Cover: Radiation by Yossy Hal

Predicting normal tissue toxicity in radiotherapy:

can we improve clinical decision-making?

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Summary

More than one in three people in the western world will be diagnosed with cancer at some point during their lifetime (1, 2). Breast cancer and prostate cancer represent the most common types of cancer with high survival rates. An important treatment modality in cancer management is radiotherapy (RT) with approximately half of all cancer patients receiving this treatment. Despite advances in technologies, radiation-induced side effects still occur and vary widely among patients which can broadly be related to radiation dosimetric variables, adjuvant cancer treatments and factors inherent to the patient, including genetics. As these side effects can impair the quality of life of cancer survivors, therapy-induced toxicity has become very important. The subject of this PhD dissertation is the identification of factors predicting or influencing the development of normal tissue toxicity and the development of integrated models that are able to predict which cancer patients are most likely to develop adverse events after RT.

RT treatment for prostate cancer can result in toxicity to the gastrointestinal (GI), genitourinary (GU) and reproductive organs. In this PhD research, only GU symptoms were evaluated in prostate cancer patients treated with high-dose primary or post-operative intensity-modulated RT (IMRT), as GI toxicity was very rare. Acute RT-induced nocturia is in our study population the predominant acute toxicity endpoint; it is a frequently occurring but under-reported GU symptom. Nocturia was recently found to be associated with a decreased quality of life and with an increased prevalence of depression because of more frequent nightly voids. A number of clinical, dosimetric parameters and SNPs in *TGFB1*, capturing all common variants in the 5' region of the gene, were tested

for association with the endpoint. The presence of mild pre-treatment complaints, treatment with primary IMRT and two polymorphisms in *TGFB1* were identified as risk factors. These results were published in a first paper, presented in **Paper I** (chapter 6).

Recent refinements of radiotherapy techniques allow for a better sparing of the rectum resulting in a minimization of GI toxicity. In contrast to the incidence of late GU symptoms that remains unchanged due to the full inclusion of the bladder neck and, in case of postoperative RT, the vesicourethral anastomosis in the high-dose region. Late radiation-induced haematuria and nocturia are the most frequently observed late GU symptoms. Models for prediction of these endpoints were build using an in-house developed statistical algorithm considering clinical, dosimetric and genetic data. The genetic data were obtained by a custom-designed Illumina GoldenGate platform containing 384 genetic variations. The variations were selected based on an extended candidate gene approach. Both integrated prediction models have acceptable predictive performance. The model predicting late haematuria and late nocturia has an AUC of 0.82 and 0.76, respectively. The paper resulting from this study is presented in **Paper II** (chapter 7).

Acute skin toxicity is assessed in breast cancer patients treated with adjuvant RT after breast-conserving surgery. The endpoints of interest are the development of moderate to severe acute dermatitis and moist desquamation. Normofractionated (25x2 Gy) or hypofractionated (15x2.67 Gy) IMRT in prone or supine position is prescribed. Systemic therapies like chemotherapy, hormone therapy and trastuzumab are administered when indicated. Eight SNPs were selected based on literature data regarding a possible involvement in toxicity after cancer therapy. BMI, large bra cup size, fractionation schedule and concurrent hormone therapy were significantly associated with the development of dermatitis and moist desquamation. Additional factors modifying the risk of dermatitis were supine IMRT, the administration of trastuzumab and the genetic variation *MLH1* rs1800734. The paper resulting from this study is presented in **Paper III** (chapter 8).

Some issues to improve research in the field of normal tissue injury can be addresses. First, a distinction should be made between individual approach (prediction) and population approach (association) which are both different in their objectives, measurements and their applicability in the clinical context. Secondly, due to a diversity in symptoms recorded by multiple scoring systems the comparison between studies and the pooling of data is hampered. In addition, predicting a dichotomised endpoint is accompanied with a loss of information but a new level of complexity is added when an ordinal endpoint is predicted. This is illustrated by the prediction of acute dermatitis. Thirdly, the added value of genetic polymorphisms, each conferring small effect sizes, in predicting a complex trait should be discussed. Together with alternative approaches for the prediction of radiation-induced toxicity, like cellular, apoptosis and gene-expression assays are overviewed.

In conclusion, the success of predicting normal tissue toxicity will depend on our efforts to collaborate in joining expertise of different research areas and in creating a standardized manner of collecting dosimetric, clinical and biological data.

REFERENCES

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2. http://www.kankerregister.org/Statistieken.

Samenvatting

Meer dan een derde van de mannen en vrouwen in de westerse wereld zal ooit de diagnose van kanker krijgen (1, 2) met borst- en prostaatkanker als de twee meest voorkomende types; beiden hebben een hoge kans op overleving. Radiotherapie (RT) is een belangrijke behandeling die wordt toegepast bij ongeveer de helft van de kankerpatiënten. Ondanks de technologische vooruitgang treden nog steeds neveneffecten op ten gevolge van de bestraling. Deze variëren sterk tussen patiënten onderling en kunnen verband hebben met de ontvangen dosis door de gezonde weefsels, met ondersteunende kankerbehandelingen en met factoren inherent aan de patiënt, zoals de genetica onder de vorm van single nucleotide polymorfismen (SNPs). Daar deze neveneffecten de levenskwaliteit van de overlevenden sterk kan beïnvloeden, is het belangrijk dit verder te onderzoeken. Het onderwerp van dit proefschrift is het identificeren van factoren die geassocieerd of voorspellend zijn voor het optreden van straling-geïnduceerde normale weefsel toxiciteit en het genereren van geïntegreerde modellen die kunnen voorspellen welke kankerpatiënten de grootste kans hebben om deze toxiciteit te ontwikkelen.

RT behandeling voor prostaatkanker kan leiden tot schade aan de gastro-intestinale (GI), genito-urinaire (GU) en de voortplantingsorganen. Aangezien het optreden van GI symptomen eerder ongewoon is, werden in dit onderzoek enkel GU symptomen onderzocht bij prostaatkanker patiënten die behandeld zijn met hoge-dosis primaire of postoperatieve intensiteit-gemoduleerde RT (IMRT). Acuut RT-geïnduceerde nycturie is in onze studiepopulatie de voornaamste vorm van acute toxiciteit; het is een veel voorkomend maar weinig gemeld GU symptoom. Nycturie werd onlangs in verband gebracht met een verminderde levenskwaliteit en met een verhoogd voorkomen van depressie

vanwege het frequenter nachtelijk opstaan. Een aantal klinische, dosimetrische parameters en SNPs in *TGFB1*, die alle varianten in de 5'-regio van het gen omvat, werden onderzocht op hun relatie met het eindpunt. De aanwezigheid van milde klachten voor de start van RT, behandeling met primaire IMRT en twee polymorfismen in *TGFB1* kwamen naar voor als als risicofactoren. Deze resultaten werden gepubliceerd in **Paper I** (hoofdstuk 6) .

Recente technologische ontwikkelingen in RT kunnen het rectum beter vrijwaren van bestraling met een minimaal voorkomen van GI toxiciteit tot gevolg. Daarentegen, het voorkomen van late GU symptomen blijft onveranderd door de volledige inclusie van de blaashals en – in geval van postoperatieve RT – de vesicourethrale anastomose, in de hoge dosis regio. Chronische straling-geïnduceerde hematurie en nycturie zijn de meest voorkomende chronische GU symptomen. Modellen die het optreden van deze eindpunten voorspellen, werden gebouwd met behulp van een intern ontwikkeld statistisch algoritme waarin klinische, dosimetrische en genetische data werden opgenomen. De genetische data werden verkregen door een op maat ontworpen Illumina GoldenGate platform met 384 genetische variaties. De polymorfismen werden geselecteerd op basis van een uitgebreide kandidaatgen benadering. Beide geïntegreerde modellen hebben een aanvaardbare voorspellende waarde. Het model dat chronische hematurie en nycturie voorspelt, heeft een AUC van, respectievelijk, 0.82 en 0.76. **Paper II** geeft deze resultaten weer (hoofdstuk 7).

Acute huidreacties werden geregistreerd bij borstkanker patiënten behandeld met adjuvante RT na borstsparende chirurgie. De eindpunten zijn het ontwikkelen van matige tot ernstige acute dermatitis en vochtige desquamatie. Normofractionering (25x2 Gy) of hypofractionering (15x2.67 Gy) IMRT in buik- of ruglig wordt voorgeschreven. Systemische therapieën zoals chemotherapie, hormoontherapie en trastuzumab worden toegediend wanneer nodig. Acht SNPs werden geselecteerd die volgens literatuurdata mogelijks betrokken zijn bij het ontwikkelen van toxiciteit na kankertherapie. BMI, grote bh-maat, fractioneringsschema en hormoontherapie gelijktijdig met RT, waren significant geassocieerd met de ontwikkeling van dermatitis en vochtige schilfering. Bijkomende factoren die het risico op dermatitis wijzigen bleken

IMRT in buiklig, de toediening van trastuzumab en de genetische variatie *MLH1* rs1800734. De paper als gevolg van deze studie wordt weergegeven in **Paper III** (hoofdstuk 8).

Het onderzoek naar normale weefsel schade ten gevolge van RT staat nog voor een aantal grote uitdagingen. Ten eerste moet een duidelijk onderscheid gemaakt worden tussen de individuele (predictie) en de algemene (associatie) benadering die beiden verschillen in hun doelstellingen, meetmethodes en hun toepasbaarheid in klinische context. Ten tweede wordt de vergelijking tussen studies en het uitwisselen van data bemoeilijkt. Dit is het gevolg van meerdere scoringssystemen die een verscheidenheid aan symptomen beoordeeld. Bovendien gaat het voorspellen van een binair eindpunt gepaard met verlies aan informatie maar meer complexiteit wordt geïntroduceerd wanneer een ordinaal eindpunt voorspeld wordt. Dit wordt aangetoond bij de predictieanalyse van acute dermatitis. Ten derde zou de toegevoegde waarde van genetische polymorfismen, die elk slechts een klein effect bijdragen, in het voorspellen van een complex kenmerk moet worden besproken. Alternatieve methodes voor de voorspelling van straling-geïnduceerde toxiciteit, zoals cellulaire, apoptose en genexpressie assays worden ook besproken.

Tot slot, het succes om normale weefsel toxiciteit te voorspellen zal afhangen van onze inspanningen tot samenwerken in het verzamelen van expertise in verschillende onderzoeksdomeinen en in het creëren van een gestandaardiseerde manier om dosimetrische, klinische en biologische gegevens te verzamelen.

REFERENTIES

1. http://seer.cancer.gov/statfacts/html/all.html.
2. http://www.kankerregister.org/Statistieken.

Résumé

Plus d'un tiers des hommes et des femmes du monde occidental sera diagnostiqué avec le cancer à un moment donné de leur vie (1, 2). Le cancer du sein et le cancer de la prostate sont les deux types de cancers les plus fréquents; les deux ont un taux de survie élevé. La radiothérapie (RT) est un traitement important qui est utilisé pour environ la moitié des patients atteints de cancer. Malgré les progrès technologiques, les effets secondaires induits par les radiations ionisantes se manifestent encore. Ceux-ci varient considérablement entre les patients et peuvent être associés à la dose reçue par les tissus sains, aux thérapies de soutiens et à des facteurs inhérents au patient, comme la génétique sous forme de polymorphisme d'un nucléotide (SNP). Étant donné que ces effets secondaires peuvent fortement affecter la qualité de vie des survivants au cancer, il est important d'étudier davantage cette question. Le sujet de cette thèse de doctorat est d’identifier des facteurs prédictifs ou des facteurs qui influencent le développement de la toxicité du tissu normal et le développement de modèles intégrés qui peuvent prédire quels patients atteints de cancer sont les plus susceptibles de développer des effets indésirables après la RT.

Le traitement RT du le cancer de la prostate peut entraîner une toxicité à l'appareil gastro–intestinal (GI), génito-urinaire (GU) et aux organes reproducteurs. Dans cette thèse, seuls les symptômes GI sont évalués dans les patients atteints du cancer de la prostate traités par une dose élevée primaire ou post-opératoire avec radiothérapie conformationelle avec modulation d'intensité (IMRT), étant donné que la toxicité gastro-intestinale était très rare. La nycturie aiguë induite par la RT, critère prédominant de toxicité aiguë dans

notre population d'étude, est un des symptômes GU commun mais sous-déclaré. La nycturie a récemment été associée à une diminution de la qualité de vie et une incidence accrue de dépression à cause des mictions nocturnes fréquentes. Un certain nombre de paramètres cliniques et dosimétriques, et les SNP de *TGFB1*, contant tous les variants communs dans la région 5' du gène, ont été testés pour la relation avec le point de terminaison. La présence de plaintes légères avant le début de la radiothérapie, le traitement primaire avec IMRT et deux polymorphismes dans TGFB1 ont été identifiés comme des facteurs de risque. Ces résultats ont été publiés dans un premier document, présenté dans **Paper I** (chapitre 6).

