Radiation biomarkers: novel insights from transcriptional studies

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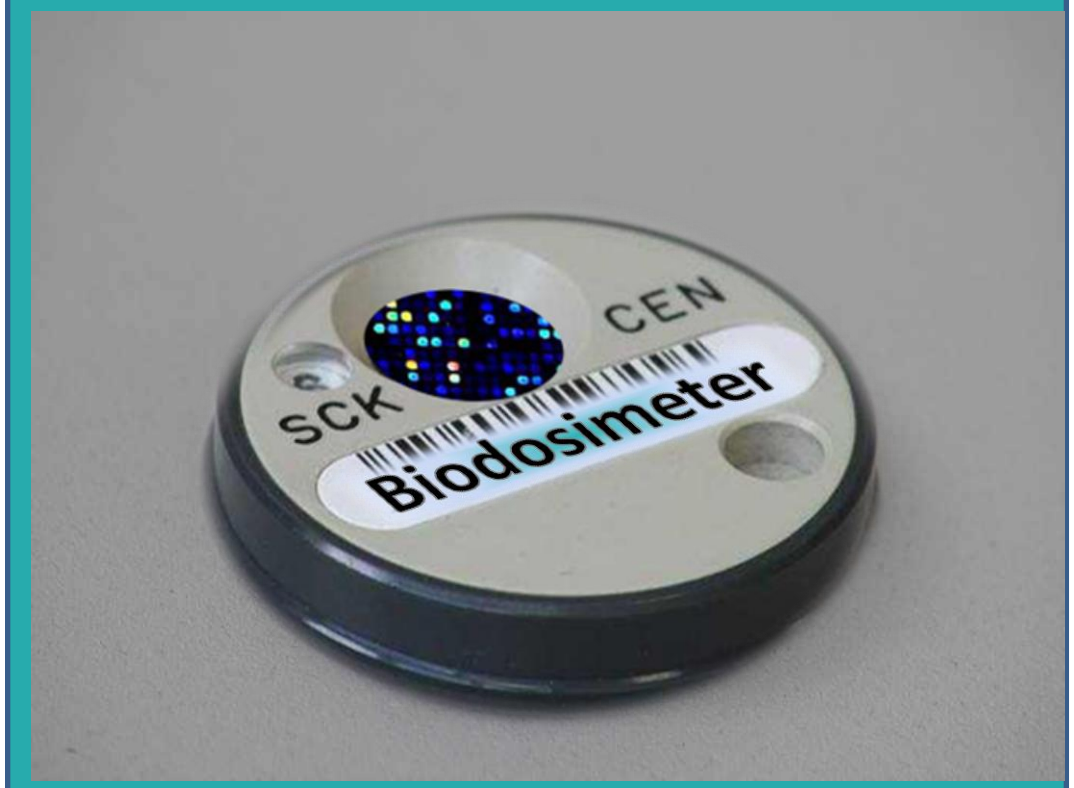

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SCK•CEN
STUDIECENTRUM VOOR KERNENERGIE
CENTRE D'ETUDE DE L'ENERGIE NUCLEAIRE

In memoriam of Prof. dr. hab. Pavel Vlad,

an eminent scientist and a loving Grandfather

and

to my wonderful family for their love and support

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Above all, don't fear difficult moments. The best comes from them.

Rita Levi-Montalcini

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Summary

In the course of evolution life has adapted to the background radiation present on Earth. But since the discovery of ionizing radiation in 1890s it became widely used in numerous applications and its impact on human health has increased. Identification of robust and reliable radiation biomarkers of exposure, which can be used as biological dosimeters following a large-scale nuclear accident or a terroristic attack are of pivotal importance for radiobiological research. On the other hand, specific biomarkers of individual radiosensitivity to different types of radiation would be of great value for improving radiotherapy treatment (personalized medicine) or for the selection of crews for long-term Space missions.

As the model for our study we chose either human peripheral blood or isolated peripheral blood mononuclear cells (PBMCs), depending on the type of the experiment.

As the first step of our study, we aimed at **identifying gene expression biomarkers of exposure to radiation with increased sensitivity to low-dose exposures.** To this end, PBMCs from healthy volunteers were exposed *in vitro* to two X-ray doses relevant for medical triage: a moderate dose of 1.0 Gy which might result in acute radiation syndrome and is associated with a high probability of long-term stochastic health effects and a low dose of 0.1 Gy which is not associated with any immediate acute health effects but might require medical follow-up as the risk of long-term effects, particularly cancer, must be taken into consideration. We performed a whole-genome microarray study and identified the biomarkers (genes and exons) most suitable for classification of the samples according to the exposure dose. Our analysis also showed that several genes, especially those that were differentially expressed, are alternatively transcribed and spliced in response to irradiation. This suggests that evaluating gene expression at the level of single exons is

of particular importance for optimal design of primer- or probe-based biodosimetric assays. We also confirmed the validity of our approach in a biodosimetry interlaboratory comparison study organized by the RENEB (Realizing the European Network of Biodosimetry) consortium.

Based on our previous microarray results we **designed a customized qRT-PCR array for biodosimetry** comprising a panel of 25 selected genes. For the genes exhibiting alternative splicing, we designed primers interrogating the most sensitive exons. This allowed us **to validate our approach using a system applicable to large-scale radiological accidents.** We used peripheral blood samples from healthy volunteers exposed to doses from 0 to 2 Gy at several time points post-irradiation from 8 to 48 hours as reference samples and the samples from other individuals were used as "blind samples". We could accurately predict dose, but also time after exposure. In this study we also compared different methodologies for RNA extraction available on the market and evaluated their applicability to emergency situations. We also used these customized qRT-PCR arrays in the second biodosimetry interlaboratory comparison organized by the RENEB consortium.

As the last step of this study we aimed to **compare the transcriptional response and DNA repair kinetics of human PBMCs after exposure to different radiation types**. Understanding the differences in response of normal cells to low- and high-LET radiation is important for several reasons and applications. First of all, in order to optimally exploit the benefits of hadron therapy, it is essential to adequately predict the treatment outcome in comparison to the conventional photon radiotherapy, taking into account not only the difference in the response of tumors but also of normal cells. Second scenario, although less common, is the exposure of the crew to heavy ions during long-term Space missions. In this respect, it is important to study whether the response of normal cells to Space radiation and low-LET radiation is comparable and that therefore the extrapolations based on

predominantly low-LET epidemiological studies used for radiation protection purposes are valid in case of high-LET exposures. Transcriptional response to high- and low-LET radiation was similar in view of identity of activated genes at least at 8 hours post-exposure. However, heavy ions showed higher potential of activation of immunity-related gene sets and more persistent activation of p53-regulated genes compared to X-rays. With an eye to possible long-term Space flights, in the present study we also investigated the potential of a core signature of genes responsive to iron ions exposure to serve as an indicator of varied DNA repair capacity of healthy astronauts, which are expected to be selected for a similar mission.

Samenvatting

Doorheen de evolutie hebben levende wezens zich aangepast aan de achtergrondstraling op aarde. Het was echter pas sinds de ontdekking van straling in de jaren 1890 dat het effectief gebruikt werd door mensen, met grote gevolgen op de gezondheid van de bevolking. De identificatie van robuste en betrouwbare biomerkers van bestraling zijn cruciaal om te kunnen gebruiken als biologische dosimeters (biodosimetrie) na een grootschalige nucleaire ramp of terroristische aanval. Bovendien zouden zulke biomerkers waardevol kunnen zijn om de individuele stralingsgevoeligheid van patiënten aan diverse stralingstypes te bepalen met het oog op gepersonaliseerde behandelingen. Verder zou deze bepaling van stralingsgevoeligheid uitermate interessant kunnen zijn om de bemanning van langete (interplanetaire) ruimtemissies te selecteren.

Perifeer bloed of geïsoleerde perifere mononucleaire cellen (PBMCs) van gezonde vrijwilligers werden gebruikt als studiemiddel, afhankelijk van het experimentele ontwerp.

Als eerste stap van de studie **identificeerden we genexpressie biomerkers van stralingsblootstelling met verhoogde gevoeligheid na een lage dosis straling.** Hiervoor werden PBMCs van gezonde vrijwilligers *in vitro* blootgesteld aan twee doses van X-straling die relevant zijn voor medische triage: ten eerste een matige dosis van 1.0 Gy, dewelke kan leiden tot acuut stralingssyndroom en een gekende rol heeft bij langetermijns stochastische effecten. Als tweede dosis werd 0.1 Gy gebruikt, die geen acute gezondheidseffecten veroorzaakt, maar waar een langere medische opvolging wel noodzakelijk kan zijn om bijvoorbeeld mogelijk gerelateerde kanker op te sporen. Op basis van een microarray experiment identificeerden we de meest geschikte biomerkers (genen en exonen) om de stalen te classificeren afhankelijk van de stralingsdosis. Onze analyse toonde verder

aan dat verschillende genen, vooral diegenen met een veranderde expressie, een alternatieve splicing en transcriptie vertoonden na bestraling. We bevestigden onze bevindingen in een onafhankelijke interlaboratorium vergelijkende studie voor biodosimetrie, georganiseerd door het RENEB (Running the European Network of biological dosimetry and physical retrospective dosimetry) consortium.

Gebaseerd op onze microarray resultaten **ontwierpen we een nieuwe qRT-PCR array voor biodosimetrie**, bestaande uit een set van 25 genen. Voor de genen met alternatieve splicing ontwierpen we primers voor de meest gevoelige exonen. Op deze manier **konden we onze strategie uitwerken in een systeem dat toepasbaar is voor grootschalige radiologische rampen.** We gebruikten bloed van gezonde vrijwilligers om kalibratiecurves op te stellen met doses van 0 tot 2 Gy op diverse tijdspunten na bestraling, nl. 8 uur tot 48 uur. Bloedstalen van andere individuen werden gebruikt als “blinde stalen”, waarvan nog de bstralingsdosis, noch de tijd na bestraling waren gekend. Op basis van onze biomerkers konden we accuraat de dosis en de tijd na bestraling achterhalen. In deze studie vergeleken we ook diverse commerciële methodes om RNA extractie uit te voeren en trachtten de toepasbaarheid ervan in noodsituaties te achterhalen. We gebruikten onze qRT-PCR arrays ook in de tweede interlaboratorium vergelijkende studie voor biodosimetrie georganiseerd door RENEB.

Als laatste stap van deze studie wilden we **de transcriptionele reactie en DNA reparatie-kinetiek bepalen van humane PBMCs na bestraling met verschillende types van straling.** Een betere kennis van hoe normale cellen reageren t.o.v. straling met een lage of hoge lineaire energietransfer (LET) is belangrijk voor diverse redenen en toepassingen. Ten eerste, om de voordelen van hadrontherapie optimaal te benutten, is het essentieel om accuraat de gevolgen van de behandeling (in vergelijking tot conventionele foton radiotherapie) te voorspellen. Dit niet enkel voor tumorcellen, maar ook voor gezonde cellen. In het tweede scenario, ook al is het minder frequent

van toepassing, is er de bestraling van bemanningsleden met zware ionen tijdens langdurige ruimtemissies. In deze context is het dan belangrijk om te weten of de reactie van cellen op kosmische straling vergelijkbaar is met die op lage-LET straling en dat daarom extrapolaties gebaseerd op lage-LET epidemiologische studies ook geldig zijn voor hoge-LET blootstellingen. De transcriptionele reactie van cellen op hoge- en lage-LET straling was grotendeels vergelijkbaar inzake de identiteit van geactiveerde genen op ten minste 8 uur na bestraling. Echter, zware ionen vertoonden een hogere capaciteit om immuniteit-gerelateerde genen te activeren en een langdurigeractivatie van p53-gereguleerde genen in vergelijking tot X-straling. Met het oog op mogelijke langetermijns ruimtevluchten bestudeerden we ook de mogelijke aanwezigheid van cruciale genen, reactief op bestraling met ijzer ionen. Deze zouden als indicator van variabele DNA herstel-mechanismes kunnen dienen bij het selecteren van gezonde astronauten.

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List of Abbreviations

(6–4)PP – 6–4 Photoproduct

26S – 26 Svedberg units

3′ SS – 3’ Splice Site

5’-UTR – 5’ UnTranslated Region

5′ SS – 5’ Splice Site

53BP1 – p53 Binding Protein 1

6-TG – 6-Thioguanine

8-oxo-dG – 8-Oxo-2'-deoxyguanosine

γH2AX – phosphorylated Histone subtype H2A isoform X

ΔCt – delta Cycle of threshold

Δm – delta Mass

Δε – delta Energy

A

ABL1 – ABL Proto-Oncogene 1, Non-Receptor Tyrosine Kinase

ACTA2 – Actin, Alpha 2, Smooth Muscle, Aorta

AMU – Atomic Mass Unit

ANKRA2 – Ankyrin Repeat Family A Member 2

ANOVA – Analysis of Variance

AP-1 – Activated Protein 1

APA – Alternative PolyAdenylation

APAF1 – Apoptotic Peptidase Activating Factor 1

APE1 – Apurinic/apyrimidinic (AP) Endonuclease 1

APTX – Aprataxin

ASCC3 – Activating Signal Cointegrator 1 Complex Subunit 3

ASTN2 – Astrotactin 2

ATM – Ataxia-Telangiectasia Mutated

ATP – Adenosine Triphosphate

ATR – Ataxia Telangiectasia and Rad3-related

ATRIP – ATR Interacting Protein

AUC – Area Under the Curve

B

B2M – Beta-2-Microglobulin

BAK1 – BCL2 homologous Antagonist/Killer 1

BAX – BCL2 Associated X apoptosis regulator

BBC3 – BCL2 Binding Component 3

BCL-X – B-cell lymphoma-extra large

BEIR – Biological Effects of Ionizing Radiation

BER – Base-Excision Repair

BIRC5 – Baculoviral IAP Repeat Containing 5

BLM – Bloom Syndrome RecQ Like Helicase

BRCA1/2 – BReast CAncer type 1/2 susceptibility protein

C

C12orf5 – Chromosome 12 Open Reading Frame 5

CASP8/9 – Caspase 8/9

CBP – Calcium-Binding Protein

CC – Correlation Coefficient

CCNG1 – Cyclin G1

CCR – Complex Chromosomal Rearrangement

CCR4 – C-C Motif Chemokine Receptor 4

CDC2 – Cell Division Cycle protein 2 homolog

CDC25C – Cell Division Cycle 25C

CDK – Cyclin-Dependent Kinase

CDKN1A – Cyclin Dependent Kinase Inhibitor 1A

c-FLIP – Cellular FLICE-Inhibitory Protein

CHEK2 – Checkpoint Kinase 2

CHK1/2 – Checkpoint Homolog Kinase 1/2

cis-Pt – cisplatin

CLPA – Chemical Ligation-dependent Probe Amplification

cm – centimeter

CNV – Copy Number Variant

CPD – Cyclobutane Pyrimidine Dimer

CREB – cAMP-Responsive Element-Binding protein

CRP – C-reactive Protein

CSR - Class Switch Recombination

CT – Computed Tomography

CtIP – Carboxy-terminal Binding Protein (CtBP)-Interacting Protein

D

D – absorbed dose

DAPI – 4',6-diamidino-2-phenylindole

DAVID – The Database for Annotation, Visualization and Integrated Discovery

DCA – Dicentric Chromosome Assay

DDB2 – DNA Damage Binding protein 2

DDIT4 – DNA Damage Inducible Transcript 4

DDR – DNA Damage Repair

DEC1 – Deleted in Esophageal Cancer 1

DMC1 – Disrupted Meiotic cDNA protein 1

DNA – DeoxyriboNucleic Acid

DNA-PKcs – DNA-dependent Protein Kinase, catalytic subunit

DSB – Double-Strand Break

$D_{T,R}$ – absorbed dose in tissue T by radiation type R

E

E – Effective dose

E2F1 – Retinoblastoma-Associated Protein 1

EDA2R – Ectodysplasin A2 Receptor

EDTA – Ethylenediaminetetraacetic acid

EGR -1 – Early Growth Response 1

EI24 – Etoposide-Induced Protein 2.4

EMT – Epithelial-Mesenchymal Transition

EU – European Union

eV – electron Volt

EXO1 – Exonuclease 1

F

FANCD2 – Fanconi Anemia Complementation Group D2

FAS – Fas Cell Surface Death Receptor

FBXO22 – F-Box Protein 22

FC – fold change

FDR – False Discovery Rate

FDXR – Ferredoxin Reductase

FEN1 – Flap Endonuclease 1

FIRMA – Finding Isoforms using Robust Multichip Analysis

FITC – Fluorescein isothiocyanate

G

G0/1/2 – Gap 0/1/2 phase

GADD45A – Growth Arrest and DNA Damage inducible Alpha

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GCR – Galactic Cosmic Rays

GEO – Gene Expression Omnibus

GLM – Generalized Linear Models

GNG11 – Guanine Nucleotide binding protein (G protein), gamma 11

GO – Gene Ontology

GSEA – Gene Set Enrichment Analysis

Gy – Gray

GYPA – glycophorin A

H

HAT – Histone AcetylTransferase

HBA1 – Hemoglobin Subunit Alpha 1

HBB – Beta-globin

HDAC2 – Histone Deacetylase 2

HPRT1 – Hypoxanthine Phosphoribosyltransferase 1

HR – Homologous Recombination

H_T – equivalent dose absorbed by tissue T

HZE – High (Z) atomic number and Energy

I

ICRP – International Commission on Radiological Protection

IL-1/2/6 – Interleukin-1/2/6

ITGA2 – Integrin Subunit Alpha 2

ITPR2 – Inositol 1,4,5-Trisphosphate Receptor Type 2

J

JAK – Janus Kinase

K

keV – kiloelectron Volt

keV/µm – kiloelectron Volt/micrometer

kV – kilovolt

L

LET – Linear Energy Transfer

LIG4 – DNA Ligase 4

M

M – Mitosis phase

M – Methyl group

MAMDC4 – MAM Domain Containing 4

MAP4K2 – Mitogen-Activated Protein Kinase 2

MCP-1 – Monocyte Chemoattractant Protein-1

MDC1 – Mediator of DNA Damage Checkpoint 1

MDM2 – Mouse Double Minute 2 homolog

MeV – megaelectron Volt

mGy – milliGray

MIAME – Minimum Information About a Microarray Experiment

miRNA – micro RiboNucleic Acid

MMC – Mitomycin C

MOMP – Mitochondrial Outer Membrane Permeabilization

MP – Modified Protocol

MRE11 – Meiotic Recombination 11 Double Strand Break Repair Nuclease

MRN – MRE11-RAD50-NBS1 complex

mRNA – Messenger RiboNucleic Acid

mSv – milliSievert

mTORC1 – Mammalian Target of Rapamycin Complex 1

N

NBS1 – Nijmegen Breakage Syndrome protein 1

NDUFAF6 – NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 6

NER – Nucleotide-Excision Repair

NF-κB – Nuclear Factor κB

NHEJ – Non-Homologous End Joining

NOXA – Phorbol-12-myristate-13-acetate-induced protein 1

NRF2 – Nuclear erythroid-derived 2-Related Factor 2

NUP133 – Nucleoporin 133

P

P – Phosphate group;

p14ARF – Alternate Reading Frame protein product of the *CDKN2A* gene

$p21^{CIP1/WAF1}$ – cyclin-dependent kinase inhibitor 1

p300 – E1A binding Protein 300

p48 – DNA damage-binding protein 48

p53 – tumour Protein 53

PAI-1 – Plasminogen Activator Inhibitor-1

PAM – Prediction Analysis for Microarrays

PAPPA-AS1 – Pregnancy-Associated Plasma Protein A Antisense RNA 1

PARP-1/2 – Poly [ADP-Ribose] Polymerase 1/2

PBMC – Peripheral Blood Mononuclear Cell

PCA – Principal Components Analysis

PCC – Premature Chromosome Condensation

PCNA – Proliferating Cell Nuclear Antigen

PF4 – Platelet Factor 4

PGE – Positional Gene Enrichment

PGK1 – Phosphoglycerate Kinase 1

PI3-K – Phosphatidylinositol 3-Kinase

PIG3 – Tumor Protein P53 Inducible Protein 3

PML – Promyelocytic Leukemia protein

PNK – Polynucleotide Kinase

POLH – DNA Polymerase Eta

pp1/2 – primer pair 1/2

PPM1D – Protein Phosphatase, Mg2+/Mn2+ Dependent 1D

PUMA – p53 Up-regulated Modulator of Apoptosis

PVT1 – PVT1 Oncogene (Non-Protein Coding)

qPCR – quantitative Polymerase Chain Reaction

qRT-PCR – quantitative Reverse Transcription Polymerase Chain Reaction

R

R – Radiation

RAD50 – Double Strand Break Repair Protein

RAD51/52 – DNA repair protein RAD51/52 homolog 1

RAD51B/C/D – DNA Repair Protein RAD51 Homolog B/C/D

RAD54 – DNA Repair And Recombination Protein RAD54

RAE – Relative Absolute Error

RBE – Relative Biological Effectiveness

RBM14 – RNA Binding Motif Protein 14

RENEB – Realizing the European Network of Biodosimetry

REV3L – Protein reversionless 3-like

RF – Random Forests

RG – Reference gene

RMA – Robust Multichip Analysis

RNA – RiboNucleic Acid

RNF8/168 – Ring Finger protein 8/168

RNGTT – RNA Guanylyltransferase and 5'-Phosphatase

rNTP – riboNucleoside Tri-Phosphate

ROS – Reactive Oxygen Species

RPA – Replication Protein A

RPS27L – Ribosomal Protein S27 Like

RRHO – Rank-Rank Hypergeometric Overlap

S

S – Synthesis phase

SAA – South Atlantic Anomaly

SC35 – splicing factor SC35

Ser – Serine

SESN1 – Sestrin 1

SFN – Stratifin

SI – International System of Units

SNP – Single Nucleotide Polymorphism

SP – Standard Protocol

SP1 – Specificity Protein 1

SPE – Solar Particle Event

SSB – Single-Strand Break

ssDNA – single-stranded DNA

STAT3/5 – Signal Transducer and Activator of Transcription 3/5

Sv – Sievert

SVM – Support Vector Machines

T

T – Threonine

T – Tissue

TC-FISH – Telomere Centromere Fluorescence in situ Hybridization

TF – Transcription Factor

TID1 – DnaJ Heat Shock Protein Family Member A3

TMEM30A – Transmembrane Protein 30A

TNFRSF10B/D – Tumor Necrosis Factor Receptor Superfamily Member 10B/D

TNFSF8 – Tumor Necrosis Factor Superfamily Member 8

TNFα – Tumor Necrosis Factor alpha

TopBP1 – Topoisomerase II Binding Protein 1

TP53 – Tumor Protein 53

TRAF2 – TNF Receptor Associated Factor 2

TRIM22 – TRipartite Motif containing 22

U

U – Ubiquitin

UCSC – University of California, Santa Cruz

UNSCEAR – The United Nations Scientific Committee on the Effects of Atomic Radiation

UV – Ultraviolet

V

V(D)J – Variable, Diversity and Joining

VWCE – Von Willebrand Factor C and EGF Domains

W

WEKA – Waikato Environment for Knowledge Analysis

WHO – World Health Organization

W_R – Radiation Weighting factor

W_T – Tissue Weighting factor

X

XLF – X-ray repair cross-complementing protein 4-Like Factor

XPC – Xeroderma pigmentosum, complementation group C

XRCC1/2/3/4 – X-ray Repair Cross-Complementing protein 1/2/3/4

Z

ZMAT3 – Zinc Finger Matrin-Type 3

ZNF79 – Zinc Finger Protein 79

1.

Introduction

1.1 Types of ionizing radiation: electromagnetic and particulate radiation

Ionizing radiation is the type of radiation which carries enough energy to eject electrons from atoms or ionize them, hence the name. The notion of ionizing radiation comprises a vast spectrum of physical phenomena. Based on the physical nature ionizing radiation can be classified into electromagnetic and particulate radiation. The latter is made up of energetic subatomic particles, atoms and ions, while the ionizing electromagnetic waves include γ-rays, X-rays and the higher ultraviolet part of the electromagnetic spectrum [1] (Figure 1). Absorption of an X-ray or γ-ray photon can cause ionization due to its high energy content, which would be in excess of at least 10 eV (considered to be the minimum photon energy capable of causing ionization) [2].

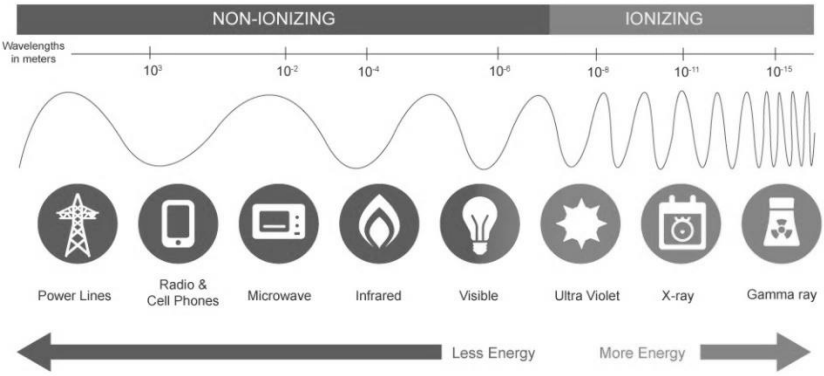

Figure 1. The scale of electromagnetic radiation, broken down into categories of ionizing and non-ionizing radiation. Available at *https://www.mirion.com/introduction-to-radiation-safety/what-is-radiation/*.

1.2 Physical origins of different types of ionizing radiation

Unstable nuclei undergo spontaneous transformation in order to convert to a stable state by emitting a part of their energy in the form of α-, β-particles and γ-rays. This process is naturally occurring and is called radioactive decay [1]. X-rays are in many aspects identical to γ-rays and the difference between the two is based on their source: γ-rays are emitted by the atomic nuclei during their natural decay, while X-rays are emitted as the result of electronic transitions and are produced in X-ray generators [1]. Besides α- and β-particles, there are other particles such as neutrons, which are produced copiously in nuclear fission and fusion and accelerated charged nuclei, which are characteristic of cosmic radiation and can be artificially produced in particle accelerators [3]. The main characteristics of the above-listed radiation types are summarized in Table 1.

Table 1. Characteristics of the main radiation types

Radiation type	Energy	Mass (AMU)	Source	Type	Range	LET, keV/μm	W_R
γ-rays	0.01-10 MeV	0	Radioactive decay	Electromagnetic waves	km in air, m in tissue	0.3 (Co^{60})	1
X-rays	1 keV-10 MeV	0	X-ray generators	Electromagnetic waves	km in air, m in tissue	2 (250 keV)	1
α-particle	3-8 MeV	4	Radioactive decay	Helium nucleus, particle	<10 cm in air; 60 μm in tissue	Variable, 65 (8 MeV), 170 (4.5 MeV)	20
β-particle	0-3 MeV	1/1836	Radioactive decay	Electron, particle	≈15 cm in air; a few mm in tissue	Variable, 0.25 (1 MeV) to 12 (1 keV)	1
neutron	0-10 MeV	1	Nuclear fission and fusion	Neutron, particle	Dependent on the energy	Dependent on the energy and method of measurement, 10-100	5-20
HZE ions	Variable, up to millions of MeV	>4	Galactic cosmic rays, particle accelerators	The nuclei of all elements heavier than helium	Dependent on the energy	Dependent on the energy	20

1.3 Contributions of different radiation types to possible human exposure scenarios

Different radiation types are particularly important in different exposure scenarios. For example, X-rays are widely used in medical imaging (projectional radiographs, computed tomography, fluoroscopy) and

conventional radiotherapy where lower energy X-ray beams are used for skin cancer treatment [4], while higher energy beams are used for treating the tumors within the body [5]. γ-rays are also used in radiotherapy (e.g., gamma-knife which is used to treat brain tumors) and nuclear medicine for imaging purposes (e.g., Single Photon Emission Computed Tomography and Positron Emission Tomography).

Another type of radiotherapy, hadron therapy, involves the therapeutic use of protons and heavier ions, such as carbon ions. Of these, proton therapy is the most common [6], though still rarely used compared to other forms of external beam radiotherapy. Exposure to protons, alpha-particles and heavier ions up to iron also occurs during Space flights [7].

Radiation exposure as a result of nuclear accidents can happen in three ways: total or partial body exposure as a result of close proximity to a radiation source, external contamination, and internal contamination [8]. In previous reactor accidents, only plant workers and emergency personnel had substantial total or partial body external exposure mainly due to β-radiation which can be a significant source of dose to skin and high-energy γ-radiation which penetrates deeply and results in exposure of internal organs [8]. External contamination occurs when the fission products settle on human body, thereby exposing skin or internal organs. Internal contamination occurs when fission products are ingested, inhaled or enter the body through open wounds. The latter is the primary exposure mechanism due to which large populations living around a reactor can be exposed to radiation in case of an accident [8]. Following a reactor accident a variety of radioisotopes are released into the environment. The health threat from each of them depends on many factors, such as the half-life, whether they are gaseous and whether they are released in substantial quantities [8]. Contamination with most of the radioisotopes results in exposure to β- and γ-radiation; some of them are also sources of α-particles [8]. Among the different radioisotopes, iodine-131 is an important source of exposure because of its prevalence in reactor discharges

and its tendency to settle on the ground. After entering the body, iodine-131 rapidly accumulates in the thyroid gland, where it can be a source of substantial doses of β- and γ-radiation [8].

1.4 Low and high linear energy transfer (LET) radiation

Linear energy transfer is the average amount of energy that is released per unit length of the radiation track. LET depends on the nature of the radiation as well as on the material it interacts with and is usually expressed in units of keV/μm [3]. Generally speaking, X-, γ-rays and β-particles are considered low-LET or sparsely ionizing radiations, while energetic neutrons, protons, α-particles and heavier charged particles are considered high-LET or densely ionizing radiations. The LET of radiation describes its energy deposition density, which largely determines the biological consequences of radiation exposure [1]. Examples of LET values of main radiation types are given in Table 1. The examples of patterns of energy deposition for different types of radiation resulting in different distribution of DNA double-strand breaks are shown in Figure 2. In contrast to photon radiations, for which the dose distribution in matter is characterized by an exponential decline in dose with depth, high-energy charged particles deposit relatively little energy as they enter the absorbing material but tend to deposit extremely large amounts of energy in a very narrow peak, the Bragg peak, as they reach the end of their track, which results in a dramatic increase of the LET [9].

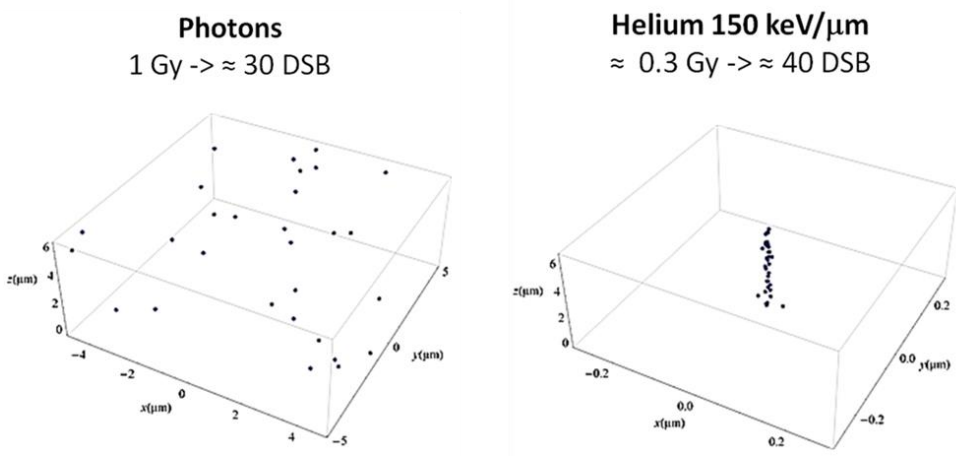

Figure 2. Simulated patterns of DNA double-strand breaks distribution after photon and ion irradiation in a typical cell nucleus (radius of ≈5 μm). Adapted from **[10]**.

1.5 Relative biological effectiveness

Even though LET is widely used to categorize radiation-induced damage, it is not a good parameter to describe the full spectrum of biological radiation effects. Multiple experiments in irradiated cultured cells and healthy and tumor-bearing animals were performed in order to define the biological effectiveness of the accelerated particles in comparison to the same physical dose of a reference radiation (250kV X-rays or ^{60}Co γ-rays) using such endpoints, as the cell survival, chromosomal aberrations induction, histological changes, median lethal dose, etc. [11]. The obtained value is known as the relative biological effectiveness (RBE). An elevated RBE has been clearly demonstrated for ions heavier than helium [12]. The RBE rises with LET increasing to 100–200 keV/μm and decreases at higher LET values - the effect known as the “over-kill” effect, i.e., more dose is deposited in a cell than is necessary to kill it [12].

1.6 Dose quantities

The *absorbed dose* (D) is the energy (Δε) absorbed in specified volume per unit mass (Δm):

$$\mathrm{D} = \frac{\Delta\varepsilon}{\Delta\mathrm{m}}$$

The SI unit used to measure *absorbed dose* is the gray (Gy). One Gy is equivalent to 1 joule per kilogram. The absorbed dose is independent of the radiation type and does not describe the biological effect of radiation.

To enable consideration of stochastic health risk (such as cancer induction) based on the different radiation qualities and organ sensitivities, the International Commission on Radiological Protection (ICRP) introduced the terms *equivalent dose* and *effective dose* [13]. The most recent guidelines on the calculations for conversion of the absorbed dose into equivalent dose which takes into account the radiation type R, and the effective dose which takes into account the irradiated tissue (T), are given in the ICRP Publication 103 [14]. The SI unit of the equivalent and the effective dose is the sievert (Sv).

The *equivalent dose* absorbed by tissue T (H_T) is calculated using the following formula:

$$H_T = \sum_R W_R \cdot D_{T,R}$$

where,

W_R is the radiation weighting factor

$D_{T,R}$ is the absorbed dose in tissue T by radiation type R

In case there are different types of radiation (R) involved, summation is performed. The weighting factors for different radiation types (W_R) are given in Table 1.

The *effective dose* (E) is calculated from the equivalent dose using the following formula:

$$E = \sum_T W_T \cdot H_T = \sum_T W_T \sum_R W_R \cdot \bar{D}_{T,R}$$

where,

W_T is the tissue weighting factor

H_T is the equivalent dose absorbed by tissue T

In case there are several organs or tissues (T) being irradiated, summation is performed. The weighting factors (W_T) for the organs and tissues considered for calculation of the effective dose are listed in Table 2. In case of total body exposure all the organs and tissues for which specific weighting factors are defined are always included in the calculation, while in case of partial body exposure only the organs at risk of exposure are taken into account. For the gonads, the arithmetic mean of the absorbed doses to ovaries and testes is used in conjunction with the weighting factor of 0.20. Absorbed doses to blood and blood vessels are not included in the calculation [15].

Table 2. Recommended tissue weighting factors from [14].

Organ or tissue	Weighting factor (W_T)
Red bone marrow, lungs, colon, stomach, breast	0.12
Gonads	0.08
Bladder, liver, oesophagus, thyroid	0.04
Skin, bone surface, salivary glands, brain	0.01
Remainder of the body*	0.12
Total	1.00
*Adrenals, extra-thoracic region, gall bladder, heart, kidneys, lymphatic nodes, muscle, oral mucosa, pancreas, prostate, small intestine, spleen, thymus, uterus/cervix	

1.7 Health risks associated with ionizing radiation

The health effects of ionizing radiation are divided in two main categories: deterministic and stochastic effects. The main difference between the two types of the effects is the mechanism of their production: stochastic effects are caused by non-lethal mutational events in cells, while deterministic effects are caused by cell killing. Radiological protection dose limits are generally set based on stochastic risks and these fall well below the level at which there is any likelihood of deterministic injury [16], while deterministic effects are of most relevance in radiotherapy, where normal tissue doses are limited to avoid these effects [17]. The approach used by ICRP to take into account the induction of stochastic effects by high-LET compared to low-LET radiation is to multiply the dose by a radiation weighting factor [14]. However, these weighting factors are generally too high to be applied to deterministic effects and therefore it is suggested that judgments of RBE should be made from a case by case inspection [18].

1.7.1 Deterministic effects

Deterministic effects occur when too many cells are killed by radiation for the body to replace within a reasonable time, usually because the stem cell pool has also been seriously depleted. Therefore deterministic effects generally occur only after high-dose acute exposure with a threshold dose below which the effect is not observed. Deterministic effects are generally observed days (e.g. prodromal syndrome, gastrointestinal syndrome, central nervous system syndrome) or weeks (e.g. haematopoietic syndrome, pulmonary syndrome) following radiation exposure; however, certain deterministic effects (e.g. cataracts, hypothyroidism) only become evident after a period of years [16].

1.7.2 Stochastic effects

Stochastic effects, which include somatic (such as cancer induction) and genetic effects, are the main late health effects of radiation exposure, with somatic risks being the most detrimental. For both somatic and genetic effects

the probability of their occurrence, and not their severity, is dependent on the radiation dose [17]. Also, in contrast to the deterministic effects, for stochastic effects it is generally accepted that there is no threshold dose for the effect to be observed, as there is not enough epidemiological evidence for setting such thresholds [19]. The data used to predict stochastic effects, such as radiation-induced cancers, comes from (a) the Japanese atomic bomb survivors, (b) medically exposed populations and (c) occupationally exposed populations [20]. BEIR VII Committees, US Environmental Protection Agency, ICRP and the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) developed multiple cancer risk models, taking into account such parameters as gender, age at exposure and time since exposure, which result in an overall average lifetime excess risk of cancer development of approximately 5%/Sv [21]. Development of a malignancy can take 20-30 years for solid tumors and 5-10 years for leukaemia. For both solid tumors and leukaemia, a reduction of excess relative risk with increasing age at exposure, and a reduction of excess relative risk with increasing time after exposure were shown [17]. The heritable radiation-induced genetic risks occur when exposed cells include reproductive ones and are estimated based on the data from animal studies combined with the baseline incidence of disease in human populations [22]. There is also emerging evidence of excess risks of non-cancer late health effects, such as circulatory, digestive and respiratory diseases, in the Japanese atomic bomb survivors [23, 24].

1.8 Sources of ionizing radiation

Exposure to ionizing radiation from natural sources is an unavoidable feature of life on the Earth and for most individuals this exposure exceeds the one from man-made sources [25]. The two main natural sources of radiation are the high-energy cosmic radiation and the radioactive nuclides originating from the Earth's crust, which are present everywhere, including the human body. Since the discovery of X-rays by Wilhelm Konrad Roentgen in 1895 and the naturally emitted radiation from uranium by Henri Becquerel in 1896, ionizing

radiation has experienced the rise and fall of its popularity. By the 1920s, accumulating evidence of the adverse effects of ionizing radiation led to the foundation in 1928 of the International X-ray and Radium Protection Committee which issued the first radiation protection guidelines, although only for professionals in the medical field. In 1950 it was restructured to take account of new uses of radiation outside the medical area, and given its present name - the International Commission on Radiological Protection.

Nowadays, man-made sources of ionizing radiation are still widely used for multiple industrial, military and medical purposes, but both occupational exposures as well as those to the general public are strictly regulated and should be limited to 20 mSv/year and 1 mSv/year whole body exposure, respectively (excluding any medical and natural background radiation doses) [26]. For the purpose of quantifying cancer risk associated with radiation exposure the ICRP used as a rule of thumb, effective doses above or equal to 1 Sv, 100 mSv, 10 mSv, 1 mSv, and 0.1 mSv to signify the terms “moderately high”, “moderate”, “low”, “very low”, and “extremely low” doses, respectively [27].

In 2008 UNSCEAR proposed the classification of radiation exposures given in Table 3 [25].

Table 3. Sources of radiation exposure from [25]

Public exposure		
Natural sources	Normal occurrences	Cosmic radiation
		Terrestrial radiation
	Enhanced sources	Metal mining and smelting
		Phosphate industry
		Coal mining and power production
		Oil and gas drilling
		Rare earth and titanium dioxide industries
		Zirconium and ceramics industries
		Application of radium and thorium
		Other
Man-made sources	Peaceful purposes	Medical applications
		Nuclear power production
		Transport of nuclear and radioactive materials
		Others
	Military purposes	Nuclear tests
		Residues in the environment
Historical situations		
Exposure from accidents		
Occupational exposure		
Natural sources		Cosmic ray exposure of aircraft and spacecraft crews
		Exposures in extractive and processing industries
		Gas and oil extraction industries
		Radon exposure in workplaces other than mines
Man-made sources	Peaceful purposes	Nuclear power industries
		Medical uses of radiation
		Industrial uses of radiation
		Miscellaneous uses
	Military purposes	Other exposed workers

Annual average doses and ranges of individual doses of ionizing radiation by source are given in Table 4.

Table 4. Annual average doses (worldwide) and ranges of individual doses of ionizing radiation by source from [25]

Source or mode	Annual average dose, mSv	Typical range of individual doses, mSv	Comments
Natural sources of exposure			
Inhalation (radon)	1.26	0.2-10	The dose is much higher in some dwellings.
External terrestrial	0.48	0.3-1	The dose is higher in some locations.
Ingestion	0.29	0.2-1	
Cosmic radiation	0.39	0.3-1	The dose increases with altitude.
Total natural	2.4	1-13	Sizeable population groups receive 10-20 mSv.
Artificial sources of exposure			
Medical diagnosis (not therapy)	0.6	0- several tens	The averages for different levels of health care range from 0.03 to 2.0 mSv; averages for some countries are higher than that due to natural sources; individual doses depend on specific examinations .
Atmospheric nuclear testing	0.005	Some higher doses around test sites still occur.	The average has fallen from a peak of 0.11 mSv in 1963.
Occupational exposure	0.005	~0-20	The average dose to all workers is 0.7 mSv. Most of the average dose and most high exposures are due to natural radiation (specifically radon in mines).
Chernobyl accident	0.002*	In 1986, the average dose to more than 300,000 recovery workers was nearly 150 mSv; and more than 350,000 other individuals received doses greater than 10 mSv.	The average in the Northern hemisphere has decreased from a maximum of 0.04 mSv in 1986 . Thyroid doses were much higher.
Nuclear fuel cycle (public exposure)	0.002*	Doses are up to 0.02 mSv for critical groups at 1 km from some nuclear reactor sites.	
Total artificial	0.6	From essentially zero to several tens	Individual doses depend primarily on medical treatment, occupational exposure and proximity to test or accident sites.

* Globally dispersed radionuclides. The value for the nuclear fuel cycle represents the maximum per caput annual dose to the public in the future, assuming the practice continues for 100 years, and derives mainly from globally dispersed, long-lived radionuclides released during reprocessing of nuclear fuel and nuclear power plant operation.

As can be seen from the numbers given in Table 4, about 80% of the annual dose worldwide is due to the natural sources of radiation. In the developed countries, however, as can be seen from Figure 3, the main contribution to the average annual exposure dose comes from medical applications. Moreover, for example, in the United States of America the dose has increased more than 5.5-fold since 1987 (Figure 3). In Belgium, since the beginning of use of man-made sources of radiation, the average annual exposure dose has doubled [28] (Figure 3). The main contribution to the total collective effective dose (47% of it) is made by computed tomography (CT) scanning, the average dose per examination in 1997-2007 was 7.4 mSv [29]. In Belgium, CT scans contribute to 59% of doses received from diagnostic X-ray examinations [28].

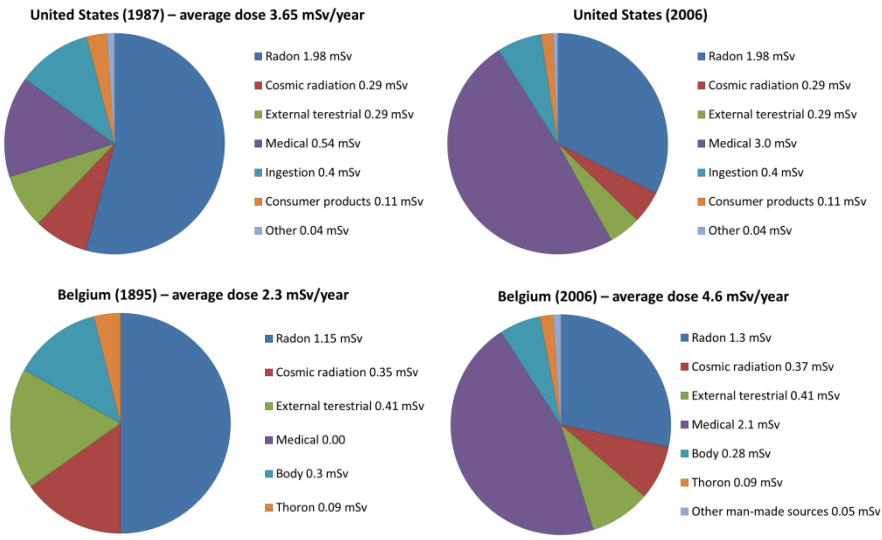

Figure 3. Upper panel: estimated contributions to public exposure from different sources of radiation in the United States in 1987 [30] and 2006 [31]. Lower panel: estimated contributions to public exposure from different sources of radiation in Belgium in 1895 and 2006 [28].

In a recent study it was shown, however, that the risks from the medical use of ionizing radiation are perceived much lower by the general population compared to the experts working with ionizing radiation. Opposite to this, the general population had a higher risk perception for nuclear waste, an accident in a nuclear installation and natural radiation, and they were more concerned about Belgian nuclear installations after the Fukushima accident [32].

