blood lymphocytes exposed to X-rays and neutrons *Int J Radiat Biol.* 2009 Jul 9:1-9

2. Wojcik A, Buraczewska I, Sommer S, Brzozowska K, Pregowski J, Witkowski A, Garmol D, Pszona S, Bulski W. Enhanced level of micronuclei in peripheral blood lymphocytes of patients treated for restenosis with 32P endovascular brachytherapy. *Cardiovasc Revasc Med.* 2008 Jul-Sep;9(3):149-55

Scholarships

Helmholtz-DAAD scholarship - PhD thesis conducted in Forschungszentrum Jülich GmbH, Department of Security and Radiation Protection, Radiation Biology Team in Germany under supervision of Dr. Sabine Schmitz since 01.2007 to 03.2010

Curriculum Vitae

Personal Information

Kinga Brzozowska
Date and place of birth: 02.02.1980 in Tarnow, Poland

Education

1999 – 2004	Warsaw University of Life Sciences, Interfaculty Study of Biotechnology, Discipline of Biotechnology for Animal Production and Healthcare; Master of sciences (21.09.2004)
1995 – 1999	T. Kosciuszki Secondary School, Lukow, Poland
1994 – 1998	Primary music school, Lukow (violoncello class), Poland
1987 – 1995	Primary school, Trzebieszow, Poland

Work experience

Jan. 2007 – Mar. 2010	Research Centre Juelich GmbH, Div. of Safety and Radiation Protection, Radiation Biology Team, 52428 Juelich, Germany; Ph.D position
Mar. 2005 – Sep.2006	Institute of Nuclear Chemistry and Technology, Department of Radiation Biology and Health Protection, Warsaw; Biologist position,
Oct. 2004 – Feb. 2005	Meat processing plant „Lmeat-Lukow S.A.”, Lukow, Poland; Microbiologist position

Training

Oct. - Dec. 2006	Institute of Medical Virology, Justus Liebig University, Giessen, Germany
Feb.2003 - Sep.2004	Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Department of Microbial Biochemistry, Warsaw, Poland; M.Sc position
Jul. – Nov. 2003	Agricultural students practice, G’s Marketing Ltd, Barway, Great Britain,
Aug. 2002	Microbiological laboratory hospital, Tarnow, Poland

Personal skills and competences

Mother tongue: polish
English: very good
German: very good
Spanish: basic
Russian: verbal skills: basic

Computer skills and competence: Word, Excel, Power Point, Origin, Metafer4, Internet
Driving licence: polish category B

Interests

Historical novels, classical and modern music, travelling, mountains walking, winter sports, karate

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Biologie und Geografie Fakultäten zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das *Thema „Molecular and cellular assays after radiation exposure*
Part 1: Influence of temperature during irradiation on the level of DNA damage
Part 2: Comparison of individual radiosensitivity of peripheral blood lymphocytes from prostate cancer patients and healthy donors“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Kinga Brzozowska befürworte.

Essen, den …….

Unterschrift d. wissenschaftl. Betreuers/Mitglieds
der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Biologie und Geografie Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstsändig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den……

Unterschrift des/der Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Biologie und Geografie Fachbereiche zur Erlangung der Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den…….....

Unterschrift des/der Doktoranden/in