Les améliorations récentes des techniques de radiothérapie permettent une meilleure épargne du rectum, résultant dans une incidence minimale de toxicité GI. Contrairement à l'incidence des symptômes tardifs GU qui reste inchangé en raison de l'inclusion du col de la vessie et, en cas de radiothérapie postopératoire, de l'anastomose vésico-urétrale dans la région à forte dose. L'hématurie et la nycturie radio-induite sont les symptômes GU chroniques les plus courants. Les modèles qui prédisent la présence de ces paramètres ont été élaborées en utilisant un algorithme statistique tenant compte des données cliniques, génétiques et dosimétriques. Les données génétiques ont été obtenues grâce à une plateforme Illumina GoldenGate conçue sur mesure avec 384 variations génétiques. Les variations ont été sélectionnées basée sur une approche de gène candidat étendue. Les deux modèles de prévision intégrés ont des performances prédictives acceptables. Le modèle qui prédit que l'hématurie chronique et la nycturie, ont une AUC de, respectivement, 0.82 et 0.76. **Paper II** montre les résultats (chapitre 7).

Des réactions aiguës de la peau ont été évaluées chez les patients de cancer du sein traitées avec la radiothérapie adjuvante après chirurgie mammaire conservatrice. Les paramètres d'intérêt sont le développement de la dermatite aiguë modérée à sévère et de desquamation. L'IMRT normo fractionnée (25x2 Gy) ou hypofractionnée (15x2.67 Gy) en position décubitus ventral ou dorsal est prescrit. Les traitements systémiques comme la chimiothérapie, l'hormonothérapie et le trastuzumab sont administrées lorsque

indiqué. Huit SNP ont été sélectionnés, sur la base de données de la littérature, qui pourrait être impliqués dans le développement de la toxicité après la thérapie du cancer. L'IMC, une grande taille de poitrine, le fractionnement et l'hormonothérapie simultanément avec la RT, étaient significativement associés avec le développement de la dermatite et de desquamation. D'autres facteurs modifiant le risque de dermatite ont été IMRT en position couchée, l'administration de trastuzumab et la variation génétique *MLH1* rs1800734. Le document qui résulte de cette étude est présenté dans **Paper III** (chapitre 8).

La recherche de toxicité tissulaire normale causée par RT est encore confrontée à plusieurs défis. Tout d'abord, il convient de faire une distinction claire entre l'approche de l''individu (prédiction) et approche globale (association) car les deux diffèrent dans leurs objectifs, méthodes de mesure et de leur applicabilité dans le contexte clinique. Deuxièmement, la comparaison entre les études et l'échange de données plus difficile. Ceci est le résultat de plusieurs systèmes de notation revue une variété de symptômes. En outre, les prévisions d'un critère binaire associée à la perte d'information, mais plus la complexité est introduite lorsqu'un point d'extrémité ordinal est prévu. En outre, la prévision d'un critère dichotomique est accompagnée d'une perte d'information, mais un nouveau niveau de complexité est ajouté quand un critère ordinal est prévu. Ceci est démontré dans la prédiction de la dermatite aiguë. Troisièmement, la valeur ajoutée des polymorphismes génétiques, chacun contribuant que peu d'effet dans la prévision d'un trait complexe, devrait être discuté. Des méthodes alternatives pour la prédiction de la toxicité induite par les rayonnements, tel que de essaies cellulaires, d'apoptose et l'expression des gènes, sont également examinés.

En conclusion, la réussite de la prédiction de la toxicité tissulaire normale dépendra de nos efforts pour coopérer à la collecte d'expertise dans différents domaines de recherche et à la création d'une méthode rationalisée pour la collecte de données dosimétriques, cliniques et biologiques.

RÉFÉRENCES

1. http://seer.cancer.gov/statfacts/html/all.html.
2. http://www.kankerregister.org/Statistieken.

Contents

List of Acronyms

A

AIROPROS	Italian Association for Radiation Oncology Group on Prostate Cancer
AP-1	jun proto-oncogene
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 related
AUA	American Urological Association
AUC	Area Under the ROC Curve

B

BER	Base Excision Repair
BMI	Body Mass Index
53BP1	Tumor Protein p53 Binding Protein 1
BRCA2	Breast Cancer 2, early onset

C

CD	Cluster of Differentiation
CDC25	Cell Division Cycle 25C
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
COGS	Collaborative Oncological Gene-environment Study
CT	Computed Tomography
CTCAE	Common Terminology Criteria for Adverse Effects
CTV	Clinical Target Volume
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9

D

DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
DSB	Double-Strand Breaks
DVH	Dose-Volume Histogram

E

ED	Erectile Dysfunction
EGFR	Epidermal Growth Factor Receptor
EM	Expectation-Maximisation algorithm
EU	European Union
EUD	Equivalent Uniform Dose

F

FDA	Food and Drug Administration
FSHR	Follicle Stimulating Hormone Receptor

G

G	Grade
GenePARE	Genetic Predictors of Adverse Radiotherapy Effects
GI	Gastrointestinal
GIANT	Genetic Investigation of ANthropometric Traits
GOF	Hosmer-Lemeshow goodness-to-fit test
GU	Genitourinary
GUH	Ghent University Hospital
GWAS	Genome-wide association study
Gy	Gray

H

(γ-)H2AX	(phosphorylated) histone subtype H2A isoform X
HER-2	Human Epidermal growth factor Receptor 2
HIF-1	Hypoxia Inducible Factor 1
HR	Homologous Recombination
HRMA	High Resolution Melting Analysis

I

IFN	Interferon
IFNK	Interferon, Kappa
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IMRT	Intensity-modulated radiotherapy
IR	Ionizing Radiation

L

Lasso	Least Absolute Shrinkage and Selection Operator
LD	Linkage Disequilibrium
LENT/SOMA	Late Effects Normal Tissues: Subjective, Objective, Management and Analytic
LHRH	Luteinizing-Hormone-Releasing Hormone
LIG1	DNA -Ligase I , ATP-dependent
LIG3	DNA Ligase III , ATP-dependent

M

MLH1	MutL Homolog 1
MMR	Mismatch Repair
MRN	MRE11/RAD50/NBN-complex

N

NADPH	2,4-dienoyl CoA reductase 1, mitochondrial
NER	Nucleotide Excision Repair
NFκB	Nuclear Factor of κ light polypeptide gene enhancer in B-cells
NHEJ	Non-Homologous End Joining
NTCP	Normal Tissue Complication Probability

O

OR	Odds Ratio

P

PARP1	Poly (ADP-Ribose) Polymerase 1
PCR	Polymerase Chain Reaction

R

RAPPER	Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy
REQUITE	Radiotherapy for Quality of life through reduced Toxicity
RFLP	Restriction Fragment Length Polymorphism
RGC	Radiogenomics Consortium
RILA	Radiation-Induced Lymphocyte Apoptosis
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RPA	Recursive Partitioning Analysis
RT	Radiation Therapy
RTOG/EORTC	Radiation Therapy Oncology Group/ European Organization for Research and Treatment of Cancer

S

SEMA3A	Semaphorin 3A
SNP	Single Nucleotide Polymorphisms
SSB	Single-Strand Breaks
STAT	Standardized Total Average Toxicity

T

TCP	Tumour Control Probability
TGFB1	Transforming Growth Factor β1
TNF	Tumor Necrosis Factor
TP53	Tumor Protein p53
TURP	Transurethral Resection of the Prostate

U

UTR	Untranslated Region

V

VEGR	Vascular Endothelial Growth Factor
VKORC1	Vitamin K epoxide Reductase Complex, subunit 1
VUS	Volume Under the ROC Surface

X

XRCC1	X-ray Repair Complementing defective repair in Chinese hamster cells 1

Part I

Patients vary in their normal tissue response to radiation

1 Radiotherapy is an important treatment modality in cancer management

Yearly, almost 700.000 women in the western world are diagnosed with breast cancer and the same number of men with prostate cancer. They represent the most common types of cancer (1). For all stages together, the 5-year survival rate for breast cancer patients is 89% and more than 90% for prostate cancer patients (2). Mammographic screening for breast cancer patients and PSA-screening for prostate cancer patients result in early diagnosis, which improves the chances of successful treatment.

This chapter outlines the use of radiotherapy as a cornerstone in cancer management, the impact of the treatment at the DNA level and the cascade of effects associated with DNA damage. The application of radiotherapy in the treatment of breast and prostate cancer is then explained in more detail.

1.1 Radiotherapy in cancer management

Just a few years after Wilhelm Roentgen discovered x-rays in 1895 and Antoine Becquerel discovered radioactivity in 1898, various forms of radiation were used to treat cancer. Immediately, it was apparent that radiation therapy (RT) held great promise as an effective therapeutic modality (3). Approximately 50% of all cancer patients receive RT at some point during the course of their treatment (4). At present, RT is the most important non-surgical modality for curative treatment of cancer. Since it accounts for only 5% of the total cost of cancer care, it is also cost effective (5).

Radiation is mainly delivered by external beam RT. An external radiation source generates ionizing radiation (IR) that is directed towards the tumour. The most frequently used form of external beam RT is high-energy x-rays generated by linear accelerators (6). Other forms are particle therapy which uses high-energy charged particles, like electrons, protons and carbon ions. In brachytherapy, a radiation source is brought into the tumour site either by implantation or an afterloader (6).

RT is based on the balance between cure and toxicity. The success of RT in cancer treatment principally depends on the total radiation dose given, which is limited by the tolerance of the normal tissues surrounding the tumour (7) This can be quantitatively described by dose-response curves for tumour control and normal tissue damage, see Figure 1.1.

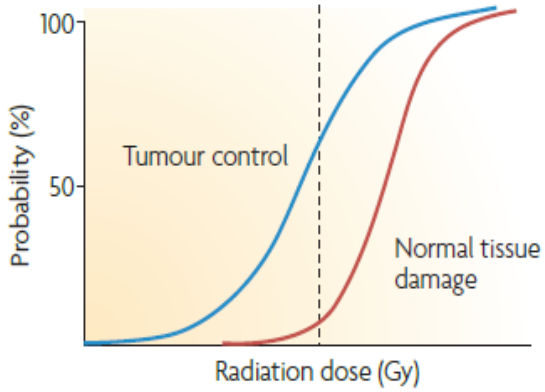

Figure 1.1: Dose-response curves for radiotherapy. Sigmoidal shaped response curves for tumour control and normal tissue damage. The probability of tumour cure increases with radiation dose with accompanying probability of severe late normal tissue damage. The dotted line shows a theoretical dose associated with ~60% tumour control and ~5% severe late toxicity. *Adapted from Barnett et al.* (7).

External beam RT is usually given over a course of multiple fractions as it maximises tumour kill and minimizes normal tissue damage. Based on empirical studies with respect to these normal tissue reactions, conventional fractionation regimens of 1.8-2 Gy per fraction at a rate of five fractions per week have been the backbone over the last decades in most institutions (8, 9).

Altered fractionation regimens and new technologies like computerized treatment planning systems, image-guided RT and intensity-modulated radiotherapy (IMRT) can substantially improve the therapeutic ratio by better tumour control and reduction of normal tissue toxicity (10, 11). Cure rates can be further improved by combining molecular targeting agents, hormone and chemotherapy with radiotherapy (5).

In this PhD dissertation, all patients were treated with IMRT, an advanced form of three-dimensional conformal RT. IMRT includes modulation of the beam intensity; this is achieved by beam modifiers, such as multileaf collimators. As a result, concave-shaped dose distributions and tight dose gradients are created to closely sculpt the 3D shape of the target. In addition, IMRT allows the

delivery of high radiation doses to the tumour while limiting the radiation dose to the normal tissues, as shown in Figure 1.2 (6, 12).