The exposure scenarios most relevant for the present study are discussed below.

1.8.1 Nuclear power plant accidents

The electricity generation by nuclear power plants grew steadily since 1956. Although the doses from nuclear power reactors to which the general public is exposed are generally very low and decrease over time because of lower discharge levels [25], the possibility of serious accidents raises serious concerns among the general public.

To date there have been two major accidents classified as the highest possible Level 7 accidents on the International Nuclear Event Scale [33]: The Chernobyl accident on the 26th of April 1986 and the Fukushima Daiichi accident on the 11th of March 2011.

The accident at the Chernobyl nuclear power plant in 1986 is the most severe in history. In total 134 plant workers and emergency personnel were exposed to high doses of radiation (0.8 – 16 Gy) and suffered acute radiation syndrome [34]. Of these, 28 died within the first three months after the accident. Besides that, about 530,000 clean-up workers (known as “liquidators”) received doses of between 0.02 and 0.5 Gy [34], but for a number of reasons, individual doses were monitored inadequately (e.g. only one dosimeter was available per group) or were not registered at all (e.g. “pre-calculated” doses were used) for many of them [35]. In the longer term,

the general population was exposed to low levels of chronic radiation but there has been no consistent evidence yet of any other radiation-related health effects in the general population, except for more than 6,000 cases of thyroid cancer among the people who were children or adolescents in 1986 [34]. The absence of demonstrated increased risk of developing other cancer types, however, is not a proof that no increase has occurred, as such an increase is difficult to detect and requires well-designed epidemiological studies [36]. According to the predictions based on the models of radiation-associated risk from epidemiological studies, mainly of Japanese atomic bomb survivors, the predicted lifetime excess of cancer and leukaemia deaths was assessed to be about 4 000 cases for the three highest exposed groups (liquidators, evacuees and residents of the strict control zones) [37]. In addition, the fears about the effects of radiation and the uncertainty about the exposure doses in the affected population caused them to perceive themselves as helpless and lacking control over their future, which may have led to further health effects [38-40]. In fact, twenty years after Chernobyl, the Chernobyl Forum concluded that the biggest public health problem from Chernobyl accident was mental health [41].

The recent Fukushima accident was the second largest nuclear disaster ever. To date, there have been no fatalities that could be directly ascribed to the radiation exposure. In May 2013 UNSCEAR reported that "radiation exposure following the nuclear accident at Fukushima Daiichi did not cause any immediate health effects. It is unlikely to be able to attribute any health effects in the future among the general public and the vast majority of workers". The only individuals who received effective radiation doses of over 100 mSv predominantly from external exposures were 173 emergency and mitigation workers, 12 of which were estimated to have received absorbed doses to the thyroid from iodine-131 intake alone of 2 to 12 Gy. The average first-year effective doses to evacuees and to the population in the non-evacuated areas most affected by the accident were estimated to be in the range from about 1

to 10 mSv for adults and about twice as much for 1-year-olds [42]. In the World Health Organization (WHO) report the effective doses in the most affected areas of Fukushima prefecture were estimated to be within a dose range of 10–50 mSv [43]. Methodological options for estimation of the lifetime risks for cancer development were consciously chosen to avoid risk underestimation, therefore they are likely to represent the upper bound of the risk and are not absolute risks for developing such cancers. The additional lifetime risk for developing solid cancers (in females) was assessed to be around 1% (normally expected risk is 29%), for developing breast cancer (in females) – 0.36% (normally expected risk is 5.53%), for developing leukaemia (in males) – 0.04% (normally expected risk is 0.60%), for developing thyroid cancer (in females) – 0.50% (normally expected risk is 0.75%). The above-mentioned estimations are valid for individuals exposed as infants [43]. The WHO report underlines that, although for the general population inside and outside of Japan, the predicted risks are very low, there is need for long-term health monitoring of those who are at high risk, along with the provision of necessary medical follow-up and support services [43]. Therefore, it can be stated that radiation exposure in general was comparatively low and certainly below the threshold of acute radiation disease albeit that possible measurement inaccuracies must be taken into consideration. For the general public the probability of receiving doses higher than 100 mSv after a similar nuclear accident is relatively low but the uncertainty about the received dose can lead to additional stress causing psychological problems and more serious health consequences [40, 44]. Adequate assessment and communication of the levels of exposure as well as possible health consequences to the general public are therefore of great importance.

In order to create a sustainable EU network in biodosimetry the Realizing the European Network of Biodosimetry (RENEB) project was launched in 2012 [45]. The goal of RENEB is to support in a coordinated way the response in case of major nuclear or radiological emergency in Europe. In order to assess

the preparedness of biodosimetric laboratories for emergency situations as well as to compare/harmonize the applied methodologies RENEB organized several biodosimetry interlaboratory comparison exercises [46-55]. The final goal of the project is to use the established network as a part of EU radiation emergency management.

1.8.2 Space radiation

Humans in Space are subjected to galactic cosmic rays (GCR) and solar particle events (SPE) which cause significant but poorly understood risks of carcinogenesis [7]. The GCR spectrum is composed primarily of high-energy protons and atomic nuclei, namely about 87% high energy protons, 12% alpha-particles and 1% heavier ions up to iron [56] of very high energies ranging from hundreds of MeV per nucleon up to 1 GeV per nucleon [57], which are very difficult to shield from with currently used materials [58]. SPE consist of low to medium energy protons and α-particles, which enables efficient shielding inside the spacecraft but not inside a spacesuit during extravehicular activities. For the orbital missions a major contribution to the exposure dose is made by the geomagnetically trapped protons and electrons of the Van Allen radiation belt present at altitudes between 200 and 600 km known as the South Atlantic Anomaly (SAA) [59]. According to measurements performed during the Euromir ’95 mission on board the Mir Space station, the maximum dose due to crossing the SAA was 0.055 mGy and the mean dose rate inside the station was calculated to be 0.012-0.014 mGy/h, half of this value being due to the SAA [60].

According to the published data, chronic exposure to GCR occurs at a dose rate of 1.3 mGy/day (4.8 mSv/day equivalent dose) [61] and up to 0.5 Sv/h during large SPE [62]. Because of their high LET, a lower physical dose of high-charge and energy particles is needed to produce a certain biological effect compared to low-LET radiation. According to available calculations, during the trip to Mars every cell in the organism would be exposed to a

proton every 3 days, to an α-particle every 30 days and at least $3.2x10^{12}$ cell nuclei would be exposed to iron ions [63-65]. Based on the measurements of the energetic particle radiation inside the spacecraft containing the Curiosity rover on its way to Mars, the dose equivalent for the shortest round-trip there of about 500 days with current shielding was found to be 0.66 ± 0.12 Sv [61]. Radiation exposures during flight are always monitored using personal passive dosimeters (e.g. thermoluminescent dosimeters and solid-state nuclear track detectors), in order to detect the different radiation types encountered in Space [57]. However, these physical measurements only provide absorbed skin doses, which are consequently combined with computerized phantoms and radiation transport codes to estimate the dose at different regions of the body [66]. Therefore, physical dosimetry is always combined with biodosimetry data obtained by means of cytogenetic methods, which is more comprehensive in terms of the effects of shielding provided by the body itself as well as the effects of all radiation types [67]. Biodosimetry can also provide valuable information on individual sensitivity to radiation in the presence of additional stress factors such as, for example, microgravity.

Many of the aspects of health dysregulations associated with long-term Space flights are not yet fully characterized and may represent a serious risk to crew members during deep Space missions. To date, about 550 persons have flown to Space the majority of which remained there for less than 30 days, therefore, few effects of return missions to e.g. Mars can be predicted. The differences in physical characteristics and biological effects of Space and low-LET radiation make the usefulness of γ- or X-ray exposure data (from atomic bomb survivors and radiotherapy patients) in predicting heavy ion effects limited [7]. Cancer development remains the most important health risk factor in astronauts [7], but circulatory diseases are likely to be of great importance in the newer risk estimates for a mission to Mars [68], especially as recent data indicate that the cardiovascular disease mortality rate among Apollo astronauts, the only humans having travelled beyond the Earth's

magnetosphere, was 4–5 times higher than in non-flight and low-orbit flight astronauts [69]. Experiments in mice performed by the same group suggest that the observed sustained vascular endothelial cell dysfunction is caused by the exposure to high-LET iron ions, and not the simulated microgravity or the combination of both [69]. Numerous studies reported that not only high dose exposure during radiotherapy treatment of e.g. lung or breast cancer [70-73], but also moderate doses of up to 2 Gy in A-bomb survivors [74] and low-dose radiation exposure due to medical procedures or occupational exposures [75-78] were shown to increase the risk of developing ischemic heart disease and cardiovascular disease. In A-bomb survivors, exposed to lower doses, the observed cardiovascular effects are mainly ischemic heart disease and hypertension, suggesting that the vascular component might be more sensitive to lower doses [78, 79]. These observations suggest that the heart and blood vessels are not highly-radioresistant and are actually among the dose-limiting organs [80, 81].

Another known health hazard of Space radiation exposure is the higher risk of induction of cataracts [82, 83]. Furthermore, several recent studies in animals suggest that high-LET radiation exposure may lead to cognitive dysfunction [84, 85] or even enhance pathological progression of Alzheimer's disease [86], which could compromise mission critical activities [87].

1.8.3 Radiotherapy

Radiotherapy, along with chemotherapy and surgery, is one of the most common cancer treatment options. Radiation therapy contributes to the cure of approximately 23% of all cancer patients [88], thus playing an important role in cancer management. The majority of patients are treated with "conventional" photon beam therapy [88].

The major improvements in the efficacy of radiation therapy were always associated with significant progress in technology, moving from ortho-voltage X-rays to ^{60}Co and high-energy linear accelerators, combined with

more sophisticated diagnostic tools and radiation delivery methods [9]. The goal of any kind of radiotherapy is to deliver a high dose of radiation to the tumor site, while minimizing the dose to the surrounding healthy tissues and organs. Recent advances in external photon beams which can be precisely delivered to irregular targets via e.g. three-dimensional conformal, intensity modulated and image-guided radiotherapies are considered to be the culmination of photon therapy [9]. The search for further improvements is therefore directed to alternative radiation modalities such as hadron therapy.

Hadron therapy is an advanced radiotherapy method, which uses beams of charged particles such as protons and carbon ions, which has become a promising radiation treatment modality for some types of cancer, such as e.g. skull-base tumors, head and neck tumors, prostate cancer, hepatocellular adenocarcinoma, bone and soft tissue sarcomas, non-small cell lung cancer and recurrent rectal cancer [89-92]. The main advantage of charged particle beams is the possibility of more precisely targeting the tumor, while the surrounding healthy tissues receive a much lower dose compared to conventional photon radiotherapy [93]. This is possible as charged particles deposit only a small dose along the way to the target, followed by a sudden increase in the dose when the particle is ultimately stopped, known as the Bragg peak. The behavior of the particle can be precisely predicted based on its physical characteristics and the beam can be directed so that the Bragg peak occurs exactly within the tumor site [94]. High-LET carbon ions, in addition, also have a higher RBE compared to conventional low-LET photon therapy [95], as particles deposit their energy in a more concentrated manner resulting in clustered DNA damage which is more lethal to the tumor cells [96].

About 5–20% patients receiving radiotherapy show abnormal tissue responses (e.g., inflammations, infections, ulcerations, fibrosis, necrosis, dermatitis and rectitis) during, early or long time after the end of radiotherapy [97]. The idea of establishing an assay capable of estimating normal tissue

radiosensitivity before the onset of the treatment is highly appealing, as, in theory, this would allow exploiting the benefits of radiotherapy dose escalation among the non-radiosensitive patients, while subjecting the radiosensitive ones to the conventional or even reduced doses [98].

1.9 Cellular and molecular response to ionizing radiation

1.9.1 Radiation-induced DNA damage and repair

"DNA is, in fact, so precious and so fragile that we now know that the cell has evolved a whole variety of repair mechanisms to protect its DNA from assaults by radiation, chemicals and other hazards" (Sir Francis Crick (1988) *What Mad Pursuit*. Basic Books: New York).

As Crick expected, cells have evolved elaborate DNA damage repair (DDR) mechanisms to respond to DNA damage. It is estimated that each of the ~10^{13} cells in the human body receives tens of thousands of DNA lesions per day as a result of a variety of genotoxic attacks [99].

The interaction of radiation with cells is extremely complex, as it can occur through direct interaction of radiation with cellular components or through indirect damage caused by elevated radiation-induced ROS production which therefore initiates a complex cellular response. Depending on the type of DNA damage, mammalian cells can activate one of the four main repair pathways – nucleotide-excision repair (NER), base-excision repair (BER), homologous recombination (HR) and non-homologous end joining (NHEJ). A general overview of the most common types of DNA damage and their sources, as well as the repair mechanisms and consequences is depicted in Figure 4.

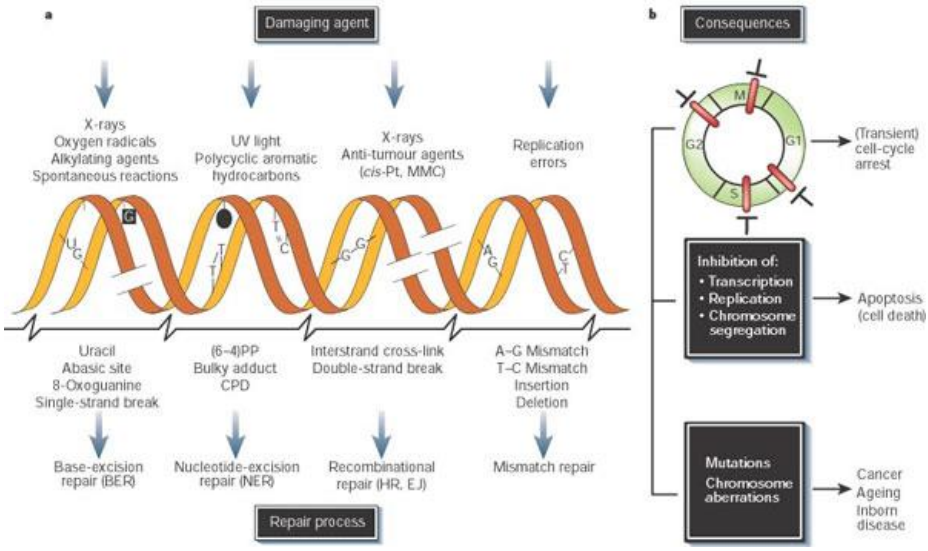

Figure 4. a. Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom). **b.** Acute effects of DNA damage on cell-cycle progression, leading to transient arrest in the G1, S, G2 and M phases (top), and on DNA metabolism (middle). Long-term consequences of DNA injury (bottom) include permanent changes in the DNA sequence (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects. cis-Pt – cisplatin, MMC - mitomycin C, (6–4)PP - 6–4 photoproduct and CPD – cyclobutane pyrimidine dimer. From [100].

The main types of DNA damage caused by ionizing radiation are (a) abnormal bases and single-strand breaks (SSBs), which are eliminated by BER, and (b) double-strand breaks (DSBs) repaired by recombination repair mechanisms (NHEJ or HR). The main components of these DDR mechanisms are listed in Table 5 [101].

Table 5. DDR mechanisms relevant for radiation-induced DNA damage and their components. From [101].

DDR mechanism	Types of lesions	Key protein components
BER and SSB repair	Abnormal DNA bases, simple base-adducts, SSBs generated as BER intermediates, by oxidative damage or by abortive topoisomerase I activity	DNA glycosylases (sensors), APE1 endonuclease, DNA polymerases (β, δ, ε) and associated factors, flap endonuclease FEN1, ligase I or ligase III. SSB repair can also involve polymerase β lyase activity, XRCC1, PARP-1, PARP-2, polynucleotide kinase (PNK) and aprataxin (APTX)
NHEJ	Radiation- or chemically-induced DSBs plus V(D)J and CSR intermediates	Sensors Ku and DNA-PKcs plus XRCC4, XLF/Cernunnos and ligase IV. Can also employ the MRE11-RAD50-NBS1 complex, Artemis nuclease, PNK, Aprataxin and polymerases μ and λ
HR	DSBs, stalled replication forks, inter-strand DNA cross-links and sites of meiotic recombination and abortive topoisomerase II action	RAD51, RAD51-related proteins (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, DMC1), RAD52, RAD54, BRCA2, RPA, FEN1, DNA polymerase and associated factors. Promoted by MRN, CtIP, BRCA1, and the ATM signalling pathway

1.9.1.1 DDR mechanisms relevant for radiation-induced DNA damage

NER is responsible for repairing a wide class of helix-distorting lesions mostly arising from exogenous sources (except for some oxidative lesions), that interfere with base pairing and obstruct transcription and replication. BER deals with small chemical alterations of bases mainly of endogenous origin which may or may not obstruct transcription and replication, although they frequently miscode. BER is therefore particularly relevant for preventing mutagenesis [100].

Whereas SSB and base damages can be usually correctly repaired, this is not always the case for DSBs. Therefore, DSBs are considered to be the most severe as they are more likely to result in chromosome aberrations, genomic instability and can ultimately lead to cancer [102, 103].

In NHEJ, DSBs are recognized by the Ku protein, which in its turn binds and activates the protein kinase DNA-PKcs, leading to recruitment and activation of end-processing enzymes, polymerases and DNA ligase IV [104]. HR is always initiated by the generation of single-stranded DNA (ssDNA) promoted by the proteins of the MRE11-RAD50-NBS1 (MRN) sensor complex. In events catalyzed by RAD51 and the breast-cancer susceptibility proteins BRCA1 and BRCA2, the ssDNA then invades the undamaged template. The following actions of polymerases, nucleases, helicases and other components result in DNA ligation and substrate resolution [105]. The mechanisms of NHEJ and HR are depicted in Figure 5.

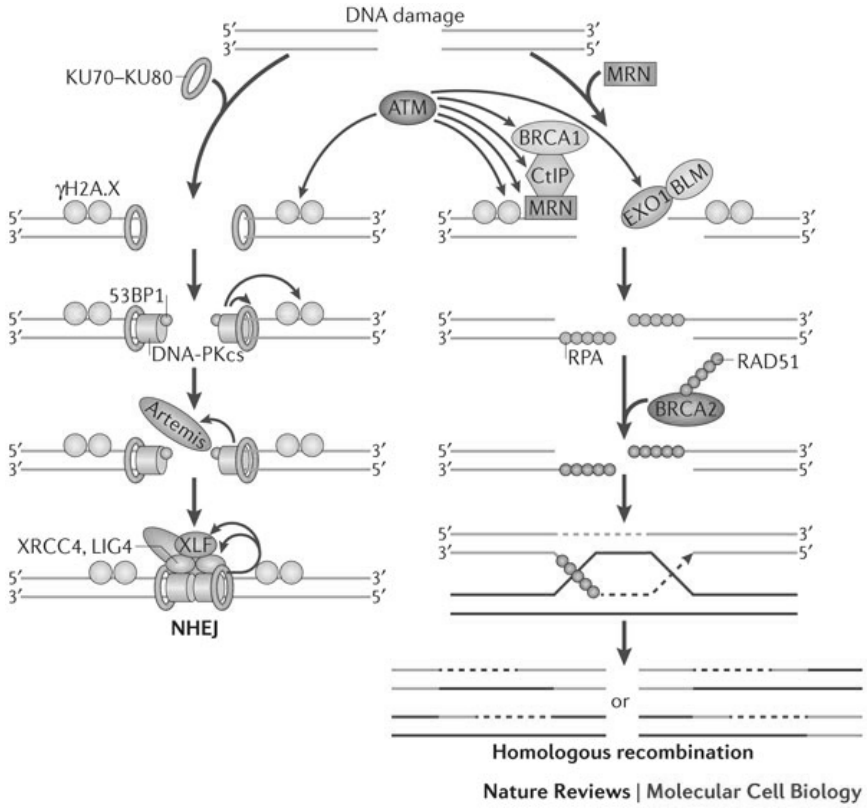

Figure 5. Mechanisms of homologous recombination and non-homologous end joining. From [106].

The relative contribution of the two DSB repair pathways varies in different cell types and in different phases of the cell cycle [107]. To a large extent this choice can be explained by the fact that HR requires the presence of an intact sister chromatid and, as sister chromatids are only available in G2 and late S-phase, this repair mechanism is clearly cell-cycle stage specific. In several studies on mammalian cells irradiated during different cell cycle phases, NHEJ was found to be important in all cell cycle phases and predominant in G1 and early S-phase, while HR is particularly important in late S/G2 phase [108, 109]. Cell cycle-dependent expression of the key repair proteins may also play a role in the DSBs repair mode. Cellular levels of several homologous recombination-specific factors such as BRCA1, RAD51 and RAD52 increase as cells progress from G1 to S phase [110]. The choice between the two DSB repair pathways is also partly determined by whether the DNA ends at the DSB require resection - the processing of DNA ends to generate 3′ single-strands, which is required for HR but inhibits canonical NHEJ in which DNA ends are protected with minimal processing before joining [111].

Protein kinases ATM and ATR, the key DDR-signalling components in mammalian cells, also activate protein kinases CHK1 and CHK2 which, together with ATM and ATR, reduce cyclin-dependent kinase (CDK) activity. Inhibition of CDKs delays or stops the cell cycle at critical stages before or during DNA replication (G1/S and intra-S checkpoints) and before cell division (G2/M checkpoint), preventing duplication and segregation of damaged DNA [112]. If the DNA repair is efficient, the resumption of normal cell functioning occurs. Alternatively, if the damage cannot be removed, chronic DDR signaling triggers apoptosis or senescence (i.e. permanent cell-cycle withdrawal).

1.9.1.2 Formation of DDR foci

In response to DSBs, PI3-K like kinases, including ATM, ATR and DNA-PKcs rapidly phosphorylate the conserved C-terminal tail of H2AX at serine-139. ATM and DNA-PKcs play an equal role in phosphorylating H2AX following ionizing radiation exposure, while ATR is more important for H2AX phosphorylation in response to DNA damage that would slow or stall replication forks [113]. The phosphorylation of H2AX creates γH2AX, which is crucial for modulation of chromatin structure and subsequent accumulation of various signalling and repair proteins to DNA breaks [114]. Several essential DNA-repair factors implicated either in HR (e.g., BRCA1, RAD51) or in both HR and NHEJ (e.g., RAD50, 53BP1) form nuclear foci that co-localize with γH2AX [115, 116]. Factors accumulating at DSB sites do not always co-localize perfectly, with some DDR proteins being present directly at damage sites, coating ssDNA resulting from DSB resection, and those associated with DSB-flanking chromatin (Figure 6) [117]. DDR proteins initially accumulate at DSB sites and then spread at distance via a positive feedback loop involving MDC1, which binds γH2AX, the MRN complex, and ATM kinase, which phosphorylates additional H2AX molecules further away from the break site [117]. Factors involved in NHEJ DSB repair are recruited within seconds upon break formation and dissociate within two hours, while HR factors show delayed and persistent recruitment to DSBs, reflecting different repair kinetics between these two pathways [117].

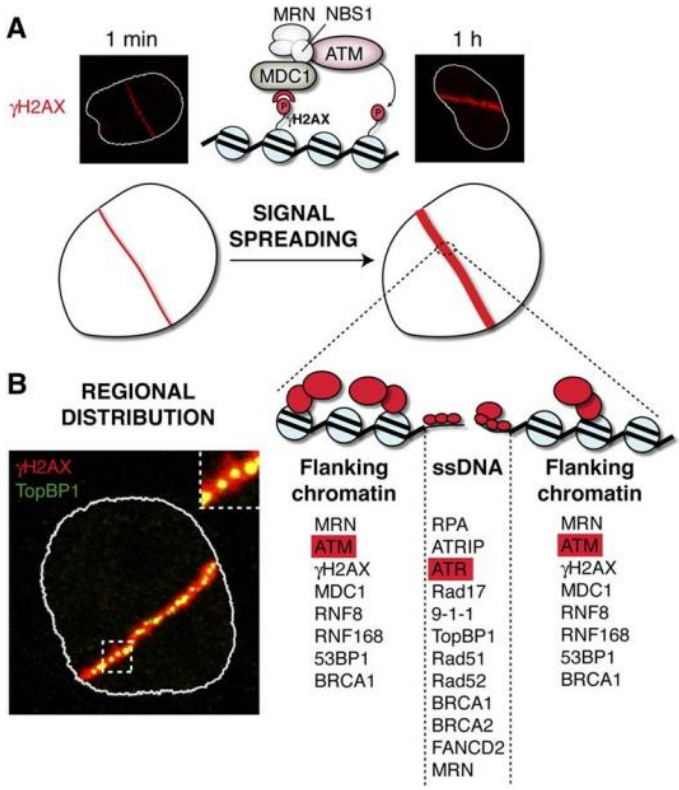

Figure 6. Spatial organization of DDR protein accumulation at DNA DSBs. **A.** DDR signal spreading via a positive feedback loop involving MDC1 and ATM kinase. **B.** Regional distribution of DDR proteins around DSBs. Factors involved in ATR signaling accumulate proximal to the break site on ssDNA generated by DNA end resection, while ATM signaling factors localize on flanking chromatin regions. From [117].

1.9.1.3 LET-dependent differences in DDR

It was demonstrated that after exposure to high- or low-LET radiation both the quality of the DNA repair as well as the kinetics differ [7, 118, 119]. In case of high-LET irradiation the high local ionization density of the particle will cause more complex DNA damage, also referred to as clustered damage [118, 120, 121]. Due to the complexity of this damage, the enzymatic activity needed for DNA repair will be retarded or might not occur at all, resulting in more cell death [118, 122]. The degree of complexity of the damage depends on the LET of radiation and reaches a maximum at the LET of 150-200 keV/µm [118,

123]. Above 200 keV/µm enough energy is deposited to obtain the same biological effect and some energy may be wasted, resulting in over-killing effect [124]. Studies of DSBs formation and repair after exposure to low- or high-LET radiation also showed that the repair kinetics is characterized by a fast component and a slow component and that these processes reflect the quality as well as the localization of the DNA damage in chromatin of different compactness [125-127]. Interestingly, DNA damage induced by the indirect effect of both γ-rays and α-particles was shown to be more efficiently repaired by NHEJ and BER compared to HR repair [128].

1.9.2 Transcriptional response to ionizing radiation: the central role of p53

DNA damage is the major consequence of radiation exposure in cells which leads to significant modulation of the transcriptome [129-132]. In response to radiation exposure several sensor molecules detect the induced DNA damage and rapidly accumulate at the damaged sites. These proteins initiate damage processing by transmitting a signal to transducers, which in their turn relay the signal to multiple downstream effectors involved in specific pathways, resulting in cell death or survival [133]. The activation of these signal transduction pathways results in altered expression of target genes.

One of the key checkpoint proteins in the DNA damage response is the transcription factor p53, which transcriptionally controls target genes involved in diverse pathways ranging from cell cycle arrest and survival to death by apoptosis [134]. In its normal state p53 has a short half-life as a result of binding to MDM2, which targets p53 to degradation through a ubiquitin-dependent pathway on nuclear and cytoplasmic 26S proteasomes [135]. Upon exposure to ionizing radiation both p53 and MDM2 are directly phosphorylated by ATM at Ser15 and Ser395, respectively [136]. In the meantime, ATM also phosphorylates CHK1 and CHK2, which in turn phosphorylate p53 at Ser20 [137]. The phosphorylation cascade leads to further phosphorylation of T18, weakening the interaction of p53 and MDM2,

ultimately resulting in stabilisation of p53 [138]. Other key transcription factors in the radiation response are nuclear factor κB (NF-κB), nuclear erythroid-derived 2-related factor 2 (NRF2), cAMP-responsive element-binding protein (CREB), activated protein 1 (AP-1), specificity protein 1 (SP1), and early growth response 1 (EGR-1) [139]. Nevertheless, p53 is considered to be the central player in the radiation response [131].

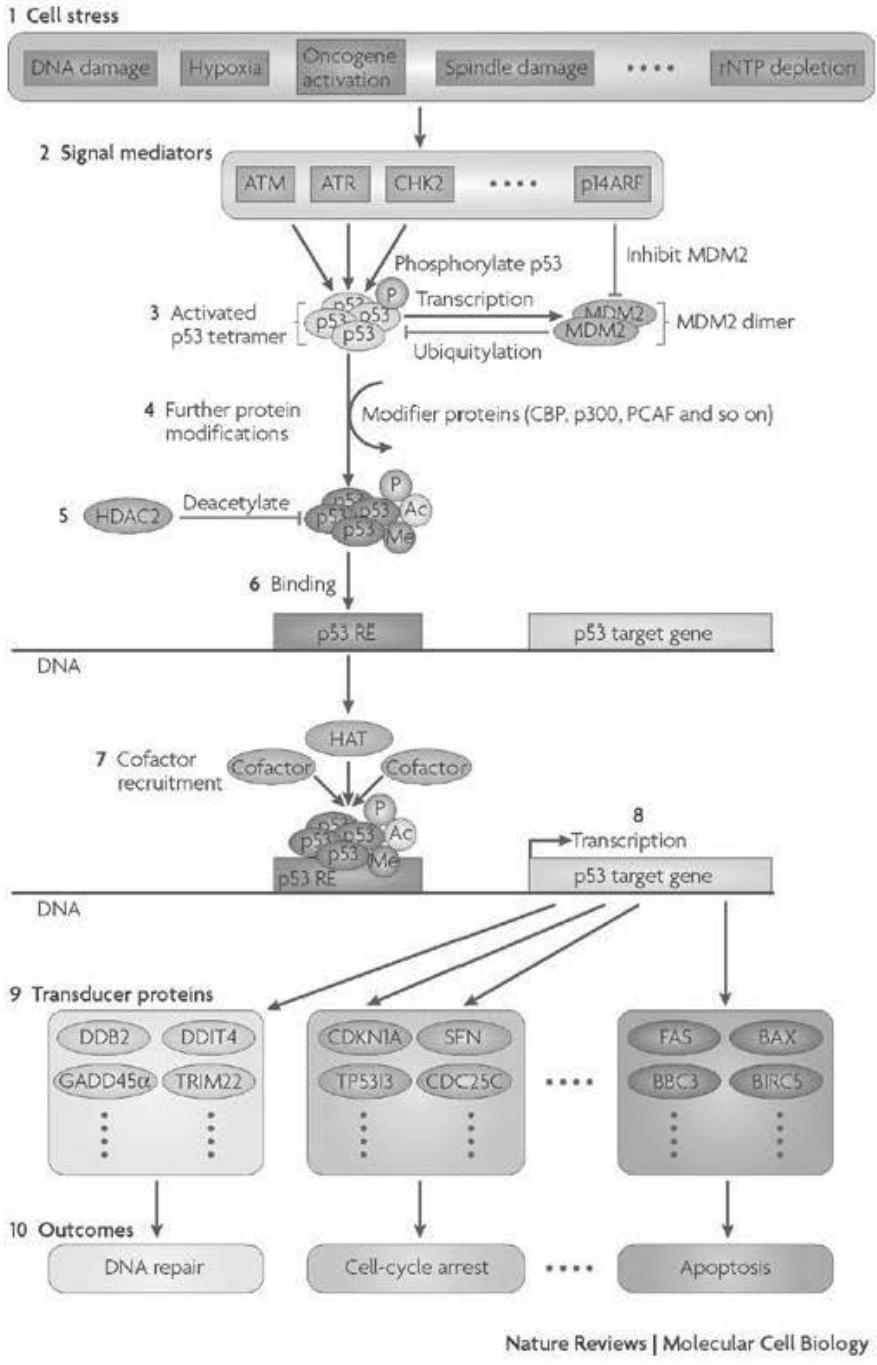

Figure 7. Mechanisms of p53 activation and regulation of downstream targets. From [140].

The p53 pathway responds to various cellular stress signals, including exposure to ionizing radiation, by transcribing a number of genes to accomplish several functions (Figure 7). The activation of p53 can have three main outcomes: cell-cycle arrest, DNA repair and apoptosis/senescence. Apoptosis and senescence are terminal for the cell, whereas cell-cycle arrest allows the repair to occur, so that the cell survives [140].

1.9.2.1 Role of p53 in DDR

p53 is also involved in DNA repair mechanisms, in both transcription-dependent and transcription-independent manner. The involvement of p53 as a transcription regulator in NER is limited to two known relevant regulatory targets - genes encoding the p48 protein (*DDB2*) and the XPC protein (*XPC*) [141, 142].

p53 has both transcription-dependent and transcription-independent functions in BER, an example being its interaction with endonuclease APE1/Ref-1, which functions during the removal of damaged bases and the subsequent repair of the resulting apurinic and apyrimidinic sites: APE1/Ref-1 modulates the trans-activation and pro-apoptotic functions of p53, while p53 in its turn seems to directly regulate transcription of the *APE1* gene [143]. In a study by Offer et al. p53 was also shown to enhance BER during G0-G1 cell cycle stages, while reducing BER and inducing apoptosis in G2-M stages [144].

Of the two DSB repair pathways, the role of p53 in NHEJ remains poorly understood. What is clear is that p53 has several genetic interactions with components of the NHEJ pathway that are manifested by downstream effects on cellular survival and cell cycle control or effects on DNA repair [143]. For example, p53 accumulation leading to cell cycle arrest and apoptosis was shown in cells depleted for Artemis endonuclease, which plays a key role in NHEJ [145].

Several studies demonstrated that p53 can regulate HR DSB repair transcriptionally by direct interaction between p53 and the *RAD51* promoter [146, 147], although its contribution was small compared to other transcription factors [148].

The specific transcriptional response to radiation is dependent on many factors, such as genetic background, cell type, radiation quality, time after irradiation, dose and dose rate [139]. It is also accepted that the severity of DNA damage is critical in directing the signaling cascade to reversible cell cycle arrest and DNA damage repair or to apoptosis [134]. Several studies also showed tissue specificity with distinct regulation of p53-induced genes in different cells and tissue compartments [149-151]. The list of p53 targets currently exceeds 100 well-validated genes and is constantly growing with dozens potential targets being identified [131, 152, 153]. The p53-regulated genes often found as radiation-responsive are, as could be expected, involved in cell cycle regulation (e.g. *CDKN1A*, *GADD45A*, *CCNG1*), DNA repair (e.g. *PCNA*, *XPC*, *DDB2*, *RAD51*, *POLH*) and apoptosis (e.g. *BAX*, *FAS*, *TNFRSF10B*, *BBC3, FDXR*) [154-159].

1.9.2.2 Role of p53 in cell cycle regulation

The key transcriptional target of p53, relevant for the G1 and G1/S checkpoint responses is the $p21^{CIP1/WAF1}$ (coded by the *CDKN1A* gene), which inhibits cyclin E and cyclin A/CDK2 required for the G1/S phase transition [160]. p21 also mediates cell cycle progression independently of cyclins and cyclin-dependent kinases by its direct binding to PCNA, another transcriptional p53 target, which leads to inhibition of DNA replication [160]. The G2/M checkpoint also partly relies on the transcriptional programmes regulated by p53, leading to the up-regulation of cell-cycle inhibitors such as p21, GADD45a and 14–3–3 sigma proteins [161]. In addition, the p53-activated p21 further blocks the phosphorylation of CDC2, thus blocking entry into mitosis [162].

1.9.2.3 Role of p53 in induction of apoptosis and senescence

In case DNA repair is not successful, p53 induces apoptosis through the intrinsic mitochondria-mediated pathway, which is predominantly mediated by mitochondrial outer membrane permeabilization (MOMP). p53-mediated MOMP is regulated by the anti-apoptotic and pro-apoptotic members of the BCL2 family proteins [163]. Several pro-apoptotic molecules important for MOMP were shown to be transcriptionally regulated by p53, e.g. Bcl-2 homologous antagonist/killer (*BAK1)* [164], B-cell CLL/lymphoma 2 (BCL2)-associated X-protein (*BAX)* [165], p53 up-regulated modulator of apoptosis (*PUMA)* [166] and *NOXA* [167]. Following MOMP, apoptogenic proteins, such as cytochrome c, are released to the cytosol. This p53-dependent process can be induced by extrinsic or intrinsic activation of effector caspases (e.g. caspase 3). The extrinsic pathway involves FAS receptor and tumour necrosis factor receptor and results in the activation of effector caspases or the initiation of the intrinsic pathway by inducing mitochondrial injury (loss of mitochondrial membrane potential or release of cytochrome C), which will eventually also lead to the activation of effector caspases [168].

Senescent cells are also characterized by enhanced expression of several p53-regulated senescence markers, e.g. p21, PML, PAI-1, and DEC1, all of which are able to induce senescence themselves [169].

1.9.3 Radiation-induced alternative transcription and splicing

As discussed above, DNA damage caused either by radiation or other factors triggers broad changes in the gene expression program of the damaged cells. A large body of evidence has shown that the expression of several genes involved in the DDR in addition to transcriptional regulation is also controlled by mechanisms regulating their splicing profile, the stability of their transcripts and/or their utilization by the translational machinery [170].

Alternative transcription and alternative splicing are responsible for the production of multiple mature mRNAs from a single gene. The human genome comprises less than 20 000 protein-coding genes, coding for almost 80 000 protein-coding transcripts and the estimated number of proteins synthesized from these transcripts is in the range of 250 000 to 1 million [171]. Alternative splicing of pre-messenger RNAs (pre-mRNAs), which is the alternative selection of exons to be included into mRNAs during splicing, is currently regarded as the main process contributing to both transcriptome and proteome diversity [172], although alternative transcription was shown to play a more important role in some cases, e.g. cerebellar development [173].

Alternative transcription initiation by the use of alternative promoters and transcription start sites leads to the formation of transcripts differing in their first exon or in the length of the 5′ untranslated region (5′-UTR). The use of alternative first exons leads to transcripts with different open reading frames giving rise to protein isoforms with alternative N termini, while transcripts with different 5′-UTR can be subject to differential translational regulation [171].

Alternative splicing comprises the following major events: exon skipping or cassette exon usage, use of alternative acceptor and/or donor sites, intron retention, and mutually exclusive exons [171] (Figure 8). Exon skipping appears to be the most common (occurs in ∼38% of mouse and human genes), while intron retention is the least common (occurs in ∼3% of the genes) [174].

Another regulatory layer of gene expression is the process of polyadenylation [175]. The use of alternative polyadenylation (APA) sites results in the formation of transcripts differing in their 3′ ends. Transcripts arising from APA may differ in either their coding region (if APA sites are located in a different exon or intron) or in the length of their 3′ untranslated region. APA can have an effect on transcript localization, stability, translation efficiency and on the nature of the encoded protein [171].

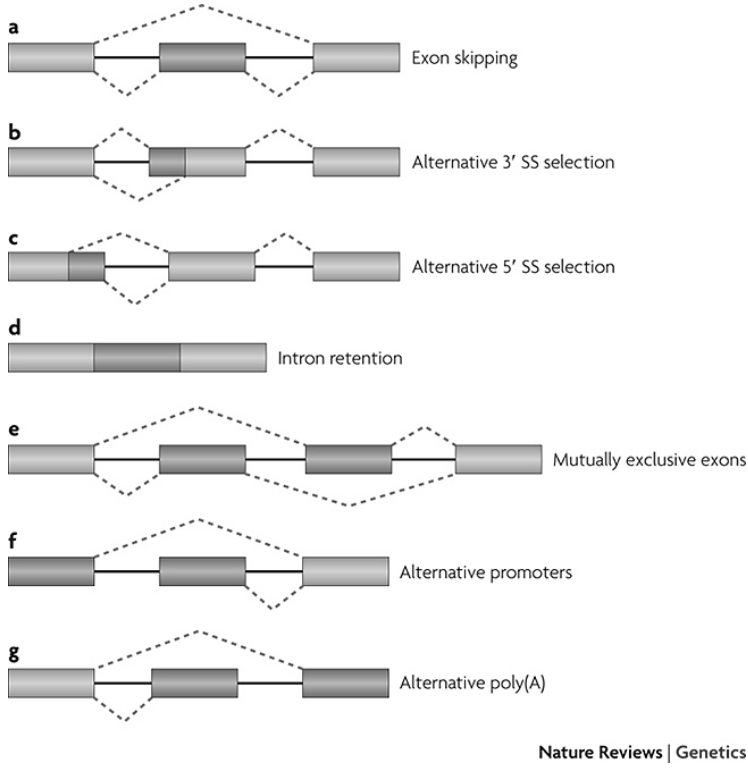

Figure 8. Alternative splicing and transcription. **(a)** Exon skipping, in which exon known as a cassette exon is spliced out of the transcript. **(b)** Alternative 3′ splice site (3′ SS) and **(c)** 5′ SS selection occur when two or more splice sites are recognized at one end of an exon. **(d)** Intron retention occurs when an intron remains in the mature mRNA transcript. **(e)** Mutually exclusive exons. (f) Alternative promoter usage**. (g)** Alternative polyadenylation. From [176].

The physiological activity of proteins encoded by different transcript variants from the same gene may, however, differ dramatically. For instance, several genes such as *BCL2* family members and *TID1*, have been shown to encode both pro- and anti-apoptotic protein isoforms [177, 178]. p53, TRAF2, APAF1, caspases 2 and 8, survivin, and PIG3 are other examples of DDR effectors regulated at the level of alternative splicing making cell survival/apoptosis decisions [179, 180]. Transcription of the *TP53* gene also involves positive and negative regulation from several promoters, and it is also subject to multiple alternative splicing events, such as e.g. intron 2 retention and an in-frame deletion of 198 bp between exons 7 and 9 [181].

In addition to modulating the relative expression of protein isoforms through AS, stress can impact on the “productivity” of gene expression, for example by inducing “unproductive” splice variants, such as those with premature stop codons, which are rapidly degraded by the nonsense-mediated decay pathway [170]. Several reports demonstrated a striking regulation of the splicing of MDM2, the main p53 negative regulator, by various genotoxic agents. The MDM2 alternatively spliced variants lack up to 8 (out of 12) exons that include the p53-binding domain and nuclear localization signal, thus allowing the increase in p53 protein levels and induction of the p53 pathway [182, 183].

Stress caused by DNA damaging agents widely modulates the alternative splicing of genes involved in the DNA repair, cell-cycle control and apoptosis, thus expanding their functional diversity [170]. For example, cisplatin favors the production of pro-apoptotic splice variants of *c-FLIP*, *CASP8*, *CASP9* and *BCL-X* through up-regulation of SC35 splicing factor by E2F1 [184]. UV irradiation of human fibroblasts induces ATM-dependent changes in alternative splicing of *ATRIP* gene, which is an essential component of DNA damage checkpoint signaling [185]. UV irradiation promotes splicing shifts in genes involved in cell cycle control, such as *CHEK2*, *MAP4K2* and *ABL1* [186].

In addition to affecting the alternative splicing of specific DDR-related genes, DNA damage was shown to play an important role in the regulation of alternative splicing in general. UV radiation affects co-transcriptional alternative splicing in a p53-independent manner through hyperphosphorylation of RNA polymerase II and inhibition of transcription elongation thereby affecting the selection of alternative exons [187]. Transcription-blocking DNA lesions trigger profound changes in spliceosome organization affecting preferentially late-stage spliceosomes [185]. Additionally, the same study showed a reciprocal regulation between ATM-controlled DDR signaling and the core spliceosome, demonstrating ATM

contribution to selection of genetic information ultimately included in mature transcript [185]. DNA damage also affects transcription, post-translational modifications (such as phosphorylation) and localization of splicing factors, which interact with exonic and intronic regions of pre-mRNA and control the recruitment and activity of the spliceosome [170].

Several recent studies have shown that a large number of genes are alternatively spliced or transcribed in response to (UV) radiation [187-190]. However, to the best of our knowledge, there have been no studies carried out so far to assess the alternative transcription and splicing initiation by high-LET radiation. In most of the cases the changes observed for other radiation types are due to alternative promoter usage by p53 [188, 190]. While the exact functional impact of these changes is not known in most of the cases, many of the affected genes are involved in DNA repair, cell-cycle control and apoptosis. The exact sequence identity of radiation-induced splice variants also remains to be identified by means of e.g. next-generation sequencing. Functional characterization of radiation-induced transcript variants would also increase the understanding of the molecular mechanisms of radiation response.

1.10 Radiation biomarkers

A biomarker is defined as “any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological”[191]. Characteristics of a good biomarker include sensitivity, specificity, reproducibility, known variability in the general population [191] and the possibility for non-invasive and simple sample collection (especially for children) [192].