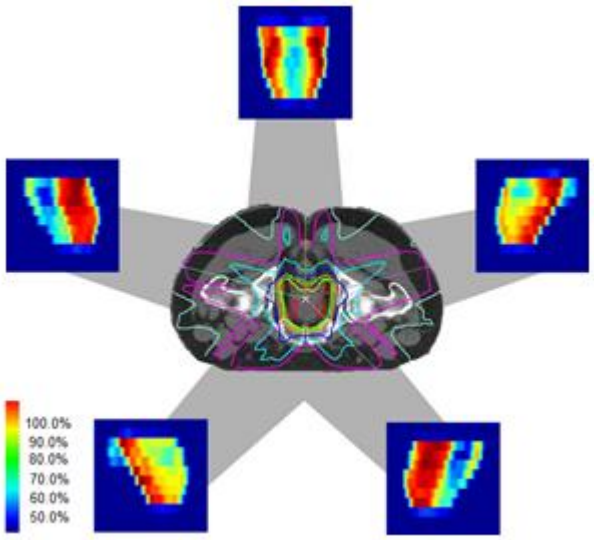

Figure 1.2: Concept of IMRT for example in prostate cancer (transversal slice of the male abdomen). In each direction, the beam is modulated by varying the intensity of its smaller units. This precise radiation dose conforms to the shape of the tumour and the amount of radiation to normal tissues surrounding the treated area is minimized. *Adapted from (13)*.

1.2 Radiotherapy – mode of action

RT is the treatment of cancer using IR. Interaction of IR with the cellular environment results in energy depositions causing ionizations (8, 14). Ionizations produce highly reactive free radicals, that have the potential to break chemical bonds. Damage to DNA is the most harmful effect to cells, for example, single-strand breaks (SSBs), double-strand breaks (DSBs), DNA crosslinks and various base modifications leading to SSBs and/or DSBs (8, 15). Of them, DSBs are biologically the most important lesions as they are more difficult to repair than other DNA lesions because the two DNA ends can separate, and accompanying base damage hampers DSB ligation (14, 16). DNA damage can be induced by direct interaction of IR with DNA or indirectly by the generation of reactive species (oxygen (ROS) and nitrogen (RNS)) in close proximity to the DNA (15).

Upon DNA damage, a complex coordinated system is triggered that determines the fate of the cell. This DNA damage response (DDR) encompasses processes of DNA repair and signal transduction mechanisms that alert the cell to the presence of DNA damage. Firstly, sensor proteins detect the sites of damage within the DNA. This signal is then amplified by a set

of proteins known as transducers. They function to relay the signal to downstream effector pathways determining cell fate, either arrest the cell cycle to allow repair of damaged DNA or, if the damage is beyond repair, initiate the cell to undergo programmed cell death or apoptosis (8, 16, 17). Figure 1.3 lists many of the key genes involved. The DDR is explained in more detail.

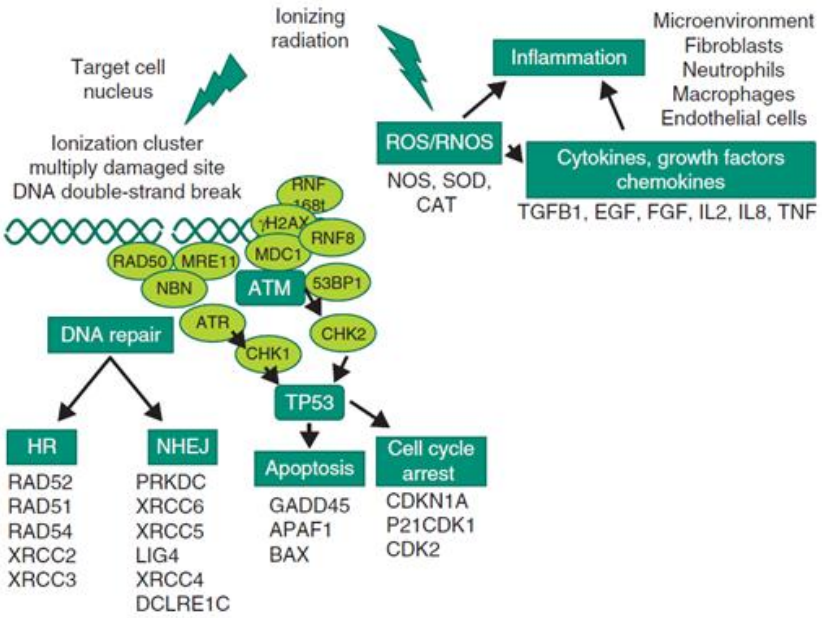

Figure 1.3: Summary of the pathways and mechanisms involved in cell response to RT. *Adapted from West et al.* (14).

ATM lies at the heart of the signalling response induced by DNA damage and is activated through the MRN (MRE11-RAD50-NBN) complex which is the primary sensor of DSBs (15). Cells rely on two major pathways to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (16). They are complementary and are used under different circumstances (17). HR requires a homologous template, usually a sister chromatid, occurs in S and G2 phases of the cell cycle and is error free. NHEJ repairs DSBs without requiring sequence homology throughout the cell cycle (16, 17). Although it was commonly believed that HR plays a major role in G2 phase, recent studies have shown that NHEJ represents the major DSB repair pathway in G2, with HR only being essential for the repair of a minor subset (~15%) of IR-induced DSBs (18). When the classical route is impeded due to missing or mutated NHEJ components, alternative NHEJ pathways can operate which rely on factors involved in HR and SSB repair like the MRN complex, PARP1, XRCC1 and LIG1 or LIG3 (17). SSBs are initially detected by PARP1 and are through ATR

activation, repaired by the mechanisms base excision repair (BER) or nucleotide excision repair (NER). The latter corrects bulky helix-distorting lesions, the BER system targets nonbulky lesions (base modifications) and abasic sites (15). It is, however, estimated that ~1% of these single-strand lesions are converted into DSBs which are repaired by NHEJ or HR through ATR and ATM activation (15). In addition, mismatch repair (MMR) removes nucleotides arising from replication errors and accumulating data suggest that MMR proteins are involved in DDR upon exposure of IR (19). ATM activation leads to phosphorylation of CHK2, TP53 and CDC25 which triggers checkpoint activation and cell cycle arrest, in G1/S and/or G2/M phase. These checkpoints induce transient cell cycle arrest, allowing sufficient time for DNA repair (8). ATR, on the other hand, signals via CHK1 to promote cell cycle arrest. If DSB repair fails, apoptosis or cellular senescence is induced via ATM/ATR signalling (14). Another function of ATM is shown to be the protection of cells from ROS accumulation by stimulating NADPH production and promoting the synthesis of nucleotides required for DSB (20).

Besides DNA damage, ROS and RNS may also damage proteins, lipids and mitochondrial DNA (21). They may spread from targeted cells to non-targeted bystander cells through intercellular communication mechanisms, where the oxidative metabolism is further disrupted (21).

1.2.1 Efficacy of radiation treatment

Splitting up radiation dose in multiple dose fractions, maximizes tumour control and minimizes normal tissue damage. The rationale behind it is explained by radiobiological factors summarized as the five Rs of RT: DNA repair, reoxygenation, repopulation, redistribution and intrinsic radiosensitivity (22). In this context, it is believed that radiation-induced lethality is primarily caused by DNA damage in targeted cells.

Fractionation spares normal tissue because it allows the cell to repopulate and to recover from the DNA damage. Tumour cells, on the other hand, proliferate faster than normal tissue leaving them less time to repair the damage and together with the many genetic changes, they are more susceptible for radiation-induced cell death. Redistribution brings with each successive

radiation dose more cells into radiosensitive phases of the cell cycle. Decreased tumour burden leads to better vascularity and oxygenation, which increases the radiosensitivity in the tumour (8). Intrinsic radiosensitivity represents the radiosensitivity of different cell types and tumour cells (9).

1.3 Radiotherapy for breast cancer and prostate cancer

Breast-conserving surgery followed by breast irradiation, is recommended as the primary treatment for early-stage breast cancer (23). RT reduces the risk of local recurrence substantially and prevents the need for mastectomy (24-26). Moderate whole breast hypofractionated regimens (42.5 Gy in 16 fractions or 40 Gy in 15 fractions) were shown to be equally effective as to the standard RT schedule of 50 Gy in 25 fractions (27, 28). This was expected based on the radiobiological model that a larger dose per fraction given over a shorter period of time is just as effective as the more traditional longer regimen (29). The use of sequential tumour bed boost improves local control but with higher rates of fibrosis (30). Introduction of modern technologies has facilitated the planning and delivery of for example simultaneous integrated boost, to further shorten course of RT (31), or, optimization of prone positioning to reduce toxicity rates (32). In addition, modalities like accelerated partial breast irradiation and extreme breast hypofractionation are currently under investigation (31).

RT is also combined with systemic treatment. Hormonal therapy under the form of tamoxifen or aromatase inhibitors can be administered to oestrogen positive-receptor breast cancer patients. Chemotherapy, preferably not given concomitantly with RT to avoid toxicity, is mostly a combination of antracyclines and taxanes (33). The targeted agent trastuzumab has been shown to improve survival in patients with HER-2 positive tumours (34).

Management options for prostate cancer are more diverse. They include radical prostatectomy, RT (external beam or brachy), and watchful waiting or closely monitoring the cancer in slowly growing or low graded prostate cancer (35). Radical prostatectomy and RT show a similar level of effectiveness (36).

Currently, most men who receive external beam RT are treated with conventionally fractionated treatment regimens to a total dose of 74-80 Gy.

Such dose escalation has shown to improve biochemical control over standard-dose RT of 64-70 Gy (37). Moreover, hypofractionated regimens (2.1-3.5 Gy) are tested in clinical trials but there is no clear evidence that those schedules improve outcomes or result in lower toxicity when compared with conventionally fractionated regimens (38). Extreme hypofractionation and high-dose rate brachytherapy are alternative approaches and are currently under investigation (37, 39, 40). Hormone therapy like LHRH-analogues or anti-androgens are often used concomitantly with RT in prostate cancer patients.

In this PhD research, breast cancer patients are treated with the standard fractionation schedule (50 Gy in 25 fractions) or with moderate hypofractionation of 40 Gy in 15 fractions. Prostate cancer patients are treated either with radical RT with three different dose levels (74 Gy in 36 fractions, 76 Gy in 37 fractions or 80 Gy in 38 fractions), or with postoperative RT after radical prostatectomy. The prostatic bed received 74 Gy in 36 fractions in the adjuvant setting and 76 Gy in 37 fractions in the salvage setting.

2 Radiation-induced side effects are inevitable

Many patients receiving curative radiotherapy will experience toxicity due to the unavoidable irradiation of surrounding healthy tissue.

The first section of this chapter describes radiation-induced toxicity in general and the specific effects in breast and prostate cancer patients. It also gives an overview of the available scoring systems to assess toxicity. The pathogenesis is explained in more detail in the second section of this chapter and the third section deals with the factors influencing the development of normal tissue toxicity. These factors can be implemented in models that predict an individual's probability for developing radiation-induced side effects.

2.1 Radiation-induced normal tissue toxicity

Normal tissue is inevitably included in the irradiated target volume to ensure coverage of the microscopic tumour burden or to anticipate upon tumour and organ movement between fractions (8, 41). The tolerance of these normal tissues to radiation dictates the dose that is prescribed which is limited by late toxicity. Typically, RT schedules are designed to ensure that the risk of severe adverse effects does not exceed 5-10%. This basically means that the dose is submaximal in the majority of the patients (7).

Dose-response relationships for normal tissues are suggested to have a threshold at low doses and saturate at high doses (Figure 2.1). There is evidence that normal tissue dose-response relationships are steep, which means that small changes in dose results in relatively large differences in toxicity (42). Normal tissue complication probability (NTCP) models have been introduced to predict the probability of a defined undesirable effect on the patient as a function of dose or biologically equivalent dose and volume. Curves for normal tissue complications are less well-defined than tumour control probability curves (TCP). They are steeper, reflecting less heterogeneity in the biology of normal tissues (8).

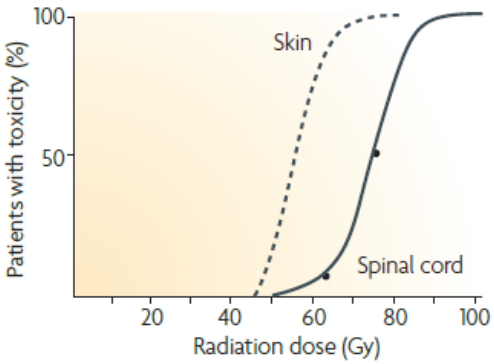

Figure 2.1: Cumulative frequency dose-response curves for skin telangiectasia and for spinal cord necrosis. *Adapted from Barnett et al*. (7).

Some tissues are thought to be functionally organised in series. The failure of a critical part of the tissue leads to a complication and largely depends on the maximum dose delivered to the organ. This is the case in nerves, gastrointestinal (GI) tract and bronchi (43). Recently, breast tissue is also shown to behave as a serial organ (44). In parallel organs, regions of an organ can be damaged without impairing global organ function; there is a 'functional reserve' which allows a certain volume fraction to lose function before a clinical unacceptable endpoint is reached. The response of such organs is dependent on the volume of the organ affected, for example lung, liver or kidney (43).

Depending on the time of symptom appearance, radiation toxicity is commonly classified as acute, consequential or late effects (Figure 2.2). Acute toxicity is observed during or within weeks after completion of RT and is usually reversible. It occurs in rapidly proliferating tissues as a result of cell death, such as in epithelial surfaces of the skin or the mucosa of the alimentary tract. Acute effects are generally manageable and transient due to proliferation and repopulation of surviving stem cells (41). Late side effects are progressive and manifest six months to many years after treatment in tissues with a slow turnover of cells. They include radiation-induced fibrosis, atrophy, vascular damage, neural damage and a range of endocrine and growth-related effects (Figure 2.2) (45). Severe late toxicity impacts negatively on the quality of life and can, in extreme cases, be life-threatening. The long-time course for their development prevents titration of dose against toxicity in individual patients. Acute reactions that fail to heal completely and persist into the late period are

consequential late effects. They are related to the severity of acute reactions (14, 41).

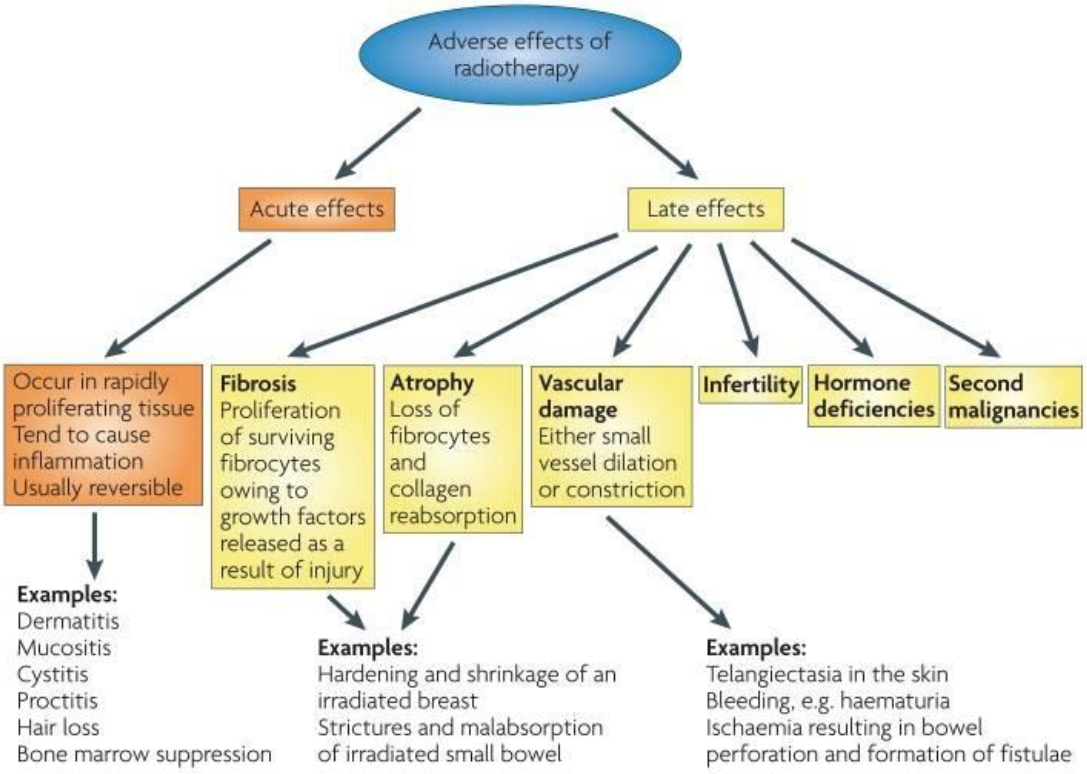

Figure 2.2: The toxicity of RT. *From Barnett et al.* (7).

In breast cancer patients, RT has a direct effect on the skin; other organs like lung, heart and coronary arteries are at risk as well. When the node region is irradiated, the shoulder, brachial plexus and axillary lymphatic are also at risk for potential injury. Acute skin reactions such as erythema, dry desquamation, hyperpigmentation and moist desquamation and the symptom of fatigue dominate the early toxicity profile. Late toxicity can be divided into two groups: the more common effects on the cosmetic appearance of the breast such as persistent breast oedema, hyperpigmentation, atrophy, telangiectasia and fibrosis, and the uncommon permanent injury to other organs such as brachial plexopathy, radiation pneumonitis, cardiac morbidity or secondary malignancy (46).

Male pelvic irradiation injury can occur in GI, genitourinary (GU) and reproductive organs. Radiation can cause functional effects in organs including the small bowel, rectum, anus, bone and bone marrow, bladder, urethra, ureter,

testicles and sexual organs. The most commonly recorded symptoms are: abdominal cramps, diarrhoea, mucus loss, rectal bleeding, faecal incontinence and urgency at the GI system and, dysuria, nocturia, pollakiuria, haematuria, urgency and incontinence at the GU system. Long-term symptoms can be seen at variable intervals following radiation (46).

2.1.1 Assessment of normal-tissue effects

Several classification systems are used to record normal-tissue reactions. The most widely used scoring systems in radiation oncology are RTOG/EORTC (Radiation Therapy Oncology Group/ European Organization for Research and Treatment of Cancer) (47), LENT/SOMA (Late Effects Normal Tissues: Subjective, Objective, Management and Analytic) (48) and the comprehensive dictionary for recording and grading side effects, the CTCAE (Common Terminology Criteria for Adverse Effects) (49). The most recent developed system is CTCAEv.4.0 (50). At the Ghent University Hospital, an in-house developed toxicity score is used which is based on RTOG, CTCAEv3.0 and LENT/SOMA toxicity scoring systems (51, 52). Toxicity is graded according to severity on a scale of none, mild, moderate or severe, with some as either none or yes. This assortment of diverse scoring systems leads however to the assessment of multiple and different endpoints which hampers comparisons across studies and pooling of data.

2.2 Pathogenesis of normal tissue side effects

The development of radiation-induced tissue injury begins with an ionizing event that results in direct damage to DNA but also initiates a cascade of events on the cellular and molecular level that is similar to the wound healing process as shown by Figure 2.3.

Until the 1990s, the pathogenesis of normal tissue effects was described through the ‘target-cell theory’, which states that radiation-induced toxicity is a direct consequence of killing parenchymal and vascular cells (53). Possible mechanisms for radiation-induced cellular lethality are apoptosis, which can be directly activated by the DDR, and, mitotic death in which cells fail to complete

mitosis correctly (8). The timing of developing the symptomatic injury depends on the proliferation kinetics of the irradiated tissue; chronic injury is caused by a delayed reduction in the number of target cells. Subsequent healing is based on the proliferation of the surviving stem cells within irradiated volume or by migration of them from non-irradiated tissue (8). This hypothesis is, however, inadequate to explain the pathogenesis of late effects but remains useful to explain the effects of the early responding tissue (45).

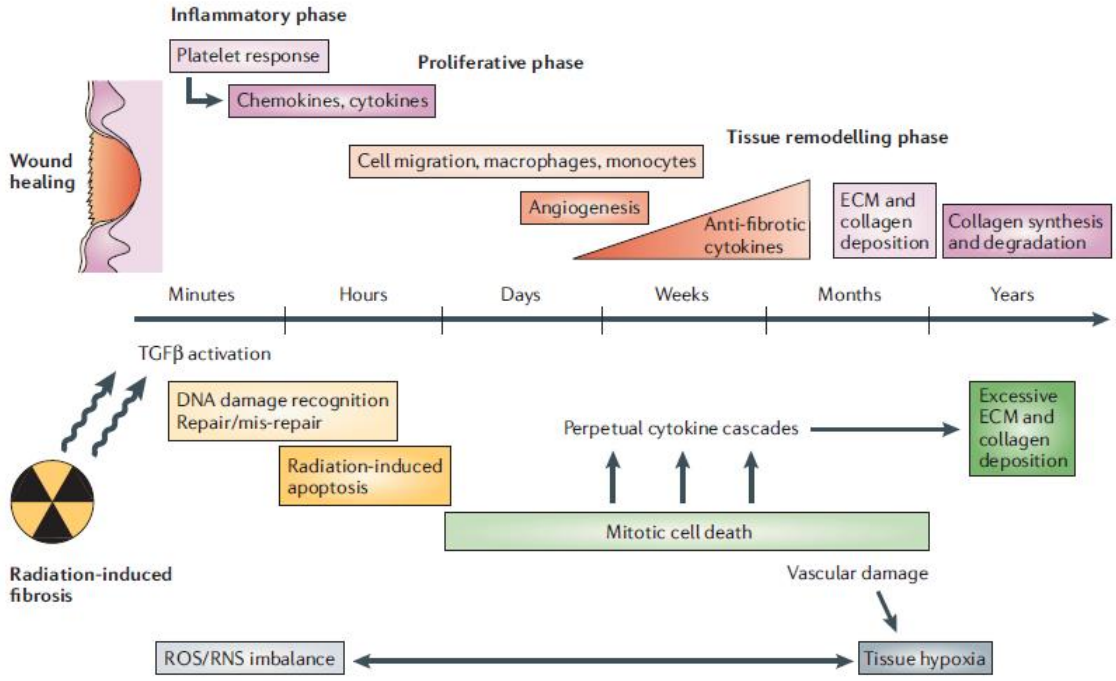

Figure 2.3: Radiation-induced fibrosis (below) has features in common with the normal wound healing response (above). *From Bentzen et al* (45).The final tissue remodeling phase in normal wound healing, which becomes less active with time, is deregulated in radiation fibrosis. Instead of resolving, a progressive increase in fibrosis occurs over many months or even years (54).

Since the mid-1990s, it became clear that radiation-induced injury is an orchestrated, active biological response which is initiated at the time of irradiation and persists until the late effects manifest clinically (55). Radiation injury includes damage to the stromal (fibrosis), the parenchymal (atrophy) and vascular compartments where cytokines play an important role, as shown in Figure 2.4. An immediate early gene response is induced by radiation with a rapid increase of the expression of pro-inflammatory cytokines such as TNFα,

IL-1, IL-6, IFN, VEGF and EGFR. They drive the formation of inflammatory lesions with changes in the local vasculature allowing infiltration of neutrophils, macrophages and lymphocytes (56). Anti-inflammatory cytokines like TGFβ, IL-10 and IL-4, restore the integrity and homeostasis through promoting angiogenesis and tissue regeneration or replacement by fibrosis with deposition of extracellular material. The involvement and impact of any cytokine will vary with cell type or tissue and with time (57).

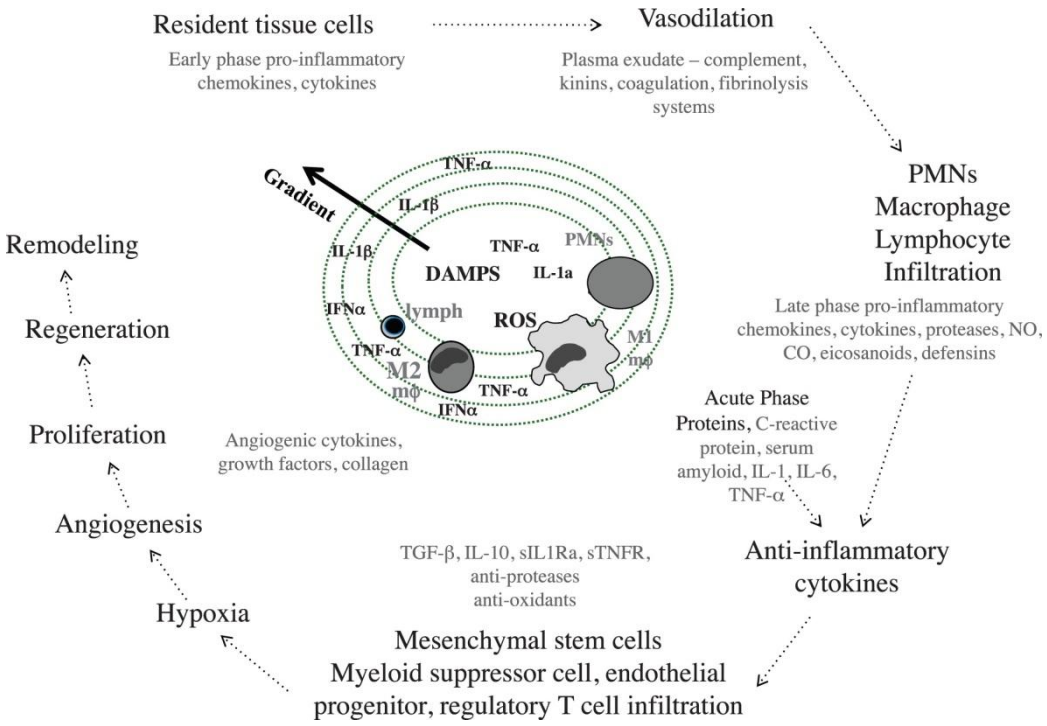

Figure 2.4: Cytokine network underlying the development of normal tissue response following radiation exposure. From *Schaue et al.* (56). DAMPs: damage-associated molecular patterns; PMN: polymorphonuclear leukocytes; Mϕ: macrophage; M1: killer macrophages; M2: repair macrophages.