Pernot et al. suggested to classify radiation biomarkers into four categories, based on temporal parameters [192] (Figure 9):

a) *biomarkers of exposure*, which are available at some point after exposure and can be used to estimate the dose received
b) *biomarkers of susceptibility or individual sensitivity*, which are expected to remain constant during the lifetime and therefore are available before, during or after exposure and can be used to predict an increased risk of radiation-induced health effects
c) *biomarkers of late effects*, which remain present and become more evident with time after exposure and can be used to assess health effects before clinical detection of the radiation-induced disease
d) *biomarkers of persistent effects*, which allow the assessment of radiation effects appearing soon after exposure and present a long time after it and at different clinical stages

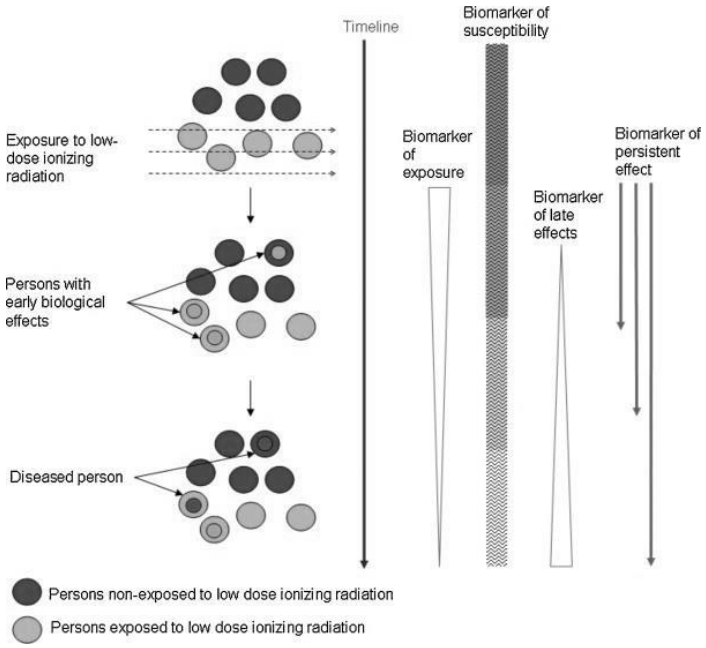

Figure 9. Timing of radiation-induced disease processes and relation with the different types of biomarkers. From **[192]**.

In the review by Pernot et al., the following biological classification of radiation biomarkers was suggested (Figure 10):

a) cytogenetic biomarkers (dicentrics, translocations, complex chromosomal rearrangements, premature chromosome condensation, telomere length and micronuclei)
b) biomarkers related to nucleotide pool damage (DNA single/double strand breaks, extracellular 8-oxo-dG)
c) biomarkers related to germline inherited mutations/variants (single nucleotide polymorphisms and inherited gene mutations, copy number variants and alterations)
d) biomarkers related to induced mutations (glycophorin A in MN blood group heterozygotes, hypoxanthine-guanine phosphoribosyl transferase gene)
e) biomarkers related to transcriptional and translational changes (changes in RNA levels, changes in protein levels, changes in cytokine levels)
f) biomarkers related to epigenetic modifications (histone modifications, DNA methylation, miRNA, phosphoproteome)
g) other (reactive oxygen species, metabolites and metabolomics, cell cycle delay, apoptosis and cell survival, biophysical markers)

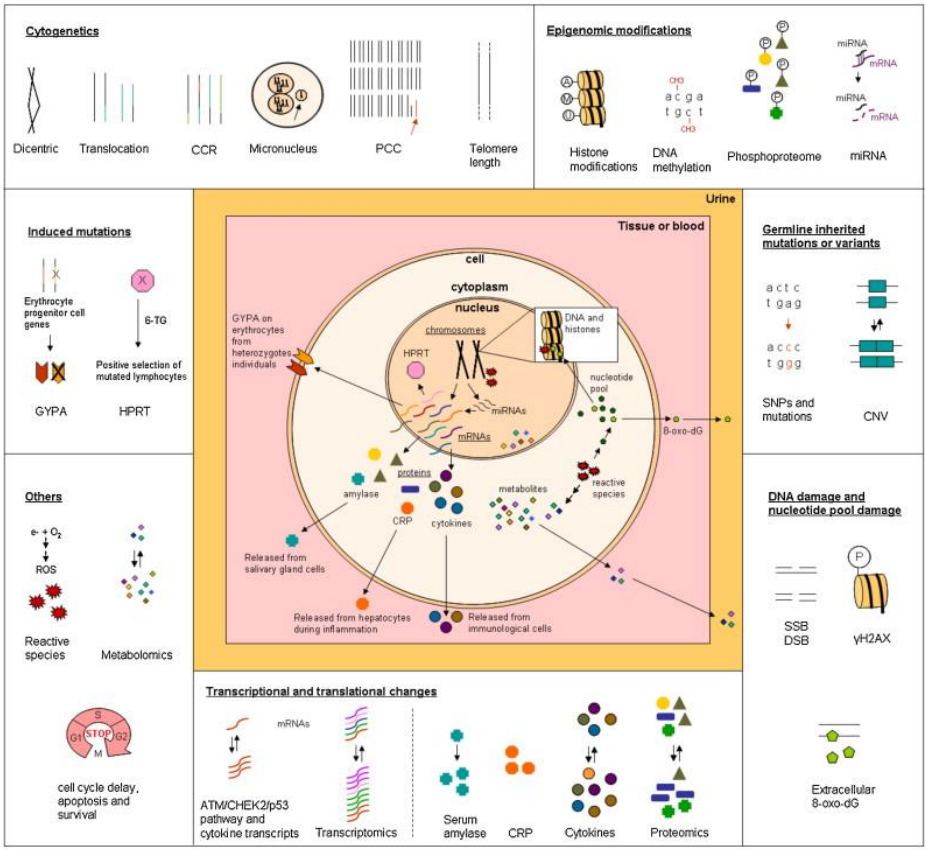

Figure 10. Biological classification of radiation biomarkers. Vertical double lines represent pairs of chromosomes and horizontal double lines represent double strands of DNA. A: acetyl group; CCR: complex chromosomal rearrangement; CNV: copy number variant; CRP: C-reactive protein; DSB: double strand break; GYPA: glycophorin A; HPRT: hypoxanthine-guanine phosphoribosyltransferase; M: methyl group; miRNA: microRNA; P: phosphate group; PCC: premature chromosome condensation; ROS: reactive oxygen species; SNP: single nucleotide polymorphism; SSB: single strand break; U: ubiquitin; 6-TG: 6-Thioguanine; 8-oxo-DG: 8-Oxo-deoxyguanosine. From [192].

The overview of different radiation biomarkers is given in Table 6.

Table 6. Overview of radiation biomarkers. Modified from [192].

Biomarker		Sensitivity	Specificity	Time window after exposure during which assays might be performed	Suitability as biomarker of exposure	Suitability as biomarker of susceptibility	Suitability as biomarker of late effects	Suitability as biomarker of persistent effects
Cytogenetics	**Dicentrics**	0.05-5 Gy	Almost exclusively induced by radiation	Before renewal of peripheral blood lymphocytes	√	P	P	P
	Translocations	0.25-4 Gy	Confounding factors: smoking; strong age effect	Years	√	P	P	√
	CCR	Unknown	High LET and heavy ion exposure	Before renewal of peripheral blood lymphocytes	√ (high-LET radiation)	P	P	√
	PCC rings and fragments	0.2-20 Gy	Specific to a large extent	PCC fragments: ideally immediately after exposure PCC rings: before renewal of peripheral blood lymphocytes	√			
	Telomere length	Unknown	Not specific: modulated by viral infection Potential confounders: age, oxidative stress	Unknown	P	P	P	P
	Micronuclei	0.2-4 Gy	Not specific: modulated by genotoxins Confounding factors: age, gender	In lymphocytes: before renewal of peripheral blood lymphocytes In reticulocytes: not yet established	√	P	P	
Nucleotide pool damage and DNA damage	**SSB/DSB**	0.1-8 Gy	Not specific: modulated by several mutagens and oxidative stress Confounding factors: age, smoking, diet	Minutes to days	√	P		
	γH2AX	0.01-8 Gy	Not specific: also formed in response to UV and other genotoxins	Minutes to days	√	P	P	P
	Extracellular 8-oxo-dG	1–100 mGy	Not specific: also formed by endogenous oxidative stress Confounding factors: unknown	1–2 h post irradiation	√ (oxidative stress)	P		

Table 6 (continued). Overview of radiation biomarkers. Modified from [192].

Biomarker		Sensitivity	Specificity	Time window after exposure during which assays might be performed	Suitability as biomarker of exposure	Suitability as biomarker of susceptibility	Suitability as biomarker of late effects	Suitability as biomarker of persistent effects
Germline inherited mutations/variants and induced mutations	SNP, CNV and inherited gene mutations	Unknown	Not specific Confounders unknown	Not time dependent		√	P	P
	CNA	Unknown		Not yet established	P			P
	GYPA	>1 Gy	Not specific: formed after exposure to other genotoxins	Years	√			√
	HPRT	>90 mGy	Not specific: formed after exposure to other genotoxins	Months	√			√
Transcriptional and translational changes	Changes in the mRNA levels of the ATM/CHK2/p53 pathway	Unknown	Not specific	Probably hours to days	√	P		
	Changes in RNAs identified by transcriptomics	5 mGy-2Gy	Unknown	1–3 days	√	P	P	P
	Serum amylase	>1Gy	Not specific	24 h after exposure	√			
	Proteins identified by proteomics	Moderate to very good	Unknown	10 min–40 weeks	P	P	P	P
	Cytokines	>1.2 mGy	Not specific	24 h after exposure	P	P	P	P
Epigenomic modifications	Histone modifications	Unknown	Unknown	Unknown	P	P	P	P
	DNA methylation	Unknown	Unknown	Unknown	P	P	P	P
	miRNA	Unknown	Unknown	Unknown	√	P	P	P
	Phosphoproteomics	Unknown	Unknown	Unknown	P	P		
Other biomarkers	ROS	Unknown	Not specific	Minutes to hours	√	P	P	P
	Metabolites and metabolomics	Unknown	Not specific	Unknown	√	P	P	P
	Cell cycle delay, apoptosis and survival	Unknown	Unknown	Unknown	P	P		
Direct dosimetry on samples	EPR/ESR	Unknown	Not specific	Days to years	√			√
	Internal emitters	>1 mGy		Days to years	√			

√: direct evidence that this biomarker could be used as such; P: potential or theoretical use; CCR: complex chromosomal rearrangement; CNA: copy number alteration; CRP: c-reactive protein; DSB: double strand break; EPR/ESR: electron paramagnetic resonance/electron spin resonance; GYPA: glycophorin A; HPRT: hypoxanthine-guanine-phosphoribosyl transferase; IR: ionizing radiation; miRNA: microRNA; PCC: premature chromosome condensation; SNP: single nucleotide polymorphism; SSB: single strand break; ROS: reactive oxygen species.

The most recent advances in radiation biomarkers research can be found in the review by Hall et al. [193]. The biomarkers relevant for the present study will be further discussed in more detail.

1.10.1 Biomarkers of exposure

Biomarkers of exposure are probably the best established and validated, especially for moderate and high doses of radiation, with multiple biological endpoints being used to assess the exposure dose. These biomarkers are particularly important for such exposure scenarios, as nuclear power plant accidents and smaller-scale accidental radiation exposures, as well as terroristic attacks, during which rapid dose assessment for the affected population is required [194].

1.10.1.1 Dicentric assay

Cytogenetic measurements, more specifically the dicentric chromosome (i.e. chromosome with two centromeres) assay, is considered to be the gold standard in biodosimetry, its main advantage being the specificity to radiation exposure [195]. In order to produce a dicentric aberration, DNA damage must be induced in two unreplicated chromosomes located in close proximity so that the damaged chromosomes can undergo exchange as a result of the misrepair of DNA strand breaks induced directly by the radiation, or as a result of misrepair during the base excision repair [195]. Dicentric chromosomes are stable in non-dividing cells such as lymphocytes but as the half-life of blood lymphocytes is in the order of weeks to years depending on the sub-population [196], this assay is the biomarker of choice for recent exposure. This well-validated method can be used to assess doses as low as 100 mGy if up to 1000 cells are analyzed and also to distinguish between partial and whole body exposures [197] and high- and low-LET radiation exposures [198]. However, this method also suffers from several drawbacks. It is time-consuming, laborious and requires special training and experience, which makes it less appropriate for screening of large cohorts of individuals in

a short time. Several automated systems allowing for faster dicentric scoring have been developed so far [199-201]. A simplification of dicentric scoring can also be achieved with the application of TC-FISH – the technique which allows to simultaneously stain telomeres and centromeres [202].

1.10.1.2 γH2AX assay

Another technique often used as biomarker of exposure to radiation is the γH2AX assay. Phosphorylation of histone H2AX which occurs within a few minutes of DNA damage provides a good marker of induction and repair of DSBs caused by radiation [203]. The number of γH2AX foci in the cell nucleus is directly proportional to the number of DSBs formed, and their dephosphorylation correlates with DSBs repair [204]. γH2AX foci formation is not specific to radiation exposure and can arise following multiple cellular processes, e.g. replication fork stalling/collapse at regions of single stranded DNA, the repair of DNA adducts, crosslinks [205], UV-induced pyrimidine dimers [206] and at later stages of apoptosis during DNA fragmentation [207]. The main advantage of the γH2AX assay is its high sensitivity. Several recent studies on patients undergoing CT-scans showed that γH2AX foci could be detected after exposure to low doses of 10 mGy [208] and of ≈3 mGy (average dose to the blood) [209] and very low doses of 0.22-1.22 mGy (average dose to the blood) [210]. It was also demonstrated that γH2AX assay is suitable for estimation of partial body exposures [211] and that it outperforms the analysis of dicentrics in this regard [212]. The spatial distribution of radiation-induced DNA breaks and the phosphorylation-dephosphorylation kinetics depends on radiation quality [213-215], therefore γH2AX assay is potentially suitable for assessing this parameter. However, extensive use of the γH2AX assay as a biomarker of exposure is limited due to the fast decline of the signal. There are also other critical features which might help increasing the reliability of the assay, such as standardization of experimental protocols, used specimens and statistical analysis [216].

1.10.1.3 Gene expression

The fast development of high-throughput transcriptomic profiling technologies since the late 1990s allowed to analyze the expression of a large number of genes simultaneously and initiated the search for mRNA biomarkers of radiation exposure. One of the earliest microarray studies investigating the effect of radiation on gene expression levels in isolated human peripheral blood lymphocytes found a dose-dependent induction of a number of genes up to three days following exposure [217]. Since then experimental data, obtained by means of microarrays [218], quantitative nuclease protection assay [219], NanoString technology [154], quantitative PCR (qPCR) [155], or chemical ligation-dependent probe amplification assay [158] proved to be efficient in providing accurate and rapid prediction of radiation exposure. Importantly, most of these studies point to a number of genes showing consistent responses in different irradiation set-ups and experimental models confirming their high potential as radiation biomarkers [220]. Most of the identified genes are known to be regulated by p53 (e.g. *MDM2*, *DDB2* [221], *FDXR* [222], *PCNA* [223], *GADD45A* [224], *RPS27L* [225], *SESN1* [226]), and involved in classical p53-mediated pathways, such as cell cycle regulation, DNA damage repair and apoptosis.

The specificity of the gene expression response as radiation biomarker is a complex issue, as these genes are responsive to DNA damage, which can be caused by other DNA-damaging agents. Multiple confounding factors, such as age, gender, infections and inflammatory diseases, smoking status and lifestyle in general might influence the induction levels of specific genes.

Recent studies have shown that gene expression analysis is suitable for determining exposure to very low doses of radiation, as some of the modulated genes are induced by doses as low as 5-25 mGy [155, 227, 228]. Nosel et al. showed dose-dependent regulation of genes involved in DNA damage repair and p53 signaling starting from 25 mGy, while other genes

involved in cellular respiration, ATP metabolic processes and chromatin organization showed constant modulation from 5 mGy exposure dose [228]. Knops et al. could identify nine genes responsible for proteolysis and apoptosis regulation which are suitable to predict doses as low as 20 mGy with a sensitivity of 86.7% [227].

Most of these studies, however, used *ex vivo* irradiated blood samples or even isolated PBMCs which might poorly reflect the *in vivo* response to irradiation. Several genome-wide studies have been undertaken to assess the *in vivo* transcriptional response to ionizing radiation using blood samples from radiotherapy patients undergoing either total body irradiation [229] or local intensity modulated radiotherapy [230]. The results of these investigations indicate that *in vivo* irradiation mainly affects genes involved in pathways that are related to the immune system and inflammatory responses, as well as p53-mediated pathways. Accordingly, induction of p53-dependent genes was observed in patients either undergoing CT scans (up to 43 mGy) or receiving (F-18)-fluoro-2-deoxy-d-glucose (6 mGy) [231]. Overall, the examined genes were induced in all samples, although differences in the *in vivo* and *in vitro* response were found, especially for doses below 50 mGy [231]. Other studies have identified *in vitro* gene signatures that could accurately predict the *in vivo* radiation exposure status [55, 158, 232, 233]. Overall, these studies have shown that the *in vitro* transcriptional radiation response is a reliable model for the *in vivo* situation. A recent study in patients undergoing treatment with radionuclides (^{131}I-labeled metaiodobenzylguanidine) also confirmed the validity of biomarkers of external exposure such as *CDKN1A*, *DDB2* and *BAX* as indicators of internal exposure [234].

Recent technological advances, such as customized qRT-PCR arrays [235] or multiplex qRT-PCR assays [154, 155], which allow rapid PCR amplification of a number of genes sufficient for dose estimation, are more appropriate for high-throughput screenings in case of a large-scale accident compared to genome-wide technologies [55]. The above-mentioned technologies allow fast

processing of a large number of samples in virtually any laboratory possessing a qPCR instrument, offering gene expression-based biodosimetry the advantage of high-throughput, in comparison with classical biodosimetry methods, such as dicentric chromosome assay.

One of the disadvantages of gene expression is that the effect is short-lived (up to a few days, depending on the dose), which should nevertheless be sufficient for use in case of a radiological accident [236]. Another disadvantage of the method is a saturation effect observed for doses higher than 2 Gy [51, 55]. In experiments in mice such saturation was observed following acute exposure to 6 Gy [237]. Importantly, both effects – the fast decline of the signal with time and the effect saturation at higher doses, might be significantly affected by *in vitro* experimental conditions. Therefore further validation of the assay *in vivo* is of pivotal importance.

1.10.2 Biomarkers of susceptibility

Defects in DNA repair mechanisms often result in abnormal radiosensitivity of cells, for example in such syndromes as ataxia telangiectasia [238], ataxia-telangiectasia like-disorder [239], radiosensitive severe combined immunodeficiency [240], Nijmegen breakage syndrome [241], and LIG4 deficiency [242]. More specifically, all the above-mentioned syndromes are caused by defects of repair of DNA DSBs, which are considered to be the most specific and severe damage caused by radiation, likely to result in chromosome aberrations and genomic instability [102, 120, 243].

The research into cellular markers predictive of clinical radiosensitivity was first focusing on colony-forming assays [244, 245], followed by reliable surrogate endpoints, such as scoring of chromosomal aberrations [246, 247]. Other studies aiming at establishing an assay for radiosensitivity prediction were focusing on the measurement of DNA DSBs repair efficiency by means of the comet assay [248-250] or the γH2AX assay [250, 251]. Nevertheless, no single cell-based assay proved to be capable of discriminating the full

range of cellular radiosensitivity, independently on the cause of it and there is not enough evidence of their utility in clinical practice [98]. It might suggest that other mechanisms such as altered cell cycle or defective apoptosis could play a critical role toward determining radiosensitivity.

Alternatively, gene expression profiling might be a promising predictive parameter for radiosensitivity [250]. Greve and co-authors identified a set of 67 genes differentially expressed in non-irradiated peripheral blood lymphocytes and those exposed to 5 Gy of γ-rays, which allowed to distinguish between the group of severely radiosensitive and non-radiosensitive breast, head and neck carcinoma patients. Most of the identified genes belong to the apoptosis or cell cycle regulation pathways [250]. Another study involving 21 prostate cancer patients with severe late radiotherapy complications and 17 patients without such symptoms identified a classifying gene signature in 2 Gy X-ray irradiated peripheral blood lymphocytes predicting radiosensitivity in 63% of the patients [252]. Rieger and co-workers used microarray gene expression profiling in lymphoblastoid cells derived from a diverse group of cancer patients with acute radiation toxicity. A set of 24 genes predicted radiation toxicity in 9 of 14 patients with no false positives among 43 controls [253]. A recent study by Forrester and co-authors identified an 8-gene signature which could potentially predict predisposition to fibrosis in patients prior to radiotherapy treatment [254]. It was suggested that radiosensitivity prediction will probably require a multi-parametric approach, as several, seemingly independent molecular pathways are likely to be involved in the development of late adverse tissue reactions [98]. In this respect, gene expression measurements are more flexible and promising than assays measuring single cellular endpoints.

2.

Scope and outline of research

Every human living on Earth is constantly exposed to very low levels of natural ionizing radiation, which are harmless. Occasionally, though, individuals are exposed to radiation doses exceeding the natural background levels, often as a result of medical diagnostic tests and treatments but sometimes through the accidental or deliberate release of radioactivity. Identification of radiation biomarkers of exposure, which can be used as biological dosimeters following a large-scale nuclear accident or a terroristic attack are of pivotal importance. At the same time, specific biomarkers of individual radiosensitivity would be of great value for personalized radiotherapy treatment or for the selection of crews for long-term Space missions.

As the model for our study we chose peripheral blood or isolated peripheral blood mononuclear cells, depending on the type of experiment, for several reasons. First, blood is a comparatively easily accessible biological sample, which can be collected from a large number of people in case of an accident. Second, there are several well-validated products for RNA extraction available on the market, which can be adapted for the needs of specific study design. Third, blood cells are among the most radiosensitive cell types of the body. And last but not least, using blood, which is a liquid tissue composed of multiple cell types suspended in plasma containing extracellular matrix, cytokines, chemokines and hormones gives a better representation of *in vivo* response to radiation. In our experiments we mainly used 250 kV X-rays which is the type of low-LET radiation historically used for biodosimetry

purposes in IAEA recommendations. In addition, we used accelerated carbon ions and iron ions, which are representative of hadron therapy and Space radiation, respectively.

The recent nuclear accident at Fukushima Daiichi in March 2011 led to recognition of the urgent need for effective biodosimetry tools which could confirm or quantify exposure to radiation in large cohorts of individuals potentially exposed to unknown doses for the purpose of triage and treatment [255]. Another important lesson learned from the Fukushima accident was that for the general public the probability of receiving doses higher than 100 mSv in a similar “low-dose scenario” is relatively low. On the other hand, the uncertainty about the exposure dose for a large number of individuals highly concerned about the possibility of radiation exposure known as “worried wells” can lead to additional stress causing psychological problems and more serious health consequences [40, 256].

Besides the development of biodosimetric assays as such, the investigation of their suitability for low-cost, fast measurements, which do not require specific training or instruments is of pivotal importance.

Studying the differences in biological response of normal cells to photon and particulate radiation is also important for several reasons and applications. First of all, adequate prediction of the treatment outcome in comparison to the conventional photon radiotherapy would allow optimal exploitation of the benefits of hadron therapy. Secondly, in view of the exposure of Space crew to heavy ions during long-term Space missions, it is important to study whether the response of normal cells to Space radiation and low-LET radiation is comparable and that therefore the radiation protection regulations based on predominantly low-LET epidemiological studies can be extrapolated in case of high-LET exposures.

In order to address the above-mentioned issues we divided our study in three main steps described below:

1. As the first step of our study, we aimed at **identifying gene expression biomarkers of exposure to low-LET radiation with increased sensitivity to low-dose exposures.** To this end, PBMCs from healthy volunteers were exposed *in vitro* to two X-ray doses relevant for medical triage: a moderate dose of 1.0 Gy which might result in acute radiation syndrome [257] and is associated with a high probability of long-term stochastic health effects and a low dose of 0.1 Gy which is not associated with any immediate acute health effects but might require medical follow-up as the risk of long-term effects, particularly cancer, must be taken into consideration [258]. We performed a whole-genome microarray study in order to identify the biomarkers most suitable for classification of the samples according to the exposure dose. We also assessed transcriptional events which might have an impact on the identified gene expression biomarkers. The results of this study are summarized in the Research article I entitled "Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry" [259] (**Chapter 3**). We also confirmed the validity of our approach in a biodosimetry interlaboratory comparison study organized by the RENEB consortium, the results of which are published in a research article by Abend et al. [55]
2. Microarrays are an invaluable tool for whole-genome radiation response studies but they are not appropriate for use in large-scale radiological accidents due to significant costs, time inefficiency and the complexity of the analysis. Therefore, as the second step of our study, based on our previous microarray results we **designed a customized qRT-PCR array for biodosimetry** comprising a panel of 25 genes. For the genes exhibiting alternative splicing, we designed primers interrogating the most sensitive exons. This allowed us **to validate our approach using a system applicable to large-scale radiological accidents.** We used peripheral blood samples from

healthy volunteers exposed to doses from 0 to 2 Gy at several time points post-irradiation from 8 to 48 h as reference samples and the samples from other individuals were used as “blind samples”. In this study we also compared different methodologies for RNA extraction available on the market and evaluated their applicability to emergency situations. The results of this study are summarized in Research article II entitled “Gene expression-based biodosimetry using customized qPCR arrays: assessment of dose and time after exposure” (**Chapter 4**). We also used these customized qRT-PCR arrays in the second biodosimetry interlaboratory comparison organized by the RENEB consortium [51, 260].

3. As the third and final step of this study we aimed to **compare the transcriptional response and DNA repair kinetics of human PBMCs after exposure to different radiation types**. To achieve this goal, we compared the transcriptional profiles of PBMCs of healthy donors after the cells were exposed to 1 Gy of X-rays, iron ions or carbon ions. To assess DNA damage induction and repair kinetics, we performed γH2AX staining at different time points after exposure. With an eye to possible long-term Space flights, we also opted for investigating the potential of a core signature of genes responsive to iron ions exposure to serve as an indicator of varied DNA repair capacity of healthy astronauts, which are expected to be selected for a similar mission. The results of this study are summarized in the Research article III entitled “Transcriptional profiling of human peripheral blood mononuclear cells following the exposure to low- and high-LET radiation” (**Chapter 5**).

The detailed results obtained in each of the above-mentioned steps of the study are described in the following Chapters 3-5. The final **Chapter 6** “General discussion and perspectives” entails the general discussion on the

importance of our findings and highlights the future perspectives on radiation biomarkers research.

3.

Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry

This chapter is modified from:

Macaeva E., Saeys Y., Tabury K., Janssen A., Michaux A., Benotmane M., De Vos W., Baatout S., Quintens R. *Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry.* Scientific Reports 6, 19251; doi: 10.1038/srep19251 (2016).

3.1 Abstract

Accurate assessment of the individual exposure dose based on easily accessible samples (e.g. blood) immediately following a radiological accident is crucial. We aimed at developing a robust transcription-based signature for biodosimetry from human peripheral blood mononuclear cells irradiated with different doses of X-rays (0.1 and 1.0 Gy) at a dose rate of 0.26 Gy/min. Genome-wide radiation-induced changes in mRNA expression were evaluated at both gene and exon level. Using exon-specific qRT-PCR, we confirmed that several biomarker genes are alternatively spliced or transcribed after irradiation and that different exons of these genes exhibit significantly different levels of induction. Moreover, a significant number of radiation-responsive genes were found to be genomic neighbors. Using three different classification models we found that gene and exon signatures performed equally well on dose prediction, as long as more than 10 features are included. Together, our results highlight the necessity of evaluating gene expression at the level of single exons for radiation biodosimetry in particular and transcriptional biomarker research in general. This approach is especially advisable for practical gene expression-based biodosimetry, for which primer- or probe-based techniques would be the method of choice.

3.2 Introduction

The recent nuclear accident at Fukushima Daiichi in March 2011 and the subsequent growing concerns about large-scale human radiation exposure have triggered the widespread recognition that there is an urgent need for effective biodosimetry tools that are capable of confirming or quantifying exposure to radiation in large cohorts of individuals potentially exposed to unknown doses for triage and personalized treatment [255].

Following the Fukushima accident, the only individuals who received effective radiation doses of over 100 mSv, were 173 emergency and mitigation workers. Despite this generally low radiation exposure, which was clearly below the threshold of acute radiation disease, about 90,000 people were evacuated as a preventive safety action. This measure reduced the levels of possible exposure but also resulted in a number of evacuation-related deaths due to stress and/or lack of medical and social welfare facilities [42]. Hence, a rapid and accurate biodosimetry method would reduce the uncertainty about received doses and may mitigate psychological and health problems related to additional stress among the individuals who may or may have not been exposed to radiation (the so-called “worried wells”) [44, 256].

Cytogenetic measurements, more specifically dicentric assays, are considered the gold standard in biodosimetry [195]. While reliable and applicable to assess doses as low as 100 mGy, this method is time-consuming and laborious, and is not amenable to rapid diagnostics. A promising alternative technique consists in using gene expression data. Indeed, experimental data obtained by means of microarrays [218], quantitative nuclease protection assay [219], NanoString technology [154], quantitative PCR [155], or chemical ligation-dependent probe amplification (CLPA) assay [158] have proven most efficient in accurately and rapidly assessing radiation exposure.

Several recent studies have shown that transcriptome analysis at the individual exon level may significantly add to our understanding of the transcriptional response to radiation exposure [188-190]. In particular, alternative transcription and alternative pre-mRNA splicing dramatically expand the translational repertoire. We hypothesize that alternative transcription and splicing analyses applied in the context of radiation exposure may generate additional radiation biomarkers with potentially increased sensitivity.

To test our hypothesis, we established gene and exon signatures that may serve as radiation biomarkers and subsequently compared their reliability and effectiveness. We opted for two X-ray doses relevant for triage purposes (0.1 and 1.0 Gy) and compared these to sham-irradiated control samples. We evaluated the predictive performance of gene and exon signatures using three different statistical models, which were further used to assess the robustness of our gene signature on an independent, publicly available dataset (Figure 1). Our results yield new insights into transcriptional biomarker identification studies using genome-wide strategies and underline the importance of investigation of gene expression at the single exon level.

3.3 Materials and Methods

Experimental procedures are schematically summarized in Figure 11.

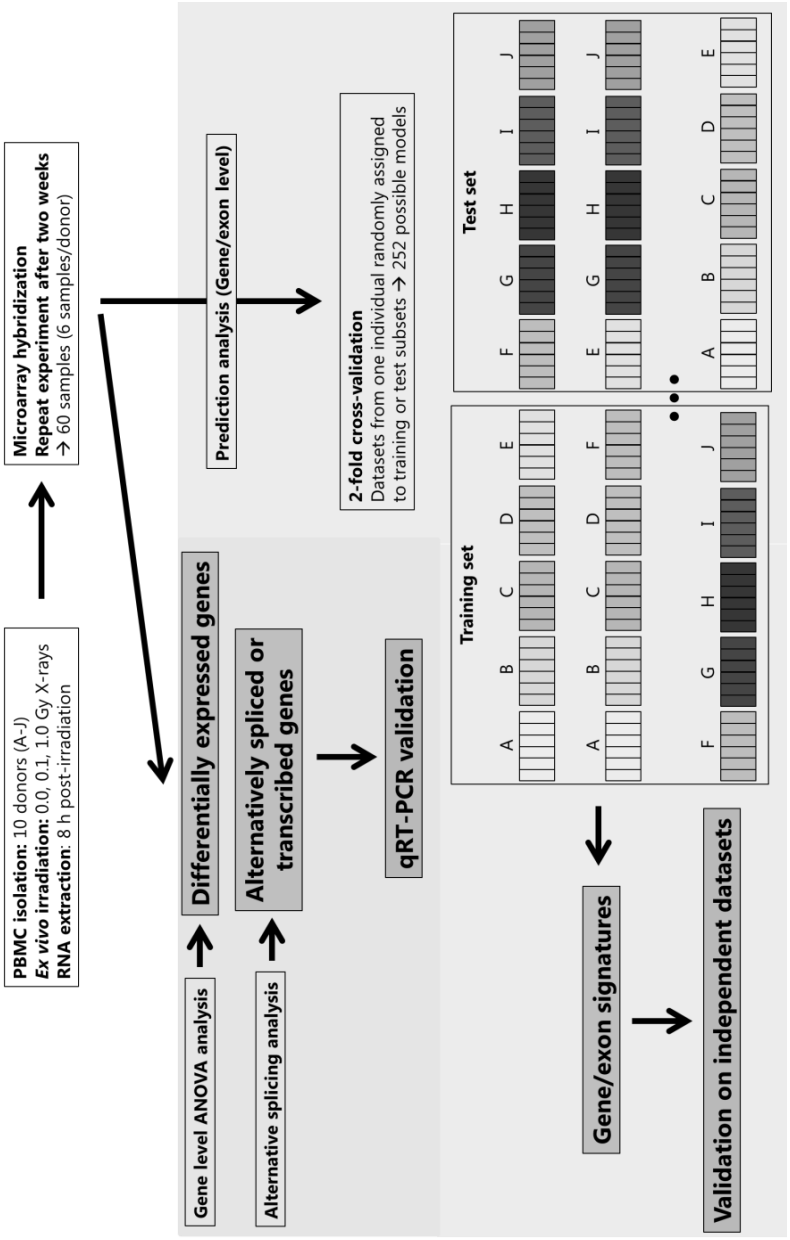

Figure 11. Outline of the strategy to identify gene and exon signatures suitable for prediction of exposure to doses of 0.1 and 1.0 Gy of X-rays. Isolated PBMCs from 10 healthy donors were irradiated and RNA was extracted 8 h after irradiation. RNA was hybridised onto Affymetrix Human Gene 1.0 ST arrays and differentially expressed and alternatively spliced genes were identified and validated using qRT-PCR. Three classification algorithms were employed to identify predictive gene and exon signatures and their predictive performance was tested using 2-fold cross-validation (boxes labeled with letters A-J represent all the samples from each out of ten donors (0.0, 0.1 and 1.0 Gy, in duplicates). The best performing gene signature as identified by the RF model was further evaluated on an independent, publicly available dataset.

3.3.1 Blood collection and PBMCs isolation

Peripheral blood samples used for microarrays were collected from 10 healthy, non-smoking Caucasian donors (5 males/5 females; age range: 23-50 years; median age: 28 years) in EDTA vacutainer tubes. All procedures followed were approved by the local SCK•CEN Ethics Committee and were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. All donors had signed an informed consent form prior to blood donation. Within 30-60 min of blood drawing, PBMCs were isolated by centrifugation on Histopaque-1077 (Sigma-Aldrich, Bornem, Belgium) density gradient according to the manufacturer's instructions. Isolated cells were suspended at a density of 10^6 cells/ml in LGM-3 culture medium (Lonza, Walkersville, MD, USA) and were allowed to equilibrate to culture conditions at 37°C in a humidified 5% CO_2 atmosphere. Two weeks later, the experiment was repeated using fresh PBMCs from the same donors, resulting in a total of 60 samples for microarray hybridization. For quantitative RT-PCR (qRT-PCR) validation, blood collected from 5 different donors (1 male and 4 females), from whom informed consent had been obtained, was subjected to identical procedures as the samples used for microarray hybridization, unless otherwise indicated. To confirm the results obtained for isolated PBMCs in blood samples, blood was collected from 3 donors (1 male and 2 females), from whom informed consent had been obtained, in EDTA vacutainer tubes, which were then directly used for irradiation.

3.3.2 *In vitro* irradiation

Cells were irradiated "free-in-air" at 21°C in a horizontal position with single doses of 0.1 and 1.0 Gy of X-rays from a Pantak HF420 RX generator at an air kerma rate of 0.26 Gy/min or were sham-irradiated. More detailed information on the irradiation setup can be found in the Supplementary Methods. Following *in vitro* irradiation, PBMCs were incubated at 37°C in a

humidified 5% CO_2 atmosphere. Blood samples were incubated on a rocking platform at 37°C without additional CO_2 supply for the indicated time points.

3.3.3 RNA extraction

RNA from irradiated and sham-irradiated PBMC samples was extracted 8 h after irradiation for microarray hybridization and 8 and 24 h for qRT-PCR validation. RNA from blood samples used for qRT-PCR validation was extracted 8 and 24 h after irradiation. For RNA isolation from PBMCs, a combined approach consisting of the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) extraction method and purification on Qiagen RNeasy columns (Qiagen, Venlo, The Netherlands), was used. More detailed information on the RNA extraction procedure can be found in the Supplementary Methods. The QIAamp RNA Blood Mini Kit (Qiagen, Venlo, The Netherlands) was used to extract RNA from blood samples. The starting quantity of blood was 1.5 ml per sample. All procedures were performed following the manufacturer's instructions. RNA concentration was measured on a NanoDrop-2000 spectrophotometer (Thermo Scientific, Erembodegem, Belgium) and the quality of total RNA samples was assessed using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). All samples had a RIN >8 and were therefore considered as suitable for further processing for microarrays and qRT-PCR.

3.3.4 Microarray hybridization

Gene expression profiling was performed using the GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA), which interrogates 28,536 well-annotated genes with 253,002 distinct probe sets, allowing expression analysis at both gene and exon level. Since each probe corresponds to one exon in most of the cases, we will further refer to probe set-level analysis as exon-level analysis. More detailed information on the microarray hybridization procedure can be found in the Supplementary Methods. All microarray data

are available in MIAME compliant format at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-3463.

3.3.5 Microarray data analysis

3.3.5.1 Gene and exon level ANOVA

The obtained microarray data were imported into Partek Genomics Suite, version 6.6 (Partek Inc., St Louis, MO, USA) as .CEL-files. Probe summarization and probe set normalization were done using the Robust Multichip Analysis (RMA) algorithm [261], which includes background correction, quantile normalization and $\log_2$ transformation. Microarray data were analyzed both at the level of probe sets and probe sets summarized to genes using a three-way ANOVA with dose, gender and batch as factors. Inclusion of batch in the model allowed correcting for differences between experiments resulting from different scanning days of the microarrays. To correct for multiple testing, we used the false discovery rate (FDR) as described by Benjamini and Hochberg [262] to adjust *p*-values (FDR < 0.05). We also performed linear contrasts between two specific groups (0.1 Gy vs control and 1.0 Gy vs control) within the context of ANOVA. The coefficients of the levels in the two compared groups add up to 0. The computations of *p*-values are based on Least-squares means, which are the means adjusted by other factors. Genes and exons were considered significantly differentially expressed between the two groups if adjusted *p*-values were < 0.05 with no fold change cutoff. Pearson's correlation coefficient was used to assess dose dependence of the gene expression levels. We used the Principal Components Analysis tool of the Partek software as an exploratory method to detect groupings in the dataset as well as to spot possible outliers. This technique is used to describe the structure of high dimensional data by reducing its dimensionality. It is a linear transformation that converts *n* original variables (genes or exons, in our case) into *n* new variables, which have three important properties: principal components are ordered by the amount of

variance explained, they are uncorrelated and they explain all variation in the data. PCA was performed at both gene and exon level using normalised expression values. The correlation method applied to calculate the dispersion matrix adjusted the data to be standardised to a mean of 0 and standard deviation of 1.

3.3.5.2 Alternative splicing analysis

To predict alternative splicing in irradiated samples compared to controls, we used three different methodologies, since it is known that alternative splicing analysis from gene arrays is prone to generate false positive results [263]. First, we performed Alternative Splicing ANOVA in Partek. A FDR-corrected *p*-value of < 0.05 was considered significant for alternative splicing events. To further reduce the number of false positives, we excluded the probe sets with $\log_2$ value < 3.0 (noise level) in all samples from analysis, except for the cases where there was a significant difference in expression of a single exon between the groups ($p < 0.05$). Next, we used two supplementary methods to perform a pairwise comparison of the samples (0.1 Gy vs 0.0 Gy and 1.0 Gy vs 0.0 Gy) to further increase the reliability of our results. Gene Array Analyzer [264] is an on-line tool that uses the Splice Index algorithm [265] and allows the user to perform more advanced filtering, i.e., removing probe sets that are not expressed in at least one group, removing genes (transcript clusters) that are not expressed in both groups, discarding probe sets with high potential for cross-hybridization and those with very large gene-level normalized intensities. Software parameters were set to default values, except for the Splice Index cutoff, which was set to 0.5. AltAnalyze [266] is an open-source software utilizing the FIRMA algorithm which is another method for detection of alternative splicing [267]. Software parameters were set to default values, except for the Minimum alternative exon score and the Maximum absolute gene-expression change, which were set to 0.5 and 50, respectively.

3.3.5.3 Positional Gene Enrichment analysis (PGE)

The PGE tool [268] is available at http://homes.esat.kuleuven.be/~biouser/pge/. We used default parameters to detect positional enrichment of radiation-responsive genes.

3.3.5.4 Prediction analysis

The following statistical models were evaluated with regard to their predictive performance and identification of a minimal list of genes and exons capable of discriminating between exposure conditions: generalized linear models, Random Forests [269] and Nearest Shrunken Centroids as implemented by the PAM (Prediction Analysis for Microarray) method [270]. A more detailed description of these models can be found in the Supplementary Methods.

To compare the predictive performance of genes and exons, two versions of the dataset were constructed: (a) a version measuring expression changes at the gene level, and (b) a version measuring expression changes at the exon level.

Cross-validation was used to assess whether classification models could be constructed to predict the different conditions. The original dataset was split into a part for model training (training set) and a part for model evaluation (test set), where both sets are disjoint. In our case, the cross-validation had to be executed at the level of individuals, since otherwise correlations between different conditions of the same biological sample might have led to overoptimistic results. This setting best mimics the true setup where new, unseen biological samples need to be classified by the model. A higher number of possible train-test combinations results in a more robust assessment of model performance, since higher numbers of models could be averaged. Therefore, we finally used 2-fold cross-validation for the prediction analyses (Figure 11).

For model hyperparameters that needed to be tuned (such as the lambda value for GLM or the threshold for PAM), an internal cross-validation on the training partition in each cross-validation loop was used. This optimal value was subsequently used to train a final model on the training partition in each cross-validation loop, and produce results for the test partition in each cross-validation loop.

Performance of the individual models was evaluated by calculating the AUC in which a value of 0.5 corresponds to random prediction behavior and a value of 1 to optimal prediction performance. This is known to be a robust estimator of model performance over different model decision thresholds.

To validate our results on an independent publicly available dataset, we retrieved data from Paul and Amundson [271] (GEO accession number GSE23515), describing a set of 95 samples from 24 individuals of different age, gender and smoking status exposed to different doses of radiation (0.0, 0.1, 0.5 and 2.0 Gy). After pre-processing, probe sets that did not map to gene symbols and probe sets containing more than 25% empty values were filtered out. After this filtering step, 23,031 probe sets were kept for further analysis. Subsequently, feature importance rankings were derived from classifiers based on RF and PAM as described above. The overlap between the top 100 genes from our study and those from Paul and Amundson was higher based on the RF ranking; therefore, the cross-validation was performed using this model.

The Venny on-line tool [272] was used to compare gene lists and create Venn diagrams: http://bioinfogp.cnb.csic.es/tools/venny/index.html

3.3.6 Reverse transcription and qRT-PCR

The following genes were selected for qRT-PCR validation: *DDB2*, *POLH*, *MDM2*, *TNFRSF10B*, *FDXR, ASTN2*, *NDUFAF6*, and *PCNA* (primer sequences can be found in Supplementary Table S1). RNA samples from 5

donors were used for cDNA synthesis using the GoScript™ Reverse Transcription System (Promega, Leiden, The Netherlands) with random hexamer primers. For each gene, qRT-PCR reactions were run in duplicate using the MESA GREEN® qRT-PCR kit (Eurogentec, Seraing, Belgium) on an Applied Biosystems® 7500 Real-Time PCR instrument following the manufacturer's instructions. To determine the efficiency and specificity of the designed primers, we ran a standard curve experiment with melt curve for every primer pair. Primer sequences and reaction efficiencies are listed in Supplementary Table S1. qRT-PCR data were analysed by 7500 Software v2.0.6 and Microsoft Excel using the Pfaffl method [273]. The relative amount of transcript of the selected genes was normalised to *PGK1* and *HPRT1* using the geometric mean of these reference genes [274]. Relative expression levels were tested for statistical significance using the paired *t*-test; *p*-values of < 0.05 were considered significant. Pearson's correlation coefficient was used to assess dose dependence of the gene expression levels.

3.4 Results

3.4.1 Low- and high-dose X-irradiation results in up-regulation of common genes

Whole-genome microarrays were used to analyze genome-wide transcriptional changes in human peripheral blood mononuclear cells (PBMCs) at 8 h after exposure to X-ray doses of 0.1 and 1.0 Gy compared to sham-irradiated control cells. We chose the 8 h post-exposure time point for our microarray experiments as in previous studies from our group a significant radiation-induced modulation of gene expression was shown at this time point [275, 276], while at earlier time points, e.g. 2 h, the transcriptional response was significantly less pronounced [155]. Microarray results for several genes were validated by qPCR for a later time point of 24 h, as shown below. Three-way ANOVA revealed 125 significantly differentially expressed genes (FDR < 0.05) between different doses of X-rays (Supplementary Table S2). Of these,

the large majority (90.4%) were dose-dependently induced (Pearson's correlation coefficients in Supplementary Table S2). Gene expression changes in response to radiation exposure were not gender-dependent (column FDR-corrected p-value (Dose*Gender) in Supplementary Table S2), as also previously suggested [271]. Unsupervised hierarchical clustering yielded a clear separation of the samples depending on the exposure dose (except for 3 out of 20 low dose-irradiated samples, which clustered together with the control samples) (Figure 12a). Similar results were obtained using principal component analysis (PCA) of the same dataset (Figure 12b). Comparison of the controls with each of the different doses yielded significantly increased expression levels following exposure to 0.1 Gy in 23 genes. Of these, 20 genes (87%) were also differentially expressed in cells irradiated with 1.0 Gy (Figure 12c). Unsupervised hierarchical clustering using this subset of overlapping genes resulted in a perfect separation of the samples by exposure dose (Figure 12d).