ROS formation, directly after irradiation, is followed by downstream activation of metabolic sources of pro-oxidant production. This includes mitochondria, nitric oxide synthases and oxidoreductase enzymes, such as the NADPH oxidases and may be secondarily linked to DNA damage response pathways (56, 58). Changes in the balance between free radicals and antioxidants (59-61) may participate in radiation injury by the activation of redox-sensitive signaling pathways. Some radiation-inducible redox-sensitive transcription factors are NFκB, Egf1 and AP-1, involved in the cytokine production, ATM, the core

protein of the DDR and HIF-1, a major contributor to angiogenic cytokine production (62-64).

Pro-inflammatory cytokines generate cellular ROS and require ROS for signal pathway activation (65, 66). In contrast, anti-inflammatory cytokines tend to inhibit ROS/RNS mediated effects and display anti-oxidant properties (67, 68). This balance of pro- and anti-inflammatory cytokines is critical in determining the outcome; it may shift back and forth for a long time after radiation exposure and it appears that the redox status of the cell is the turning point (56).

2.3 Factors influencing the development of radiation-induced toxicity

A substantial degree of variability among patients in the response to a standard course of RT has been observed for a long time. A variety of factors influence the likelihood of a patient developing toxicity; these can broadly be related to dosimetry, adjuvant cancer treatments and factors inherent to the patient.

Dosimetry-related factors include total dose, dose per fraction, irradiated volume and dose inhomogeneity (7). Late effects tend to be more sensitive to changes in fraction size, and are less sensitive to changes in overall treatment time than early responses. In this respect, an increase of fraction size must be accompanied with a reduction of the total dose (29). The volume of normal tissues exposed to high radiation doses will also affect development of toxicity and depends on the organizational structure (parallel vs. serial) and the radiosensitivity of the critical components (functional subunits) (69, 70).

Interaction with other treatment modalities, typically surgery and/or systemic therapy such as chemotherapy or hormone therapy, may influence the pattern of toxicity after RT (71, 72).

Age, body weight, pre-existing symptoms, use of cigarettes are all factors that can possibly affect the development of normal tissue toxicity (72-75). In addition, patients with certain underlying conditions or diseases may be more susceptible for the development of adverse events. Case reports suggest that patients with co-morbid conditions like collagen vascular diseases, diabetes or

hypertension and inflammatory bowel disease are at greater risk for developing normal tissue toxicities. However, retrospective studies have generally not found substantial increases in the risk for toxicity (76-79).

2.3.1 Factors associated with toxicity in breast and prostate cancer patients

Large breasts and dose inhomogeneity are established risk factors for acute and late skin toxicity after whole-breast RT (80, 81). Additionally, post-operative infection and boost to the tumour bed have previously been shown to be associated with the development of late skin toxicity (80, 82). A recent study suggests, however, that the development of breast fibrosis depends more on the maximum RT dose instead of the effect of treated breast volume (44). Cardiac disease after breast cancer RT, especially present in patients with left-treated breasts, is found to be associated with the mean dose to the heart, with a 7.4% increase rate in major coronary event per Gy (83). Women with pre-existing cardiac risk factors are at higher absolute risk than other women (83).

Established risk factors for acute and late rectal radiation-induced toxicities for prostate cancer include prior abdominal surgery, concomitant androgen deprivation and previous co-morbid conditions as diabetes mellitus, haemorrhoids, or inflammatory bowel disease (78, 84-86). Development of acute rectal toxicity is also associated with an increased risk of developing late rectal complications (87-90). The volume of rectal tissue exposed to high doses of RT has been shown to be associated with the development of rectal toxicity which is consistent with the serial behaviour of the GI tract (8, 89, 91).

Factors associated with GU toxicity are pre-treatment GU complaints, prior transurethral resection of the prostate (TURP) and the presence of acute GU toxicity (84, 86, 92-94). Evaluation of the dosimetry and the relationship with GU complications is difficult due to highly variable bladder filling (95) and differences in bladder contouring: for some studies the bladder is a solid organ, containing the bladder wall and its entire contents (51, 93), whereas others contour the bladder wall alone (96-98). Prostate cancer patients treated at GUH undergo daily medical imaging to verify bladder filling and the bladder is

contoured as a solid organ. Acute symptoms are suggested to be related to swelling and inflammation of the prostatic urethra (99); late toxicity is possibly related to damage to the bladder neck and dose to the trigone region (100). Recently, image-guided RT using implanted prostatic fiducial markers showed to reduce the dose to this region together with the levels of urinary toxicity (94). Late bladder toxicity typically manifests many years after rectal toxicity with increasing rates over time (89).

Erectile dysfunction (ED) is a relatively common complication in prostate cancer treatment and the occurrence of spontaneous erections before treatment is the best indicator for the preservation of erections sufficient for intercourse (101). Other factors associated with decline in erectile function are higher age, worsening co-morbid conditions and androgen deprivation therapy (102, 103). Moreover, the use of magnetic resonance imaging permits, by vessel-sparing RT, a reduction in the dose delivered to vascular structures critical for erectile function (104). At the start of this PhD research, erectile function prior to RT was not standard recorded and was therefore not analysed.

2.3.2 Different approaches for prediction modelling

The parameters mentioned above can be used to develop predictive models which would enable us to calculate the individual's probability to develop radiation-induced side effects in order to personalize RT treatment. Their predictive value can be evaluated applying several approaches. The most commonly used methods for predictor selection are logistic regression for binary outcome and Cox regression for time-to-event outcome. A brief overview of the clinically usable models with applied methodology is given.

Predictive models are created in prostate cancer patients with the focus on rectal toxicity symptoms such as rectal bleeding and faecal incontinence. Within the AIROPROS 0102 trial, a number of prediction models, displayed as nomograms, were constructed that deal with clinical and dosimetric factors (105-107). The nomograms were developed based on forward and backward multivariate logistic regression analysis, incorporating covariates associated with univariate analysis ($p \leq 0.20$), to yield an individualised estimation of the

toxicity risk. The performance of the models was quantified by AUC and the sensitivity and specificity. For some models, calibration was assessed and bootstrapping was applied to correct for overfit (106). The authors were able to develop nomograms for the different rectal symptoms (105-107). The most recent model that predicts mean faecal incontinence is given as example (107), see Figure 2.5.

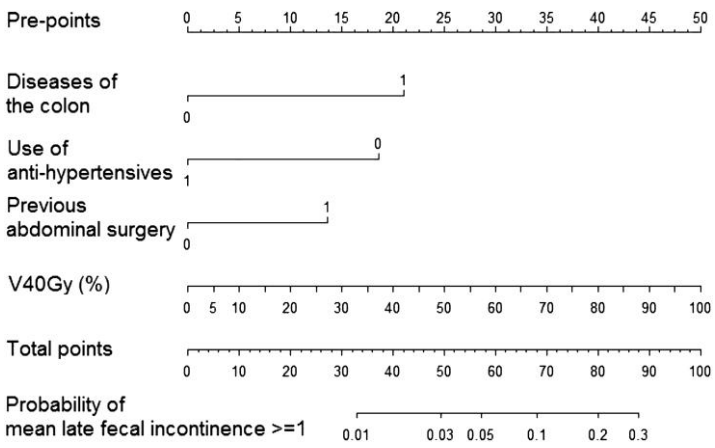

Figure 2.5: Nomogram for late mean faecal incontinence (longitudinal definition) according to Fiorino *et al (2012)* (107). V40 Gy (%): the percentage of the rectum receiving 40 Gy or more. This model has an AUC of 0.73 with sensitivity = 66.7% and specificity = 74.0%. Use of the nomogram: each predictor represents a number of points, achieved by drawing a straight upwards line to the 'Pre-points' axis. Subsequently, the points for each predictor are summed and this sum is located on the 'Total points' axis. Then, draw a line straight downwards to find the patients probability of developing late faecal incontinence.

The EORTC trial could demonstrate that an additional RT-boost in breast cancer patients reduces the risk of local recurrence but increases the rate of development of fibrosis at 10 years of follow-up (30). For guiding clinicians in their decision of delivering a boost, the authors proposed nomograms to predict the risk of moderate or severe fibrosis at 10 years. Therefore, the dataset was split in a model development dataset and in a validation dataset. Models were developed applying multivariate Cox analysis including only the factors univariately significant at the 0.20 level, via backward elimination at the 0.10 statistical significance level. Furthermore, bootstrap resampling for model calibration and for internal validation, provided a bias-corrected estimate of the

c-index. Sensitivity and specificity of the models were not calculated but according to the total points derived from the nomogram, patients were classified in subgroups, as shown in Figure 6.2.

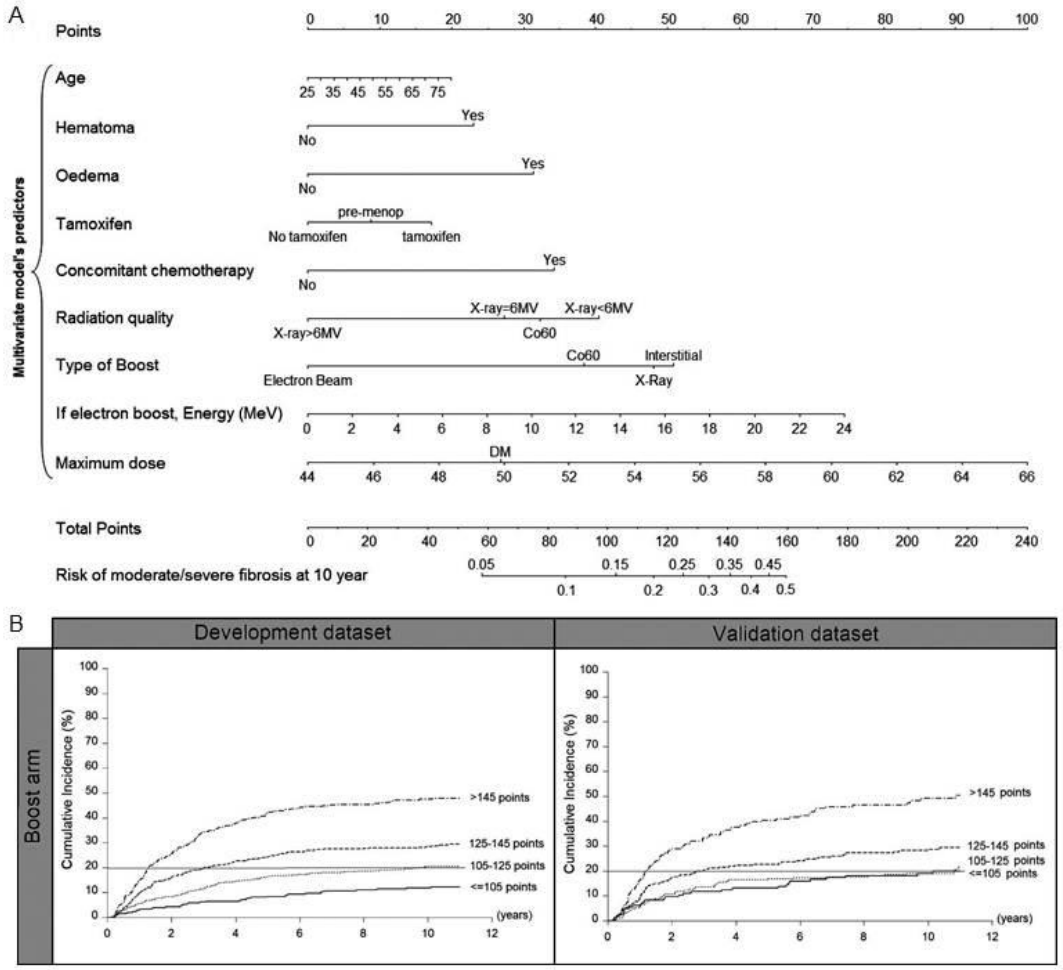

Figure 2.6: Prediction of the 10-year risk of moderate or severe fibrosis when treated with a boost according to *Collette et al (2008)* (30). (A) Nomogram; the c-index of the model is 0.66 in the development set and 0.62 in the validation set. (B) The cumulative incidence according to the total prognostic score derived from the nomograms. The horizontal line indicates 20% cumulative incidence of moderate to severe fibrosis. The model is able to discriminate the patients with >125 points on the nomogram (high-risk subgroup), the patients that show a low risk of fibrosis (<125 points) are not well discriminated (30).