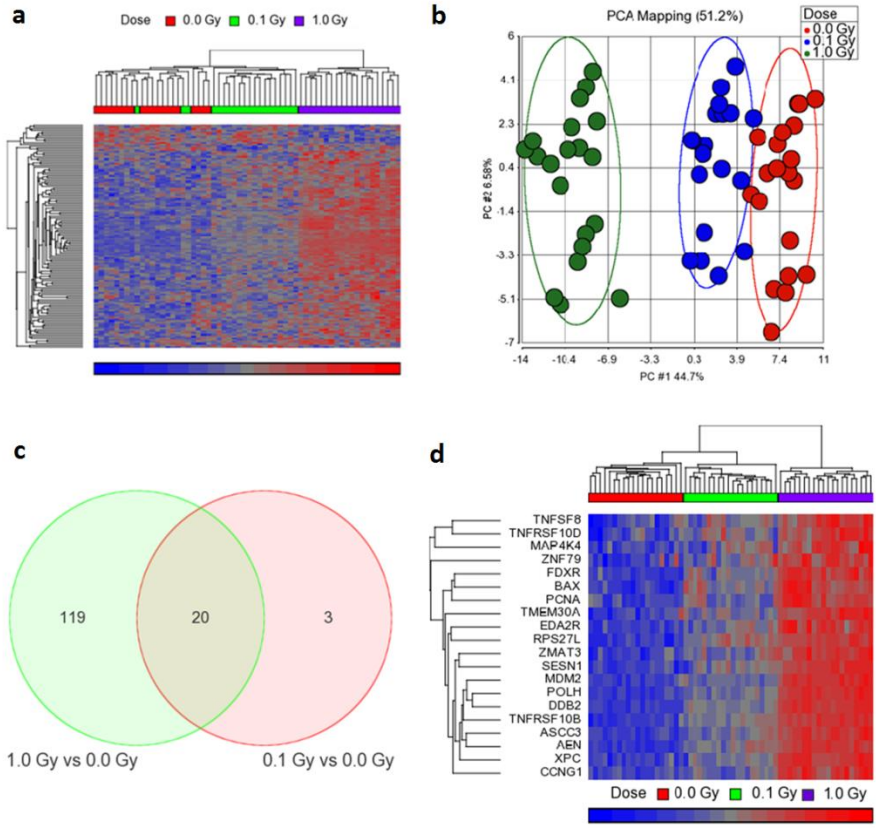

Figure 12. Gene expression changes in PBMCs in response to irradiation at 8 h after exposure. In total, 125 genes were identified as differentially expressed by ANOVA (FDR corrected *p*-values<0.05) between 0.1 Gy and 1.0 Gy samples and sham-irradiated controls. **a.** Unsupervised hierarchical clustering analysis of these 125 genes (plotted on y-axis) showed good separation of samples (x-axis) depending on the dose of exposure. Red: high gene expression, blue: low gene expression. **b.** 2D Principal Components Analysis (PCA) also separated samples depending on the dose. Each circle represents the expression profile of the 125 significantly differentially expressed genes in one sample. The percentage of the variance explained by the first and the second principal components is 44.7% and 6.58%, respectively. Ellipses represent two standard deviations. **c.** Venn diagram showing the overlap of differentially expressed genes after exposure to 0.1 and 1.0 Gy of X-rays. **d.** Unsupervised hierarchical clustering analysis of the 20 overlapping genes (plotted on x-axis) showed perfect separation of samples (y-axis) depending on the dose of exposure. Red: high gene expression, blue: low gene expression.

3.4.2 X-irradiation induces alternative transcription and splicing

Considering the well-documented ability of ionizing radiation to induce alternative gene splicing/transcription [187, 188], we first performed an Alternative Splicing ANOVA to identify which genes produced alternative transcripts following X-ray irradiation (Supplementary Table S3). Our results were in accordance with those of Sprung and co-authors [188] for the most significant genes, despite differences in experimental models (e.g. PBMCs versus lymphoblastoid cell lines) and conditions (doses, time points and gene expression platforms). The Splice Index algorithm with additional filtering identified much less alternatively spliced genes (3 genes after 0.1 Gy exposure and 17 after 1.0 Gy) (Supplementary Table S4). The FIRMA algorithm results were in keeping with those obtained by the Splice Index (37 genes after 0.1 Gy exposure and 39 after 1.0 Gy) (Supplementary Table S5) with 15 genes being identified as alternatively spliced in response to 1.0 Gy by both algorithms. The same 15 genes were also identified as highly significant by the Partek Alternative Splicing ANOVA algorithm, with 13 of them among the 30 most significant ones according to their p-values (Supplementary Table S3) and, importantly, all 15 genes were differentially expressed (Supplementary Table S2). It has been shown before that radiation-induced alternative splicing occurs predominantly in genes that are differentially expressed at the gene level [188, 190].

Although it is not possible to infer the exact sequence identities of specific transcript variants from the gene array results, it was clear that different alternative splicing and transcription mechanisms had been activated in response to radiation exposure. For example, we found evidence of transcription from alternative promoters (e.g. *ASTN2*, *NDUFAF6*, *FDXR* and *PCNA*), alternative transcription initiation (e.g. *ASTN2*), alternative splicing (e.g. *ASTN2* and *FDXR*), and use of alternative 3'-UTRs (e.g. *ASTN2*) (Figure 13a-d and Supplementary Figure S1). The observed variation in the expression levels between different transcripts was validated by qRT-PCR

using transcript-specific primers (Figure 13). We found significant differences in radiation-induced expression of different variants of *ASTN2* (Figure 13a), *FDXR* (Figure 13c) and *PCNA* (Figure 13d) at 8 h after exposure to 0.1 and 1.0 Gy, while this difference was not significant for *NDUFAF6* after exposure to 1.0 Gy, possibly because of large interindividual variations in the transcriptional response of this gene (Figure 13b).

Furthermore, several of the probe sets among the 125 genes that were differentially expressed (Supplementary Table S2) have not yet been annotated to a gene. Mapping of their sequences to the mouse genome showed that most of them hybridize to intronic sequences of the *PVT1*, *EI24*, *REV3L*, *RNGTT* and *ITPR2* genes (Supplementary Figure S2). Two other probe sets were found to map to a sequence downstream of *PCNA* and upstream of *REV3L*, respectively. Interestingly, *EI24*, *REV3L*, *ITPR2* and *PCNA* were among the identified radiation-responsive genes (Supplementary Table S2), whereas *Pvt1* was recently identified as a radiation-responsive gene in the embryonic mouse brain [190]. Our data suggest that these probe sets actually identify currently unknown exons of these radiation-responsive genes.

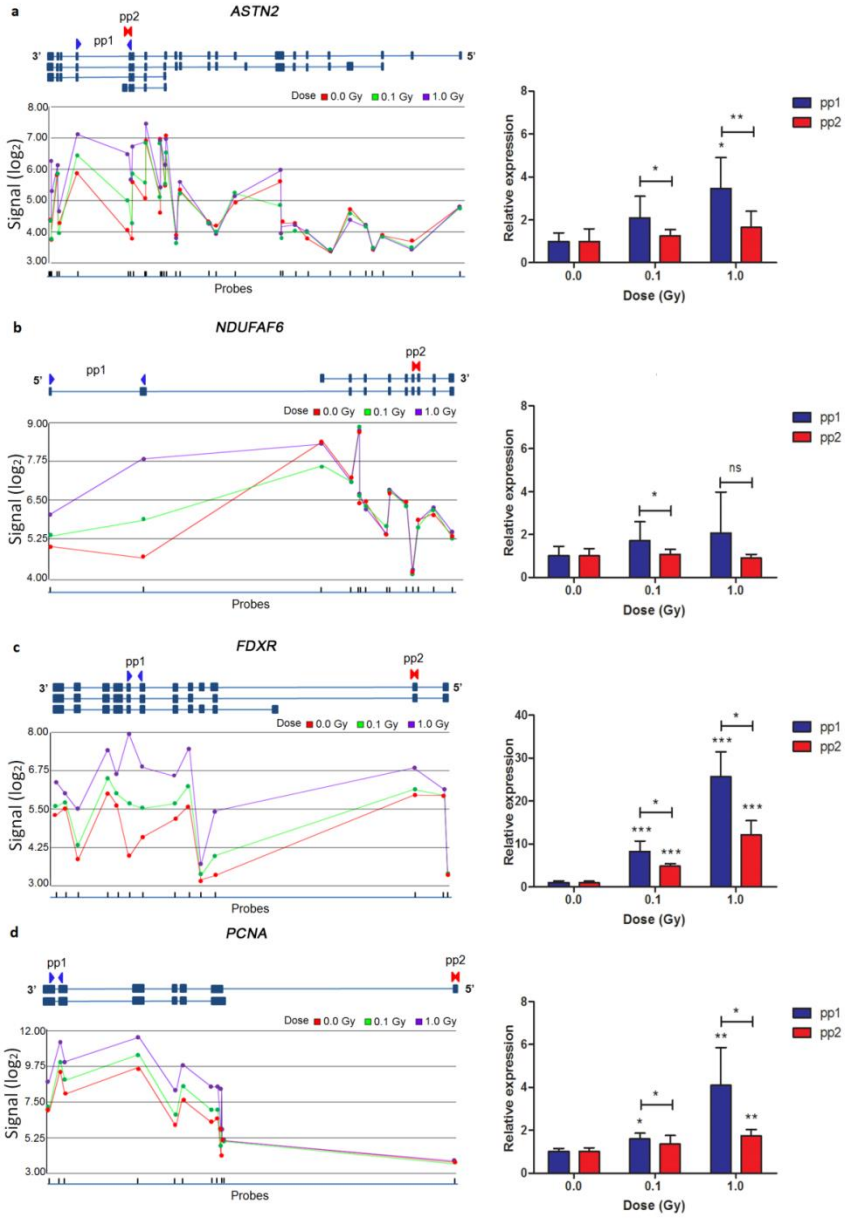

Figure 13. Radiation-induced alternative splicing. **a-d.** Upper panels show genomic organization of a few transcript variants from the UCSC database of the *ASTN2* (a), *NDUFAF6* (b), *FDXR* (c) and *PCNA* (d) genes. Each blue box represents an exon and the interconnecting lines represent introns. Lower panels show the $\log_2$ normalized intensity signals for each microarray probe, located on each specific exon shown above (error bars were left out to increase clarity). Arrows indicate the location of the primer pairs (pp) used for qRT-PCR validation. qRT-PCR validation results for each primer pair are shown on the right. Graphs represent mean + standard deviation. Statistical comparison was performed using the paired *t*-test (**p*-value < 0.05, ***p*-value < 0.005, ****p*-value < 0.0001, ns: not significant).

3.4.3 Radiation exposure induces expression of neighboring genes

The above results indicate that although radiation exposure leads to exon skipping and the use of alternative splice junctions, the mechanism that was most often observed to result in transcript variation was the expression of transcripts from alternative promoters. Since most of these genes are regulated by p53, we hypothesize that the DNA damage response, which is activated after irradiation, induces the expression of p53-dependent transcript variants, as shown previously in both lymphoblastoid cell lines [188] and the embryonic mouse brain [190]. Interestingly, we also observed that a significant proportion (23 out of 129 annotated genes; 17.8%) of the genes that were differentially expressed after irradiation with 1.0 Gy are genomic neighbors, several of which are transcribed from bidirectional promoters (Supplementary Figure S3). This finding aligns well with a study in which human lung fibroblasts were treated with the p53 activator 5-fluorouracil [277]. Chromatin immunoprecipitation with a p53 antibody followed by next-generation sequencing revealed that about 4% of the high-confidence peaks were located at bidirectional promoters [277], including some that are identical to those observed by us (e.g. *FAS-ACTA2* and *ASTN2-TRIM32*).

3.4.4 Differential expression of distinct exons is more pronounced compared to entire genes

For several genes, individual exons responded much stronger to the irradiation than others. This suggested that signatures of highly responsive exons might be more sensitive and would have greater predictive value as a biomarker of radiation exposure compared to genes, whose expression signals are averaged over the totality of their exons. This observation led us to perform ANOVA at the exon level as well, revealing 706 differentially expressed exons (FDR <0.05) between different doses of radiation exposure (Supplementary Table S6), with 157 exons being differentially expressed after exposure to both 0.1 and 1.0 Gy (Supplementary Table S7). Comparison of the distributions of fold changes in expression between genes and exons

confirmed that the changes in the exon expression levels were more pronounced compared to the genes on a generic basis, especially at the higher dose of 1.0 Gy (Figure 14a, 14b). Average fold changes for significant genes and exons after exposure to 0.1 Gy were 1.58 and 1.71, respectively (Figure 14c), increasing to 1.72 and 2.21 after exposure to 1.0 Gy (Figure 14d).

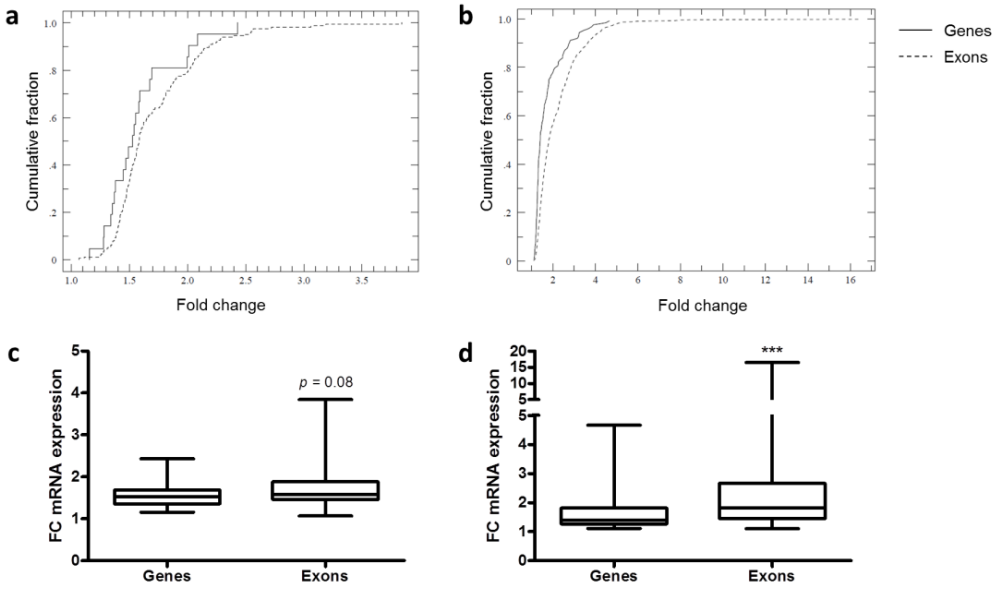

Figure 14. Fold-change induction of exon expression is more pronounced compared to gene expression. **a-b.** Cumulative distributions of fold changes in expression for significantly differentially expressed genes and exons after exposure to 0.1 Gy (a) and 1.0 Gy (b) of X-rays. *p*-values for the difference between distributions for genes and exons according to the Kolmogorov-Smirnov test were 0.195 and 0.000 for 0.1 Gy and 1.0 Gy, respectively. **c-d.** Box plots depicting fold changes in expression for the same data as in a, b. (c) Genes and exons upregulated at 0.1 Gy. (d) Genes and exons upregulated at 1.0 Gy. Centerlines show the median, boxes represent the range between the first and third quartiles and whiskers represent the highest and lowest values. ****p*-value < 0.0001 (Two-tailed Mann-Whitney test).

Clustering of the samples based on the expression levels of the 706 differentially expressed exons using unsupervised hierarchical clustering (Figure 15a) and PCA (Figure 15b) resulted in perfect separation of the

samples according to radiation dose. Together, these results suggest that exons might be more sensitive radiation biomarkers.

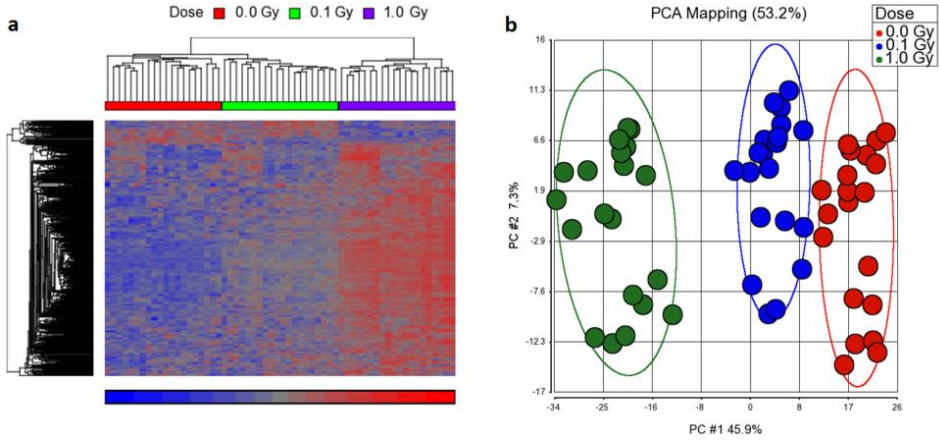

Figure 15. Probe (exon) expression changes in PBMCs in response to irradiation. In total, 706 exons were identified as differentially expressed by ANOVA (FDR corrected *p*-values < 0.05) between different irradiation doses. **a.** Unsupervised hierarchical clustering analysis of the 706 exons (plotted on y-axis) showed perfect separation of samples (x-axis) depending on the dose of exposure. Red: high gene expression, blue: low gene expression. **b.** 2D Principal Components Analysis (PCA) also separated samples depending on the dose. Each circle represents the expression profile of the 706 significantly differentially expressed exons in one sample. The percentage of the variance explained by the first and the second principal components is 45.9% and 7.3%, respectively. Ellipses represent two standard deviations.

3.4.5 Prediction analysis of transcriptional markers for radiation exposure

To identify signatures of genes and exons that distinguish between different irradiation doses, we used three supervised classification models: generalized linear models (GLM), Random Forests (RF) and Nearest Shrunken Centroids as implemented by the PAM (Prediction Analysis for Microarrays) algorithm. Additionally, we assessed the suitability of the above-mentioned models for classification of the samples according to exposure dose.

Table 7 shows the results of all models for gene and exon level analysis in the 2-fold cross-validation setting. Both PAM and the RF models attained a very

high performance even with a small number of features, and both models outperformed the GLM model. A combined PAM-RF model (features selected by PAM combined with classification by RF) achieved perfect classification with only two gene features (Table 7).

Table 7. Predictive performance at gene (G) and exon (E) levels using 2, 5, 10, 20, 50, 100 or all features as measured by the AUC.

Model	2		5		10		20		50		100		ALL	
	G	E	G	E	G	E	G	E	G	E	G	E	G	E
GLM Net	0.985	0.942**	0.965	0.926**	0.932	0.922**	0.949	0.946	0.953	0.952	0.952	0.951**	0.959	0.953
RF	0.985	0.968**	0.998	0.993**	1.000	0.998**	1.000	1.000	1.000	1.000	1.000	1.000**	0.918	0.955
PAM	0.999	0.997	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.997	1.000**	0.999	1.000**
PAM +RF	1.000	0.997	1.000	0.999	1.000	1.000	1.000	1.000	1.000	0.999**	1.000	1.000	0.917	0.953

GLM: Generalized Linear Models; **RF**: Random Forests; **PAM**: Nearest Shrunken Centroids
**p < 0.01 for comparison between gene and exon level (Mann-Whitney U-test)

Similar results were obtained for classification at the exon level (Table 7). Here, the PAM and RF models outperformed the GLM model more clearly. Comparison between exon and gene level analysis gave slightly inferior results for exons, with more features being needed to obtain optimal predictive performance. On the other hand, exons performed better than genes when 100 or all features were used. The genes/exons that were selected as the top 20 most important features for each of the classifiers are listed in Table 8. Of these, 12 genes were identified as differentially expressed according to ANOVA and suitable for class prediction by both RF and PAM (*AEN*, *BAX*, *DDB2*, *EDA2R*, *FDXR*, *MDM2*, *POLH*, *RPS27L*, *SESN1*, *TNFRSF10B*, *XPC*, *ZMAT3*).

Table 8. Top 20 most important features selected by PAM and Random Forests models for the data at gene and exon level.

	Random Forests		PAM	
			Genes	Exons
1.	**DDB2**	ASCC3 exon 43 (8128473)	**DDB2**	DDB2 exon 4 (7939743)
2.	**POLH**	**DDB2 exon 5 (7939744)**	**POLH**	DDB2 exon 5 (7939744)
3.	**AEN**	XPC exon 15 (8085487)	**MDM2**	FDXR exon 8 (8018242)
4.	**TNFRSF10B**	**TNFRSF10B exon 9 (8149735)**	**TNFRSF10B**	DDB2 exon 8 (7939747)
5.	**RPS27L**	RPS27L exon 1 (7989499)	**RPS27L**	POLH exon 5 (8119864)
6.	**FDXR**	PCNA exon 2 (8064851)	**AEN**	AEN exon 3 (7985775)
7.	**MDM2**	XPC exon 2 (8085501)	PCNA	TNFRSF10B exon 9 (8149737)
8.	**ZMAT3**	ASCC3 exon 41 (8128476)	**XPC**	**POLH exon 11 (8119870)**
9.	**XPC**	ASCC3 exon 6 (8128513)	ASCC3	DDB2 exon 3 (7939742)
10.	**C12orf5**	**POLH exon 11 (8119871)**	**ZMAT3**	RPS27L exon 3 (7989497)
11.	**EDA2R**	ASCC3 exon 14 (8128504)	**GADD45A**	MDM2 exon 4 (7956994)
12.	NUP133	**POLH exon 10 (8119869)**	**SESN1**	DDB2 exon 9 (7939748)
13.	FBXO22	MDM2 exon 10 (7957001)	**C12orf5**	POLH exon 4 (8119863)
14.	**GADD45A**	POLH exon 1 (8119860)	**EDA2R**	MDM2 exon 7 (7956997)
15.	ZNF79	RPS27L exon 2 (7989498)	**FDXR**	DDB2 exon 6 (7939745)
16.	**BAX**	TNFRSF10B exon 7 (8149739)	CCNG1	**POLH exon 10 (8119869)**
17.	**SESN1**	ASCC3 exon 13 (8128505)	**BAX**	PCNA exon 3 (8064850)
18.	ACTA2	TNFRSF10B exon 5 (8149756)	PPM1D	**TNFRSF10B exon 9 (8149735)**
19.	ITGA2	PHPT1 exon 2 (8159444)	FAS	NDUFAF6 exon 2 (8147426)
20.	TMEM30A	ASCC3 exon 37 (8128481)	PAPPA-AS1	DDB2 exon 7 (7939746)

Features are listed according to their internal weight (importance) in every model
The numbers in brackets in the Exons columns indicate corresponding Affymetrix Probe set IDs
Overlapping genes and exons are indicated in **bold**

To evaluate the robustness of gene expression signatures for practical radiation biodosimetry, we tested the predictive performance of our signature on an independent dataset from a study in which whole blood samples from male and female smokers and non-smokers were irradiated with similar doses to those used in our study, i.e., 0.1 Gy, 0.5 Gy and 2.0 Gy [271]. Using ten of our best predictive markers, we were able to classify these independent samples with 97% accuracy (Figure 16a), which overall is similar to the accuracy obtained in the original publication [271]. Next, we ran the RF model on the dataset of Paul and Amundson, and used the ten best predictors for cross-validation on our samples. This resulted in 100% accuracy (Figure 16b), i.e., identical to what we found using our 10 best gene predictors (Table 7). Unfortunately, we were not able to independently validate our exon signatures because this dataset did not contain exon-level information.

a

Dose (Gy)	Sensitivity	Specificity
0.0	1.000	1.000
0.1	1.000	0.960
0.5	0.916	0.956
2.0	0.956	0.956

b

Dose (Gy)	Sensitivity	Specificity
0.0	1	1
0.1	1	1
1.0	1	1

c

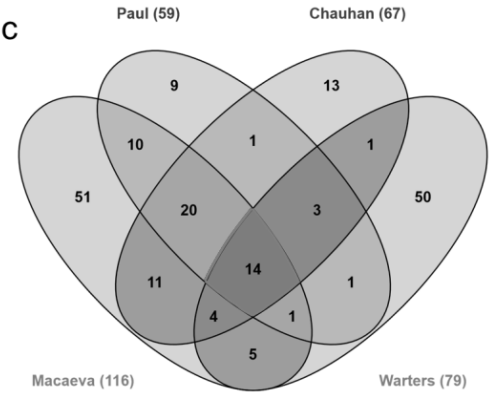

Figure 16. Gene signatures are robust predictive biomarkers of exposure to radiation. **a.** Five-fold cross-validation of our 10 best predictive genes on the dataset of Paul and Amundson [271] resulted in 97% accuracy for sample classification. **b**. Five-fold cross-validation of the 10 best predictive genes from the dataset of Paul and Amundson [271] as identified by the Random Forests model resulted in 100% accuracy for classification of our samples. **c.** Overlap between differentially expressed genes from this study and gene signatures identified by Paul and Amundson [218], Chauhan and co-authors [235] and Warters and co-authors [278]. Common genes between all datasets are *DDB2*, *POLH*, *MDM2*, *RPS27L*, *FDXR*, *CCNG1*, *TRIAP1*, *SESN1*, *FBXO22*, *PPM1D*, *ANKRA2*, *CDKN1A*, *TRIM22*, and *BBC3*.

Furthermore, we compared our results with those of three other studies in which different subjects, radiation doses (up to 8 Gy), dose rates, radiation qualities, time points, cell types and gene expression platforms were used. The specific characteristics of these studies are listed in Supplementary Table S8. Our comparative analysis revealed a very high degree of overlap in radiation-responsive genes between the different experiments, especially between those in which peripheral blood or PBMCs were used (Figure 16c). Nevertheless, 27 out of 79 genes (34%) that were found to be radiation-responsive in keratinocytes and fibroblasts [278] were also identified in at least two other studies (Figure 16c).

Together, these results hint at the existence of a core signature of genes that may be applicable for radiation biodosimetry for a wide range of doses, dose rates, and cell types/specimens after exposure to different radiation qualities.

3.4.6 Validation of gene and exon expression using qRT-PCR

qRT-PCR was used to validate the expression changes of several identified biomarker genes in PBMCs and peripheral blood. In general, the majority of the examined genes showed a dose-dependent up-regulation (Pearson's correlation coefficients ranging between 0.82 and 0.99) after X-irradiation (Figure 17), although clear differences in the transcriptional response were observed between different genes. For example, most of the tested genes (*ASTN2*, *MDM2*, *NDUFAF6*, *POLH*, *TNFRSF10B*) showed a 2- to 3-fold induction in expression at 8 h after exposure to 1.0 Gy of X-rays, while *DDB2*, *PCNA* and *FDXR* expression levels were 4-, 5- and 25-fold induced, respectively. Furthermore, most of these genes showed significant differences in expression at 8 h after exposure to a dose of 0.1 Gy, demonstrating their sensitivity for this radiation dose at this time point.

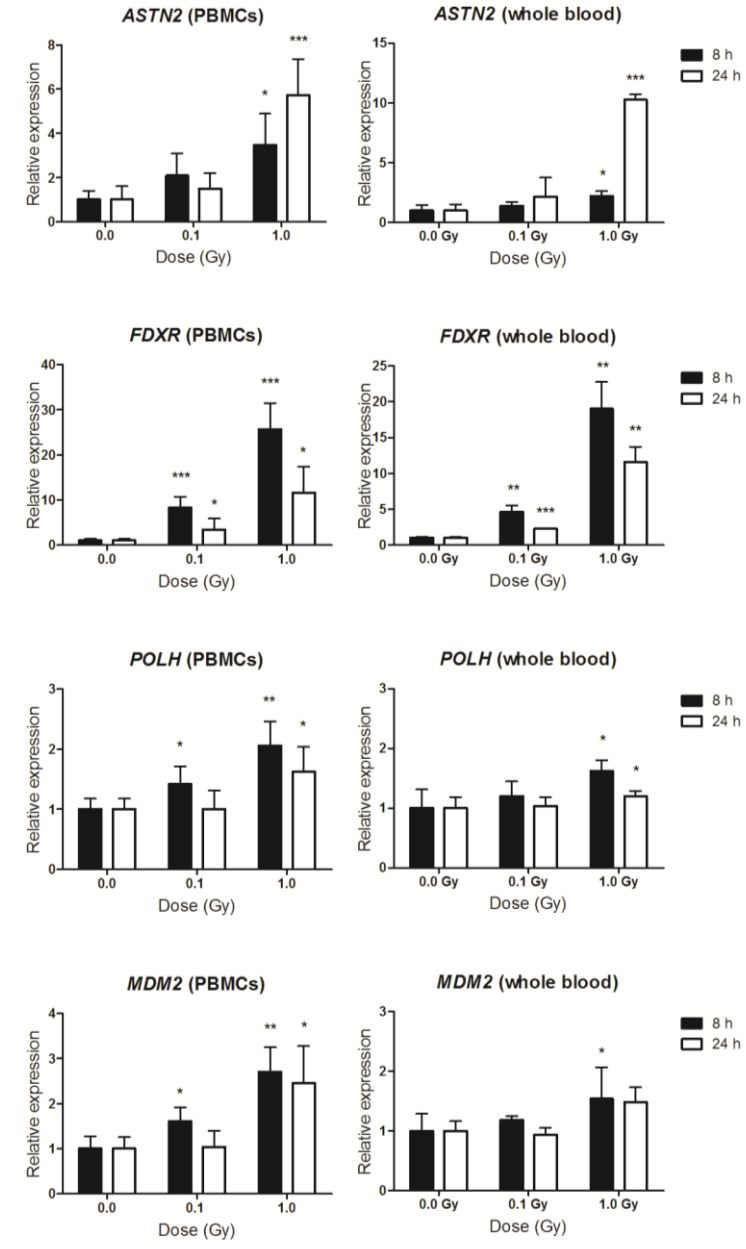

Figure 17. qRT-PCR validation of the microarray results. qRT-PCR results for *ASTN2, FDXR, POLH* and *MDM2* genes at 8 and 24 h after irradiation of PBMCs and whole blood. Graphs represent mean + standard deviation. Statistical comparison was performed using the paired *t*-test (**p*-value < 0.05, ***p*-value < 0.005, ****p*-value < 0.0001).

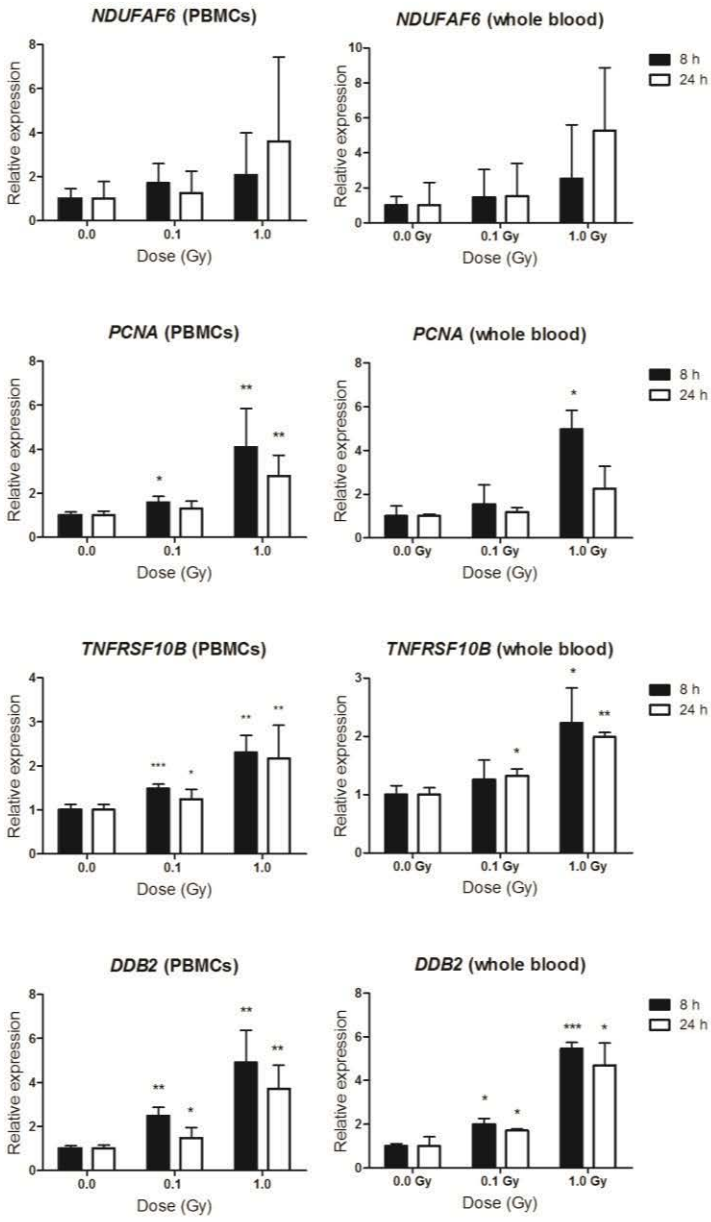

Figure 17 (continued). qRT-PCR validation of the microarray results. qRT-PCR results for *NDUFAF6, PCNA*, *TNFRSF10B* and *DDB2* genes at 8 and 24 h after irradiation of PBMCs and whole blood. Graphs represent mean + standard deviation. Statistical comparison was performed using the paired *t*-test (**p*-value < 0.05, ***p*-value < 0.005, ****p*-value < 0.0001).

To confirm the obtained results, we also assessed the expression levels of these genes at a later time point, i.e., 24 h following irradiation, which is more applicable to emergency situations. Our results point to differences in the kinetics of the transcriptional response of these radiation-induced genes: reduced expression levels – but no complete return to basal expression levels - for *FDXR,* similar expression levels for *DDB2*, *MDM2*, *PCNA*, *POLH* and *TNFRSF10B,* and further increased expression levels for *ASTN2* and *NDUFAF6* after 24 h compared to 8 h (Figure 17). In many cases, however, statistical significance of expression changes after exposure to 0.1 Gy was lost after 24 h.

In addition, we performed a similar qRT-PCR validation experiment using peripheral blood samples exposed to the same doses. Overall, the obtained results were very similar to those observed in PBMCs (Figure 17), demonstrating that PBMCs are a suitable model for the transcriptional radiation response of blood.

3.5 Discussion

Prompted by the rapid development of high-throughput genomic profiling technologies, several groups have explored the potential of gene expression signatures as biomarkers of (low dose) exposure [154, 218, 227, 235, 279-281]. Most of the genes identified in these studies are known to be regulated by p53 and are involved in cell cycle regulation, DNA damage repair and apoptosis and some are already induced after exposure to doses as low as 5-25 mGy [155, 227, 228, 231]. Furthermore, some of these genes allow to discriminate between ionizing radiation response profiles and those induced by inflammation [159]. Several genome-wide studies have been undertaken to assess the *in vivo* transcriptional response to ionizing radiation using blood samples from radiotherapy patients undergoing either total body irradiation [229] or local intensity modulated radiotherapy [230]. The results of these investigations indicate that *in vivo* irradiation mainly affects genes involved in pathways that are related to the immune system and inflammatory responses,

as well as p53-mediated pathways. Accordingly, induction of p53-dependent genes was observed in patients either undergoing CT scans (up to 4.3 cGy) or receiving (F-18)-fluoro-2-deoxy-d-glucose (0.6 cGy) [231]. Overall, the examined genes were induced in all samples, although differences in the *in vivo* and *in vitro* response were found, especially for doses below 5 cGy[231]. Other studies have identified *in vitro* gene signatures that could accurately predict the *in vivo* radiation exposure status [158, 232, 233]. Overall, these studies have shown that the *in vitro* transcriptional radiation response is a reliable model for the *in vivo* situation. Another possibility for biodosimetry studies is the use of animal models. For instance, it was demonstrated that radiation-responsive genes in mice show a response that is similar to that of homologous genes from *ex vivo* human studies [232, 280, 282]. On the other hand, gene expression profiles developed through analysis of murine blood radiation responses alone were found to be inaccurate in predicting human radiation exposures [158].

Unlike the moderate to high radiation doses used in most other studies dealing with transcriptional radiation biomarkers, the X-ray doses applied in this study are low to moderate but nonetheless relevant for medical triage. The moderate dose of 1.0 Gy represents the lower limit of doses that result in acute radiation syndrome [257] and is associated with a high probability of long-term stochastic health effects. The low dose of 0.1 Gy is not associated with any acute health effects but might require medical follow-up since the risk of long-term effects, particularly cancer, cannot be excluded [258]. To the best of our knowledge, only two studies aimed at identifying a predictive gene signature based on genome-wide data have used doses of 0.1 Gy or below [227, 271]. However, no cross-validation at the individual donor level was performed in either of these studies, which may have positively biased the results.

One of the initial steps in our study consisted in a gene-level analysis of the microarray data, resulting in a list of genes capable of discriminating between

the exposure conditions with high accuracy. Furthermore, a substantial fraction of the radiation-responsive genes were located in close physical proximity on the genome (often as neighbors with bidirectional promoters). We propose that these genes are co-regulated, most likely via activation by p53, or via chromatin loops which can bring promoters in close proximity, thereby exposing them to the same regulatory proteins. This co-regulation may be related to the nature of the stress inflicted on the cells by radiation exposure (i.e. DNA damage) since the frequency of bidirectional promoters is enriched in DNA repair genes compared to other gene classes [283, 284]. This observation may also be instrumental in identifying currently undiscovered radiation-responsive transcripts. One such new gene we identified as a predictive marker is *PAPPA-AS1* (Table 7), which is a long non-coding RNA transcribed from the opposite strand of *PAPPA*, presumably from a shared bidirectional promoter with *ASTN2*.

The specific microarray platform we used, interrogates the vast majority of exons from multi-exon genes, allowing to analyze the expression data at the exon level as well. Although we could not draw definite conclusions about the exact mechanisms underlying these events, our data are suggestive of the activation of different alternative splicing mechanisms (exon skipping, alternative splice sites, alternative polyadenylation) in response to irradiation. However, the most utilized mechanism appeared to be alternative promoter usage. Importantly, such events result in significant differences in the expression of single exons, while changes in the expression of the gene itself are less pronounced. Forrester and Sprung even proposed that dose prediction could be improved by the use of radiation-responsive transcript variants as biomarkers in combination with unresponsive intragenic controls [285]. However, these authors evaluated only three genes, one of which turned out unsuitable for dose prediction [285].

To the best of our knowledge, comparison of gene and exon signatures for class prediction is a novel approach in biodosimetry, and has only rarely been

applied even in general biomarker research. Tian et al. showed that exons outperformed genes as biomarkers of Tourette syndrome [286]. In another study gene and exon signatures performed equally well in predicting of overall survival in neuroblastoma patients [287]. Likewise, our results are indicative of an overall comparable prediction performance of gene and exon signatures.

From our results, and those from other groups [218, 227, 235, 271, 279, 281], it is now clear that there is a core of approximately 20 genes that can be regarded as robust biomarkers for radiation exposure to a wide range of doses. As such, genome-wide expression studies are undoubtedly highly informative to identify accurate dose-prediction signatures. Nevertheless, using microarrays for mass casualty screening in a radiological emergency situation is not a very realistic approach, due to high costs, limited availability of infrastructures equipped for performing these assays, the rather long response time and the complexity of the analysis. A more cost- and time-efficient alternative would be to use primer- or probe-based assays (such as qRT-PCR) that measure the expression of a limited number of *a priori* identified biomarkers. However, these methods, in contrast to exon-specific microarrays, do not allow to measure the expression of the entire gene but only cover a relatively short region of one or a few exons. Therefore, selection of the most appropriate exons is imperative prerequisite for using primer- or probe-based assays. We validated the expression profiles of some of the identified genes that were also alternatively spliced in response to irradiation by qRT-PCR using variant- and exon-specific primers for transcripts with different radiation responses, and, for many of the tested genes, we only found a significant difference in expression in low dose-exposed samples with primer pairs amplifying the most sensitive exons. This suggests that these exons may be more sensitive markers for prediction of similar low doses and possibly also those below 0.1 Gy, i.e., doses at which combined exon signals (i.e. gene level) may be no longer predictive. This further highlights the importance of always obtaining prior knowledge about expression levels at

the exon level when primer- or probe-based assays are used to perform "gene-level" expression analysis.

Our study has a few limitations. First, only two radiation doses and one time point after irradiation were used to identify the predictive signatures. However, a comparative analysis with previously published studies, as well as validation of the predictive performance of our signatures on an independent dataset containing two additional doses, revealed that our signature also applies to higher doses and longer time points. Second, the gene expression profiles applied in our study stemmed from isolated PBMCs and not from whole blood. To address this, we validated gene expression using qRT-PCR on *ex vivo* irradiated peripheral blood samples, revealing highly similar transcriptional responses to radiation in PBMCs and blood for the investigated genes.

In conclusion, we have shown that gene and exon signatures are equally performing in predicting exposure to radiation doses within the 0.1-1.0 Gy range at 8 h after exposure. We have generated a robust fingerprint for predictive biodosimetry and especially triage of individual radiation casualties. Implementation of a dedicated assay based on the identified biodosimetric panel may lead to improved point-of-care diagnostics for radiological accidents. Finally, we have shown the importance of evaluating gene expression at the level of single exons for transcriptional biomarker discovery in general.

3.6 Acknowledgements

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3.7 Author Contributions Statement

E.M., K.T., A.J., and A.M. performed experiments. E.M., Y.S., and R.Q. analysed data. K.T., M.A.B., S.B., and R.Q. designed the study. M.A.B., W.D.V., S.B. and R.Q. directed the work. E.M. and R.Q. wrote the manuscript. All authors revised and approved the manuscript.

3.8 Competing financial interests

The authors declare no competing financial interests.

3.9 Supplementary material

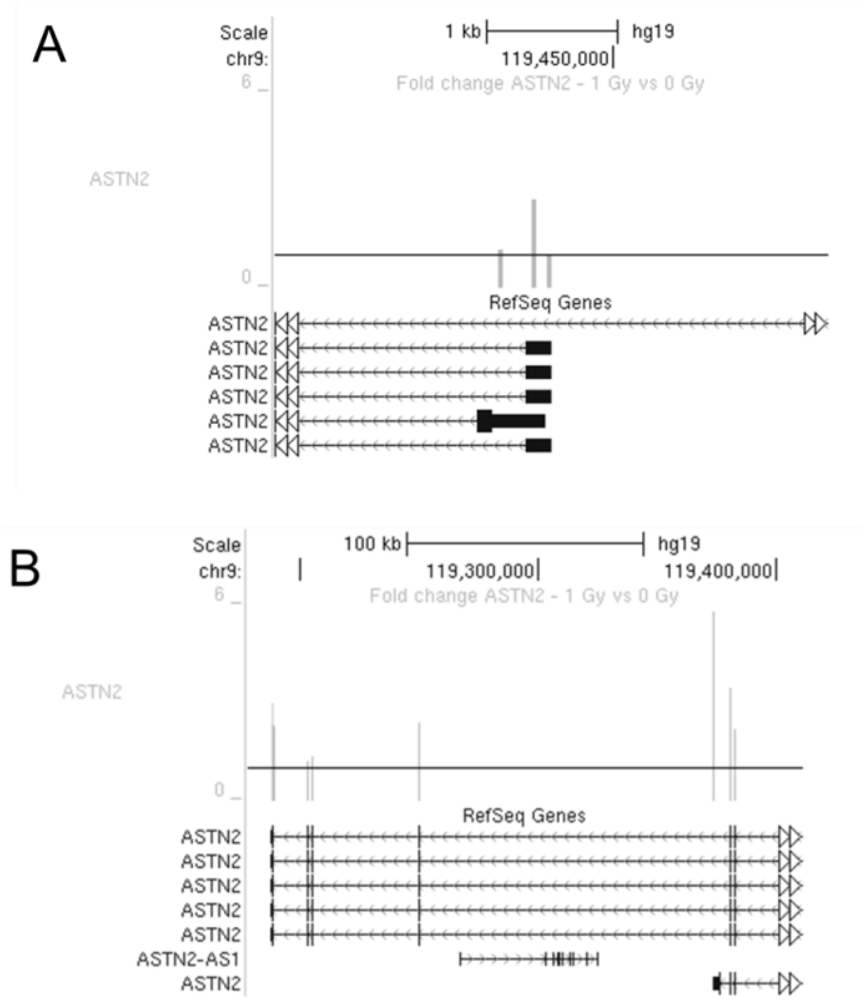

Figure S1. Possible alternative splicing events responsible for differences in expression signals for different probes of the *ASTN2* gene. (A) Radiation-induced expression of a truncated first exon of the short ASTN2 variant. (B) Radiation-induced splicing of two cassette exons and expression of an alternative 3' exon. In both panels top tracks indicate fold changes for individual probes (green lines) in 1.0 Gy samples compared to 0.0 Gy. The vertical line indicates no change. Bottom track shows different transcript variants. Arrows indicate the 5' to 3' orientation of the gene.