Another methodology was applied for the prediction of esophagitis after RT in non-small cell lung cancer patients (108). Again, patients were divided into a training set and a validation set. Multivariate logistic regression models were

generated on the training set by the use of forward-stepwise selection procedures. Factors included in the model needed to meet three criteria: they should be statistically significant, they should increase the discriminating ability of the model and the model should be well-calibrated. Then, three risk groups (low, intermediate, high) were created by recursive partitioning analysis (RPA) including the significant predictors from multivariate analysis. The performance of the model, under the form of c-statistic, and the RPA were evaluated using the validation set, see Figure 2.7.

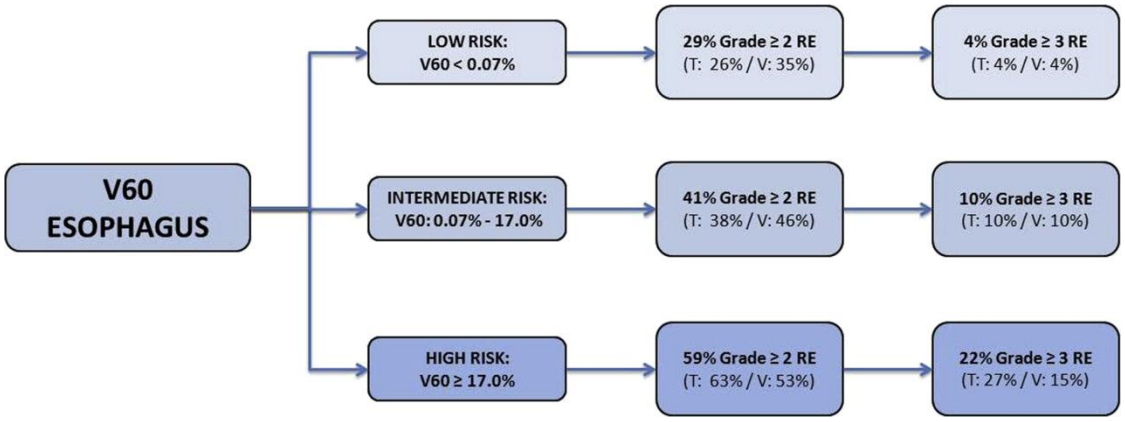

Figure 2.7: Recursive partitioning analysis (RPA) for radiation esophagitis (RE) grade ≥2 and grade ≥3 according *to Palma et al. (2013)*. T: Training set; V: Validation set. The percentage of the volume of the oesophagus receiving 60 Gy (V60) is the only factor with good discrimination score (*c* > 0.60). The c-statistic of the predictive model for grade ≥2 was 0.58 and for grade ≥3 was 0.66 (108).

In this PhD, associations between different types of parameters (clinical, treatment and dosimetric) and the endpoint of interest were investigated by applying logistic regression. Prediction models were developed applying the least absolute shrinkage and selection operator or Lasso method. Additionally, we add genetic data under the form of genetic polymorphisms, as it is suggested that they play an important role in influencing the susceptibility for development of radiation injury and, thus, may enrich the predictive performance of models (109, 110). This genetic part is discussed in more detail in the following chapter.

3 Radiosensitivity is influenced by genetic factors

The study of genetic variation on radiation response is called radiogenomics; it focuses on uncovering the underlying genetic causes of individual variation in sensitivity to radiation. The most common source of variation between humans are single nucleotide polymorphisms or SNPs.

In this chapter, we start by describing the suggested allelic architecture of radiosensitivity and explain the unique features of SNPs. Furthermore, different approaches to select polymorphisms and genotyping assays are discussed.

3.1 Radiosensitivity heritability and allelic architecture

Radiosensitivity can result in different observations. This can be (i) in cellular context (measured in the laboratory using a clonogenic, chromosome damage, DNA damage or apoptosis assay) or (ii) in clinical setting, described by differences in toxicity after RT with some tissues more radiosensitive than others, or (iii) susceptibility to radiation-induced cancer (111, 112). The high heritability of enhanced chromosomal and cellular radiosensitivity has been shown by many studies with values ranging from 58% to 78% (113-117). In contrast, data to assess the heritability of clinical radiosensitivity based upon family history are not available, but, is perhaps somewhat lower than for chromosomal and cellular radiosensitivity (117). One study comparing intra- and interpatient variability, estimated that about 80% of the total variation in the development of skin telangiectasia was attributed to patient-related factors, such as genetics and physiology (118). Cellular and clinical radiosensitivity follow an approximately Gaussian distribution as is the case for height, which has a strong heritability component (111, 119, 120).

Radiosensitivity is considered to be a complex polygenic trait (7, 110). It is previously proposed that the allelic architecture of this trait includes a spectrum of sequence alterations ranging from rare highly penetrant alterations to common alterations with small relative risks (1.1 – 1.5), as shown in Figure 3.1 (111, 120, 121). Earlier studies identified rare homozygous mutations resulting in large effects on clinical radiosensitivity with a high relative risk (>10) (121), for example in patients with severe syndromes such as ataxia telangiectasia,

Blooms syndrome and Nijmegen breakage syndrome (122). However, it is unknown whether high-risk radiosensitivity alleles exist outside patients with genetic Mendelian syndromes.

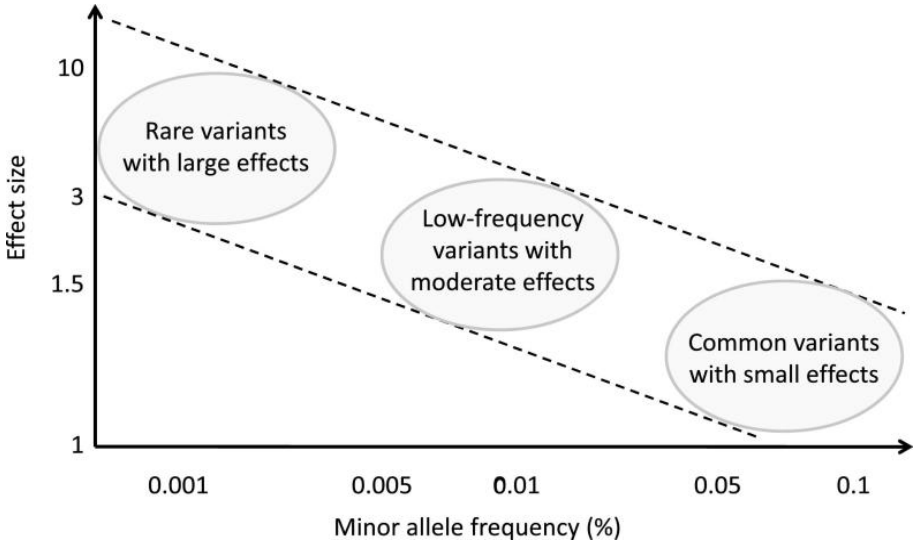

Figure 3.1: Allelic architecture of radiosensitivity. From West et al. (111). Allelic architecture refers to the number, type, effect size and frequency of susceptibility variants in relation to a specific trait. Rare high-risk alleles are typically discovered by family studies and linkage analysis, rare intermediate-risk alleles by re-sequencing in case–control studies and common low risk alleles by candidate gene SNP studies and GWAS (123).

3.2 Single Nucleotide Polymorphisms

A SNP is a single base substitution and may occur every 100 to 300 bases among the 3 billion base-pair genome; they are generally described as allelic variants that occur in the population with a frequency of >1% and are typically characterized as low-penetrance variants. This is in contrast to mutations, which are usually rare, but associated with high penetrance. The allele frequencies may differ between ethnic groups or in different geographical regions, usually as a result of genetic drift or natural selection (111, 124). The human genome is organised into haplotype blocks separated by recombination hot spots. Within each block, alleles of multiple SNPs are inherited together as a single unit; they are in linkage disequilibrium (LD).

In genetic studies, SNPs can serve as genomic markers for their association with complex diseases and traits and for predicting disease susceptibility and drug response, contributing to personalised medicine. They are also used in studies of human migration and evolution (124). Depending on their position, genetic variants can have an impact on function; SNPs in coding regions can have an effect at the protein level, with altered protein stability or catalytic activity. SNPs in regulatory regions of the genome can affect gene expression, and those in non-coding sequences can influence splicing, RNA cleavage, stability or export (125). Identification of the variants causing the disease or trait may bring more insights into disease aetiology. However, most SNPs do not cause disease, they rather represent a physical location to pinpoint the disease on the human genome map. Hence, most SNPs that are statistically associated with the disease from genetic association studies, are likely in LD with the true causative alleles. The next step to investigate the causal role are additional functional and mechanistic studies (126). Additionally, SNPs can be used to determine the likelihood that an individual will develop a disease which can serve as the basis for a prediction assay. These assays might enable us to permit an earlier intervention to prevent development of the disease of interest. For example, in pharmacogenomics, the FDA recommendation of the dosage of warfarin, given to prevent systemic embolism, is based on a SNP profile in the *CYP2C9* gene and the *VKORC1* gene (127).

3.2.1 Different genetic inheritance models

Bi-allelic polymorphisms can occur under the form of three genotypes; wild type and homozygous variant genotype when two copies of the, respectively, major or minor alleles are present and the heterozygous genotype when one of each allele is present. To assess the genetic effect of each genotype, different genetic inheritance models can be defined. The most commonly used genetic models are the recessive, dominant, additive and codominant models. In the recessive model, patients carrying the homozygous variant genotype are considered to be different from the group of patients with the wild type and heterozygous genotype. In the dominant model, patients containing at least one minor allele are grouped and compared with patients carrying the wild type. In

the additive model, it is assumed that the effect of the heterozygous genotype is in between the effects of both homozygous genotypes in a dose-dependent manner. A comparison of the three genotypes is performed in the codominant model, with usually the wild type as a reference compared with the heterozygous and homozygous variant genotype separately. A special case is the over-dominant model where the risk conferred by the heterozygous genotype falls outside both homozygous risks (128). This can be tested by comparing the heterozygous genotypes to all homozygous genotypes.

In the present PhD research, the dominant, recessive and codominant models are tested for association of SNPs with the phenotype. Testing multiple hypotheses at the conventional significance level of 0.05 may lead to inflated false-positive results and requires multiple-testing correction. In this work, multiple testing is performed by the Benjamini-Hochberg procedure.

3.3 How are SNPs selected?

Genetic studies can be performed using different approaches, the candidate gene approach and the genome-wide approach.

In candidate gene association studies, there is *a prior* knowledge of the genes based on the pathogenesis of the phenotype. The idea is that maximising the biological plausibility would increase the chance of success. This approach, however, is limited by its reliance on the existing knowledge to identify candidate genes (126) but can be broadened by selecting genes that participate in the entire pathway of interest. In this way, the variability in that pathway is explored without restricting the analysis to a single gene (129). Subsequently, different criteria can be applied to perform the SNP selection. Some criteria are listed: the validation status of the SNP, to increase the certainty of selecting genuine polymorphic variants, the position of the variant in the different gene regions (3’ or 5’ near gene or untranslated region (UTR), introns or exons), the functionality of the polymorphism, which can already be extensively studied or examined *in silico* by different web-based tools, like SNPs3D (130). SNPs can also be selected within evolutionary conserved sequences within different species which are likely of functional importance (129).

Genome-wide association studies (GWASs) are primarily designed to provide an unbiased survey of the effects of common genetic variants (131). LD is exploited to tag the most common haplotypes which are extracted from the International HapMap Project data set. However, despite the density of the SNPs on the arrays, it covers only a fraction of the total variation in the genome (131). The drawback of this method is the large dataset necessary to identify SNPs related to the phenotype at a certain confidence level taking into account the multiple testing correction.

3.4 SNP genotyping methods

There is a diversity of high-fidelity SNP genotyping techniques, with different strategies; some of them are preceded by a polymerase chain reaction (PCR). The restriction fragment length polymorphism (RFLP) technique involves restriction endonucleases and their affinity to bind and cleave unique and specific restriction sites. The single base extension reaction (SnapShot®) is based on primer extension incorporating a single fluorescently labelled dideoxynucleotide by DNA polymerase and TaqMan Assays rely on the 5'-3' nuclease activity of the Taq polymerase and fluorophore-based detection (132). The High Resolution Melting Analysis (HRMA) technique measures the differential fluorescence of double-strand specific DNA intercalating dyes while the DNA amplicon is melted. SNP platforms are based on direct hybridisation to allele-specific oligonucleotides or on the combination of primer extension and ligation or by aggregating both techniques for example in the Affymetrix GeneChip, Illumina GoldenGate and Infinium Beadchips arrays. Other arrays using mass spectrometry (iPlex), denaturing high performance liquid chromatography or quantitative-PCR are also available but are not further discussed.

The predominantly used genotyping techniques in this PhD research are PCR-RFLP and HRMA. Additionally, a custom Illumina GoldenGate platform containing 384 SNPs was designed by applying the extended candidate gene approach. After a comprehensive literature search, genes within an entire pathway were selected. The pathways are involved in the early response to IR,

DNA checkpoint control and repair of DNA damage upon exposure to IR, ROS metabolism and hormonal metabolism. SNPs in those genes were selected either based on evolutionary conservation or on evidence for functionality. They were supplemented by SNPs previously shown to be associated with cancer predisposition in GWA case-control studies.

4 Aim of the research

A substantial degree of variability exists among patients in their response to RT (118). Although long-term severe, sometimes life-threatening, side effects are present in only a minority of the patients, more patients experience moderate toxicity which can seriously impair patients' quality of life. Examples are poor cosmetic outcome following breast irradiation or rectal and urinary complaints after prostate irradiation. Acute toxicity can cause pain and discomfort (81). In addition, there is growing clinical evidence that acute reactions are associated with the development of late toxicity; in breast cancer patients, telangiectasia seem to be late sequelae of moist desquamation and acute erythema seems to be a risk factor for poor cosmetic outcome (133, 134).

The first aim of this thesis was to investigate the influence of patient-, treatment-, dosimetric parameters and genetic variation on the risk of developing acute radiation-induced toxicity in breast and prostate cancer patients.

The second aim was to develop integrated predictive risk models for late toxicity in prostate cancer patients that allows a patient individualized estimation of its pre-treatment risk. Such models are clinical applicably to guide the allocation of patients to treatment groups based on their probability of severe RT-induced toxicity and simultaneously improve the therapeutic ratio.

5 Outline of the research

External beam RT is a standard treatment modality for localized and locally advanced prostate cancer. More recent technologies such as IMRT allow for the delivery of high doses to the prostate, together with a better sparing of the rectum which results in a low rate of severe GI complications. Due to the full inclusion of the bladder neck and the vesicourethral anastomosis in the high-dose region, the risk of developing severe GU toxicity remains, however, unchanged (88). In this PhD research, we have chosen to break down the overall toxicity to specific symptoms that are likely to reflect a specific radiation pathophysiology. As GI toxicity is very rare in our study cohort of prostate cancer patients, the analysis was performed for GU symptoms only: dysuria, incontinence, haematuria, urgency, nocturia and increased daily frequency.

Acute RT-induced nocturia is in our study population the predominant acute toxicity endpoint in prostate cancer patients treated with primary or postoperative high-dose IMRT. A number of clinical and dosimetric parameters, together with five SNPs in the *TGFB1* gene, capturing all common variants in the 5' region of the gene (135), were tested for association with the endpoint. The polymorphic sites were examined by PCR-RFLP and HRMA. The results of this study are presented in a first paper (chapter 6) entitled ACUTE RADIATION-INDUCED NOCTURIA IN PROSTATE CANCER PATIENTS IS ASSOCIATED WITH PRETREATMENT SYMPTOMS, RADICAL PROSTATECTOMY, AND GENETIC MARKERS IN THE TGFB1 GENE.

Late radiation-induced haematuria and nocturia are the most frequently observed late GU symptoms. Models for prediction of these endpoints are developed containing clinical, dosimetric and genetic data. The genetic data were obtained by a custom-designed Illumina GoldenGate platform containing 384 genetic variations. To deal with missing data and the high number of predictors, the EMLasso, an in-house developed and validated method, was applied. The results of this study are presented in a second paper (chapter 7) entitled INTEGRATED MODELS FOR THE PREDICTION OF LATE GENITOURINARY COMPLAINTS AFTER HIGH-DOSE INTENSITY-MODULATED RADIOTHERAPY FOR PROSTATE CANCER: MAKING INFORMED DECISIONS.

The current breast cancer cohort is treated with adjuvant RT after breast-conserving surgery. Normofractionated (25x2 Gy) or hypofractionated (15x2.67 Gy) IMRT in prone or supine position is prescribed. Systemic therapies like chemotherapy, hormone therapy and trastuzumab are administered when indicated. The endpoints of interest are the development of acute dermatitis and moist desquamation. In this PhD research, treatment- and patient-related factors such as bra size cup, body mass index (BMI) and smoking status, supplemented with eight SNPs are investigated for the association with the endpoints. Five of the eight SNPs were selected based on their putative effect on the expression levels of radiation-responsive genes (136). The other SNPs were chosen based on their previous association with toxicity induced by RT or methylating agents (137-140). The polymorphic sites were examined by PCR-RFLP, HRMA and single base extension technique. The results of this study are represented in the third paper (chapter 8) entitled FACTORS MODIFYING THE RISK FOR DEVELOPING ACUTE SKIN TOXICITY AFTER WHOLE-BREAST INTENSITY MODULATED RADIOTHERAPY.

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Part II

Identification of factors modifying or predicting the risk to develop radiation-induced toxicity

6 Paper I

Acute Radiation-Induced Nocturia in Prostate Cancer Patients Is Associated With Pretreatment Symptoms, Radical Prostatectomy, and Genetic Markers in the *TGFB1* Gene

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SUMMARY

Severe nocturia occurs in approximately 25% of the prostate cancer patients as a side effect of radiation therapy, and thus sleep disturbance diminishes patients' quality of life. This study of 322 patients demonstrates that clinical factors such as prior radical prostatectomy and the presence of mild pretreatment symptoms, as well as genetic markers in the *TGFB1* gene, contribute to the development of severe radiation-induced nocturia.

ABSTRACT

Purpose: After radiation therapy for prostate cancer, approximately 50% of the patients experience acute genitourinary symptoms, mostly nocturia. This may be highly bothersome with a major impact on the patient's quality of life. In the past, nocturia is seldom reported as a single, physiologically distinct endpoint, and little is known about its aetiology. It is assumed that in addition to dose-volume parameters and patient- and therapy-related factors, a genetic component contributes to the development of radiation-induced damage. In this study, we investigated the association among dosimetric, clinical, and *TGFB1* polymorphisms and the development of acute radiation-induced nocturia in prostate cancer patients.

Methods and Materials: Data were available for 322 prostate cancer patients treated with primary or postoperative intensity modulated radiation therapy (IMRT). Five genetic markers in the *TGFB1* gene (-800 G>A, -509 C>T, codon 10 T>C, codon 25 G>C, g.10780 T>G), and a high number of clinical and dosimetric parameters were considered. Toxicity was scored using an symptom scale developed in-house.

Results: Radical prostatectomy (P<.001) and the presence of pretreatment nocturia (P<.001) are significantly associated with the occurrence of radiation-induced acute toxicity. The -509 CT/TT (P=.010) and codon 10 TC/CC (P=.005) genotypes are significantly associated with an increased risk for radiation-induced acute nocturia.

Conclusions: Radical prostatectomy, the presence of pretreatment nocturia symptoms, and the variant alleles of *TGFB1* -509 C>T and codon 10 T>C are identified as factors involved in the development of acute radiation-induced nocturia. These findings may contribute to the research on prediction of late nocturia after IMRT for prostate cancer. © 2013 Elsevier Inc.

INTRODUCTION

External beam radiation therapy (RT) is a standard treatment modality for localized and locally advanced prostate cancer (1). Modern technologies such as intensity modulated radiation therapy (IMRT) allow for the delivery of high doses to the prostate while lowering the dose to the neighboring organs at risk

(2, 3). This combination is of importance because a higher dose to the prostate improves local tumor and biochemical control (3). The use of modern radiation technology is needed to avoid excessive late toxicity with higher doses, as has been shown in randomized trials (4).

However, even with IMRT, up to 50% of the patients treated with doses >70 Gy experience bladder or bowel symptoms during treatment – so-called acute toxicity (5). Clinical variables such as any pretreatment genitourinary (GU) symptoms, androgen suppression, and prior transurethral resection of the prostate (TURP) appeared to be important prognostic factors for radiation-induced acute GU toxicity (2, 6). Although late toxicity is reported more frequently, acute toxicity has been found to be an independent predictor for late toxicity (7). Radiation-induced GU toxicity is frequently scored using the Radiation Therapy Oncology Group (RTOG) scoring scale (8). This grading method includes criteria such as increased urinary frequency, nocturia, dysuria, urgency, and haematuria.

A frequently occurring and under-reported GU symptom, occurring during or shortly after radiation therapy is nocturia (2, 3). Nocturia was recently found to be associated with a decreased quality of life and with an increased prevalence of depression because of more frequent nightly voids. Quality of life and well-being are already affected in patients with a nocturnal voiding frequency of ≥2 times (9). Nocturia is a storage problem of the bladder with a dynamic and irritative character (10) and is suggested to result from radiation-induced inflammation of the prostatic urethra (11). The majority of clinical studies do not evaluate nocturia as a specific endpoint or use grading systems other than RTOG to score nocturia (6, 12). In addition, many studies combine multiple symptoms, which are all suggested to differ in aetiology (10), into a single toxicity score (1, 6, 7).

Because extrinsic factors, such as RT planning and delivery, are better controlled today, factors intrinsic to the patient arise as potentially more important in the development of radiation-induced toxicity. Identification of genes that possess genetic markers associated with clinical radiosensitivity may lead to a better understanding of the molecular pathogeneses underlying normal tissue injury and may allow a more rational approach to prevent radiation toxicity (13).

The multifunctional cytokine transforming growth factor-β1 (TGFB1) triggers a wide diversity of radiation responses depending on the genetic makeup and environment of the target cell. It is considered a biomarker of inflammatory and fibrotic responses to RT and has also been shown to play a key role in the cellular response to radiation-induced DNA damage (14). The 5' region of the *TGFB1* gene is highly polymorphic and likely to have an impact on the pathogenesis of numerous diseases through altered TGFB1 expression (15). Several studies have claimed associations between polymorphisms in the *TGFB1* gene and acute or late adverse effects of RT in lung, prostate, breast, and gynecological cancer patients (13, 16, 17). Up to now, no studies have considered the relationship between *TGFB1* polymorphisms and radiation-induced nocturia in prostate cancer.

Because dosimetric and patient-related risk factors add variability in radiation toxicity outcome, it is necessary to take these factors into account when trying to link genotype with a clinical phenotype (13). Therefore, we examined the effects of dose parameters, clinical, and individual genetic variations in *TGFB1* to the development of radiation-induced nocturia during RT or within 3 months after RT in prostate cancer patients treated with high-dose IMRT.

MATERIALS AND METHODS

Patients

The study population consisted of 322 Caucasian men treated with IMRT as primary or postoperative treatment for prostate cancer at the Ghent University Hospital between 1999 and 2010. All patients had a follow-up of at least 3 months to be considered eligible for this study.

The dose was prescribed as the median dose to the planning target volume, and the maximal rectal dose (R) was used as hard constraint. Prescription doses of 74, 76, or 80 Gy were delivered in, respectively, 36 (74R72), 37 (76R74), or 38 (80R76) fractions with 18-MV photons of an Elekta linear accelerator (Crawley, United Kingdom) as described previously (2, 3, 18). Two hundred twenty-two patients were treated with primary IMRT, and 100 patients were treated with postoperative IMRT after radical prostatectomy (Table 1).

Table 1: Overview of prescription doses and RT-induced nocturia in primary and post-operative IMRT setting.

Treatment Prescription group		All (n=322)	No RT-induced nocturia (n=240)	RT-induced nocturia (n=82)
Primary IMRT (n = 222)				
	P74R72	13 (5.9)	7 (53.8)	6 (46.2)
	P76R74	24 (10.8)	21 (87.5)	3 (12.5)
	P80R76	185 (83.3)	122 (65.9)	63 (34.1)
Post-operative IMRT (n = 100)				
Adjuvant	A76R74	51 (51.0)	47 (92.2)	4 (7.8)
Salvage	S74R72	49 (49.0)	43 (87.8)	6 (12.2)

Abbreviations: RT = radiotherapy; IMRT = intensity-modulated radiation therapy; P = prescription; A = adjuvant; S = salvage; R = maximal rectal dose.
Data in parentheses are percentages.