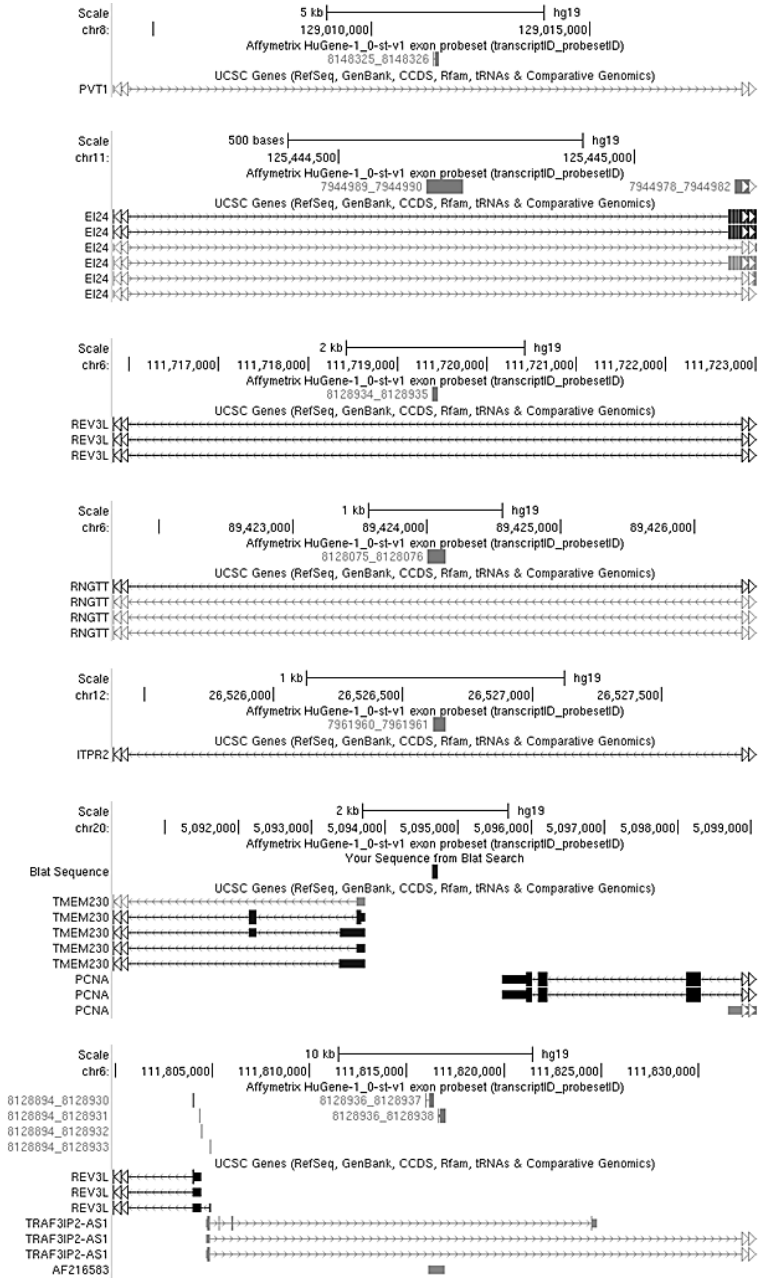

Figure S2. Mapping of the probesets currently not annotated to a gene from Table S1. In each panel, top tracks indicate Affymetrix probesets while bottom tracks indicate known splice variants. Arrows indicate 5' to 3' orientation of the gene.

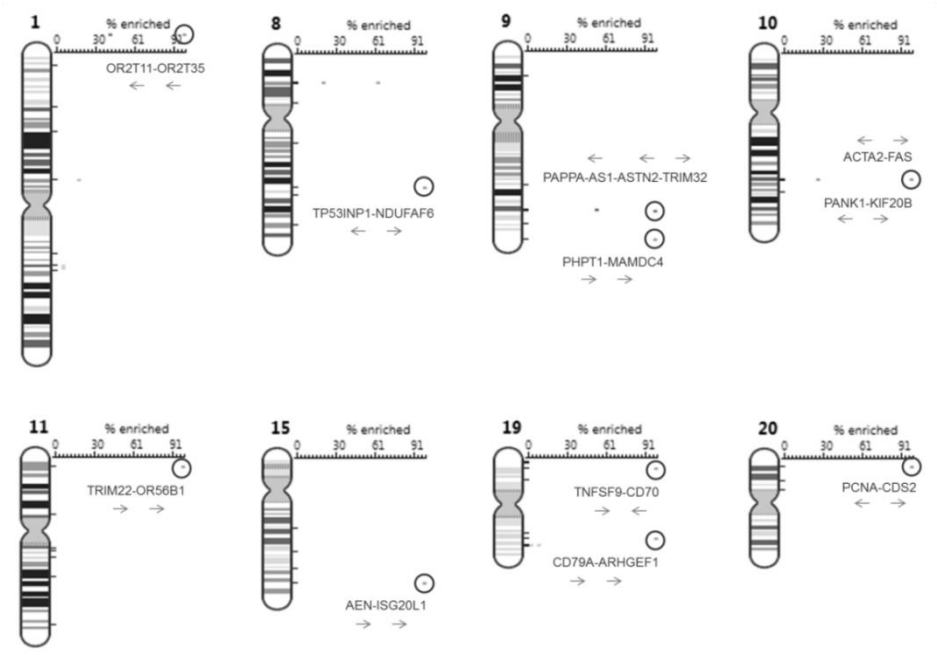

Figure S3. Positional gene enrichment analysis shows significant co-localisation of radiation-responsive genes. Scale bar indicates percentage of enrichment with 100% enrichment corresponding to genomic neighbors. Arrows indicate the 5' to 3' orientation of the genes. Please note that the separate clusters *ACTA2-FAS* and *PANK1-KIF20B* are in close proximity (< 1 Mb) on chromosome 10.

Supplementary Table S1. Primer sequences for qRT-PCR			
Gene	**Forward primer (5' à 3')**	**Reverse primer (5' à 3')**	**Reaction efficiency (%)**
ASTN2 (pp1)	TCTGCAGCATAAGAAAGTGGATGA	CAATCAAGTTGGCTTCATCCCTG	95
ASTN2 (pp2)	TCTGCAGCATAAGAAAGTGGATGA	GACCAGCTGCTACACAAGATGT	103
DDB2	TGAACATGGACGGCAAAGA	TGGCCAGGAACCAATCAC	93
FDXR (pp1)	GCAAGTGGCCTTCACCATTAAG	CCTTGATCTTGTCCTGGAGACC	88
FDXR (pp2)	GCTTCTGCCACCATTTCTCC	CTTTAGCAGGTGTTGGGCC	104
HPRT1	TCAGGCAGTATAATCCAAAGATGGT	AGTCTGGCTTATATCCAACACTTCG	96
MDM2	CGATTATATGATGAGAAGCAACAAC	CTCTTTCACAGAGAAGCTTGG	88
NDUFAF6 (pp1)	AAAGAGAGACTGGAGCCACCT	GAGGAATGTGAAATGCTGATTGGC	94
NDUFAF6 (pp2)	GCAACACCATATCATGGGAGCA	CTTGGTTCCTCCGTAGAAAGTCC	97
PCNA (pp1)	GCACTGAGGTACCTGAACTT	TCTTCATCCTCGATCTTGGG	109
PCNA (pp2)	CCCTGGTTCTGGAGGTAAC	CAGGTCAGCAAGCATTTGTC	104
PGK1	CAAGAAGTATGCTGAGGCTGTCA	CAAATACCCCCACAGGACCAT	102
POLH	GGGAAGCCAGTGTTGAAGT	CTTGTACAGCACTGGTCAGAT	94
TNFRSF10B	GTGGATGGAACATCCTGTAACT	AGTACGCACAAACGGAATGA	93

Supplementary Tables S2-S7 are available in electronic format only (as Excel files).

Supplementary Table S8: Characteristics of studies used for comparison analysis

Reference	Cell type	Radiation quality	Radiation dose (Gy)	Dose rate	Time points	Array platform
This study	PBMCs	X-rays	0; 0.1; 1.0	0.26 Gy/min	8 h	Affymetrix Human Gene 1.0 ST array
Paul and Amundson[1]	Peripheral blood	γ-rays	0; 0.5; 2; 5; 8	0.82 Gy/min	6 h, 24 h	Agilent Whole Human Genome Oligo Microarrays
Chauhan et al.[2]	PBMCs	α-particles	0; 0.5; 1.0; 1.5	0.98 Gy/h	24 h	Illumina human-12 v2 RNA Bead Chips
Warters et al.[3]	Keratinocytes, fibroblasts	γ-rays	0; 0.1; 1; 5	0.47 Gy/min	4 h	Agilent 44K arrays

1. Paul, S. & Amundson, S. A. Development of gene expression signatures for practical radiation biodosimetry. *International journal of radiation oncology, biology, physics* **71**, 1236-1244, doi:10.1016/j.ijrobp.2008.03.043 (2008).

2. Chauhan, V., Howland, M. & Wilkins, R. Identification of gene-based responses in human blood cells exposed to alpha particle radiation. *BMC medical genomics* **7**, 43, doi:10.1186/1755-8794-7-43 (2014).

3. Warters, R. L., Packard, A. T., Kramer, G. F., Gaffney, D. K. & Moos, P. J. Differential gene expression in primary human skin keratinocytes and fibroblasts in response to ionizing radiation. *Radiation research* **172**, 82-95, doi:10.1667/RR1677.1 (2009).

Supplementary Methods

In vitro irradiation

The beam quality can be approximated to H-250 (ISO4037): 250 kV, 15 mA, 1.2 mm Al equivalent inherent filtration and 1 mm Cu additional filtration. The K_{air} at the reference position was measured using a NE2571 ionisation chamber (SN309) connected to a Farmer 2500 electrometer. The chamber, together with the electrometer, was calibrated in terms of K_{air} and the traceability to the international standards was assured. The reference point of the ionisation chamber was placed at the same distance with the reference position of the samples. The ionisation chamber was always placed in the beam, next to the samples, for a precise measurement of the time integrated K_{air}. The stability of the X-ray generator during the irradiation was verified in this way.

RNA extraction

For RNA isolation from PBMCs a combination of the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) extraction method and the purification on Qiagen RNeasy columns (Qiagen, Venlo, The Netherlands) was used. Briefly, $5x10^6$ cells were lysed in 1 ml of TRIzol® reagent and further processed following the manufacturer's recommendations. Following the RNA precipitation with isopropanol, the obtained pellet was resuspended in 1 ml of ethanol and transferred to the RNeasy column. Further purification was done according to the manufacturer's instructions.

Microarray hybridisation

Ten µg of cRNA, synthesised and purified from 0.25 µg of total RNA using the Ambion® WT Expression kit (Ambion, USA) was used for cDNA synthesis, followed by cDNA fragmentation and labeling with the GeneChip® Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA). Fragmented and labeled cDNA was hybridised to Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) using the GeneChip® Hybridization, Wash and Stain kit (Affymetrix, Santa Clara, CA, USA) (hybridization module) and hybridization controls (Affymetrix, Santa Clara, CA, USA) with rotation at 45°C for 16 hours. After hybridization, arrays were washed and stained using GeneChip® Hybridization, Wash and Stain kit (stain module) after which the arrays were immediately scanned using an Affymetrix GeneChip® Scanner.

Predictive analysis

Generalized linear models were trained using the R glmnet package. These methods build a regularised linear model which uses the lasso penalty to perform feature selection, resulting in only relevant features to receive nonzero weights. A multinomial model was used to model the three-class classification problem, and an internal five-fold cross-validation was used to tune the model's internal parameter lambda. Feature importance measures

were then derived from the weights of the linear model. The Random Forest based classification model uses an ensemble of randomised decision trees to perform classification. We used a collection of 1000 decision trees to build these models, and subsequently used the internal feature importance mechanism based on entropy reduction to obtain feature importance values. The Nearest Shrunken Centroid Classifier gradually shrinks the average gene expression centroids of the two groups to the overall centroid. The non-differentially expressed genes are removed first as the distance between the centroids of two groups is small in this case and the group centroids of these genes will therefore quickly reach the overall centroid. Differentially expressed genes, in contrast, will "survive" the shrinkage much longer and will have a higher probability of being used for classification. The optimal level of shrinkage is determined with ten-fold cross-validation, which is used to select the number of genes for class prediction. Finally, the centroids of these genes are used to classify the new samples to the nearest centroid.

While each of these classifiers has an internal mechanism to select informative features based on their internal weight or importance, we also experimented with explicitly reducing the number of features describing the data. To this end, internal model information was used to weigh features and keep only the most important ones. We explored the following number of features: 2, 5, 10, 20, 50, 100 most important features, and finally also the traditional setting where all features were used. To perform feature selection in an unbiased way, we again only selected the most important features from the training partition in each cross-validation loop. Subsequently, a model with only the selected number of features was retrained on the full training partition, and executed on the test partition within each cross-validation loop.

4.

Gene expression-based biodosimetry using customized qPCR arrays: assessment of dose and time after exposure

This chapter is modified from:

Macaeva E., Mysara M., De Vos W., Baatout S., Quintens R. *Gene expression-based biodosimetry using customized qPCR arrays: assessment of dose and time after exposure.* Manuscript submitted to *International Journal of Radiation Biology*.

4.1 Abstract

The poor suitability of the currently used biodosimetry methods for mass-casualty events gives rise to the development of new, time- and cost-efficient assays, such as detection of gene expression changes. In the present study, we tested the usefulness of gene expression signature integrated in a qRT-PCR array for the prediction of exposure dose but also the time elapsed since irradiation. We used peripheral blood samples from seven healthy volunteers as reference samples (doses: 0, 25, 50, 100, 500, 1000, and 2000 mGy; time points: 8, 12, 24, 36 and 48 h) and samples from five other individuals as “blind samples” (20 in total). Our analysis showed that ΔCt values normalized to the reference gene without normalization to the unexposed controls contained sufficient information for discrimination of the samples between the doses with a correlation coefficient between the true and the predicted doses of 0.86. Importantly, we could also classify the samples according to the time point with a correlation coefficient between the true and the predicted time point of 0.96. In a real accident situation this feature will be of critical importance for adequate gene expression-based dose prediction. In this study we also compared different methodologies for RNA extraction and chose the one most suitable for emergency. Our results represent an important advance in the application of gene expression for biodosimetry purposes.

4.2 Introduction

Biodosimetry is the dose estimation after exposure to ionizing radiation by means of changes in biological endpoints, or biomarkers. In case of large-scale radiological accidents, when physical dosimetry is not available for all the individuals at risk of exposure, these biomarkers could be used to detect individual exposure cases. In such situations triage decisions have to be undertaken as soon as possible in order to split the exposed subjects into different categories, depending on the exposure dose and radiosensitivity [288]. This triage will allow focusing the medical staff and facilities only on the subjects in need of urgent medical assistance [288].

As yet, the gold standard method in biodosimetry is the detection of dicentric chromosomes in peripheral blood lymphocytes (dicentric chromosome assay or DCA) [195]. Besides its sensitivity to doses down to 20 mGy [289], this method has many other advantages, such as high specificity to ionizing radiation, possibility to detect partial body exposure and possibility of exposure assessment even months after irradiation [195]. However, DCA is low in throughput: it is time-consuming, laborious and requires highly trained personnel for scoring. In 2010, the total capacity of European Union laboratories specialized in biodosimetry for DCA was estimated to be 1493 samples in the triage mode and 187 samples in the full mode per week, excluding the 48 h time needed for lymphocyte culturing [290]. This would be insufficient in case of a large-scale accident with thousands of potentially irradiated subjects.

A promising new approach for biodosimetry that offers superior time-efficiency, is the analysis of changes in gene expression levels. Several genes, which respond to radiation exposure have been studied using different methodological approaches, such as whole genome microarray methods [218, 227, 259, 280, 281, 291] or quantitative PCR [154, 155, 292-294]. Most of the identified genes are known to be regulated by p53 (e.g. *MDM2*, *DDB2* [221], *FDXR* [222], *PCNA* [223], *GADD45A* [224], *RPS27L* [225], *SESN1*

[226]), and are involved in cell cycle regulation, DNA damage repair and apoptosis. Because some of them are induced after exposure to doses as low as 5-25 mGy [155, 227, 228, 231], it can be stated that in terms of sensitivity gene expression outperforms DCA, for which the threshold of sensitivity is about 20 mGy when scoring a few thousands metaphases [289]. As a result, the development of a biodosimetry gene signature and associated assays which can be configured as devices suitable for low-cost, "point-of-care" measurements make an appealing strategy [219, 294, 295].

One of the main difficulties in using changes in gene expression as a biomarker of exposure is the highly dynamic nature of the signal. The expression of every single gene following radiation exposure is affected not only by the dose, but also by the time, and the kinetics of expression is specific for every gene [155]. This means that knowledge of the time span between exposure and measurement is pivotal for correct dose prediction, or methods have to be devised that turn static snapshots into temporal information. Using a signature of genes rather than one single gene may allow assessing the time after exposure based on the combination of their expression profiles. In addition, finding the right methodological approach to monitor gene expression as early as possible following exposure is also important. Possible solutions to this include immediate snap freezing of blood in liquid nitrogen or dry ice, which might be challenging in field conditions, or addition of special whole blood preservation buffers [296], which would also solve the problem of effective preservation of easily-degradable RNA. Another challenge of using blood for gene expression studies is the heterogeneity of blood cells. About 99% of blood cells are red blood cells, including immature reticulocytes, which contain high levels of globin mRNA (accounting for ~ 70% of all mRNA in blood), which can compromise the detection of other specific mRNAs from white blood cells [297]. Although qPCR is less affected by globin mRNA contamination, this parameter is highly important for such techniques as microarrays [298] and next generation sequencing [296].

Recently, we used microarrays to analyze the expression response of human peripheral blood mononuclear cells (PBMCs) exposed *ex vivo* to radiation doses of 0.0, 0.1, and 1.0 Gy and we observed that many differentially expressed genes were also alternatively transcribed/spliced in response to radiation [259]. We thereby identified a signature of genes and exons that showed high performance in dose prediction [259]. We now used the most radiation-sensitive exons to design a qPCR array for biodosimetry. Notably, the genes used for these qPCR arrays are also responsive to high-LET radiation, such as carbon and iron ions (see Chapter 5). In addition, we also included three genes (*PF4*, *GNG11* and *CCR4*) which were shown to be up-regulated in response to low-dose exposure (0.05 Gy) and down-regulated by higher dose (1 Gy) at 6 h post-irradiation [276].

In the present study we investigated the potential of this assay to predict both dose and time after exposure. In addition, we compared different RNA extraction protocols and assessed their applicability to an emergency situation.

4.3 Materials and methods

4.3.1 Comparison of RNA extraction methods

As the first step of the present study, the performance of two RNA extraction kits specifically designed for RNA extraction from blood samples - QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) and PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) - was tested under different conditions (Figure 18).

Peripheral blood samples were collected from five healthy donors with informed consent and ethical approval from the local SCK•CEN Ethics Committee. The procedure was carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 2000.

RNA extractions were performed following the manufacturer's instructions, unless mentioned otherwise (see Figure 18 for details). RNA concentration was measured on a Trinean Xpose instrument (Trinean, Gent-Brugge, Belgium) and the quality of total RNA samples was assessed using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

Globin mRNA contamination was assessed using qRT-PCR with primers specific to *HBA1* and *HBB* genes using isolated PBMCs as a reference. PBMCs isolation was performed as described in [259]. RNA extraction from isolated PBMCs was performed using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions.

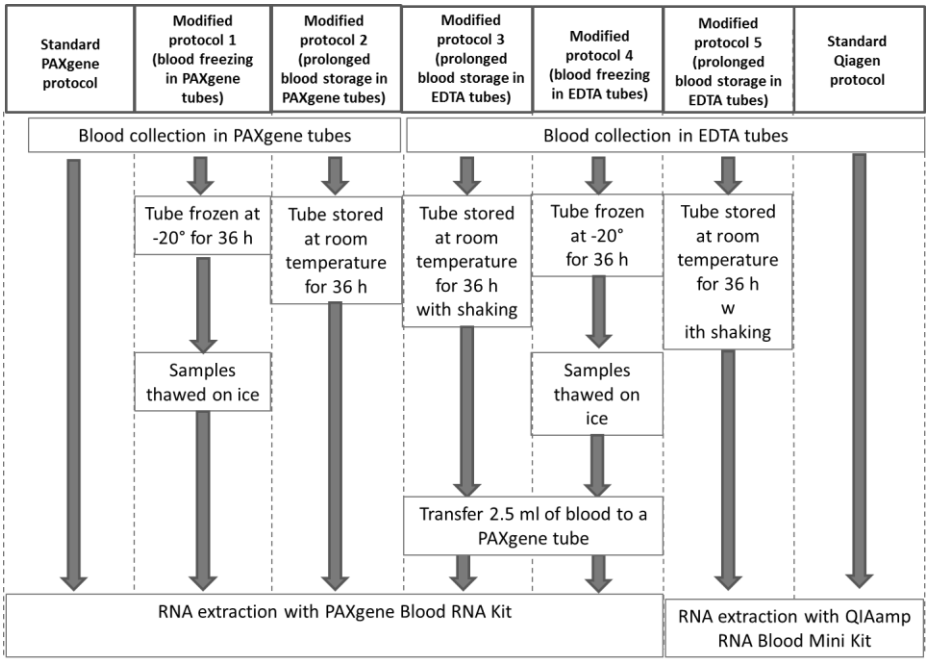

Figure 18. Schematic representation of the experimental procedures.

cDNA synthesis was performed using the GoScript™ Reverse Transcription System (Promega, Leiden, The Netherlands) with random hexamer primers.

For each gene, qRT-PCR reactions were run in duplicate using the MESA GREEN® qRT-PCR kit (Eurogentec, Seraing, Belgium) on an Applied Biosystems® 7500 Real-Time PCR instrument following the manufacturer's instructions. qRT-PCR data were analyzed by 7500 Software v2.0.6 and Microsoft Excel using the Pfaffl method [273]. The relative amount of transcript of the selected genes was normalized to *PGK1* and *HPRT1* reference genes (RGs) using the geometric mean of these reference genes [274].

4.3.2 Blood collection and *in vitro* irradiation

Peripheral blood samples were collected in EDTA coated tubes from healthy donors with informed consent and ethical approval from the local SCK•CEN Ethics Committee. All procedures were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. Half of the samples were used as reference samples, the other half was used as blind samples. Donor information can be found in Table 9.

Table 9. Donor information

Donor	Gender	Age	Smoking status	Recent viral/bacterial infection	Reference/blind sample
D1	Male	24	No	No	Reference
D2	Female	24	No	No	Reference
D3	Male	29	No	No	Reference
D4	Male	23	No	Yes	Reference
D5	Female	28	No	No	Reference
D6	Female	27	No	No	Reference
D7	Female	46	No	No	Reference
D8	Male	56	Recently stopped	No	Blind
D9	Male	58	No	No	Blind
D10	Male	54	No	No	Blind
D11	Male	49	No	No	Blind
D12	Male	40	No	No	Blind
D13	Male	56	Yes	No	Blind
D14	Male	30	No	Yes	Blind

Each blood sample was collected in an EDTA-coated tube and aliquoted in either 5 ml (reference samples) or 2 ml (blind samples) tubes for irradiation. Reference samples were irradiated with 0, 25, 50, 100, 500, 1000, and 2000 mGy, after which the tubes were placed on a rocking platform in an incubator at 37°C without CO_2 supply. The irradiations were performed at room temperature with an Xstrahl machine (250 kV, 1.4 mm Cu + 3.8 mm Al filtration) at a dose rate of 0.14 Gy/min. At 8, 12, 24, 36 and 48 h after irradiation an 1 ml aliquot was taken from each sample and used for RNA extraction. The doses and fixation time points for blind samples were assigned randomly and are given in Table 10.

Table 10. Information on doses and time points assigned to blind samples

Donor	Code	Dose, mGy	Time point, h
D8	B	0	8
D8	R	200	8
D8	P	900	12
D9	I	700	8
D9	C	60	12
D9	L	0	24
D10	S	1200	12
D10	A	30	24
D11	W	100	8
D11	F	1600	36
D11	M	0	48
D12	Q	60	36
D12	T	200	36
D12	G	2000	36
D13	Y	400	24
D13	U	400	48
D13	D	1200	48
D14	E	100	12
D14	J	0	24
D14	O	700	48

4.3.3 RNA extraction, quantification and quality control

The QIAamp RNA Blood Mini Kit was used to extract RNA from the blood samples used in the biodosimetry part of the present study. All procedures were performed following the manufacturer's instructions, unless stated otherwise. RNA concentration was measured on a Trinean Xpose instrument and the quality of total RNA samples was assessed using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). All samples had a RIN >8 and were therefore considered as suitable for further processing.

4.3.4 Reverse transcription and qRT-PCR

cDNA synthesis on the samples used for biodosimetry part of this study was performed using RT^2 First Strand Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. qRT-PCR was run using custom RT^2 Profiler PCR Arrays (Qiagen, Germantown, MD, USA). In the present study a standard 96-well plate-format arrays were used, including 25 genes of interest, four RGs (*HPRT1*, *PGK1, GAPDH* and *B2M*), positive PCR control, human genomic DNA contamination control and reverse transcription control. Each 96-well array could therefore be used to run three samples. The list of genes present on the arrays is given in Supplementary Table S9. Based on our previous results [259], for the genes alternatively transcribed/spliced in response to irradiation primers were designed to target the most responsive regions (Supplementary Table S9). qRT-PCR was run using RT^2 SYBR Green Mastermix (Qiagen, Germantown, MD, USA) following the manufacturer's instructions on an Applied Biosystems® 7500 Real-Time PCR instrument. qRT-PCR arrays data were analyzed using the dedicated software available at:

http://www.qiagen.com/be/shop/genes-and-pathways/data-analysis-center-overview-page/custom-rt2-pcr-arrays-data-analysis-center/

After comparing the variability of expression (standard deviations and variances of Ct values in 245 reference samples) of the four RGs, it was

decided to use only *B2M* to normalize the relative amounts of transcript of the genes of interest. The obtained ΔCt values to RG were used to train the prediction models. Relative expression levels were tested for statistical significance using 2-way ANOVA with Bonferroni *post-hoc* test.

4.3.5 Classification

Two models were built to predict both the radiation dose and time point of each blind sample based on the gene expression profiles. Two separate datasets were used for training and testing. For training the models, the data acquired from seven donors (reference samples, Table 8) were used, while for testing we used the data of seven different donors (blind samples, Table 8). Training both models consists of five consecutive steps, 1) identify the genes most likely responsible for the prediction of the radiation doses and time points (i.e. feature identification), 2) selecting the most informative features (i.e. feature selection), 3) building both models using the classifiers available (i.e. model training), 4) testing the models with a database unexposed to the training step (i.e. model testing), 5) selecting the best performing model for both problems (i.e. model nomination).

Feature (gene) identification was performed in our previous study [259] (see Chapter 3). Both dose and time point were treated as a regression variable ranging from 0 mGy to 2000 mGy and 0 h to 48 h, respectively. Importantly, the testing dataset was not only constructed from new subjects but also new intermediate doses such as 30, 60, 400, 700, 900, 1200 and 1600 mGy not used for training the model were included. This is particularly important to illustrate the independency of the model from the set of training instances.
Secondly, we attempted to perform a feature selection step, important to reduce the complexity and increase the accuracy of the model. To investigate if all features were necessary for the predictive model, we used the WEKA software implementation to get an idea of the importance of all attributes. Interestingly, however, performing the principle component analysis for

feature pre-selection illustrated the necessity for all features to describe 95% of the variance within the training dataset (data not shown). Consequently all features were included for training both models using a wide range of classifiers available in WEKA [299].

Different classification and regression models were considered including Support Vector Machines (SVM), linear regression, Multi-Layer Perceptron (a neural network), nearest neighbour, and three decision tree models: M5Base (implementing base routines for generating M5 Model trees and rules), decision stump and Fast decision tree learner as implemented in WEKA [299]. Next, each of the trained classifiers was tested using the test dataset, during the fourth step (model testing). To select the optimal classifier, we trained and tested various models. We reported for each classifier Pearson correlation coefficient and the relative absolute error (RAE) calculated for dose prediction as following:

$$\boldsymbol{RAE_{Dose}} = \sum_{i=1}^{n} |\boldsymbol{x_i} - \boldsymbol{y_i}| \,/\, \sum_{i=1}^{n} |\boldsymbol{z} - \boldsymbol{y_i}|$$

where $\boldsymbol{x_i}$ is the predicted dose, $\boldsymbol{y_i}$ is the true dose, $\boldsymbol{z}$ is the average of *x* values, for every *n* cases.

For time point prediction RAE was calculated as following:

$$\boldsymbol{RAE_{Time}} = \sum_{i=1}^{n} |\boldsymbol{x_i} - \boldsymbol{y_i}| \,/\, \sum_{i=1}^{n} |\boldsymbol{z} - \boldsymbol{y_i}|$$

where $\boldsymbol{x_i}$ is the predicted time point, $\boldsymbol{y_i}$ is the true time point, $\boldsymbol{z}$ is the average of *x* values, for every *n* cases.

Finally, we nominated the best performing model for each case (predicting the radiation doses and time points) achieving the lowest mean absolute error while having the highest correlation coefficient.

4.4 Results

4.4.1 Optimization of RNA extraction protocols for emergency situations

To optimize RNA extraction for emergency situations, different protocols were compared and the total RNA content, integrity and contamination was quantified.

In this part of the study a few modifications of the standard protocols of two commercially available kits, which might be useful for a mass-casualty event, were tested: PAXgene tubes, which are specifically designed for direct collection of blood and preservation of RNA for up to 3 days at room temperature and up to 50 months at -20°C and QIAmp kit intended for molecular biology applications. PAXgene system showed consistently high yields (3.11-3.78 µg of RNA per ml of blood on average) of high-quality RNA (average RIN 8.22-9.42), in case blood was directly collected in PAXgene tubes (Figure 18, Standard PAXgene protocol, Modified protocols 1 and 2). Alternatively, the option of collecting the blood in standard EDTA-coated tubes followed by transfer into PAXgene tubes was also explored (Figure 18, Modified protocol 3). This approach resulted in extraction of high-quality RNA (average RIN 9.2), however RNA yields were lower compared to the standard PAXgene procedure (2.47 µg of RNA per ml of blood on average). Importantly, sufficient RNA (1.16 µg of RNA per ml of blood on average) of acceptable quality (average RIN 6.84) could also be extracted from the blood samples which were frozen at -20°C and transferred into PAXgene tubes after thawing (Figure 18, Modified protocol 4). QIAmp protocol does not allow processing of frozen blood, therefore the options of protocol modifications were limited (Modified protocol 5), but both tested protocols involving this kit resulted in high yields of RNA (3.29-3.59 µg of RNA per ml of blood on average) of high quality (average RIN 9.7-9.9) (Figure 18, Modified protocol 5 and QIAmp standard protocol). Another parameter which was tested is the contamination of RNA samples with excessive globin mRNA. In this respect, QIAmp kit clearly outperformed PAXgene, showing low globin mRNA

contamination results comparable to those obtained for isolated PBMCs, used as a reference.

4.4.2 Robust dose- and time-dependent response of the biodosimetric gene panel

In order to identify the best endogenous controls consistently expressed across the sample population, we compared the variability of expression of *PGK1*, *HPRT1*, *GAPDH* and *B2M*. The standard deviations of Ct values for *B2M* were lower than for *PGK1*, *HPRT1*, and *GAPDH* (Table 11). Also the differences between the minimal and maximal registered Ct values for B2M were the lowest (~5-fold compared to ~10-fold for the other genes). Therefore, in the subsequent analyses, the Ct values of target genes were normalized to the Ct values of *B2M*.

Table 11. Descriptive statistics of Ct values for the four potential reference genes from 245 calibration samples (7 donors × 7 doses × 5 time points)

	Mean Ct	Median Ct	Minimum Ct	Maximum Ct	Standard deviation
PGK1	23.12	22.74	19.81	29.19	1.67
HPRT1	27.93	27.88	21.03	31.74	1.21
GAPDH	24.32	23.88	20.78	30.3	1.85
B2M	18.51	18.47	16.68	21.87	1.04

Blood samples from seven healthy volunteers exposed to X-ray doses ranging from 25 to 2000 mGy were used as reference samples at five different time points following exposure: 8, 12, 24, 36 and 48 hours. In total, 25 genes were assessed (Figure 19). Overall, it was clear that for many genes in a similar *in vitro* experiment 36 h time point is the limit as at 48 h a significant up-regulation was observed also in non-exposed samples. At 48 h post-irradiation, some genes, e.g. *AEN*, did no longer demonstrate differential expression with respect to non-exposed samples. This fading effect could be observed to some extent for the majority of radiation-responsive genes, albeit

with different kinetics. An exception was *CDKN1A*, the expression of which in control samples increased with a peak at 24 h and gradually decreased up to 48 h (Figure 20).

This effect was more pronounced (i.e. the effect of radiation was totally masked at 48 h) and sometimes observed at earlier time points for the genes involved in apoptosis (e.g. *TNFRSF10B,* Figure 20). Other genes, such as *FDXR,* showed stable dose-dependent up-regulation at all the time points (Figure 20). A few genes, e.g. *SESN1,* did not show a consistent dose-dependent response in contrast to what was expected (Figure 20). Three genes (*PF4*, *GNG11* and *CCR4*) which were previously shown to differentially respond to low- and high-dose exposures [276], in our set up did not show significant differences in response with respect to dose, however, the expression of *GNG11* and *CCR4* was clearly affected by the time after exposure.

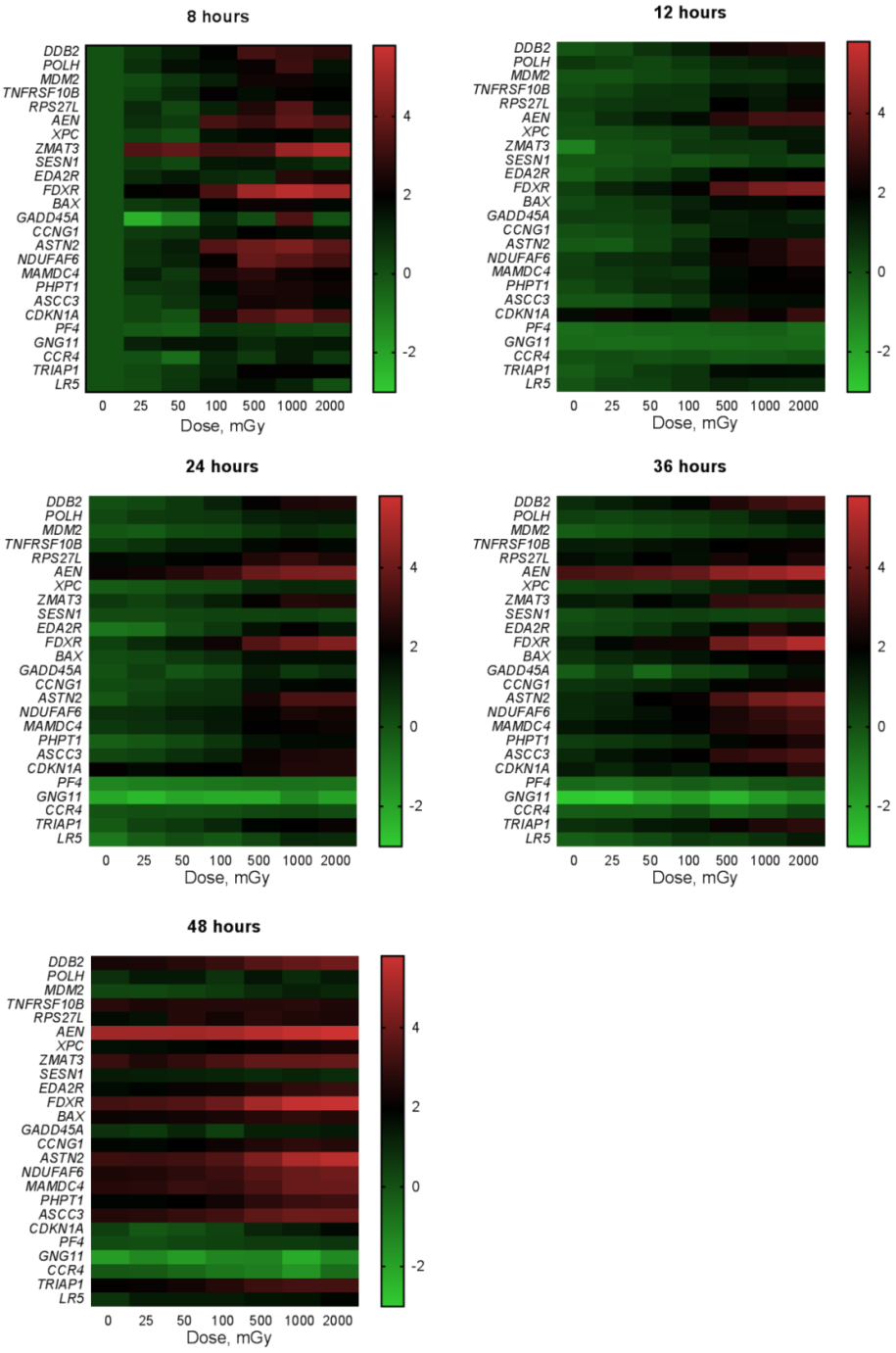

Figure 19. Heatmaps showing expression levels (relative to control samples at 8 h, log_2 transformed) for all the 25 genes (shown in rows) included in the qPCR arrays in response to exposure to different doses (shown in columns) at five time points.

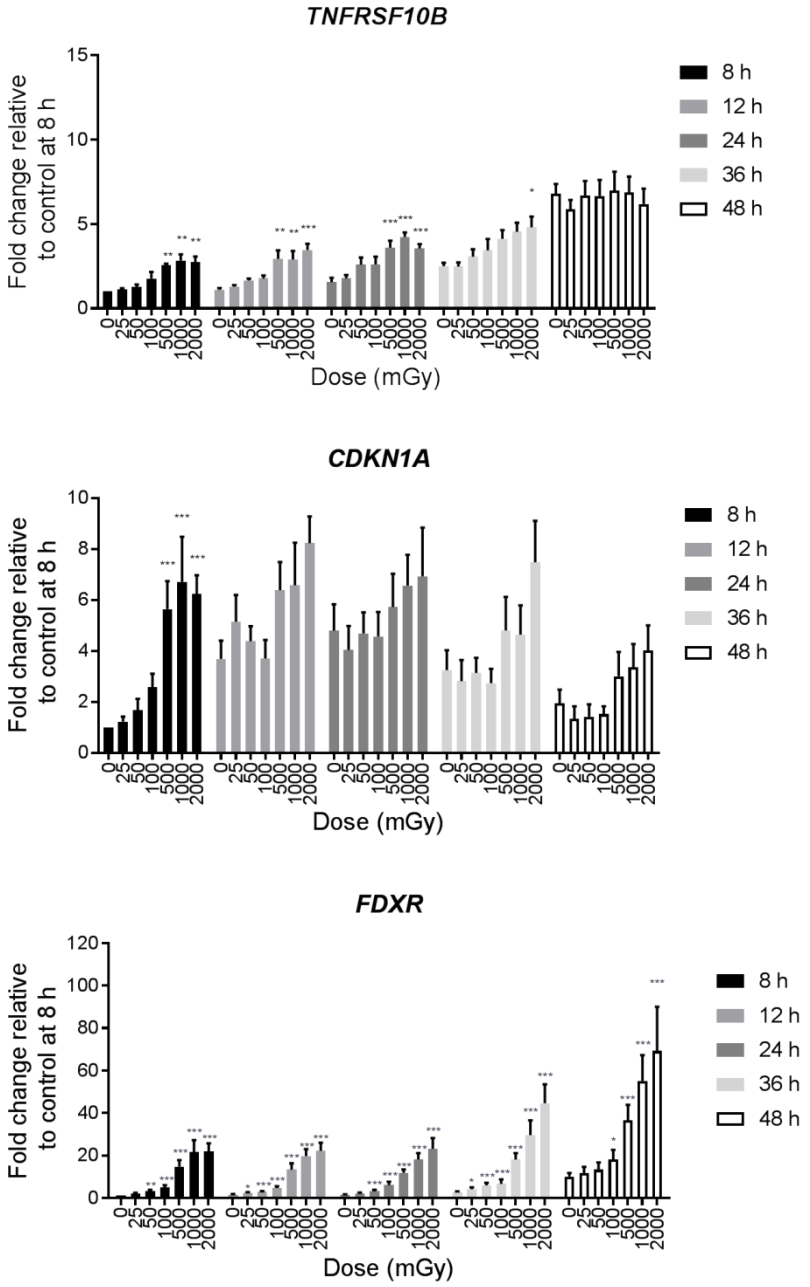

Figure 20. Changes in expression of several genes (*TNFRSF10B*, *CDKN1A, FDXR*) present on qPCR arrays. Data are shown for blood samples of seven healthy donors irradiated *in vitro* with doses from 0 to 2000 mGy and fixed at 5 time points after exposure. Error bars represent standard deviations. Statistical comparison was performed using repeated measures 2-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).

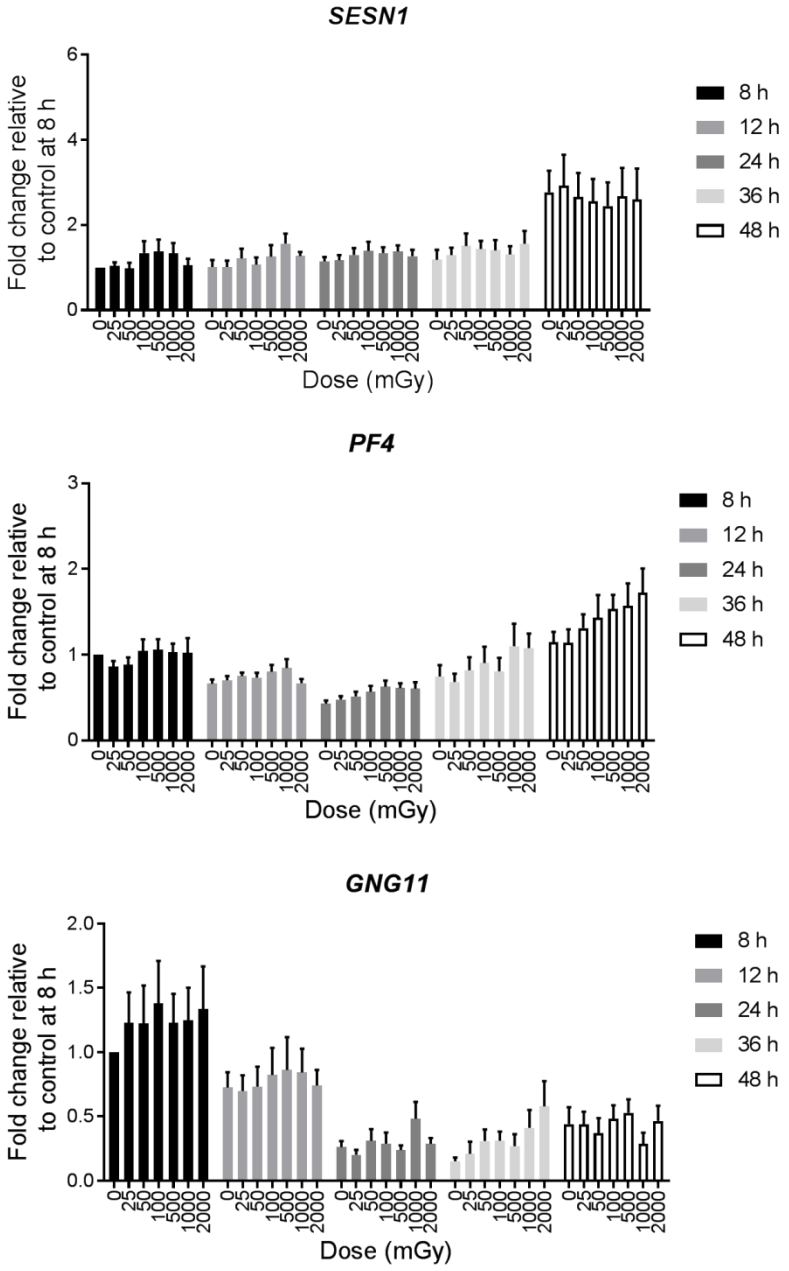

Figure 20 (continued). Changes in expression of several genes (*SESN1*, *PF4, GNG11*) present on qPCR arrays. Data are shown for blood samples of seven healthy donors irradiated *in vitro* with doses from 0 to 2000 mGy and fixed at 5 time points after exposure. Error bars represent standard deviations. Statistical comparison was performed using repeated measures 2-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).

4.4.3 Dose and time after exposure can be predicted from changes in gene expression

The ΔCt values of all the reference samples were used as the training dataset for building the dose and time point prediction models. Following this, the selected model was used to predict the dose and the time after exposure of the blind samples. The obtained results are shown in Table 12 and Figure 23.

Table 12. Dose and time point prediction results for blind samples

Sample code (with remark)	True time point, h	Predicted time point, h	Error, h	True dose, mGy	Predicted dose, mGy	Error, mGy
A	24	23.3	-0.7	30	20	-10
B (ex-smoker)	8	9.5	+1.5	0	20	+20
C	12	10.2	-1.8	60	20	-40
D (smoker)	48	44.0	**-4.0**	1200	900	-300
E (infection)	12	8.9	-3.1	100	30	-70
F	36	39.6	+3.4	1600	1370	-230
G	36	43.2	**+7.2**	2000	1370	**-630**
I	8	4.5	-3.5	700	410	-290
J (infection)	24	22.1	-1.9	0	20	+20
L	24	15.5	**-8.5**	0	20	+20
M	48	42.7	**-5.3**	0	120	+120
O (infection)	48	43.9	**-4.1**	700	900	+200
P (ex-smoker)	12	14.0	+2.0	900	1370	+470
Q	36	39.0	+3.0	60	120	+60
R (ex-smoker)	8	10.5	+2.5	200	420	+220
S	12	18.4	**+6.4**	1200	420	**-780**
T	36	40.1	**+4.1**	200	120	-80
U (smoker)	48	41.0	**-7.0**	400	900	**+500**
W	8	10.0	+2.0	100	230	+130
Y (smoker)	24	21.4	-2.6	400	410	+10

Bold: time point predictions with more than 4 h difference from the true time point and dose predictions with more than 500 mGy difference from the true dose.

For the dose prediction, the fast decision tree learner (implemented as REPTree in WEKA) was found capable of achieving the highest Pearson correlation coefficient between the true and the predicted doses of 0.86 with the lowest RAE of 42%. The merit of this classifier is that it builds the decision based on the information gained and prunes the tree branches using reduced-error pruning (with backfitting) (Figure 21).

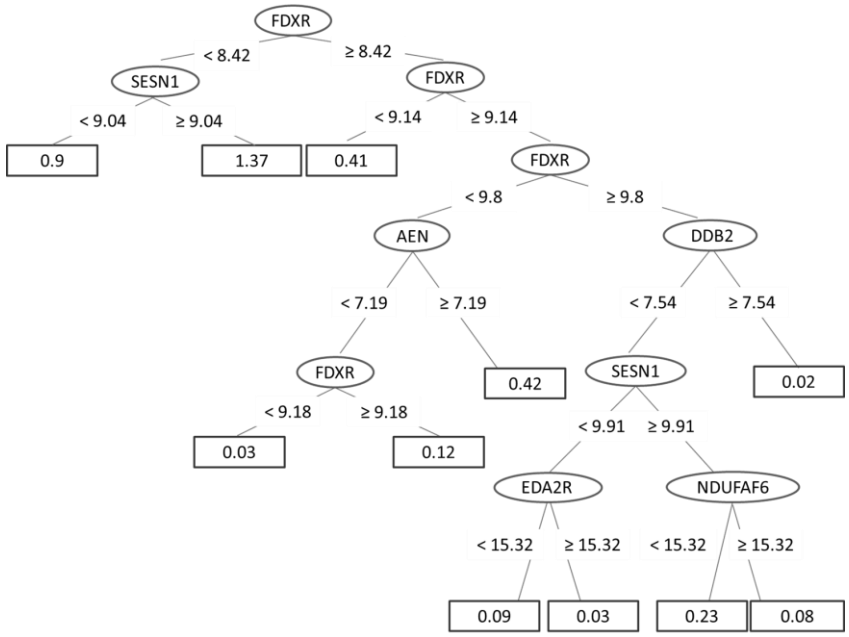

Figure 21. The decision tree for the REPTree model built for dose prediction, ending with 11 "leaves" (for each the dose prediction is calculated).

Dose prediction was more accurate for doses below 0.5 Gy (Pearson correlation 0.85) compared to higher doses (Pearson correlation 0.55), probably resulting from the well-known plateau effect of the gene expression response at high doses. Although most of the genes were required to explain the variability within the data, set of four genes including *MDM2, FDXR, ASCC3* and *CDKN1A* was considered particularly important for dose prediction. For the time point prediction, the M5Base decision tree classifier (implemented in WEKA as M5P) was able to achieve a correlation coefficient between the true and the predicted time points of 0.96 (with lowest RAE of 28%). M5Base implements base routines for the generation of the M5 model [300, 301]. It functions as a decision tree by splitting the data into branches and leaves based on few parameters, but instead of assigning the "time points" for each leave, a linear regression function is calculated for each leave (enabling a continuous numeric prediction) (Figure 22).

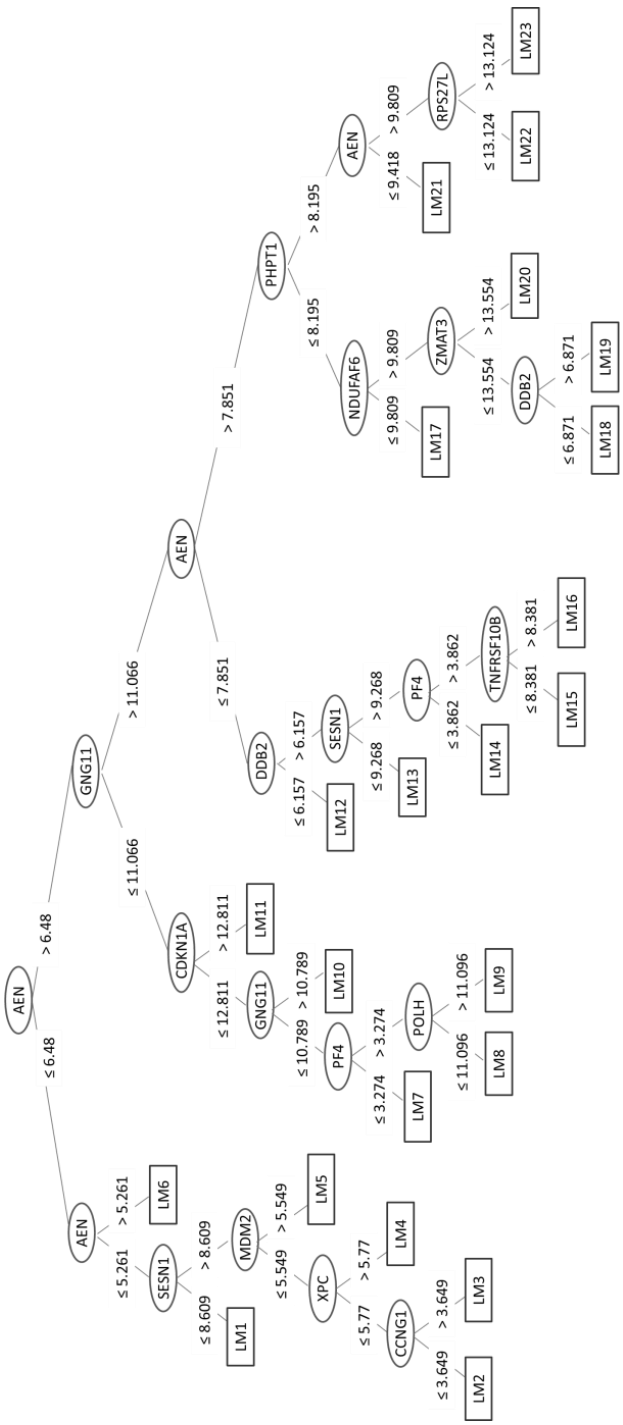

Figure 22. The decision tree for the M5Base model built for time point prediction, ending with 23 "leaves" (for each a linear model LM1-LM23 has been calculated).

The latest time point, 48 h, seemed to be the most difficult to predict with the highest number of predictions out of the ±4 h interval (Table 12). The most discriminative genes were *AEN, ASCC3, CDKN1A, GNG11* and *CCR4*, although all genes were found necessary to describe the dissimilarity within the data. In case of both dose and time point prediction using only the most important genes did not result in significantly better model performance (data not shown). All three samples misclassified according to the true dose were also misclassified according to the true time point. Among the individuals who donated blood for blind samples, one was a smoker, one recently stopped smoking and one individual reported a recent infection. Prediction of the doses or time points for these individuals was not particularly more problematic, however, a trend for dose overestimation could be observed (Figure 23). Also, the median age of donors whose blood was used for reference samples (27 years) was lower, than that of the blind sample donors (54 years), nevertheless dose prediction was still efficient.

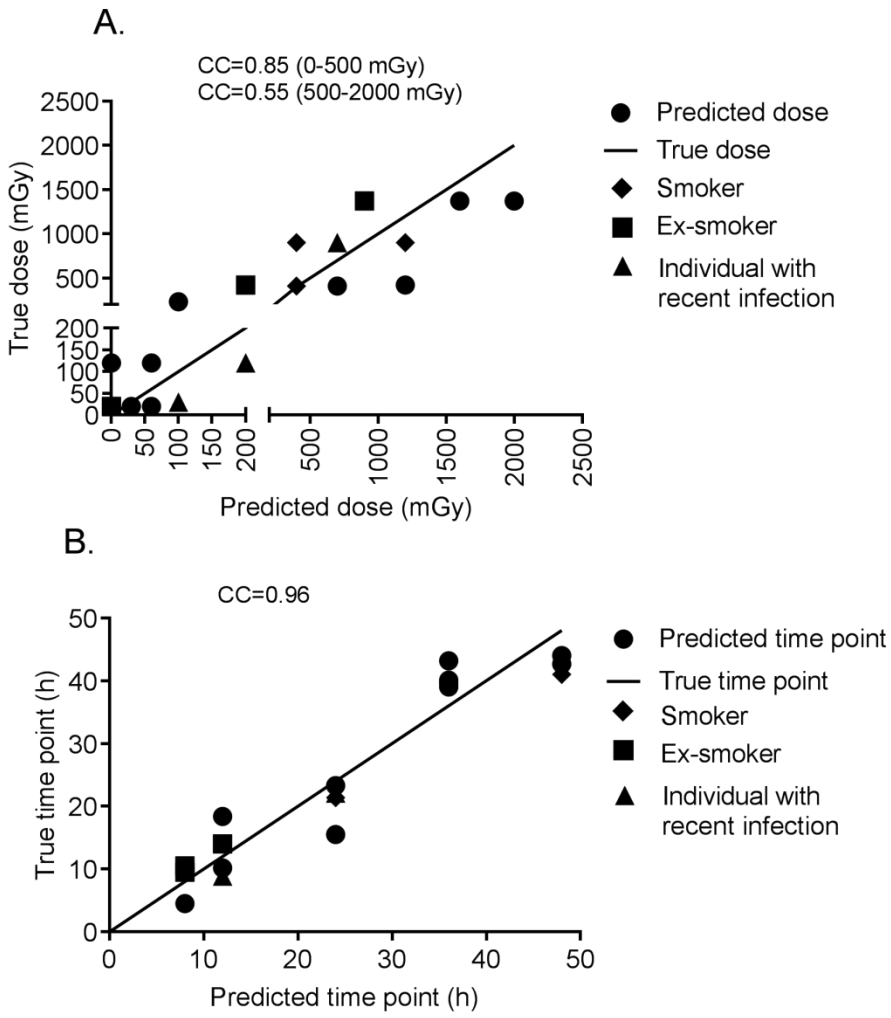

Figure 23. Dose (A) and time point (B) prediction results for the blind samples. CC – correlation coefficient.

4.5 Discussion

Gene expression is emerging as a highly powerful readout for biodosimetry. Several studies demonstrated the usefulness of microarray technology for analyzing large sets of transcripts for dose prediction [218, 232, 259, 280].

However, as was demonstrated in a recent biodosimetry exercise, the analysis of a short list of genes, or even one gene by means of qPCR technology, is far more straightforward and cheaper than the microarray analysis, while the accuracy of dose prediction is essentially similar [55]. Many of the genes included in our biodosimetry panel were also previously tested by other groups [155, 231, 292, 293], suggesting their robustness as radiation biomarkers. Nevertheless our panel composition is unique because we could select the most radiation-sensitive exons of the respective genes for qRT-PCR analysis based on our previous results [259].

Gene expression changes are a highly dynamic process, therefore knowing the point in time at which sample is taken following the radiation exposure is highly important for correct dose estimation. The classification method used in our study allowed to classify the samples according to the time elapsed since exposure with high precision (errors ≤ 4 h). The importance of time factor is also highlighted by the fact that all three samples misclassified according to the true dose were also misclassified according to the true time point. It is important to note, however, that the gene expression kinetics might be different following *in vivo* exposure, as a result adaptation of the methodology for *in vivo* exposure situation might be required.

Another difficulty of any biodosimetric analysis is the correct choice of the unexposed controls to be used for comparison with the exposed samples. It is particularly critical because confounding factors, such as age, gender, infections and inflammatory diseases, smoking status and lifestyle in general might influence the expression levels of specific genes. In this regard, using a signature of genes rather than one gene, in our opinion, could already help solving the problem and allow for effect compensation. Experiments performed by Tucker et al. in mice and by Budworth et al. in *ex vivo* irradiated human blood samples showed that although the expression of some biodosimetry-relevant genes (e.g. *CDKN1A*, *FDXR*, *BBC3*) is affected by the bacterial endotoxin lipopolysaccharide, they could still be used for dose

prediction even in the presence of inflammatory stress [159, 302]. The performance of a dose prediction gene signature was also found to be unaffected by gender or smoking status of blood donors [271]. In our case, dose prediction for samples of smokers or a donor with a recent infection was also efficient, however, these results need further validation on a larger cohort of donors. Importantly, our approach allowed efficient identification of unexposed blind samples even in the absence of pre-exposure samples from the same individuals.

Also, in many studies published so far fold change in expression between irradiated and control samples were used [154, 155, 231]. However, this approach will not be applicable to a real large-scale accident as the data for control samples from each potentially exposed individual will not be available. Therefore, in the present study, we opted to use ΔCt values compared to the RGs. This approach was previously used by Tucker et al. [292] and Brzoska and Kruszewski [293], as well as in two biodosimetry exercises organized by the RENEB consortium [51, 55].

The dose prediction was not confounded by the time that elapsed after exposure and *vice versa*. This indicates that even at later time points after irradiation, dose prediction based on a signature of genes is still possible. The transcriptional biodosimeters used in the present study were chosen based on our previous microarray results. However, not all of the tested genes responded to ionizing radiation as was expected. This highlights the importance of accurate validation of transcriptional biomarkers identified in microarray studies. A few genes from our panel were statistically most important parameters for dose (*MDM2, FDXR, ASCC3* and *CDKN1A*) and time point (*AEN, ASCC3, CDKN1A, GNG11* and *CCR4*) prediction, however, they do not capture all variability within the data, therefore relying only on these few genes would reduce the accuracy of the prediction. Out of the three genes (*PF4*, *GNG11* and *CCR4*) which were previously shown to differentially respond to low- and high-dose exposures [276], in our set up only *PF4*

followed a similar expression profile. Importantly, these chemokine-related genes showed time-dependent changes in expression profile, most probably induced by *in vitro* culture conditions, contributing to time point prediction in blind samples.

For most of the studied genes we consistently found a plateau in expression for high doses (1000 and 2000 mGy), which probably contributed to less precise dose prediction in blind samples. This saturation effect at doses higher than 2 Gy was also previously observed during RENEB biodosimetry exercise [55] and in our preliminary experiments (data not shown). This feature may reflect a saturation of the response, but it might also be the result of *in vitro* culture conditions. In radiotherapy patients undergoing total body irradiation several genes included in our panel (e.g. *CDKN1A*, *FDXR*, and *PHPT1*) showed further up-regulation after 3.75 Gy compared to 1.25 Gy [233]. Nevertheless, our approach has good potential to allow correct identification of the “worried wells” from the individuals with relatively low-level radiation exposure (below 100 mGy) and those who might manifest the acute radiation syndrome symptoms, which occur after whole-body or significant partial-body irradiation of greater than 1000 mGy delivered at a high-dose rate [303]. In experiments in mice this saturation effect was observed at acute exposure to 6 Gy [237].

Although a more common approach for similar studies is diluting the blood samples either 1:1 or 1:3 with appropriate culture medium supplemented with fetal bovine serum, which still results in about 47% of unstimulated lymphocytes undergoing apoptosis after 48 h in culture [304],we opted for using undiluted blood samples for our experiments. We realize that this protocol might not be optimal, especially for longer incubation time points, such as 36 and 48 h, but our goal was to interfere as little as possible with the samples, as gene expression changes might be affected by culture conditions, and more specifically by addition of fetal bovine serum [305, 306]. A similar approach was used by Manning and co-authors, and cell viability of

approximately 99% was found in blood samples incubated for 24 h at 37°C. In our study the genes, most affected by incubation time were those involved in apoptosis (e.g. *AEN* and *TNFRSF10B*), suggesting the increase in number of dying cells with time. Our results allow us to set a threshold of feasibility of 24 or 36 h, depending on the gene, for similar *in vitro* experiments. *In vivo* irradiation experiments performed by Tucker and co-authors in mice confirmed the possibility of dose prediction based on gene expression for at least 7 days after exposure, which is very promising, but whether the same is true in humans remains to be investigated [237]. Studies involving radiotherapy patients showed that prediction of *in vivo* radiation dose using gene signatures was possible for at least 24 h following the first fraction of 2 Gy local exposure to the pelvis [55] and after one and three 1.25-Gy fractions of total body exposure [233].

A highly important step in biodosimetric triage of casualties of a nuclear accident is collecting and preserving the blood samples in an appropriate way for further analysis. For gene expression measurement the time elapsed from exposure to blood collection and sample fixation is critical for correct dose estimation, as gene expression in non-frozen and non-stabilized blood would still be subjected to change. Several approaches to solve this issue were previously tested. Brzoska and Kruszewski extracted RNA for gene expression analysis from blood which was frozen and stored at -75°C, therefore allowing for preservation of gene expression signature at the moment of blood collection [293]. However, this approach might still not be practical in field conditions due to the lack of very low temperature freezers. An alternative approach, tested during the recent RENEB biodosimetry exercise, includes the addition of RNA stabilization reagents which protect RNA from degradation allowing for sample storage and transportation at room temperature for several hours or even days [51]. However, this methodology still requires the availability of significant quantities of such reagents at the accident site. In addition, the further processing of the samples might be

affected by transport conditions [51]. Therefore, in our study we tested another approach, consisting in blood freezing at -20°C, which would allow blood preservation without any specific equipment or reagents. Upon delivery to the biodosimetric laboratory, blood samples would be thawed and transferred to PAXgene tubes, allowing for recovery of sufficient RNA of acceptable quality.

Taken together, in the present study, we proved the usefulness of a biodosimetric panel of genes in predicting both dose and time after *in vitro* exposure. Our results confirm that the analysis of expression of these genes, which can be carried out in virtually any laboratory possessing a qPCR instrument, can certainly provide sufficient information for triage purposes in comparatively short amount of time. The validity of our customized biodosimetric qPCR arrays was recently confirmed in interlaboratory comparison exercise organized by the RENEB consortium [51]. Although in this exercise different experimental procedures, including different dose assessment approach were used, we could still achieve good dose prediction [51], confirming the flexibility and versatility of our method. We also suggest a blood preservation method allowing for further RNA extraction feasible for field conditions. Further research is needed to confirm the time- and dose-wise validity and applicability of our signature for *in vivo* situation, as well as to reveal the potential impact of confounding factors on the reliability of the gene expression-based biodosimetry.

4.6 Acknowledgements

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4.7 Conflict of interest statement

Authors declare no competing interests.

4.8 Supplementary material

Supplementary Table S9.

Gene name	Ensembl gene ID	Location of primers	Ensembl transcript used for exon numbering
DDB2	ENSG00000134574	Any exon	N/A
POLH	ENSG00000170734	Any exon	N/A
MDM2	ENSG00000135679	Any exon	N/A
TNFRSF10B	ENSG00000120889	Any exon	N/A
RPS27L	ENSG00000185088	Exon 2-3	Transcript: RPS27L-001 ENST00000330964
AEN	ENSG00000181026	Any exon	N/A
XPC	ENSG00000154767	Exon 2 or 16	Transcript: XPC-001 ENST00000285021
ZMAT3	ENSG00000172667	Exon 2	Transcript: ZMAT3-001 ENST00000311417
SESN1	ENSG00000080546	Exon 2-10	Transcript: SESN1-001 ENST00000436639
EDA2R	ENSG00000131080	Any exon	N/A
FDXR	ENSG00000161513	Exon 6-8	Transcript: FDXR-002 ENST00000442102
BAX	ENSG00000087088	Exon 3-4	Transcript: BAX-001 ENST00000293288
GADD45A	ENSG00000116717	Exon 3-4	Transcript: GADD45A-001 ENST00000370986
CCNG1	ENSG00000113328	Exon 2-3	Transcript: CCNG1-001 ENST00000340828
ASTN2	ENSG00000148219	Exon 18-19	Transcript: ASTN2-001 ENST00000361209
NDUFAF6	ENSG00000156170	Exon 1-2	Transcript: NDUFAF6-001 ENST00000396111
MAMDC4	ENSG00000177943	Exon 25-26	Transcript: MAMDC4-005 ENST00000317446
PHPT1	ENSG00000054148	Exon 2-5	Transcript: PHPT1-003 ENST00000371661
ASCC3	ENSG00000112249	Any exon	N/A
CDKN1A	ENSG00000124762	Exon 2-3	Transcript: CDKN1A-201 ENST00000405375
PF4	ENSG00000163737	Any exon	N/A
GNG11	ENSG00000127920	Any exon	N/A
CCR4	ENSG00000183813	Any exon	N/A
TRIAP1	ENSG00000170855	Exon 2	Transcript: TRIAP1-001 ENST00000546954
LRP5	ENSG00000162337	Exon 3-7	Transcript: LRP5-001 ENST00000294304

5.

Transcriptional profiling of human peripheral blood mononuclear cells after exposure to equivalent doses of photons and heavy ions

This chapter is modified from:

Macaeva E., Tabury K., Van Walleghem M., Michaux A., Janssen A., Averbeck N., Moreels M., De Vos W., Baatout S., Quintens R. *Transcriptional profiling of human peripheral blood mononuclear cells after exposure to equivalent doses of photons and heavy ions.* Manuscript under revision for resubmission to *Oncotarget* journal.

5.1 Abstract

Understanding the differences in the cellular response to low- and high-LET radiation is important in order to optimally exploit the benefits of particle therapy, as well as to adequately apply radiation protection measures to astronauts during Space missions. To address this need, we compared the transcriptional profiles of freshly isolated peripheral blood mononuclear cells after exposure to 1 Gy of X-rays, iron ions or carbon ions. While all radiation types induced a p53-dependent gene expression response at 8 h after exposure, heavy ions exposure triggered a prolonged activation of p53-mediated genes after 24 h as compared to X-rays. This coincided with a stronger induction of DNA damage repair and larger residual DNA damage as evidenced by γH2AX immunostaining. Despite a common p53 signature between low-LET and high-LET irradiation, specific gene sets related to immune response and epithelial-mesenchymal transition were significantly enriched in cells irradiated with heavy ions. In addition, irradiation, and in particular exposure to carbon ions, promoted radiation-induced alternative splicing. Classification of DNA repair-related gene signature revealed a strong correlation with radiation type, timing and especially the donor, suggesting

that it may serve as a sensitive indicator of individual DNA damage repair capacity.

In conclusion, we have shown that both low- and high-LET irradiation induce similar transcriptional pathways, albeit with variable amplitude and timing, but also elicit radiation-type specific events that may have implications for cancer progression and treatment.

5.2 Introduction

The use of charged particles is a promising modality of cancer therapy. At the same time, exposure to charged particles represents a significant risk factor for chronic and late effects in astronauts. Particle therapy, which uses focused beams of charged particles such as protons and carbon ions, has become the treatment of choice for targeting specific solid tumors [9]. The main advantage of charged particle beams is the possibility of more precise tumor targeting, while the surrounding healthy tissues receive a much lower dose compared to conventional photon radiotherapy [9]. High-LET carbon ion radiation also has a higher relative biological effectiveness (RBE) compared to conventional low-LET photon therapy [95], as particles deposit their energy in a more concentrated manner and therefore result in more complex and clustered DNA damage which is more lethal to the tumor cells [96]. However, other endpoints but cell death, such as chromosome aberrations, genetic alterations and normal tissue damage, also show an increased RBE for high-LET radiation [122]. Normal tissue injury is a complex process, which is not solely caused by cell death. Radiation-caused DNA damage triggers changes in the microenvironment through chemokines and cytokines, altered cell-cell interactions, influx of inflammatory cells and the induction of restorative processes [307]. Genes involved in DNA damage repair, apoptosis, proliferation and inflammatory processes may play a role in the normal tissue response to irradiation [308].

Astronauts are at increased risk for high-LET radiation exposure in space. The more feasible and realistic long-term and interplanetary space missions and commercial space flights become, the more concern they raise about possible health risks due to exposure to cosmic radiation. Humans in Deep space would be subjected to galactic cosmic rays (GCR) and solar particle events (SPE), which result in levels of radiation hundreds of times higher than on Earth. The GCR spectrum is composed of about 87% high energy protons, 12% alpha-particles and 1% of heavier ions up to iron [56] which are extremely penetrating and difficult to shield [309]. SPE consist of low to medium energy protons and alpha-particles. Up to now, the assessment of radiation risk for astronauts is almost completely based on extrapolation from epidemiological data on low-LET exposures. Therefore, comprehensive models and radiobiological studies comparing the biological response to different radiation types are needed to validate this approach [310].

The particles and energies which are most often used for particle therapy partially overlap with the lower range of charge and energies of the ions commonly related to space applications (Z=1-26 and approximately 100-1000 MeV/nucleon). Understanding the cellular radiation response and the processes governing individual sensitivity to high-LET radiation is of pivotal importance in rational choice of radiotherapy treatment schemes. The same holds true for the risk assessment of astronauts and the development of effective protection measures.

Radiobiological transcriptional studies can bring valuable results in this regard, revealing the biological basis of the cellular response to different radiation types [311]. However, there have been only a limited number of studies comparing gene expression profiles following exposure to low- and high-LET radiation in isolated human peripheral blood mononuclear cells (PBMC) exposed *in vitro* to α-particles and X-rays [235] and human peripheral blood exposed *in vitro* to neutrons and X-rays [312] or mouse blood cells exposed *in vivo* to neutrons and X-rays [313].

To gain more insight into the cellular response to low- and high-LET radiation, we compared the transcriptional profiles of PBMCs of healthy donors after the cells had been exposed to X-rays, carbon ions and iron ions. We identified biological processes over-represented as a response to heavy ions exposure or X-ray exposure, as well as processes shared by both types of radiation. Our results provide an important basis for further detailed investigations of differential normal tissue responses to high- and low-LET radiation.

5.3 Materials and methods

5.3.1 Blood collection and PBMCs isolation

Peripheral blood samples were collected from healthy donors in 9 ml EDTA vacutainer tubes (10 individuals were involved in the X-ray, 12 individuals in the carbon ions and 6 in the iron ions experiment). Blood collection was approved by the local SCK•CEN Ethics Committee and were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. Prior to blood donation all the donors involved in the present study signed an informed consent form. Within 30-60 min of blood drawing, PBMCs were isolated by centrifugation on Histopaque-1077 density gradient (Sigma-Aldrich, Bornem, Belgium) according to the manufacturer's instructions. Isolated cells were suspended at a density of 10^6 cells/ml in LGM-3 culture medium (Lonza, Walkersville, MD, USA) and allowed to equilibrate to culture conditions at 37°C in a humidified 5% CO_2 atmosphere.

5.3.2 *In vitro* irradiation

X-ray, carbon and iron ion irradiations were performed independently, on different days. X-ray irradiation experiments were performed at the irradiation facility at the Belgian Nuclear Research Centre, Mol, Belgium (for microarrays) and at GSI, Darmstadt, Germany (for qRT-PCR validation and γH2AX staining). At SCK•CEN, PBMCs were exposed to 1.00 Gy of X-rays, using a Pantak HF420RX machine (250 kV, 15 mA, dose rate of 0.26 Gy/min)

as described in [259]. For samples that were irradiated at GSI, freshly isolated PBMCs were transported for 4 h by car to GSI using a transportable incubator. Irradiation with heavy ions was done at GSI's heavy-ion synchrotron SIS. Carbon ion exposure (0.25 and 1.00 Gy) was performed in the middle of a 25 mm spread-out Bragg peak (center depth 42.5 mm, realized with a PMMK bolus), obtained by active energy variation of the beam in the range of 114.6 – 158.4 MeV/u. Accordingly, the dose averaged LET at the proximal and distal part of the samples (5-ml plastic tube, inside diameter 10 mm) was 60-80 keV/µm. Irradiation with iron ions (0.25 and 1.00 Gy) was performed with a monoenergetic beam (1 GeV/u; LET 155 keV/µm). X-ray exposures were performed using an IV320-13 X-ray tube (250 keV, 16 mA, dose rate of 0.5 Gy/min; Seifert, Germany) at 0.25 and 1.00 Gy. Sham-irradiated samples were always subjected to exactly the same procedures as the irradiated ones, except for the radiation exposure itself. After *in vitro* irradiation, cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for the indicated time until further processing.

5.3.3 RNA extraction

RNA from irradiated and sham-irradiated PBMCs samples was extracted 8 and 24 hours after irradiation with X-rays and carbon ions and after 8, 12 and 24 hours after exposure to iron ions. For RNA isolation, a combination of the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) extraction method and the clean-up on Qiagen RNeasy columns (Qiagen, Venlo, The Netherlands) was used. Briefly, 5 x 10^6 cells were lysed in 1 ml of TRIzol® reagent and further processed following the manufacturer's recommendations. Following the RNA precipitation with isopropanol, the obtained pellet was re-suspended in 1 ml of ethanol and transferred to the RNeasy column. Further purification was done according to the manufacturer's instructions. RNA concentration was measured on a NanoDrop-2000 spectrophotometer (Thermo Scientific, Erembodegem, Belgium) and the quality of total RNA samples was assessed using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA,

USA). Only samples with an RNA integrity number >7 were considered as suitable for further analysis. For the X-ray experiment the samples from 10 donors were used for further microarray hybridization, in case of carbon and iron ions samples from 4 donors were used.

5.3.4 Microarray hybridization

Gene expression profiling was performed using the GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) which interrogates 28,536 well-annotated genes with 253,002 distinct probe sets, allowing expression analysis at both gene and exon level. Ten µg of cRNA, synthesized and purified from 0.25 µg of total RNA using the Ambion® WT Expression kit (Ambion, USA) was used for cDNA synthesis, followed by cDNA fragmentation and labeling with the GeneChip® Terminal Labeling kit [263]. Fragmented and labeled cDNA was hybridized to Human Gene 1.0 ST arrays [263] using the GeneChip® Hybridization, Wash and Stain kit [263] (hybridization module) and hybridization controls [263] with rotation at 45°C for 16 hours. After hybridization, arrays were washed and stained using the GeneChip® Hybridization, Wash and Stain kit (stain module) after which the arrays were immediately scanned using an Affymetrix GeneChip® Scanner.

5.3.5 Microarray data analysis

The obtained microarray data were imported into Partek Genomics Suite, version 6.6 (Partek Inc., St Louis, MO, USA) as .CEL-files. The probe summarization and probe set normalization were done using the Robust Multichip Analysis (RMA) algorithm (Wu and Irizarry 2004)[261] which includes background correction, quantile normalization and $\log_2$ transformation. Microarray data were analyzed using ANOVA with dose, donor and time point (whenever applicable) as factors. To correct for multiple testing, we used the false discovery rate (FDR) as described by Benjamini and Hochberg [262] to adjust *p*-values (FDR < 0.05). Genes were considered significantly differentially expressed between the two groups if adjusted *p*-

values were < 0.05. In some cases, a more stringent additional cut-off of fold-change ≥|2| was used, as explained in the text.

We also performed Alternative Splicing ANOVA in Partek to detect genes which were alternatively spliced in response to different radiation types. A FDR-corrected *p*-value of < 0.05 was considered significant for alternative splicing events. To further reduce the number of false positives, the probe sets with $\log_2$ values below the noise level in all samples were excluded from analysis, except for the cases where there was a significant difference in expression of a single exon between the groups ($p < 0.05$).

The Venny on-line tool [272] was used to compare gene lists and create Venn diagrams: http://bioinfogp.cnb.csic.es/tools/venny/index.html

The Gene Ontology Biological Processes enrichment analysis was performed using the on-line tool DAVID [314]:

https://david.ncifcrf.gov/home.jsp

5.3.6 Reverse transcription and qRT-PCR

The following genes were selected for qRT-PCR validation: *PCNA, GADD45A, RPS27L, ASTN2, NDUFAF6, FDXR, MAMDC4*. The same RNA samples as those used for microarray hybridization (n=6), whenever possible, were used for cDNA synthesis with the GoScript™ Reverse Transcription System (Promega, Leiden, The Netherlands) with random hexamer primers. For each gene, qRT-PCR reactions were run in duplicate using the MESA GREEN® qRT-PCR kit (Eurogentec, Seraing, Belgium) on an Applied Biosystems® 7500 Real-Time PCR instrument following the manufacturer's instructions. To determine the efficiency and specificity of the designed primers, a standard curve experiment with melt curve was run for every primer pair. qRT-PCR data were analysed by 7500 Software v2.0.6 and Microsoft Excel using the Pfaffl method [273]. The relative amount of

transcript of the selected genes was normalized to *PGK1* and *HPRT1* using the geometric mean of these reference genes [274].

5.3.7 Rank-Rank Hypergeometric Overlap (RRHO) analysis

The RRHO algorithm allows for the comparison of two microarray datasets. Each dataset is processed as a ranked list based on expression differences between two classes of samples (0 Gy and 1 Gy, in our case). RRHO analysis [315] was performed using the on-line tool (http://systems.crump.ucla.edu/rankrank/index.php).

As this algorithm only allows the comparison of two gene lists at a time, the following comparisons were performed: X-rays vs carbon ions, X-rays vs iron ions and carbon ions vs iron ions using a step size of 100.

5.3.8 Transcription factor and Gene Ontology terms enrichment analysis

Transcription factor and Gene Ontology terms enrichment analysis was performed using the Enrichr on-line tool (http://amp.pharm.mssm.edu/Enrichr/) [316, 317] which uses input gene lists to calculate enrichment of genes based on different databases of chromatin immunoprecipitation experiments and Ontologies. We used the “ENCODE and ChEA Consensus TFs from ChIP-X” and “GO Biological Process 2015” databases to calculate enrichment of transcription factor binding and biological processes, respectively.

5.3.9 Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis [318] was performed using default settings: the significance of the normalized enrichment score for each gene set was assessed through 1000 gene set permutations. Gene sets with a FDR q-value < 0.25 were considered significant, as suggested by the GSEA tutorial. For each radiation type, 1-Gy and sham-irradiated samples analyzed at 8 h after exposure were used for comparison. To have a general view of response to

each radiation type, Hallmark Gene Sets collection of the Molecular Signatures Database was used. This collection consists of 50 gene sets representing specific well-defined biological states and processes, which helps to reduce noise and redundancy in different available databases and provides a better delineated biological space for GSEA.

5.3.10 γH2AX foci detection using fluorescent microscopy

PBMCs from four donors were fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) at 0.5, 2, 6 and 24 h following irradiation with X-rays and carbon ions. For iron ions an additional time point of 12 h was used. Following the fixation step, cells were cytospun on glass slides using Shandon™ EZ Double Cytofunnels™ (Thermo Fisher Scientific) and permeabilized with 0.25% Triton X (Sigma Aldrich, Belgium) for 5 min, blocked with 3% bovine serum albumin (Sigma Aldrich, Belgium) for 30 min and incubated overnight at room temperature with monoclonal mouse anti-γH2AX (phospho S139) antibody [3F2] (ab22551, Abcam, Cambridge, MA, USA) at 4°C. Cells were then incubated for 1 h with polyclonal goat anti-mouse secondary antibody coupled to FITC (F2012, Sigma Aldrich, Belgium) at 37°C and then mounted in Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Between each of the previous steps, the slides were washed with phosphate-buffered saline.

An automated inverted fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan), equipped with a motorized XYZ stage was used for the image acquisition of the immunostained slides. Images were acquired with a 40X Plan Fluor oil objective (Numerical aperture 1.3) and an Andor iXon3 camera (Andor Technology, South Windsor, CT, USA), providing images with a lateral resolution of 0.2 µm/pixel. For each sample, 25 fields were acquired on 7 Z-planes (separated by 1 µm). The obtained images were analyzed with the CellBlocks.ijm script [319], written for FIJI image analysis freeware [320], essentially as described before [321]. In brief the image analysis workflow

starts by segmenting each nucleus in the DAPI channel, using an automatic thresholding algorithm, after noise reduction and flat field correction. Subsequently, γH2AX foci are selectively enhanced by means of a multiscale Laplacian and segmented by means of automatic thresholding. Within each nucleus, the intensity of the γH2AX channel is measured along with the number of γH2AX foci and the foci occupancy, *i.e.,* the total projected area of the nucleus that is occupied by spots (total spot area divided by the nucleus area). On average, 500 nuclei were analyzed per sample.

5.4 Results

5.4.1 Gene level analysis shows a high degree of overlap between genes affected by low- and high-LET irradiation

To compare the effects of high- and low-LET radiation exposure on gene expression in human PBMCs, microarray analysis was performed at 8 h after exposure to 1 Gy of X-rays, carbon ions or iron ions. This time point was chosen because we observed a prominent gene expression response after 8 h in our previous studies [259, 276]. Sixty-nine, 95 and 78 differentially expressed genes (FDR-corrected $p < 0.05$) were detected after exposure to X-rays, carbon ions or iron ions, respectively, compared to control samples (Figure 24A-E; Supplementary Tables S10-12). The majority of these genes was induced after irradiation (Figure 24A-E), including 30 genes that were differentially expressed in response to all radiation types. Of these, 14 genes were up-regulated more than 2-fold (Figure 24E). Based on this analysis, the overlap between X-rays and carbon ions (p = 3.1e-96) and X-rays and iron ions (p = 5.2e-95) was more significant compared to the overlap between the two types of heavy ions (p = 9.1e-79).

When comparing two independent high-throughput gene expression experiments with different sample numbers, threshold-free methods outperform threshold-based ones in providing reliable results [315]. Thus, to obtain a better impression of the overall similarity in gene expression after

exposure to different radiation types, the Rank-rank Hypergeometric Overlap algorithm was used. This revealed a very significant degree of overlap among the top up-regulated genes for the comparisons between X-rays and carbon ions (Figure 24F) as well as X-rays and iron ions (Figure 24G). For the comparison between the two high-LET ions (Figure 24H) the degree of overlap was not only more significant, but also more vast, comprising the

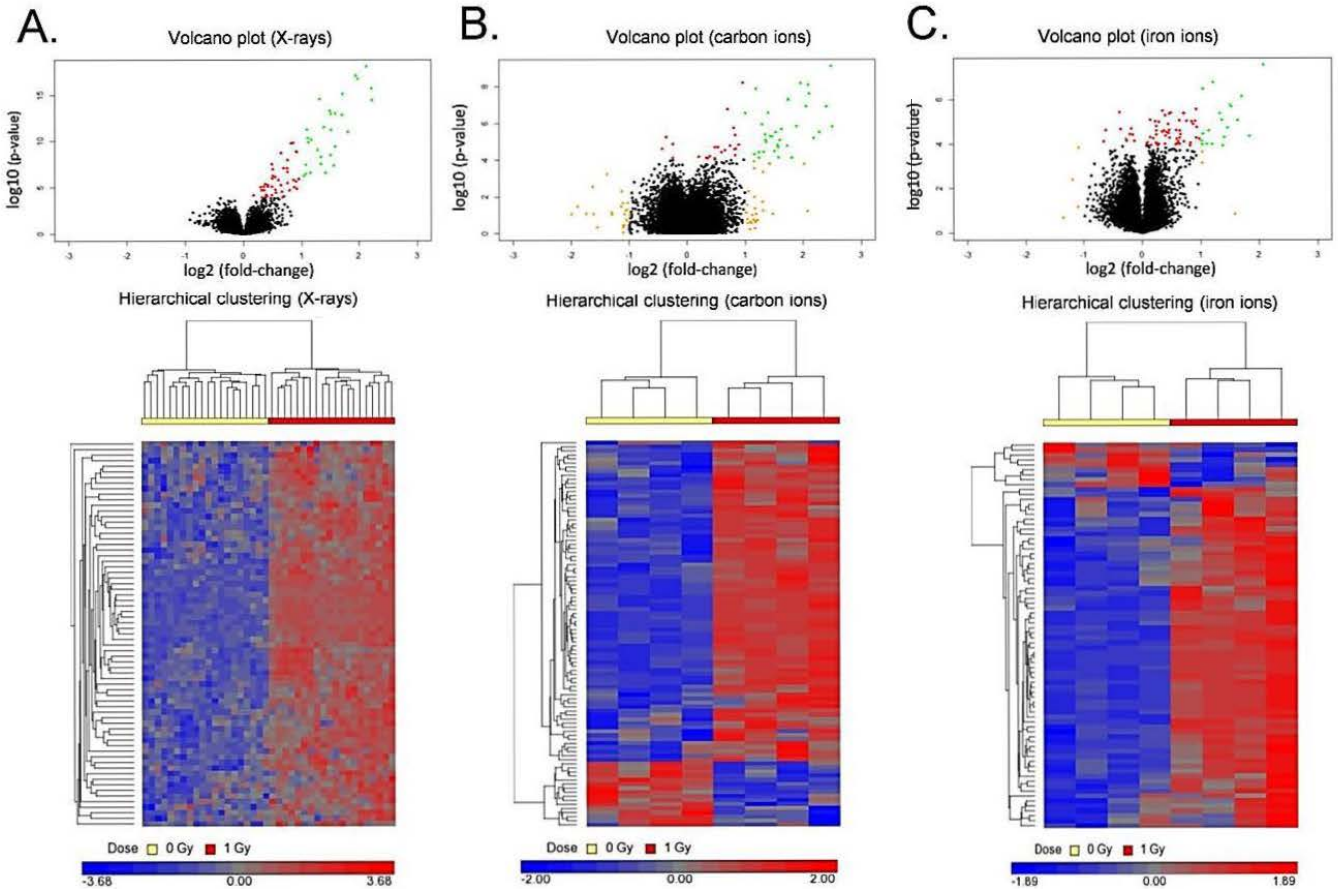

Figure 24. Changes in gene expression in PBMCs after exposure to X-rays, carbon ions and iron ions. **(A-C)** Volcano plots and heatmaps of gene expression changes between controls and cells irradiated with X-rays (A), carbon ions (B) and iron ions (C) at 8 h after exposure. Red points on volcano plots indicate genes with FDR <0.05, orange points indicate genes with |FC| >2 and green points indicate genes with FDR <0.05 and |FC| >2. Heatmaps show expression profiles of differentially expressed genes with a FDR <0.05

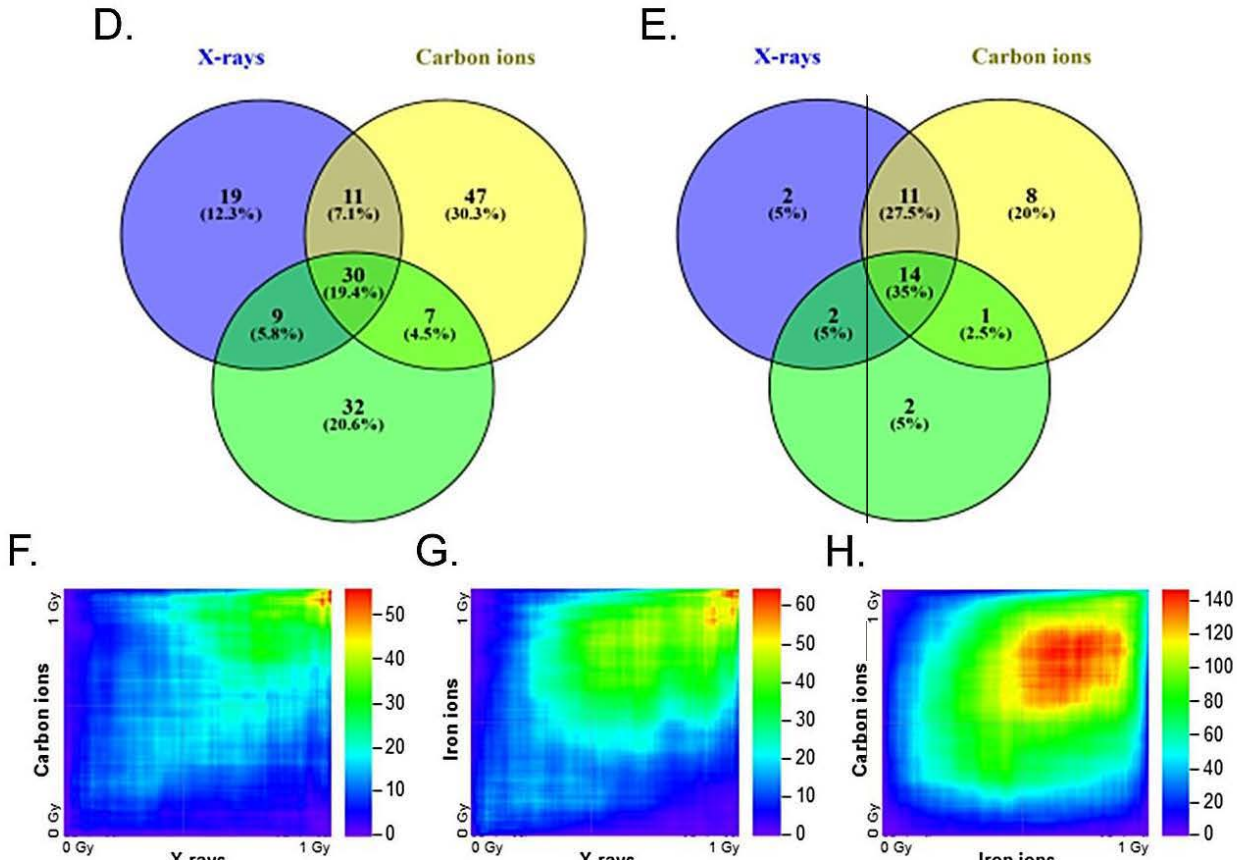

Figure 24 (continued). Changes in gene expression in PBMCs after exposure to X-rays, carbon ions and iron ions. **(D, E)** Venn diagrams showing overlap in differentially expressed genes with FDR <0.05 (D) or FDR <0.05 and |FC| >2 (E) between the different radiation types. **(F-H)** Rank-rank hypergeometric overlap heatmaps indicating overlap in gene expression changes between X-rays and carbon ions (F), between X-rays and iron ions (G), and between carbon ions and iron ions (H). Color scale bars indicate the $\log_{10}$-transformed hypergeometric *p*-values.

majority of up-regulated genes in both conditions. Together, our data show that irrespective of the radiation type, the large majority of the affected genes are up-regulated after exposure, and that the identity of these genes is highly similar, although some radiation type-specific genes do seem to exist.

According to the transcription factor enrichment analysis, for all radiation types, the affected genes were most likely transcriptionally regulated by p53 (Figure 25, left panel), and they were enriched in functions related to canonical p53-dependent pathways such as response to (UV) radiation, negative regulation of the cell cycle, DNA repair and apoptosis (Figure 25, right panel), as shown by the Gene Ontology (GO) terms enrichment analysis.

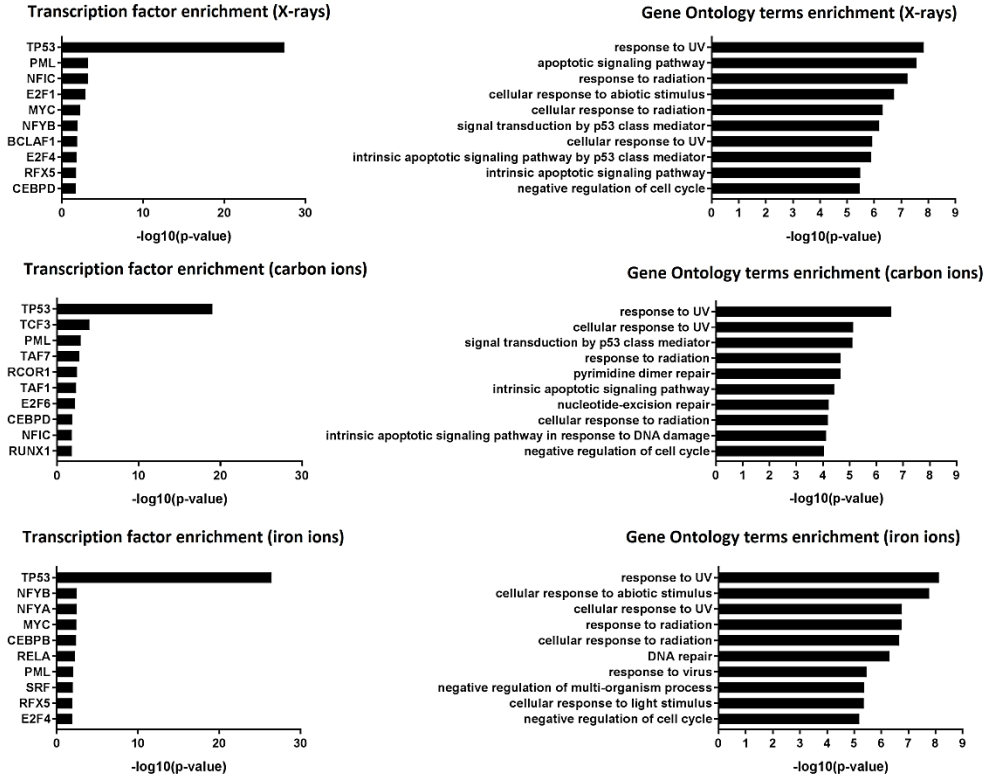

Figure 25. Transcription factor enrichment and GO term enrichment. Left panel: transcription factor enrichment results following exposure to X-rays, carbon ions and iron ions. Right panel: Biological processes that are mostly affected following exposure to X-rays, carbon ions and iron ions, based on gene level analysis.

5.4.2 GSEA reveals stronger enrichment of inflammation and epithelial-mesenchymal transition [322] gene sets by high-LET radiation

In contrast to gene set analysis methods, such as GO, which search for enriched terms in *a priori* defined lists of differentially expressed genes, GSEA is a threshold-free method using all the analyzed genes, ranked by their expression ratio, to get a list of terms enriched in all genes weighting their ranks. GSEA is more powerful in detecting modest but coordinated changes [323].

Gene set enrichment analysis for all the samples exposed to 1 Gy of all radiation types showed a classical DNA damage response, with p53-pathway, apoptosis and DNA damage repair-related gene sets being very significantly enriched (Table 13, Figure 26A). Interestingly, especially after exposure to heavy ions, also several immune response-related gene sets were identified as significantly enriched in irradiated samples (Table 13, Figure 26). For instance, genes related to the inflammatory response showed no preferential enrichment in either sham- or X-irradiated PBMCs. In contrast, exposure to heavy ions, especially iron ions, resulted in a significant up-regulation of these genes (Figure 26B). Similarly, the radiation effect on genes involved in TNFα signaling was more pronounced after heavy ion irradiation compared to X-irradiation (Figure 26C). Another gene set common for all radiation types was the EMT (Table 13, Figure 26D). Together, these results corroborate the observation that exposure of PBMCs to heavy ion irradiation induces generally more pronounced changes in gene expression compared to X-rays, while certain pathways, especially those related to inflammation are particularly affected by heavy ions.

Table 13. GSEA based on Hallmark Gene Sets

X-rays			Carbon ions			Iron ions		
Gene Set	**FDR q-value**	**NES**	**Gene Set**	**FDR q-value**	**NES**	**Gene Set**	**FDR q-value**	**NES**
p53 pathway	0.00	2.96	p53 pathway	0.00	2.60	p53 pathway	0.00	2.64
DNA repair	0.00	2.14	**IL2-STAT5 signaling**	0.00	1.88	**TNFα signaling via NF-kB**	0.00	2.30
Apoptosis	0.00	2.00	Cholesterol homeostasis	0.00	1.82	**IL6-JAK-STAT3 signaling**	0.00	2.00
Oxidative phosphorylation	0.04	1.61	**TNFα signaling via NF-kB**	0.00	1.81	DNA repair	0.00	1.99
Adipogenesis	0.05	1.58	**IL6-JAK-STAT3 signaling**	0.01	1.66	Apoptosis	0.00	1.94
TNFα signaling via NF-kB	0.15	1.42	Apoptosis	0.01	1.66	**Inflammatory response**	0.00	1.85
Epithelial-mesenchymal transition	0.18	1.37	DNA repair	0.01	1.65	Coagulation	0.00	1.83
mTORC1 signaling	0.24	1.31	**Myc targets V2**	0.03	1.55	Epithelial-mesenchymal transition	0.01	1.70
			Inflammatory response	0.05	1.48	**Interferon gamma response**	0.04	1.51
			Epithelial-mesenchymal transition	0.07	1.42	**Interferon alpha response**	0.08	1.43
			Wnt/β-catenin signaling	0.09	1.39	**Allograft rejection**	0.22	1.30
			Estrogen response early	0.09	1.37			
			Interferon gamma response	0.17	1.29			
			Notch signaling	0.23	1.25			
			Allograft rejection	0.23	1.24			
			Estrogen response late	0.23	1.23			
			Hypoxia	0.23	1.22			

Bold: gene sets related to the immune system and inflammation.
NES – normalized enrichment score

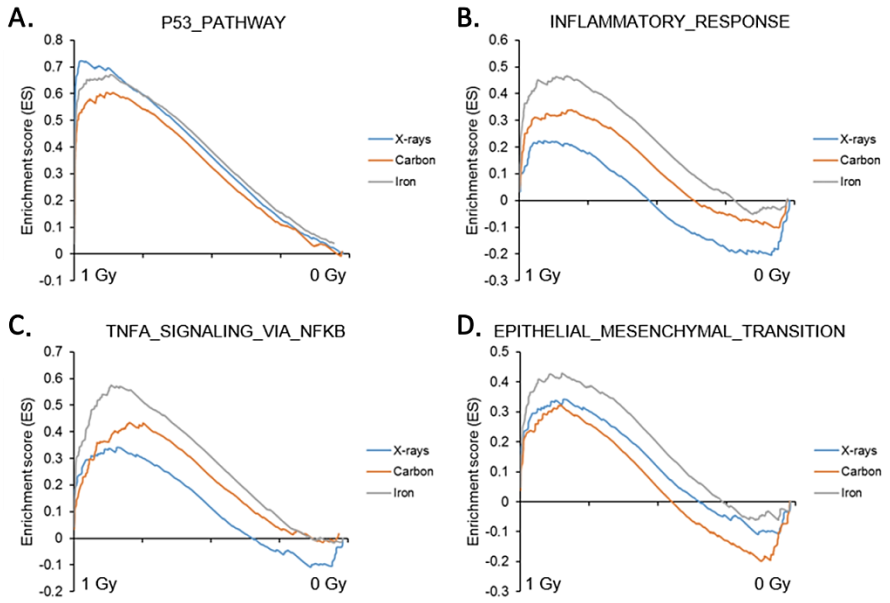

Figure 26. GSEA analysis. GSEA enrichment plots for four gene sets following exposure to X-rays, carbon and iron ions. Gene sets with a distinct peak at the beginning or the end of the ranked list are generally the most relevant, indicating that this specific gene set is enriched in up- or down-regulated genes, respectively.

5.4.3 qRT-PCR analysis shows radiation type- and time-dependent gene expression response

Seven genes (*PCNA*, *GADD45A*, *RPS27L*, *ASTN2*, *NDUFAF6*, *FDXR*, *MAMDC4)* that were significantly up-regulated in response to all radiation types were selected for qRT-PCR validation. To obtain better insight in the dose- and time-dependence of these genes, a lower dose (0.25 Gy) as well as an additional time point (24 h) were included (Figure 27). For the selected genes, the expression patterns at 8 h after exposure were in general comparable for all radiation types, confirming the microarray results. However, while all of these genes, except *MAMDC4*, reduced in expression with time in X-irradiated cells, their up-regulation was in general retained, or often even further induced in cells exposed to heavy ions, especially in the case of carbon ions.

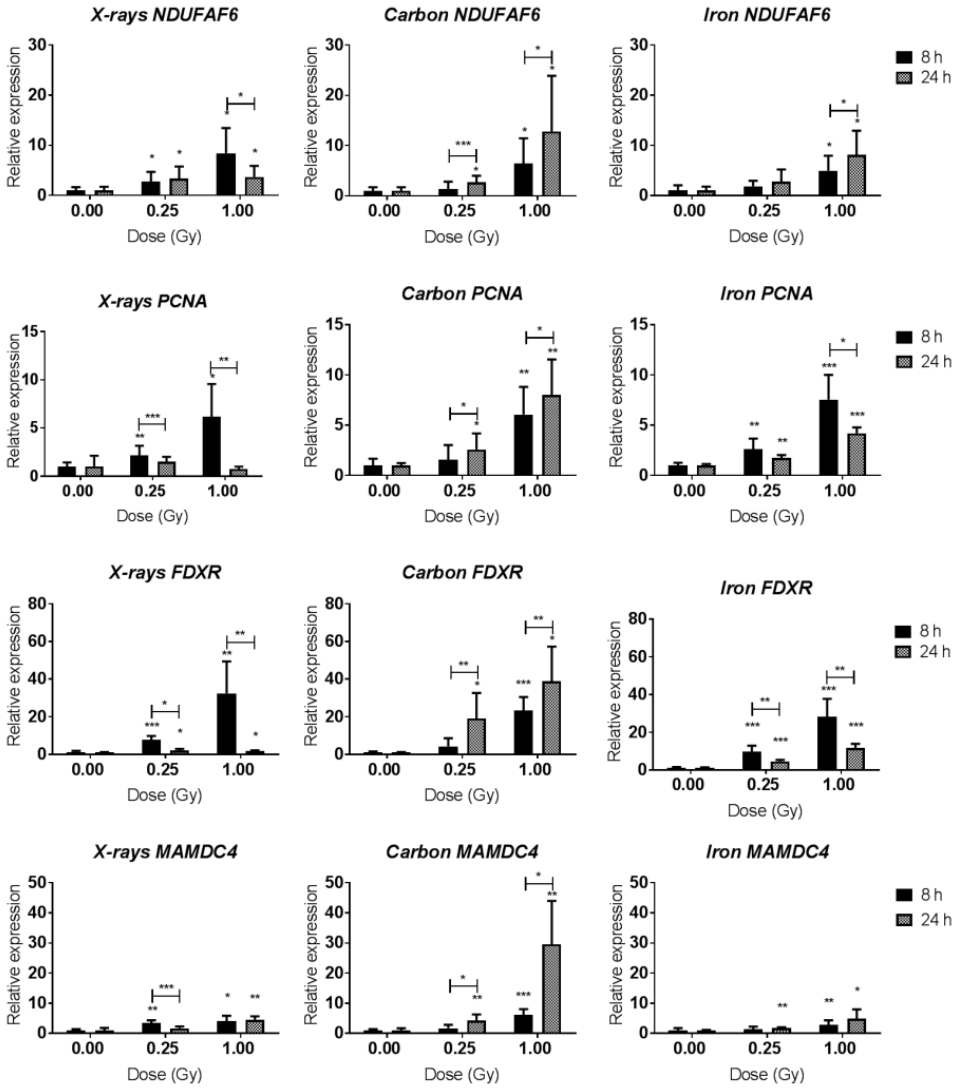

Figure 27. qRT-PCR validation of the microarray results. qRT-PCR results for *NDUFAF6, PCNA, FDXR, MAMDC4* genes (shown in rows) at 8 and 24 h after irradiation with 0.25 and 1.00 Gy of X-rays, carbon or iron ions (shown in columns). Graphs represent mean of six biological replicates + standard deviation. Statistical comparison was performed using 2-way ANOVA with Bonferroni *post-hoc* test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).

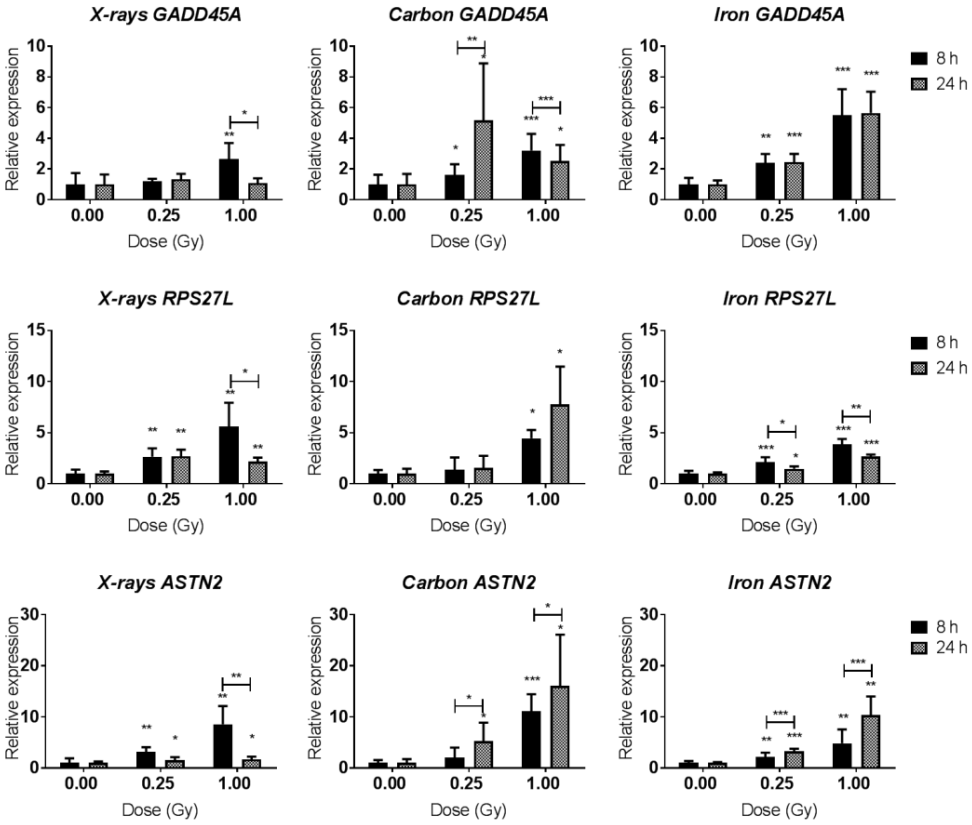

Figure 27 (continued). qRT-PCR validation of the microarray results. qRT-PCR results for*GADD45A, RPS27L,* and *ASTN2* genes (shown in rows) at 8 and 24 h after irradiation with 0.25 and 1.00 Gy of X-rays, carbon or iron ions (shown in columns). Graphs represent mean of six biological replicates + standard deviation. Statistical comparison was performed using 2-way ANOVA with Bonferroni *post-hoc* test (*$p < 0.05$, **$p < 0.005$, ***$p< 0.0001$).

5.4.4 Low- and high-LET radiation both induce production of alternative transcripts

Exposure to low-LET radiation not only changes gene expression as such but also triggers the production of alternative transcripts (due to alternative splicing or transcription) [188, 189, 259]. Here, a core signature of the genes alternatively spliced in response to all radiation types was identified (Figure 29A), the majority of which were also significantly differentially expressed at the gene level (36 out of 46), aligning with our previous results [259]. More overlap was observed between iron and carbon ions – 47.6% of the genes were in common, while between the X-rays and each of the heavy ions types the overlap was only about 15%. We also compared the number of differentially expressed exons between different radiation types in order to assess the levels of induction of transcript variants. Exposure to 1 Gy of X-rays resulted in significant (FDR < 0.05) up-regulation of 724 exons, to 1 Gy of carbon ions – of 511 exons and to 1 Gy of iron ions – of 708 exons (Figure 29B, Supplementary Table S13). In this case, more overlap was observed between iron ions and X-rays – 39.7% of exons were in common (Figure 29B). When comparing the fold-changes in expression of the overlapping 246 exons (Supplementary table 13), the highest induction levels were shown by carbon ions (Figure 29C). In addition, changes in expression of the 20-exon signature identified in [259] as particularly responsive to X-rays and important for classification of the samples depending on the exposure dose were compared between different radiation types (Supplementary Table S13). This comparison revealed that most of the above-mentioned 20 exons are in general less responsive to heavy ions compared to X-irradiation (Figure 29D). The detailed results for four genes overlapping for all radiation types (*PCNA*, *VWCE*, *FDXR* and *MAMDC4*) are shown in Figure 29E-H). In this case, the most pronounced alternative splicing response was observed after carbon ions exposure, especially this was the case for *MAMDC4* and *VWCE*. The Gene Ontology Biological Processes terms enriched in alternatively spliced genes common for all radiation types were predominantly related to apoptosis

and DNA damage repair (Supplementary Table S14). The detailed results of the Gene Ontology Biological Processes terms enrichment for genes identified as alternatively spliced specifically after exposure to X-rays, carbon ions and iron ions can be found in Supplementary Table S14.

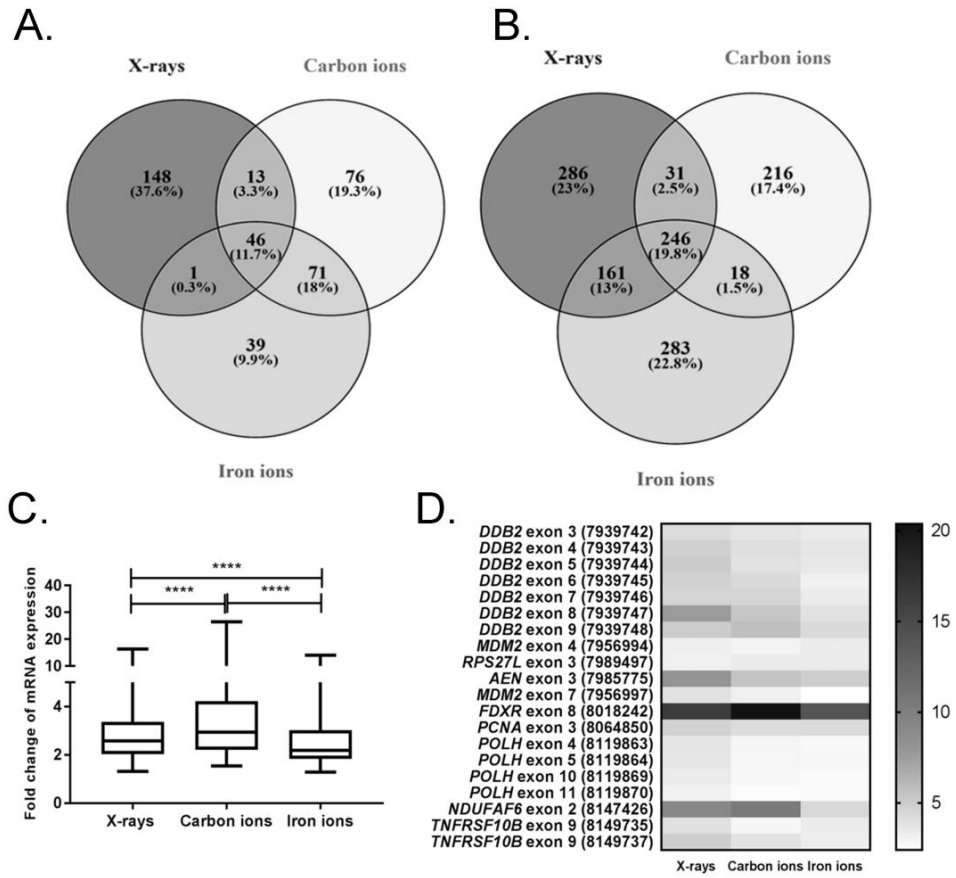

Figure 28. Radiation-induced alternative splicing. **A**. Venn diagram showing the number of alternatively spliced genes with FDR-corrected p-value < 0.05 at 8 h after exposure to 1 Gy of X-rays, carbon or iron ions. **B.** Venn diagram showing the number of differentially expressed exons with FDR-corrected p-value < 0.05 at 8 h after exposure to 1 Gy of X-rays, carbon or iron ions**. C.** Changes in exon expression induced at 8 hours after exposure to 1 Gy of different radiation types. Centerlines show the median, boxes represent the range between the first and third quartiles and whiskers represent the highest and lowest values. Statistical comparison was performed using Wilcoxon matched-pairs signed rank test (****p*-value < 0.0001). **D.** Heatmap showing fold-changes in expression of the 20 exon signature (probe set numbers are shown in brackets) identified as particularly responsive to X-ray exposure in **[259]** 8 hours after exposure to 1 Gy of different radiation types.

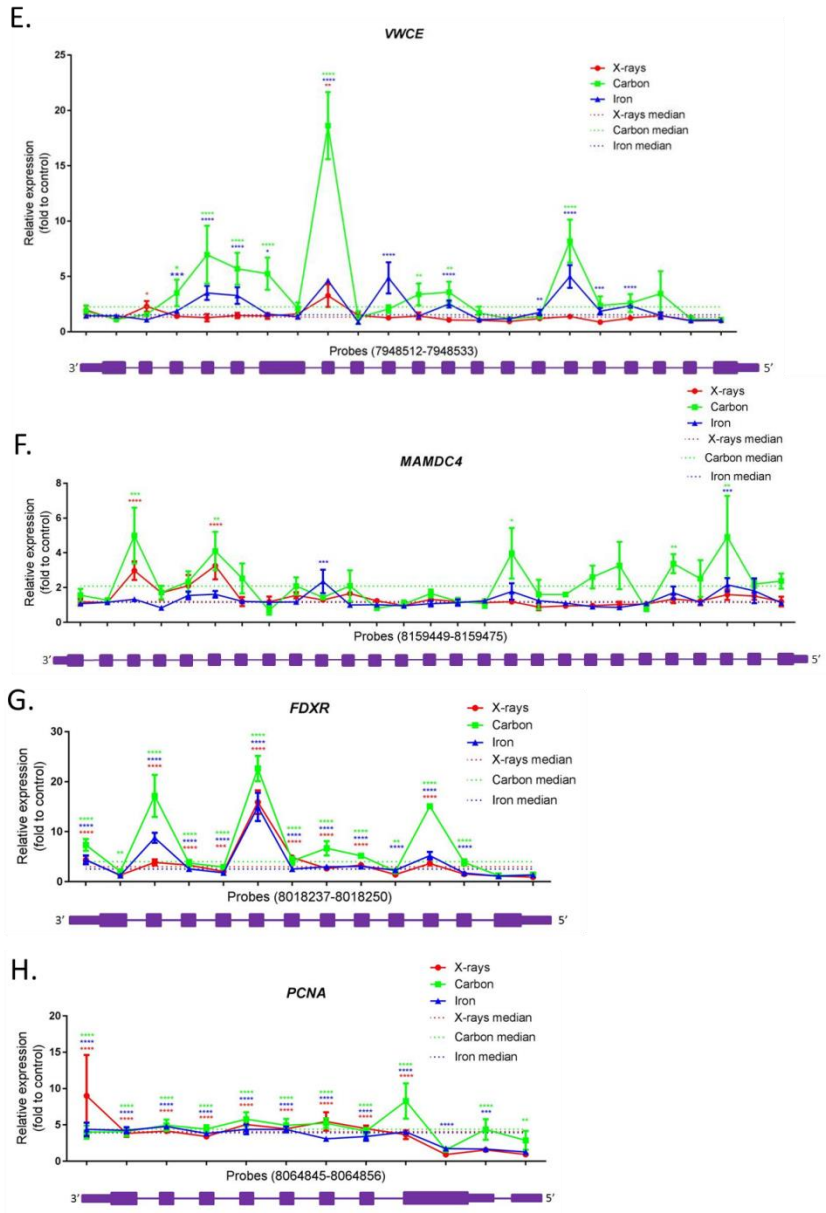

Figure 28 (continued). Radiation-induced alternative splicing. **E-H.** Alternative transcription/splicing of *VWCE*, *FDXR*, *MAMDC4* and *PCNA* genes at 8 h after exposure to 1 Gy of X-rays, carbon or iron ions. Genomic organization of each gene is shown below the graph in purple; every box represents an exon of the gene, schematic representation of the exons does not correspond to their actual size. Fold-changes to control values are shown for every probe set specific to each exon of the gene. Median fold-change to control value for each radiation type is shown with the dotted line. Error bars represent SEM (n = 10 for X-rays, n = 4 for carbon and iron ions). Statistical comparison was performed using repeated measures 2-way ANOVA with Sidak's *post-hoc* test (*p < 0.05, **p < 0.005, ***p<0.001, ****p< 0.0001).

5.4.5 Heavy ions exposure results in clustered DNA damage and slower DNA damage repair compared to X-rays

Quantification of DNA damage is commonly performed by counting the number of nuclear γH2AX foci. For diffuse radiation such as X-rays, this has been shown to be a robust readout [324, 325]. However, high-LET radiation induces strongly clustered breaks along the track of the beam that may result in few microscopic foci, but with large relative size when a cell is visualized perpendicular to the orientation of the beam track (Figure 30A). Thus, γH2AX spot occupancy (Figure 30C) better reflects the severity of the damage caused by heavy ions compared to X-rays [321]. Indeed, when calculated as the number of foci per nucleus, the absolute number of unrepaired breaks after 24 h was similar for all radiation types (Figure 30B). However, when considering spot occupancy, the amount of unrepaired DNA DSBs was 23% for X-rays, 42% for carbon ions and 31% for iron ions. When considering the spot occupancy per nucleus, the amount of damage still present 24 h after exposure to iron ions was comparable to the amount of damage observed in X-irradiated cells at 0.5 h (Figure 30C). The severity of DNA damage as assessed by the spot occupancy of γH2AX foci was therefore clearly LET-dependent. For X-rays and iron ions the maximal foci occupancy was detected at 0.5 h post-irradiation, while for carbon ions this peak was observed at 2 h post-irradiation.

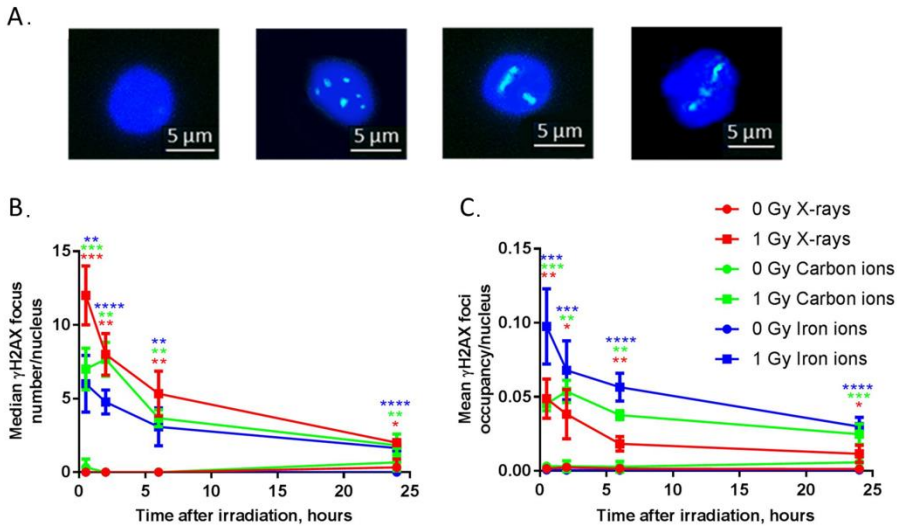

Figure 29. DNA repair kinetics after exposure to different types of radiation. **A.** Representative examples of immunostained γH2AX foci in PBMCs 6 h following (from left to right) sham-irradiation, exposure to 1 Gy of X-rays, iron ions and carbon ions. **B.** The number of γH2AX foci per nucleus after exposure to 1 Gy of X-rays, carbon and iron ions at different time points (median of three biological replicates for X-rays and carbon ions and four biological replicates for iron ions, error bars represent standard deviations). **C.** The occupancy of γH2AX foci per nucleus (average of three biological replicates for X-rays and carbon ions and four biological replicates for iron ions, error bars represent standard deviations) after exposure to 1 Gy of X-rays, carbon and iron ions at different time points. Statistical comparison was performed using unpaired *t*-test (*p < 0.05, **p < 0.005, ***p<0.001, ****p< 0.0001).

5.4.6 Gene expression may serve as a proxy for DNA damage repair efficiency

To compare the changes in gene expression with DNA repair kinetics at the level of individual donors, samples from four individuals that were irradiated with iron ions were used. All four individuals showed a clear time-dependent kinetic of DNA repair (Figure 31E). The percentages of DNA DSBs repaired after 24 h was calculated for every donor. For Donor 1 it was about 65%, Donor 2 – 72%, Donor 3 – 76%, Donor 4 – 70%, thus the DNA repair rate of Donor 1 was the lowest.

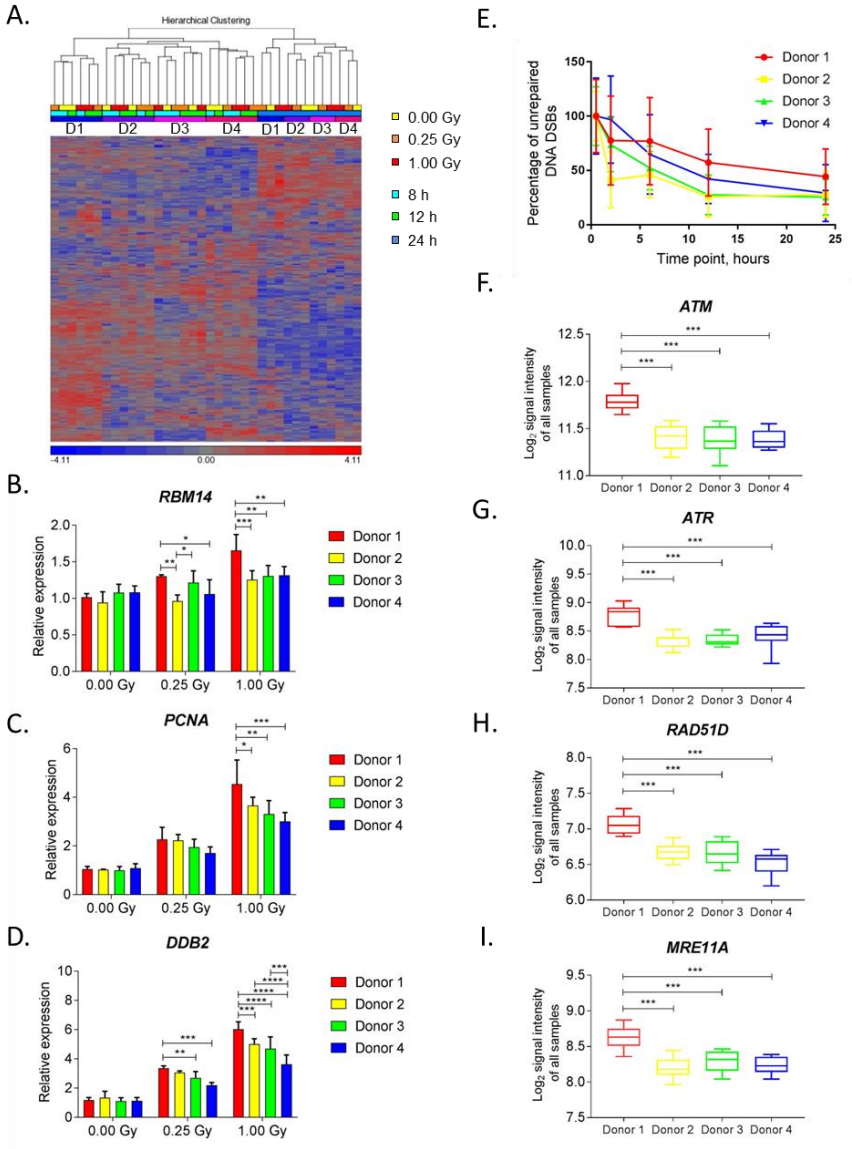

Figure 30. Individual differences in DNA damage repair kinetics and gene expression induced by exposure to iron ions**. A.** Hierarchical clustering of DNA repair genes shows time- and subject-dependent expression. **B-D**. Dose-dependent expression of selected DNA repair genes shows higher induction in Donor 1 compared to other donors. Bars show the mean of three time points, error bars - SD. Statistical comparison performed using repeated measures 2-way ANOVA with Tukey's *post-hoc* test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001). **E.** Individual DNA repair kinetics of four donors as percentage of γH2AX foci occupancy compared to 1 Gy-sample at 0.5 h . Error bars - SEM of 2 technical replicates. **F-I.** Expression levels of DNA repair genes show overall higher expression in Donor 1 compared to other donors. Box plots show the mean of all samples (all doses and time points), whiskers show minimal and maximal values. Statistical comparison was performed using unpaired t-test (***p< 0.001).

Hierarchical clustering of the gene expression profiles of DNA repair-related genes showed time- and subject-dependent expression. This resulted in two major clusters of samples depending on the time point, with 24-h samples segregating from 8-h and 12-h samples (Figure 31A). Within each time cluster, expression profiles of Donor 1 clustered separately from those of the other three subjects (Figure 31A). Some DNA repair genes (e.g. *PCNA*, *DDB2*, *RBM14*) showed an enhanced radiation response in Donor 1 compared to other donors, especially after a high dose (Figure 31B-D). This donor also showed overall elevated levels of expression of several DNA damage response-related genes, (e.g. *ATM, ATR, RAD51D, MRE11A*) independent of the irradiation dose and time point (Figure 31F-I). This indicates that individual differences in the overall and radiation-induced expression levels of DNA repair genes exist, which may explain individual differences in DNA repair kinetics.

5.5 Discussion

In the present study we investigated and compared genome-wide transcriptional response of human PBMCs after acute exposure to three radiation types with different LET characteristics: X-rays, carbon and iron ions. An equal dose of 1 Gy was used, as our main goal was to identify the differences in response caused by high- and low-LET radiation rather than comparing RBE-weighted doses. It was also previously suggested to compare equal rather than equitoxic doses of high- and low-LET radiation in the context of gene expression analysis [326]. In addition, we analyzed the DNA repair kinetics after exposure to the above-mentioned radiation types. It should also be noted, that PBMCs include multiple cell sub-populations, the vast majority of which are in G0 phase of the cell cycle. This fact is particularly important for the genes involved in cell cycle regulation, but also for those involved in the DNA damage repair, as these cells can only use non-homologous end-joining and not homologous recombination to repair the DSBs [327]. In addition, differences in DNA damage response in G0 cells

seem to be strictly cell type-specific and depend on the physiological context [328, 329].

X-rays and heavy ions induce a similar p53-dependent gene expression response, albeit with different amplitude and dynamics

In our study, we found an overall very similar primary p53-dependent response to all radiation types at 8 h after exposure. A similar observation was made in normal human fibroblasts following γ-radiation and decays of high-LET-like (125)I [330]. A study by Kurpinski and co-authors showed that most of the differentially expressed genes which were in common after exposure to 1 Gy of X-rays and iron ions in human mesenchymal stem cells were involved in cell cycle and DNA damage response and repair, which is in accordance with our observations [331]. Although we also found several genes “unique” to a specific radiation type, it is likely that many of them would also respond to the other radiation types in a different experimental set-up (i.e. time-dose combination). Some of the observed differences, however, may also be explained by the different nature of X-rays (photons) and heavy ions (particles). The DNA damage caused by particles is more complex and difficult to repair compared to X-rays, as confirmed by slower DNA repair kinetics shown in our study as well as in endothelial cells exposed to nickel ions [332]. qPCR validation of gene expression changes at later time points also showed differences in kinetics of expression of several genes in response to exposure to high- and low-LET radiation.

Heavy ions exposure results in more significant enrichment of immune response-related gene sets as compared to X-rays.

Interestingly, GSEA identified several immune response-related gene sets as significantly enriched specifically in samples irradiated with particles. Among these, NF-kB plays a central role in regulation of the expression of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-2, IL-6 and

MCP-1 [333, 334]. It is clear that different radiation types have the potential to induce different immune alterations [334]. In a study by Baumstark-Khan high-LET argon ions (272 keV/µm) induced a stronger NF-kB-dependent reporter gene expression compared to X-rays [335]. A later study from the same group showed that carbon ions (33 and 73 keV/µm) and X-rays activate NF-kB-dependent gene expression in HEK293 cells 4 h after exposure. However, activation by carbon ions was induced by 1.3 Gy while activation by X-rays required a higher dose of 16 Gy [336]. These results indicate that a lower dose of high-LET radiation than of low-LET radiation is required to activate NF-kB. This observation suggesting overall increase of carcinogenic potential related to NF-kB activation [337, 338] might have implications for both radiotherapy patients and astronauts on long-term Space missions. However, there are two sides of the coin. Carbon ions were also shown to induce anti-tumor immunity in a murine model [339]. Another study examining five human cancer cell lines showed that comparable levels of high mobility group box 1, which plays an important role in activating anti-tumor immunity were detected after irradiation with equitoxic doses of X-rays and carbon ions, meaning that a lower dose of carbon ions was needed to achieve the same effect [340]. These results suggest that carbon ion therapy might activate the immune system to a greater extent than conventional radiotherapy, even when equivalent doses are used.

Irradiation, and in particular exposure to carbon ions, promotes alternative transcription and splicing.

Another important aspect of the transcriptional response to ionizing radiation [188, 190, 259] and other genotoxic agents [185-187, 341, 342] is alternative splicing and transcription. Exposure to low and moderate doses of low-LET ionizing radiation initiates alternative splicing and transcription of a large number of genes [188, 190, 259]. In the present study, we observed a more pronounced response after exposure to heavy ions, especially carbon ions.

The exons most extensively regulated in response to X-ray exposure were not the most regulated after particle exposure, suggesting specificity in response. The most significant alternative transcription/splicing induction observed after exposure to carbon ions might be, at least in part, explained by the higher fluence used for the carbon irradiation compared to iron ions resulting in cells being hit by more ions.

Although it is not possible to draw any definite conclusions on the biological relevance of this observation from the microarray data, it is tempting to further study the role of alternatively transcribed/spliced genes in the response to different radiation types. In a very recent study exposure to UV was shown to trigger a shift from protein-coding mRNA of the *ASCC3* gene, which was alternatively spliced in response to heavy ions exposure in our set up, to a shorter non-coding isoform [343]. The non-coding ASCC3 isoform, in fact, counteracts the function of the protein-coding isoform and has an opposite effect on transcription recovery after UV-induced DNA damage [343].

Gene expression may serve as a sensitive indicator of individual DNA damage repair capacity.

Defects in DNA repair mechanisms often result in abnormal radiosensitivity of cells [238-242]. Studies aiming at establishing an assay for predicting radiosensitivity focused on colony-forming assays [244, 245] or the measurement of DNA DSBs repair efficiency by means of the comet assay [248, 249, 344] or the γH2AX assay [344, 345]. However, no single DNA damage-based assay proved to be capable of discriminating the full range of cellular radiosensitivity [98]. A possible explanation is that radiosensitivity can also be associated with differences in cell cycle and apoptosis pathways regulation [346, 347]. In this regard, transcriptional changes, which allow not focusing on one single cellular aspect, were suggested to be a promising predictive parameter for radiosensitivity [252, 344]. Greve and co-authors identified a set of 67 differentially expressed genes in peripheral blood lymphocytes exposed to 5 Gy of γ-rays, which allowed distinguishing between

the group of severely radiosensitive and non-radiosensitive breast, head and neck carcinoma patients [344]. Rieger and co-workers used microarray gene expression profiling in lymphoblastoid cells derived from a diverse group of cancer patients with acute radiation toxicity. A set of 24 genes predicted radiation toxicity in 9 of 14 patients with no false positives among 43 controls [253].

In our study, we integrated the two approaches mentioned above based on the data of four donors after exposure of PBMCs to iron ions. It is important to mention that all the subjects involved in this study were apparently healthy, without any known abnormal variations in radiosensitivity. Our main goal was not to assess the radiosensitivity of these individuals as such, but rather to explore the potential of gene expression signatures in reflecting the efficiency and kinetics of DNA repair as measured by γH2AX assay. As could be expected, we did not find any significant differences in DNA repair efficiency of the four studied donors. However, we could make an interesting observation. Donor 1, who showed the lowest rate of DNA DSBs repair, also had a distinct gene expression profile observed for some genes as highest levels of up-regulation compared to control and for the others as higher overall expression in all samples. Interestingly, a recent study comparing transcriptional response of radiosensitive and radioresistant immortalized B-lymphocytes also showed a greater and prolonged response in radiosensitive cells [348]. Genes regulated by p53 and involved in DNA damage response and apoptosis were still up-regulated in the radiosensitive cells and not in the radioresistant ones 24 h post-exposure to 2 Gy of γ-rays [348].

Although at this stage we performed a small-scale pilot study, our results could be of interest for assessing the DNA repair efficiency and overall response to radiation in Mars mission crew members and, potentially, radiotherapy patients. Moreover, gene expression measurements are more straight-forward and are technically less affected by such factors as radiation type compared to the γH2AX assay. At the same time, measuring gene

expression for radiosensitivity assessment has another advantage of allowing to have a broader look at it rather than focusing on DNA damage repair as such, as virtually any gene can be included in the assay.

In conclusion, we have shown that both low- and high-LET irradiation induce similar transcriptional pathways, albeit with variable amplitude and timing, but that high-LET also elicits specific and more persistent transcriptional events that may exacerbate the carcinogenic potential or, on the other hand, induce immune response against tumour cells. Our results imply that more detailed investigations of transcriptional response could bring new insight into differential normal tissue responses to high- and low-LET radiation and might have implications for the development of particle therapy treatment and radiation protection.

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5.7 Conflict of interest statement

Authors declare no competing interests.

5.8 Supplementary material

Supplementary Tables S10-S14 are available in electronic format only (as Excel files).

6.

General discussion and perspectives

The importance of radiation biomarker research

Every human living on Earth is constantly exposed to very low levels of natural ionizing radiation, which are harmless. Occasionally, though, individuals are exposed to radiation doses exceeding the natural background levels, often as a result of medical diagnostic tests and treatments but sometimes through the accidental or deliberate release of radioactivity. The fact that exposure to radiation doses higher than 100 mGy increases the risk of cancer development and, at higher doses, can even cause cardiovascular diseases and cataracts is already well-known. Recent epidemiological studies show that even exposure to low doses of ionizing radiation increases the risk of cancer development. The study on a 300,000-cohort of nuclear industry workers demonstrated increased risks of leukaemia and solid tumour development after exposure to cumulative doses below 100 mSv [349, 350]. Even more concerns are raised by reports showing increased risks of leukaemia and brain tumours in paediatric patients following doses of 30-50 mGy from CT scans [351, 352]. The accumulated scientific data led to a drastic shift in public perception of radiation technologies: from the use of shoe-fitting fluoroscopes until about 1950s [353] to the recognition of the need for regulation, justification and reduction of the doses resulting even from medical diagnostic procedures [354, 355].

In case exposure to ionizing radiation is justified or unavoidable the knowledge of the exposure dose becomes important in order to take all the necessary measures to prevent or timely diagnose and treat the potential

health consequences. Radiation biomarkers of exposure investigated in the present study play a crucial role in this regard.

Biological response to ionizing radiation is a very complex process. However, it can also be regarded as something very simple – radiation damages DNA, and all the following events are the result of this. In fact, almost all the radiation biomarkers of exposure developed so far are in one way or another based on this simplistic assumption, but given the real complexity of DNA damage response, the list of potential end points to be used as biomarkers is enormous [192, 193]. For example, the idea behind the well-known dicentric assay is very simple: the higher the exposure dose – the higher the number of DNA breaks – the higher the number of dicentrics. It is probably the simplicity of the approach that makes this assay so robust

However, the development of new techniques opened new horizons for radiation biomarkers development. Gene expression measurements used as the main technique in the present study are one of the examples. If regarded from a simplified point of view, the gene expression changes observed in normal cells, such as PBMCs used in our study, are the direct result of DNA damage caused by ionizing radiation. Again, the higher the exposure dose – the higher the number of DNA breaks – the higher the amplitude of gene expression changes. However, more sophisticated methodologies bring about the need for more sophisticated analysis and assay validation.

Validity of *in vitro* models

In the present study we used human PBMCs or peripheral blood as *in vitro* model for radiation response. Isolated PBMCs were used in transcriptome-wide microarray studies as this allowed to avoid the necessity to perform an additional globin reduction step. Isolated PBMCs also showed better viability in our set up (data not shown), which was particularly important for heavy ion irradiations performed at GSI. For the qPCR arrays part of the study we used peripheral blood as this would be more applicable to emergency situations

and it is likely to better reflect the *in vivo* response. Although *in vitro* models are not fully representative of the more complex *in vivo* situation, *in vitro* experiments represent an essential step preceding the *in vivo* validation of the results. *In vitro* experiments offer several practical advantages, for instance, they are usually cheaper, less complex and time-consuming and make it possible to strictly define the experimental conditions. For obvious reasons it is also very hard if not impossible to obtain human blood samples exposed *in vivo* to specific doses. However, it is possible to validate the results obtained in *in vitro* experiments using the samples of patients undergoing radiotherapy or medical diagnostic procedures involving exposure to radiation.

Transcriptional studies in radiation biomarkers research

Several genome-wide studies have been conducted so far to assess the *in vivo* transcriptional response to ionizing radiation using blood samples from radiotherapy patients undergoing either total body irradiation [229] or local intensity modulated radiotherapy [230]. These investigations showed that *in vivo* irradiation mainly affected genes involved in pathways related to the immune system and inflammatory responses, but also p53-mediated pathways such as the cell cycle and DNA damage and repair, which were also found in *ex vivo* irradiated blood and PBMCs. This corresponded to the finding of induction of p53-dependent genes in patients undergoing either CT scans (up to 4.3 cGy) or administration of (F-18)-fluoro-2-deoxy-D-glucose (0.6 cGy) [231]. Overall, an increased expression of the examined genes was measured in all the samples, however, differences in response *in vivo* and *in vitro* were found especially for doses below 5 cGy [231]. Other studies have identified *in vitro* gene signatures which could accurately predict *in vivo* radiation exposure status [158, 232, 233]. In a recent RENEB inter-laboratory comparison exercise gene-expression calibration curves obtained from *in vitro* irradiated blood samples could be used to distinguish blood samples of prostate cancer patients exposed to 0.009–0.017 Gy (first fraction only, partial body exposure) [55].

In all, these studies have shown that the *in vitro* transcriptional radiation response is a good model for the *in vivo* situation. Another possibility for biodosimetry studies is the use of animal models as it was demonstrated that radiation-responsive genes in mice exposed to physiologically relevant radiation doses show a similar response to homologous genes from *ex vivo* human studies [232, 280, 282], although gene expression profiles developed via analysis of murine blood radiation response alone were found to be inaccurate in predicting human radiation exposures [158].

Alternative transcription and splicing of radiation-responsive genes

One of the most important findings made in the first step of the present study is the identification of the many radiation-responsive genes regarded as potential radiation biomarkers as alternatively transcribed and/or spliced in PBMCs in response to radiation. One of the examples of alternatively spliced genes found in our study is the *FDXR* gene, which is probably the most well-validated gene in the biodosimetry field [55, 155, 227]. FDXR is a mitochondrial flavoprotein, which is involved in several p53-mediated processes and ROS-associated apoptosis [222, 356]. *FDXR* is one of the most radiation-responsive genes, showing up to 46-fold up-regulation 24 h after 4 Gy irradiation in human blood [155], making it especially suitable for discrimination between high- and low-dose exposures. However, different studies applying qRT-PCR show significantly different expression levels of this gene, for example, ≈16-fold up-regulation shown in [159] and ≈40-fold up-regulation shown in [155] 24 h following exposure to 2 Gy of X-rays. This discrepancy might be explained by radiation-induced expression of transcript variants, shown in our study, and interrogation of more or less responsive exons in different studies. The choice of optimal primer location has therefore the potential of increasing the sensitivity of the assay, which is particularly important for low- or very low-dose exposures. This observation has direct implications for practical gene expression-based biodosimetry which will most probably rely on primer or probe-based methods. Identification of the exact

sequence identity of radiation-induced splice variants, for example, using next-generation sequencing and further functional studies would contribute to understanding of the molecular mechanisms of radiation response. Although it might not be immediately useful in view of biomarkers of exposure, it might point to potential biomarkers of individual response.

Alternative transcription and splicing are universal mechanisms essential for many cellular processes, as confirmed by the determinative role of disrupted splicing in human disease [357]. The biological relevance of splicing is also emphasized by its presence in species throughout the phylogenetic tree, although its prevalence and characteristics vary considerably [176]. It is difficult to distinguish functionally significant alternative splicing events based on transcriptional studies, as not every observed transcript necessarily encodes a functional product. In addition, transcription and splicing are error-prone processes and erroneous transcripts that might occur are eliminated by pathways such as nonsense mediated decay [358].

Nevertheless, the results obtained in the present study, can be considered reliable and should serve as the basis for further functional studies as similar observations were made in murine brain cells [190] and fibroblasts and lymphoblastoid cells [188] exposed to ionizing radiation. Our data suggest that alternative transcription initiation from alternative promoters was the most common event, although the activation of other alternative splicing mechanisms (e.g. exon skipping, alternative splice sites and alternative polyadenylation) was observed as well. Our findings are also supported by the fact that alternative splicing is considered to be a key player in DDR as suggested by e.g. the large number of apoptotic genes that are alternatively spliced, with the generated isoforms often playing antagonistic roles [179].

Radiation exposure activates the expression of neighboring genes

Furthermore, we observed that a substantial fraction of the radiation-responsive genes are located in close physical proximity on the genome

(often as neighbors with bidirectional promoters). This observation is important for identification of new radiation-responsive genes. One such example is the long non-coding *PAPPA-AS1* gene which is transcribed from the opposite strand of the *PAPPA* gene, presumably from a shared bidirectional promoter with *ASTN2*. To the best of our knowledge, this is the first time that this gene has been identified as a potential radiation biomarker. The observed co-regulation may be related to radiation-induced DNA damage and might be required for a more coordinated response to it, since the frequency of bidirectional promoters is enriched in DNA repair genes compared to other gene classes [283]. In addition, it was suggested that transcriptional activation has a ripple effect (i.e. intensive transcription at one locus frequently “spills over” into its physical neighboring loci), which may be advantageous for coordinated expression [359]. This might explain the seemingly unexplainable activation by radiation of some genes which do not have (yet) a defined role in DDR. It is important to mention, though, that even if the activation of some of the identified radiation-responsive genes is in the end not biologically relevant, eventually this does not affect their suitability as biomarkers of radiation exposure.

Gene and exon signatures are equaly suitable for prediction of low to moderate radiation doses

We also observed that radiation-induced differences in expression were more pronounced at the exon level compared to genes, suggesting that exon signatures may show increased sensitivity to doses below 0.1 Gy. In our set up, gene and exon signatures from PBMCs were equally suitable for 100% accurate classification of the samples either sham-irradiated, irradiated with a low dose (0.1 Gy) or a high dose (1 Gy) of X-rays.

Gene expression measurements applicable to emergency situations: tackling the issue of confounding factors and high dynamicity of gene expression by using gene signatures

Microarray studies represent an essential step for identification of radiation-responsive genes and exons, however, their usefulness for large-scale exposure scenarios is very limited due to the complexity of the analysis, high costs and comparatively long time needed to obtain the results [55]. qRT-PCR represents an attractive alternative to microarrays. This technique is widely used, and allows automation and miniaturization of scale making it an attractive method for biodosimetry applications. For instance, it can be multiplexed (i.e. multiple targets are amplified in a single tube). Multiplex qRT-PCR minimizes the amount of required starting material and saves time by increasing throughput and decreasing sample handling. This technique was successfully used in a study by Kabacik et al. [154].

In the present study we explored another method, the qRT-PCR arrays. Based on our previous microarray results we selected a panel of 25 gene biomarkers, of which the most sensitive exons were interrogated, whenever applicable. Gene expression changes are a highly dynamic process and the exact kinetics depend strongly on every specific gene [155], therefore knowing the point in time at which sample is taken following the radiation exposure is highly important for correct dose estimation. In an emergency situation such information might not be precise or might not be available at all. To address this issue, we opted for testing the potential of our gene signature not only in predicting the exposure dose, but also the time point following irradiation, which, to the best of our knowledge, has never been tested before. Time point prediction would not be possible if using only one gene as its expression would be affected by both dose and time. Therefore, this can only be done based on a signature of genes. We also suggest that using a signature of genes has a stronger potential in overcoming issues related to individual differences in response caused by such possible confounding factors, as age, gender, smoking status and health status (e.g., inflammation). Experiments performed by Tucker et al. in mice and by Budworth et al. in *ex vivo* irradiated human blood samples showed that although the expression of

some biodosimetry-relevant genes (e.g. *CDKN1A*, *FDXR*, *BBC3*) is affected by the bacterial endotoxin lipopolysaccharide, used to mimic inflammatory stress, the gene expression analysis may still be useful for dose prediction [159, 302]. Paul and Amundson also achieved very accurate classification of the samples according to the exposure dose independent of the smoking status or gender of the subjects [271].

Accurate prediction of both dose and time point achieved using qPCR arrays technology

The classification method used in our study allowed to classify the samples according to the time elapsed since exposure with high precision (errors ≤ 4 h). Dose prediction was very accurate as well, even though pre-exposure controls were not available for blind samples. It is important to note, however, that the gene expression kinetics might be different following *in vivo* exposure, as a result adaptation of the methodology for *in vivo* exposure situation might be required. In the present study we used blood cells as comparatively easily accessible biological samples and suggested blood preservation and RNA extraction methodology suitable for field conditions, but one of the further improvements of the method would be the exploration of non-invasive sampling, allowing samples to be collected by the potentially exposed individuals unassisted, e.g. the use of hair follicles. This would require further confirmation of the validity of gene expression signature identified as activated in blood cells in hair follicle cells.

The importance of dose rate for further studies

Another factor, which might have an effect on radiation-induced gene expression, is the dose rate. Depending on the exposure situation following a radiological accident, protracted low-dose rate exposures, resulting from e.g., fallout or ground shine, might constitute much of the total dose. As low dose rate exposures can significantly decrease the extent of injury compared to an acute dose [124], assessing the rate of exposure might also be important for

adequate triage of the casualties. In our study in all the experiments high dose rates of 0.14 or 0.26 Gy/min were used. From the obtained results, differences in dose rates seemed to have no significant effect on gene expression changes at least for the tested doses, however, there might be a difference if higher doses are used. Although this issue was not addressed in the present study, it is tempting to test whether our biodosimetric panel of genes used for dose and time point prediction would also allow discrimination between acute and chronic exposures. Several studies addressing this issue were performed by the group of Sally Amundson in mice [360] and *ex vivo* irradiated human blood [361]. Overall, these studies showed that a large number of the same genes responded to low dose rate and acute exposures with typical p53-related gene responses also observed at lower dose rates, however, for some genes the magnitude of response was lower after low dose rate exposures [360, 361]. In both studies classification models were used to discriminate between different dose rates which resulted in very efficient classification both in case of *in vivo* irradiated mice (97% accuracy) [360] and *ex vivo* exposed human blood (100% accuracy) [361]. These very promising results suggest that it may be possible to develop a gene expression-based signature that can not only detect protracted exposures, but also discriminate them from acute exposures. This is particularly important as while cytogenetic methods were shown to effectively reconstruct individual total doses from chronic occupational or environmental exposures [362, 363], cytogenetic approaches cannot distinguish these from acute radiation exposures.

Coordinated actions in case of emergency – the key to efficient management of radiation casualties

Another important point in efficient management of radiation casualties is the well-developed and operated network of laboratories providing biodosimetry services, as no single laboratory would be capable of handling thousands of samples in a short period of time. RENEB - Realizing the European Network

of Biodosimetry - is a good example of such initiative. It started as a project aiming at “creating a sustainable network in biodosimetry that involves a large number of experienced laboratories throughout European Union which will significantly improve dose assessment capacity”. In the course of the project, several biodosimetry interlaboratory comparison exercises were organized in order to assess and harmonize the biodosimetry methods used by different laboratories. Currently the name of the initiative was changed from the “Realizing the European Network of Biodosimetry” to “Running the European Network of Biological Dosimetry and Physical Retrospective Dosimetry”, reflecting the broadening of the initial scope and the efforts in maintaining and strengthening the built network.

The importance of comparison of low- and high-LET radiation effects for biomarker studies

Other possible situations apart from nuclear power plant accidents which might result in radiation mass casualties are attempts towards the malevolent use of radiation. These include the use of a radiological dispersal devices (*i.e.* “dirty bombs”), which would result in potential contamination of a large number of people or placement of strong radiation sources (*i.e.* radiological exposure devices), which would potentially result in exposure of a large number of people to high doses of radiation [364]. In the event of use of one of the above-mentioned devices, the radiation exposure would consist of an unknown combination of photons, neutrons, α-particles and heavier ions, which would contribute to the total dose [365]. Therefore, it is of paramount importance to have “universal” biomarkers able to estimate the exposure dose to both low- and high-LET radiation. The vast majority of genes identified as radiation-responsive in our study are transcriptionally regulated by the p53 protein and common for the radiation types used in the present study (X-rays, carbon ions and iron ions), increasing their potential as biomarkers of exposure. At the same time, as biological effects of low- and high-LET radiation differ, finding biomarkers capable of discriminating exposures to

different radiation types, is also of high interest. In this regard it might be possible to further explore the temporal differences in gene up-regulation following the exposure to X-rays and heavy ions observed in our study. A recent study comparing the gene expression response of human peripheral blood exposed *in vitro* to neutrons and X-rays suggests that gene expression may serve as a means to differentiate between total dose and the neutron component [312].

While the biomarkers of exposure certainly benefit from the simplicity of the approach the biomarkers which can be potentially used for radiosensitivity or individual response assessment would certainly benefit from more nuanced research into the details of transcriptional response to radiation. In addition, exposure to different types of radiation can also trigger different pathways, contributing to the complexity of the response. To date, there have been only a limited number of studies comparing gene expression profiles in mouse or human blood cells following exposure to low- and high-LET radiation. A microarray study on isolated PBMCs exposed to α-particles identified 29 responsive genes associated with signaling pathways centered around p53 and GADD45A [235]. Although all genes modulated in cells exposed to α-particles were also differentially expressed 24 h after X-ray exposure, the degrees of induction were different [235]. A recent study by Broustas and co-authors on blood samples obtained from mice irradiated with X-rays and neutrons reported a total of 34 genes being differentially expressed (25 genes were uniformly downregulated, the remaining 9 genes were downregulated at day 1 post-irradiation and upregulated at day 7) in response to all exposures [313]. Gene ontology analysis revealed that cell cycle-related genes were significantly down-regulated at day 1 and significantly up-regulated at day 7. Most of the genes which were downregulated starting at day 1 and showed persistent reduction of expression at day 7 in both neutron and X-ray exposures were related to immune response, and B and T cell physiology [313].

Particularity of PBMCs as *in vitro* model for transcriptional studies in radiation biology

It should be noted that PBMCs include several cell sub-populations, the majority of which are in G0 phase of the cell cycle, therefore any extrapolations of the obtained results to other cellular systems should be made with care. The fact that PBMCs are quiescent is particularly important for the genes involved in cell cycle regulation, but also for those involved in the DNA damage repair, as these cells can only use non-homologous end-joining (NHEJ) to repair the DSBs because homologous recombination (HR) requires the presence of intact sister chromatid and resolves DSBs during the S and G2 phases of the cell cycle [327]. Differences in DNA damage response in G0 cells seem to be strictly cell type-specific and depend on the physiological context. For example, DSBs occurring in G0-arrested MCF10A epithelial cells were not repaired, although G0-arrested cells showed similar expression levels of DDR factors when compared to proliferating cells, with the exception of RAD51 involved in HR [328], while in our study we observed efficient DNA DSBs repair. Another study showed that terminally differentiated astrocytes show strongly attenuated expression of most of DDR genes in response to irradiation compared to proliferating progenitors [329]. Nevertheless, astrocytes retained the expression of NHEJ genes and are DNA repair proficient [329].

Earlier study from our group also showed that different lymphocyte sub-populations differ in their gene expression response to ionizing radiation [366], suggesting that using a more radioresponsive cell population might increase the sensitivity of the method. Another study using apoptosis induction as the endpoint showed that a minor CD8(+) subset of natural killer cells could be used as a radiosensitive lymphocyte population the disappearance of which correlated with the received dose (doses ranging from 3 to 20 Gy) [367]. However, in our opinion, using all PBMCs or whole blood rather than a specific blood cell population requiring sophisticated cell

sorting is more applicable to emergency situations. This is also supported by the fact that dose reconstruction in PBMCs required fewer genes and appeared more efficient compared to isolated CD4(+) lymphocytes [368].

Combining gene expression and RBE: a complex issue

In general, comparing the effects of radiations with different LET characteristics often comes to the differences in their RBE. However, while RBE does depend on the LET of the radiation used, precise RBE predictions cannot simply be made on the basis of LET information and require detailed knowledge of the beam composition, of fluency, charge, and velocity of the particles [9]. In addition, RBE also depends on the biological endpoint and biological system being studied [11]. For instance, carbon ions are more efficient than photons in cell killing [369, 370] and induction of apoptosis in normal cells which coincided with a differential effect on p53 phosphorylation [370]. However, the same group showed in two cell lines that induction of profibrotic PAI-1 is the same after high- and low-LET irradiation [370].

Defining RBE for gene expression changes is a difficult and questionable task. First of all, gene expression cannot be regarded as a true endpoint, such as cell survival or chromosomal aberrations induction, as it is a dynamic process. In theory, one could define the dose needed to induce the same level of up- or down-regulation of a gene at a certain time point after exposure and thus calculate the RBE, but it is very probably that if another time point is chosen the RBE value would be different. Obviously, RBE values would also be very different if different genes are investigated. In addition, PBMCs include multiple cell types with varying radiosensitivity [371, 372] and, therefore, defining a single RBE value for this *in vitro* model is not feasible. Therefore in our transcriptome-wide study we decided to use equal rather than equitoxic doses of all radiation types.

X-rays and heavy ions induce up-regulation of similar p53-dependent genes, albeit with different amplitude and dynamics

In our study we compared the transcriptional profiles of freshly isolated peripheral blood mononuclear cells of healthy donors after the cells were exposed to 1 Gy of X-rays, iron ions and carbon ions. Overall, the identity of the affected genes at 8 h post-exposure was very similar between all the radiation types, with most of the genes being regulated by p53 and involved in DNA repair, apoptosis and cell cycle regulation. In our opinion, this observation suggests that no matter what radiation type it is, in essence it causes DNA damage which triggers the activation of the above-mentioned processes. However, qRT-PCR validation of gene expression changes at 24 h showed differences in kinetics of expression of several genes in response to exposure to high- and low-LET radiation. This observation is most probably the result of the more complex DNA damage caused by heavy ions which is more difficult to repair compared to X-rays, as confirmed by slower DNA repair kinetics shown in our study. This discrepancy in expression kinetics of different genes might also illustrate the RBE issue described above. Although we also found several genes “unique” to a specific radiation type, it is likely that many of them would also respond to the other radiation types in a different experimental set-up (i.e. time-dose combination).

Heavy ions induce more significant enrichment of epithelial-mesenchymal transition and immune response-related gene sets as compared to X-rays

We also performed the enrichment analysis of different pathways induced by different radiation types. The most obvious discrepancy was observed for the gene sets involved in the immune response. Among these, NF-kB plays a central role in regulation of the expression of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-2, IL-6 and MCP-1 [333]. Several studies

show that a lower dose of heavy ions is needed to activate NF-kB compared to low-LET radiation [335] [336]. Because NF-kB is involved in regulation of apoptosis, angiogenesis, proliferation and adhesion, its activation and interplay with p53-regulated pathways may play a critical role in cellular response to radiation, and especially heavy charged particles [139]. Interestingly, a study on 3D oral mucosa model showed that equivalent doses of both X-rays and carbon ions induced activation of NF-kB, although with different kinetics [373]. Same study showed 2- to 3-fold higher levels of pro-inflammatory cytokine IL-6 and chemokine IL-8 after irradiation with X-rays and carbon ions in 3D mucosa model co-cultures including PBMCs compared to mucosa models without PBMCs [373]. This observation is highly relevant for our study and might have implications for both radiotherapy patients and astronauts on long-term Space missions, not only in view of possible mucositis initiation but also overall increase of carcinogenic potential related to NF-kB activation [337]. It is clear that different radiation types have the potential to induce different immune alterations [334]. However, there are two sides of the coin. Carbon ions were also shown to induce anti-tumor immunity in a murine model [339]. Another study examining five human cancer cell lines showed that comparable levels of high mobility group box 1, which plays an important role in activating anti-tumor immunity were detected after irradiation with iso-survival doses of X-rays and carbon ions, meaning that a lower dose of carbon ions was needed to achieve the same effect [340]. These results suggest that carbon ion therapy might activate the immune system to a greater extent than conventional radiotherapy, even when equivalent doses are used.

Another important gene set identified as significantly enriched by GSEA after exposure to all radiation qualities was related to EMT. Importantly, the enrichment of this gene set was more significant after exposure to carbon and iron ions. This gene set was also particularly enriched in up-regulated genes after exposure to iron ions. Although this process which contributes

pathologically to fibrosis, cancer progression and metastasis is not characteristic to the blood cells as such, these cells produce cytokines and growth factors which might trigger EMT [374]. Another explanation for this observation might be the partial overlap of the genes present in the EMT gene set with those from e.g. inflammation-related gene sets or p53-pathway gene set. In fact, EMT is tightly connected to inflammation, which has been recently identified as a key inducer of EMT during cancer progression [375]. Activation of EMT-related genes in response to different radiation types is highly important and needs further investigation and experimental confirmation as EMT has been proposed to be one of the critical mechanisms for the acquisition of malignant phenotypes by epithelial cells [376]. Several studies also suggest that EMT can be induced by radiation and is involved in radioresistance [377-380].

Exposure to heavy ions, and in particular to carbon ions, promotes alternative transcription and splicing

As described for the X-ray exposures above, in the present study we also observed induction of alternative transcription and splicing by high-LET radiation, and the observed effect was more pronounced after exposure to heavy ions, especially carbon ions. The exons most extensively regulated in response to X-ray exposure were not the most regulated after heavy ions exposure, suggesting specificity in response. Although it is not possible to draw any definite conclusions on the biological relevance of this observation from the microarray data, it is tempting to further study the role of alternatively transcribed/spliced genes in the response to different radiation types. In a very recent study exposure to UV was shown to trigger a shift from protein-coding mRNA of the *ASCC3* gene, which was alternatively spliced in response to heavy ions exposure in our set up, to a shorter non-coding isoform incorporating alternative last exon, of which the RNA, rather than the encoded protein, is critical for the eventual recovery of transcription [343]. The non-coding ASCC3 isoform, in fact, counteracts the function of the protein-

coding isoform and has an opposite effect on transcription recovery after UV-induced DNA damage [343].

Gene expression as a promising biomarker of radiosensitivity

In view of the continuing search for biomarkers of sensitivity to (high-LET) radiation, in the present study we also investigated the potential of a signature of genes responsive to iron ions exposure to serve as an indicator of varied DNA repair capacity. However, it is important to stress that at this stage we performed a small-scale pilot study involving apparently healthy subjects, without any known abnormal variations in radiosensitivity. As a result, the differences in DNA repair efficiency of the four studied donors were not significantly different. However, the subject who showed the lowest rate of DNA DSBs repair, also showed a distinct gene expression profile observed for some genes as highest levels of up-regulation compared to control and for the others as higher overall expression in all samples. Interestingly, a recent study comparing transcriptional response of radiosensitive and radioresistant immortalized B-lymphocytes also showed a greater and prolonged response in radiosensitive cells with genes involved in DNA damage response and apoptosis still up-regulated 24 h post-exposure to 2 Gy of γ-rays [348].

The ATM/CHEK2/p53 pathway plays a crucial role in cell cycle regulation (e.g. through the transcription of *CDKN1A*), DNA repair (e.g. through the transcription of *DDB2*) and apoptosis (e.g. through the transcription of *PUMA*) following radiation exposure and was suggested as a promising biomarker of susceptibility and late effects [193]. For example, Atm/Chek2/p53 pathway activity assessed by the response of *Puma* gene showed a good correlation with cancer induction in mice with different *Trp53* gene copy number [381]. The data obtained in mice was further validated in human mitogen stimulated T-lymphocyte cultures from healthy donors, ATM mutation carriers and Li Fraumeni Syndrome patients following irradiation [381]. The ATM mutation carriers showed very weak up-regulation of *PUMA,* while Li Fraumeni Syndrome patients showed an intermediate response compared to healthy

individuals. These data confirm that monitoring radiation-induced changes in key p53 regulated genes can serve as a proxy of the overall DDR pathway activity, pointing to variation in susceptibility.
Taking into account the higher potential of high-LET particles in inducing biological effects, it might be advisable to protect the individuals with aberrant gene expression response from long-term exposure to Space radiation and make extrapolations from low-LET radiation exposures data for radiation protection purposes with caution.

As a general conclusion of our study it can be stated that low- and high-LET radiation induces similar processes in normal blood cells but heavy ions have a higher potential of their activation and the effect is more persistent. Our results imply that further more detailed investigations of transcriptional response could bring new insight into differential normal tissue responses to high- and low-LET radiation and might have implications for the development of hadron therapy treatment and radiation protection.

To summarize, we would like to highlight the following main results and perspectives of this study:

1. We identified gene and exon signatures suitable for precise classification of the *in vitro* irradiated samples based on the exposure dose. Genes and exons showed very similar dose prediction capacity for doses higher than 0.1 Gy. The next step would be the validation of the identified signatures *in vivo*, e.g. in radiotherapy patients, and the exploration of potential of exon signatures in predicting very low (below 0.1 Gy) dose exposures, which might also be done in individuals undergoing diagnostic CT scans.
2. We demonstrated that exposure of human PBMCs to ionizing radiation induces alternative transcription and splicing. This observation has direct implications for any biodosimetry method relying on primer or probe-based assays. Functional studies on

radiation-induced splice variants would contribute to our understanding of the molecular mechanisms of radiation response and might point to potential biomarkers of individual response.

3. We designed and tested a qRT-PCR array suitable for prediction of both dose (from 25 to 2000 mGy) and time (from 8 to 48 h). However, the validation of the method *in vivo* is of paramount importance. In addition, the potential of the present biodosimetric panel for assessment and discrimination of acute and chronic exposures could be investigated. The potential improvement of the methods would also be the exploration of non-invasive sampling (for example, collection of hair follicles). Another important point is the strengthening the network of biodosimetry laboratories and standardization and harmonization of the methods and protocols used. Further attention should be paid to validation of the new promising techniques, such as gene expression measurements used in our study.
4. Transcriptional response to high- and low-LET radiation was similar in view of identity of activated genes at least at 8 h post-exposure. However, heavy ions showed higher potential of activation of immunity-related gene sets and more persistent activation of p53-regulated genes compared to X-rays. More resolved mechanistic studies focusing on specific pathways would further contribute to the development of hadron therapy treatment and radiation protection of astronauts.
5. We showed the potential of gene signatures, although in a very small-scale pilot study, in assessing slight differences in DNA repair capacity following the exposure to iron ions of apparently healthy individuals. Further validation of the obtained results in larger cohorts of subjects, and, possibly, radiotherapy patients with abnormal radiosensitivity, would significantly improve our knowledge on gene expression biomarkers of individual radiosensitivity.

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Acknowledgements

My PhD adventure started in October 2012, when I arrived to Boeretang around midnight... At first, it came a bit as a shock of how isolated this place was. In addition, leaving my family and friends behind for such a long time was a difficult experience. Luckily, I met a lot of great people who helped me start (and eventually finish) this new chapter in my life.

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As a newbie PhD student, I've been first sharing the "roof office" with the stagiers, of whom I would like to particularly thank **Nerea Talavera Moreno**, an Erasmus Master student from Spain, a lovely girl with whom we became friends and who helped me so much in my dormitory life.

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The next generation of PhD students, **Bjorn Baselet**, **Bo Byloos** and **Merel Van Walleghem** came and brought the atmosphere of "action" to the lab. So many new after-work activities were organized and so many traditions started! Bjorn Baselet, besides being ready to organize great after-work activities, was also extremely active in the lab and helped me so-so much with my experiments. Merel, who shared with me the sleepless nights and night-time irradiations at GSI and 3 am processing of the cosmonauts' blood samples.

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Acknowledgements

Curriculum Vitae

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Nationality	Republic of Moldova
Date of birth	14.02.1987
Gender	female

Work experience

Dates	October 2012 – July 2017
Occupation or position held	PhD student
Main activities and responsibilities	Experimental laboratory work, including optimization of available experimental protocols and mastering of new techniques, literature studies, thesis preparation, teaching activities (lectures for European MSc course and Summer School in Radiobiology at SCK, practicals for ManaMa students in Space Science and Namur University students)

Name and address of employer	SCK•CEN 200, Boeretang, B-2400 Mol, Belgium Ghent University 25, Sint-Pietersnieuwstraat, B-9000 Ghent, Belgium
Dates	September 2011 – September 2012
Occupation or position held	Trainee Research Fellow
Main activities and responsibilities	Experimental biochemical work (protein extraction, purification, enzymatic cleavage, SDS-electrophoresis, chromatography)
Name and address of employer	Moldova State University Laboratory of Plant Biochemistry 60, A. Mateevici str, Chisinau, Republic of Moldova
Dates	October 2010 – May 2011
Occupation or position held	Senior Laboratory Technician
Main activities and responsibilities	Practical training, cell culture techniques mastering, analyzing and processing of experimental data, microscopy, cell culture irradiation
Name and address of employer	Joint Institute for Nuclear Research, Laboratory of Radiation Biology 6, Joliot-Curie str, Dubna, Russian Federation
Dates	February 2010 – September 2010
Occupation or position held	Trainee Research Fellow
Main activities and responsibilities	Experimental biochemical work (protein extraction, purification, enzymatic cleavage, SDS-electrophoresis, chromatography)
Name and address of employer	Moldova State University Laboratory of Plant Biochemistry 60, A. Mateevici str, Chisinau, Republic of Moldova
Dates	September 2009 – December 2009
Occupation or position held	Trainee Research Fellow

Main activities and responsibilities	Experimental biochemical work (protein extraction, purification, enzymatic cleavage, SDS-electrophoresis, chromatography)
Name and address of employer	Moldova State University Laboratory of Plant Biochemistry 60, A. Mateevici str, Chisinau, Republic of Moldova
Dates	February 2009 – August 2009
Occupation or position held	Category II Technician
Main activities and responsibilities	Experimental biochemical work (protein extraction, purification, enzymatic cleavage, SDS-electrophoresis, chromatography)
Name and address of employer	Moldova State University Laboratory of Plant Biochemistry 60, A. Mateevici str, Chisinau, Republic of Moldova

Training

Dates	16 - 20 September 2013
Course name	FLAMES Summer School on Methodology and Statistics
Principal subjects/occupational skills covered	Basics of methodology and statistics, including practical training
Name and type of organisation providing education and training	Flanders Training Network for Methodology and Statistics
Dates	22 April - 3 May 2013
Course name	A two-week course on “Cellular effects of low doses and low dose-rates with focus on DNA damage and stress response”
Principal subjects/occupational skills covered	Cellular effects of low doses and low dose rates and methods used to detect such phenomena as adaptive response and bystander effect.

Name and type of organisation providing education and training	Stockholm University; Sponsored by the European NOE consortium DoReMi
Dates	10 - 21 December 2012
Course name	A two-week course on “Inter-individual variability of radiation-sensitivity: Mechanisms and Biomarkers”
Principal subjects/occupational skills covered	Molecular consequences of low dose and low dose rate exposures to ionizing radiation; the usefulness of certain endpoints for use in molecular epidemiological studies and the necessary logistics and ethical considerations for collecting and storing biological samples
Name and type of organisation providing education and training	Institut Curie & CEA, France; Sponsored by the European NOE consortium DoReMi
Dates	19 - 23 November 2012
Course name	A one-week course on “Radiation Protection”
Principal subjects/occupational skills covered	Basic knowledge on Radioactivity and nuclear physics, dosimetry, interaction of radiation with matter, applications of ionizing radiation, biological effects of ionizing radiation, ethical aspects of radiological risks
Name and type of organisation providing education and training	SCK•CEN
Dates	September 2011 - March 2012
Course name	Advanced Training Course Nano-Bioengineering
Principal subjects/occupational skills covered	Human anatomy and physiology, Tissues and molecular engineering, Medical bioinstrumentation, Signal and image processing, Nanotechnologies in biomedicine, Biomaterials and biocompatibility
Name and type of organisation providing education and training	FP7 project MOLD-ERA, Technical University of Moldova

Education

Dates	2009 - 2011
Title of qualification awarded	Master Degree in Natural Science, final score 9.57 out of 10.0
Principal subjects/occupational skills covered	The management and rational administration of natural resources, Bioconversion of Organic wastes, Sustainable development principles, Ecological technologies / Interpretation of modern ecological theories in the field of biodiversity and natural resources protection, Application of the methods of ecological analysis in the professional activity, Elaboration of research projects in the field of ecology
Name and type of organisation providing education and training	Moldova State University
Dates	2006 - 2009
Title of qualification awarded	Licentiate Degree in Natural Science, final score 9.61 out of 10.0
Principal subjects/occupational skills covered	Botany, Zoology, Microbiology, Genetics, Biochemistry, Cytology, Plant and Animal physiology, Biotechnology / Applying theoretical knowledge in practical activities, Collecting, interpreting and analyzing the relevant data from classical and modern biology, Demonstrating fundamental knowledge in Biology
Name and type of organisation providing education and training	Moldova State University
Dates	1994 - 2006
Title of qualification awarded	Baccalaureate degree, final score 9.6 out of 10.0
Principal subjects/occupational skills covered	Mathematics, Informatics, Chemistry, Biology, Physics

Name and type of organisation providing education and training

N. Gogol Lyceum, Chisinau

Personal skills and competences

Mother tongue(s)

Russian

Self-assessment	Understanding		Speaking		Writing
European level (*)	Listening	Reading	Spoken interaction	Spoken production	
Romanian	C2 (Proficient user)	C1 (Proficient user)	B2 (Independent user)	B2(Independent user	B2(Independent user
English	C2 (Proficient user)	C2 (Proficient user)	C1 (Proficient user)	C1 (Proficient user)	C2 (Proficient user)
French	A2 (Basic user)	B1(Independent user	A2 (Basic user)	A2 (Basic user)	A2 (Basic user)
Dutch	A2 (Basic user)	A2 (Basic user)	A2 (Basic user)	A2 (Basic user)	A2 (Basic user)

(*) Common European Framework of Reference for Languages

Social skills and competences

I am a sociable person, my communicatory capabilities have been well developed during student extracurricular activities, conferences and professional activities in scientific research teams, I am also used to living in an international environment

Organisational skills and competences

I can easily get adapted to a new team, can organize collective activities, can give workshops and trainings

Technical skills and competences

DNA, RNA and protein extractions, qPCR, PCR, DNA-microarrays, DNA and protein electrophoresis, Western blotting, light and fluorescent microscopy, cell culture techniques, flow cytometry, immunofluorescent staining and analysis, ELISA, shRNA- and siRNA-based transfections, chromatin immunoprecipitation, lentiviral transduction

Computer skills and competences	Microsoft Office, bioinformatics instruments and databases, genome-wide gene expression analysis tools (Partek GS, GSEA, PAM, AltAnalyze, Gene Array Analyzer, RRHO), image processing and analysis tools (Photoshop, ImageJ, Bio1D), statistical analysis tools (GraphPad Prism, SPSS, basic use of R)
Artistic skills	Hand-made jewellery

Additional information

Papers published

1. Macaeva E.. Shutov A. Peculiarity of sunflower 11S seed storage globulin: tertiary structure modeling. Studia Universitatis, ser. "Ştiinţe ale naturii". 2009, 6(26), 118-120. ISSN 1857-1735.
2. Quintens R., Moreels M., Tabury K., Macaeva E., Michaux A., Averbeck N., Choukèr A., Baatout S. - The ESA IBER-3 project - Gene expression and cytokine monitoring for biodosimetry and radiation sensitivity screening (GYMBRASS).- In: SP-706 Life in Space for Life on Earth, Aberdeen, United Kingdom, 18-22 June 2012 / European Space Agency (ESA), Noordwijk, Netherlands, ESA Communications, 2013
3. Arena C., De Micco V., Macaeva E., Quintens R.- Space radiation effects on plant and mammalian cells.- In: Acta Astronautica, 104:1(2014), p. 419-431.- ISSN 0094-5765
4. Macaeva E., Saeys Y., Tabury K., Janssen A., Michaux A., Benotmane M., De Vos W., Baatout S., Quintens R. Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry. Scientific Reports 6, 19251; doi: 10.1038/srep19251 (2016).
5. Abend M., Badie C., Quintens R., Kriehuber R., Manning G., Macaeva E., Njima M., Oskamp D., Strunz S., Moertl S., Doucha-Senf S., Dahlke S., Menzel J., Port M. Examining radiation induced in vivo and in vitro gene expression changes of the peripheral blood in different laboratories for biodosimetry purposes - First RENEB gene expression study -. Radiation Research 185[382]:109-23. doi: 10.1667/RR14221.1 (2016).
6. Manning G., Macaeva E., Majewski M., Kriehuber R., Brzóska K., Abend M., Doucha-Senf S., Oskamp D., Strunz S., Quintens R., Port M., Badie C. Comparable dose estimates of blinded whole blood samples are obtained independently of culture conditions and analytical approaches - Second RENEB gene expression study. - International Journal of Radiation Biology 93(1):87-98 (2017). doi: 10.1080/09553002.2016.1227105.
7. Ainsbury E., Badie C., Barnard S., Manning G., Moquet J., Abend M., Bassinet C., Bortolin E., Bossin L., Bricknell c., Brzoska K.,

Čemusová z., Christiansson M., Cosler G., Dellamonaca S., Desangles F., Discher M., Doucha-Senf S., Eakins J., Fattibene P., Gregoire E., Guogyte K., Kriehuber R., Lee J., Lloyd D., Lyng F., Macaeva E., et al. Integration of new biological and physical retrospective dosimetry methods into EU emergency response plans – joint RENEB and EURADOS inter-laboratory comparisons -. International Journal of Radiation Biology 93(1):99-109 (2017). doi: 10.1080/09553002.2016.1206233.

8. Hall J., Jeggo P., West C., Gomolka M., Quintens R., Badie C., Laurent O., Aerts A., Anastasov N., Azimzadeh O., Azizova T., Baatout S., Baselet B., Benotmane M., Blanchardon E., Guéguen Y., Haghdoost S., Harms-Ringhdahl M., Hess J., Kreuzerd M., Laurier D., Macaeva E., Manning G., Pernot E., Ravanat J., Sabatier L., Tack K., Tapio S., Zitzelsberger H., Cardis E. Ionizing radiation biomarkers in epidemiological studies – an update. - Mutation Research-Reviews in Mutation Research 771: 59–84 (2017). doi: 10.1016/j.mrrev.2017.01.001

Oral presentations

1. Macaeva E. Identification of new possible gene and exon biomarkers for high- and low-LET radiation exposure: update and perspectives. Seminars in Radiobiology, Mol, Belgium, 27 September 2013.
2. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Averbeck N., Choukèr A., Baatout S. Gene and exon signatures as high-potential biomarkers of exposure and individual radiosensitivity to low- and high-LET radiation. 41st Annual Meeting of the European Radiation Research Society ERR2014, Rhodes, Greece, 14-19 September 2014.
3. Macaeva E. Identification and functional analysis of high- and low-LET radiation biomarkers: update and perspectives. Seminars in Radiobiology, Mol, Belgium, 23 February 2015.
4. Macaeva E. Radiation biomarkers: the easy and the hard. Scientific seminar at the Molecular Biotechnology Department meeting, Ghent University, March 21, 2016, Ghent.
5. Macaeva E. Biomarkers of exposure and sensitivity to high- and low-LET radiation. 1st Day of the PhDs 2016, April 20, 2016, Mol, Belgium

Poster presentations

1. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Averbeck N., Choukèr A., Baatout S. Identification of new possible gene and exon biomarkers for high and low LET radiation exposure. MELODI Workshop, Brussels, Belgium, 7-10 October 2013.
2. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Averbeck N., Baatout S. Identification of new

transcription-related biomarkers for high- and low-LET radiation exposure. 2nd Day of the PhDs 2013, Mol, Belgium, 23 October 2013

3. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Averbeck N., Choukèr A., Baatout S. Gene and exon signatures as high-potential biomarkers of exposure and individual radiosensitivity to low- and high-LET radiation. 41st Annual Meeting of the European Radiation Research Society ERR2014, Rhodes, Greece, 14-19 September 2014.
4. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Averbeck N., Baatout S. Gene and exon signatures as biomarkers of exposure and individual radiosensitivity to low- and high-LET radiation. 2nd Day of the PhDs 2014, Mol, Belgium, 23 October 2014.
5. Macaeva E., Saeys Y., De Vos W., Janssen A., Michaux A., Tabury K., Benotmane R., Baatout S., Quintens R. Exon and gene expression signatures as robust biomarkers of exposure to low and moderate doses of X-rays. Sixth International MELODI Workshop, Barcelona, Spain, 7-9 October 2014.
6. Macaeva E., Quintens R., De Vos W., Moreels M., Tabury K., Michaux A., Janssen A., Averbeck N., Baatout S. Mining the transcriptome in search for biomarkers of sensitivity to low- and high-LET radiation. 15th International Congress of Radiation Research, Kyoto, Japan, 25-29 May 2015.
7. Macaeva E., Quintens R., Saeys Y., Tabury K., Michaux A., Janssen A., Benotmane R., Moreels M., De Vos W. Baatout S.- Gene expression-based biodosimetry.- Day of the PhD's.- Mol, Belgium, 29 October 2015.
8. Macaeva E., Saeys Y., Tabury K., Janssen A., Michaux A., Benotmane R., De Vos W., Baatout S., Quintens R. - Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry.- 7th MELODI Workshop "Next Generation Radiation Protection Research" .- Munich, Germany, 9-11 November 2015.
9. Macaeva E., Saeys Y., Tabury K., Janssen A., Michaux A., Benotmane R., De Vos W., Baatout S., Quintens R. - Radiation-induced alternative transcription and splicing events and their applicability to practical biodosimetry. BNS Young Generation Scientific Contest February 25, 2016, Brussels.

Awards	1. Four-year SCK•CEN/UGent PhD grant. 2. Young Investigator Award for participation to the 41st Annual Meeting of the European Radiation Research Society (ERRS 2014), Rhodes, Greece, 14 - 19 September 2014. 3. Second poster prize at the BNS Young Generation Scientific Contest February 25, 2016, Brussels.
Courses given	1. Radiation biomarkers: an overview. European MSc course in Radiation Biology. Mol, Belgium, 17-28 February 2014 2. Macaeva E.- Radiation biomarkers.- Summer school in radiobiology, SCK•CEN, Mol, Belgium, August 18, 2014. 3. Macaeva E., Baatout S.- Radiation biomarkers: an overview.- Two-week training course on radiation-induced effects with particular emphasis on genetics, development, teratology, cognition as well as space-related health issues, SCK•CEN, Mol, Belgium, March 17, 2015. 4. Macaeva E.- Measurement of gamma H2AX foci formation as a marker of ionizing radiation induced DNA damage.- UGent/KU Leuven/SCK•CEN, Mol, Belgium, November 17, 2015.- (Master in Space Sciences. Course in Life Sciences in Space. Site visit to SCK•CEN). 5. Macaeva E.- Measurement of gamma H2AX foci formation as a marker of ionizing radiation induced DNA damage.- University of Namur/SCK•CEN, Mol, Belgium, December 11, 2015.- (Cours SPHYM201 Travaux pratiques cours "Interactions des radiations avec la matière"). 6. Macaeva E. Radiation biomarkers: an overview.- Two-week training course on radiation-induced effects with particular emphasis on genetics, development, teratology, cognition as well as space-related health issues, SCK•CEN, Mol, Belgium, May 9, 2016.
Student guidance	1. Julie Broos. Bachelier – Technologue de laboratoire médical, Haute école Louvain en Hainaut. L'expression de gènes comme biomarqueur des effets des radiations dans les cellules immunitaires humaines, 9 February – 22 May 2015.