To check the relationship between dosimetric parameters and acute nocturia, the following dose-volume parameters were investigated: the maximal bladder dose (B_{max} [Gy]), the median dose to the clinical target volume (CTV) as surrogate for urethral dose (CTV_{median} [Gy]), the maximal dose to the CTV (CTV_{max} [Gy]), and the CTV volume (CTV_{Vol} [cc]).

Androgen deprivation therapy, consisting of administration of a luteinizing hormone releasing hormone analogue, was prescribed for 194 patients.

A fixed questionnaire was used to register patients' medical and surgical history and pretreatment GU symptoms for each patient (3).

During treatment, patients were seen on a weekly basis and on the last treatment day. Afterward, follow-up was performed at 1 and at 3 months after treatment. Acute toxicity was recorded as the maximal score during radiation or within 3 months after the end of RT. In the present study, patients suffered from RT-induced GU haematuria, dysuria, urgency, nocturia, and increased frequency. However, because of the low incidence of the other symptoms, only RT-induced nocturia was included in the study. Grading was performed prospectively following the grading system proposed by De Meerleer et al (3). Pretreatment nocturia was taken into account to avoid overgrading. In brief, grade 1 nocturia was defined as 2-3 mictions overnight. Grade 2 nocturia was defined as 4-6 mictions overnight, a doubling of the pretreatment nocturia frequency, or the need for medication (tamsulosin or terazosin). Grade 3 nocturia was defined as >6 mictions overnight. For this study, RT-induced

nocturia was defined as an increase in toxicity according to grade 2 or 3 in the toxicity scale.

Genomic DNA was obtained from fresh blood using the Puregene genomic DNA purification kit (Gentra Systems, Minneapolis, MN). The study was approved by the local ethical committee and all study patients provided written informed consent.

Genotyping analysis

The single nucleotide polymorphisms (SNPs) -800 G>A, -509 C>T, codon 10 T>C, codon 25 G>C, and g.10780 T>G in the *TGFB1* gene were selected to capture all common variants in the 5' region of the gene, according to (15). The polymorphic sites at position -800 (c.1638G>A; rs1800468), -509 (c.1347C>T; rs1800469), and codon 25 (Arg/Pro, c.74G>C; rs1800471) in the *TGFB1* gene were examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis as described previously by (19). The codon 10 (Leu/Pro, c.29T>C; rs1800470) and the intronic g.10780 (T>G; rs2241717) SNPs were determined by high-resolution melting curve analysis (HRMA). Primer sequences and restriction-enzymes can be found in Tables e1 and e2 in the Supplement. The HRMA assays were performed on an Applied Biosystems 7500 Fast Real-Time PCR system (Life Technologies, Gent, Belgium). Using the Applied Biosystems HRM v2.0 software, melt data and output profiles were generated.

Statistical analysis

Patients with or without RT-induced nocturia were compared by means of the Mann-Whitney test for continuous variables and the χ^2-test for categorical variables. Tests for Hardy-Weinberg equilibrium (HWE) were conducted using the observed genotype frequencies and the χ^2 test with 1 degree of freedom (P>.0001). Estimation of haplotypes and calculation of the linkage disequilibrium (LD) coefficient r^2 was performed as described previously (19). To assess the independent effect of each polymorphism, unconditional logistic regression analyses were performed to calculate crude odds ratios (ORs). In addition, multiple unconditional logistic regression analyses with adjustment for

possible confounders were performed to calculate adjusted ORs. To correct for possible interaction between variables in the multivariate analysis, variance inflation factors (VIFs) are calculated to assess multicollinearity. VIF >10 indicates multicollinearity. The Benjamini-Hochberg procedure was used for multiple testing (ie, 33 tests: 18 genetic and 15 clinical parameter tests). Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL).

RESULTS

Pretreatment nocturia

Overall, 137 of 322 patients (43%) presented with a form of nocturia existing pretreatment. Of them, 78% had grade 1 nocturia (ie, 2-3 times/night), 19% had grade 2 nocturia (ie, 4-6 times/night or needing medication), and 3% had grade 3 nocturia (>6 times/night).

Evolution of pretreatment nocturia

Data concerning the presence of radiation-induced nocturia were available for all patients. Of these, 82 patients (25%) developed acute radiation-induced nocturia; 73 patients developed grade 2, and 9 patients developed grade 3 radiation-induced nocturia. The occurrence of radiation-induced nocturia was not significantly different between the prescription doses for both treatment regimens (Table 1), but patients receiving postoperative RT seem to be less prone to the development of RT-induced acute nocturia (P<.001; Table 2). Only 8% of the patients with grade 2 pretreatment nocturia and none of the patients with grade 3 pretreatment nocturia experienced worsening, whereas 49% of the patients with grade 1 pretreatment nocturia had an increase in grade.

Table 2: Associations between patient- and therapy-related characteristics and RT-induced acute nocturia.

	All patients				Primary IMRT group			
	All (n=322)	No RT-induced nocturia (n=240)	RT-induced nocturia (n=82)	P-value	All (n=222)	No RT-induced nocturia (n=150)	RT-induced nocturia (n=72)	P-value
Age (y)								
Median	66.0	65.0	66.0		67.0	67.0	68.0	
Range	49.0-82.0	49.0-81.0	51.0-82.0	.076	51.0-82.0	51.0-81.0	51.0-82.0	.398
Missing	*0*	*0*	*0*		*0*	*0*	*0*	
Nicotine abuse (n)								
Former + never	267 (82.9)	198 (74.2)	69 (25.8)		183 (82.4)	123 (67.2)	60 (32.8)	
Current	54 (16.8)	42 (77.8)	12 (22.2)	.576	39 (17.6)	27 (69.2)	12 (30.8)	.807
Missing	*1 (0.3)*	*0*	*1*		*0*	*0*	*0*	
Diabetes mellitus (n)								
No	282 (87.6)	210 (74.5)	72 (25.5)		191 (86.0)	129 (67.5)	62 (32.5)	
Yes	39 (12.1)	29 (74.4)	10 (25.6)	.988	30 (13.5)	20 (66.7)	10 (33.3)	.924
Missing	*1 (0.3)*	*1*	*0*		*1 (0.5)*	*1*	*0*	
Hypertension (n)								
No	227 (70.5)	174 (76.7)	53 (23.3)		155 (69.8)	109 (70.3)	46 (29.7)	
Yes	95 (29.5)	66 (69.5)	29 (30.5)	.178	67 (30.2)	41 (61.2)	26 (38.8)	.182
Missing	*0*	*0*	*0*		*0*	*0*	*0*	
Hypercholesteremia (n)								
No	188 (58.4)	138 (73.4)	50 (26.6)		126 (56.7)	83 (65.9)	43 (34.1)	
Yes	71 (22.0)	52 (73.2)	19 (26.8)	.979	49 (22.1)	33 (67.3)	16 (32.7)	.853
Missing	*63 (19.6)*	*50*	*13*		*47 (21.2)*	*34*	*13*	

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Table 2 *(continued)*

	All patients				Primary IMRT group			
	All (n=322)	No RT-induced nocturia (n=240)	RT-induced nocturia (n=82)	P-value	All (n=222)	No RT-induced nocturia (n=150)	RT-induced nocturia (n=72)	P-value
Abdominal surgery (n)								
No	182 (56.5)	136 (74.7)	46 (25.3)		115 (51.8)	74 (64.3)	41 (35.7)	
Yes	139 (43.2)	103 (74.1)	36 (25.9)	.899	106 (47.7)	75 (70.8)	31 (29.2)	.288
Missing	*1 (0.3)*	*1*	*0*		*1 (0.5)*	*1*	*0*	
TURP (n)								
No	271 (84.2)	198 (73.1)	73 (26.9)		176 (79.3)	112 (63.6)	64 (36.4)	
Yes	49 (15.2)	40 (81.6)	9 (18.4)	.206	44 (19.8)	36 (81.8)	8 (18.2)	**.022**
Missing	*2 (0.6)*	*2*	*0*		*2 (0.9)*	*2*	*0*	
Nocturia pre-treatment (n)								
Grade 0	185 (57.5)	157 (85.4)	28 (14.6)		124 (55.9)	98 (79.0)	26 (21.0)	
Grade 1+	137 (42.5)	83 (60.6)	54 (39.4)	**<.001**	98 (44.1)	52 (53.1)	46 (46.9)	**<.001**
Missing	*0*	*0*	*0*		*0*	*0*	*0*	
Lymph node dissection (n)								
No	195 (60.6)	142 (72.8)	53 (27.2)		149 (67.1)	101 (67.8)	48 (32.2)	
Yes	122 (37.9)	95 (77.9)	27 (22.1)	.314	69 (31.1)	46 (66.7)	23 (33.3)	.870
Missing	*5 (1.5)*	*3*	*2*		4 (1.8)	*3*	*1*	
Androgen deprivation (n)								
No	126 (39.1)	96 (76.2)	30 (23.8)		77 (34.7)	52 (67.5)	25 (32.5)	
Yes	194 (60.3)	142 (73.2)	52 (26.8)	.549	143 (64.4)	96 (67.1)	47 (32.9)	.952
Missing	*2 (0.6)*	*2*	*0*		2 (0.9)	*2*	*0*	

continued on next page

Table 2 *(continued)*

	All patients				Primary IMRT group			
	All (n=322)	No RT-induced nocturia (n=240)	RT-induced nocturia (n=82)	P-value	All (n=222)	No RT-induced nocturia (n=150)	RT-induced nocturia (n=72)	P-value
Radical prostatectomy (n)								
No (primary IMRT)	222 (68.9)	150 (67.6)	72 (32.4)		-	-	-	
Yes (post-operative IMRT)	100 (31.1)	90 (90.0)	10 (10.0)	**<.001**	-	-	-	**-**
Missing	*0*	*0*	*0*					
$Bladder_{max}$ (Gy)								
Median	79.0	78.0	79.0		79.0	79.0	79.0	
Range	10.0-87.0	10.0-87.0	73.0-83.0	**.001**	10.0-87.0	10.0-87.0	75.0-83.0	.561
Missing	*1 (0.3)*	*0*	*1*		*1 (0.5)*	*0*	*1*	
CTV_{median} (Gy)								
Median	78.0	77.0	78.0		79.0	79.0	79.0	
Range	72.0-86.0	72.0-86.0	72.0-83.0	**<.001**	74.0-86.0	74.0-86.0	74.0-83.0	.302
Missing	*1 (0.3)*	*0*	*1*		*1 (0.5)*	*0*	*1*	
CTV_{max} (Gy)								
Median	81.0	80.0	82.0		82.0	82.0	82.0	
Range	70.0-89.0	74.0-89.0	70.0-88.0	**<.001**	77.0-89.0	77.0-89.0	77.0-88.0	.135
Missing	*2 (0.6)*	*1*	*1*		*2 (1.0)*	*1*	*1*	
CTV_{Vol} (cc)								
Median	41.0	39.0	49.5		53.0	52.5	53.5	
Range	7.0-129.0	7.0-129.0	13.0-124	**.003**	17.0-129.0	19.0-129.0	17.0-124.0	.648
Missing	*4 (1.2)*	*2*	*2*		*3 (1.5)*	*1*	*2*	

Abbreviations: RT = radiotherapy; TURP = transurethral resection of the prostate; IMRT = intensity-modulated radiation therapy; CTV = clinical target volume. Data in parentheses are percentages.

Association between clinical and dose parameters and RT-induced acute nocturia

The associations between dose and clinical parameters and RT-induced acute nocturia are represented in Table 2. All studied dose parameters, radical prostatectomy, and the presence of pretreatment nocturia were significantly associated with the development of acute nocturia in univariate analysis. The difference in dose between patients with and without radiation-induced nocturia is statistically significant but clinically irrelevant. This difference is mainly driven by the patients in the postoperative setting who are treated with a lower dose prescription. Because the occurrence of acute nocturia was 3 times higher in patients treated with primary IMRT, the analyses were repeated in the primary IMRT group (Table 2). In this group, only TURP in patient's medical history and presence of pretreatment symptoms were statistically significantly associated with the development of acute nocturia.

Association between TGFb1 polymorphisms and RT-induced acute nocturia

The minor allele frequencies of the TGFb1 polymorphisms in all patients with and without RT-induced acute nocturia are represented in Fig. e1 in the Supplement. All SNPs were in HWE. Univariate logistic regression analysis revealed that the *TGFB1* -509, codon 10, and g.10780 polymorphisms were statistically significantly associated with an increased risk for RT-induced acute nocturia (Table 3). All associations hold after Benjamini-Hochberg procedure.

Table 3: Associations between *TGFB1* genotypes and RT-induced acute nocturia.

	All patients			
	No RT-induced nocturia	RT-induced nocturia	OR	BH adjusted p-value
TGFB1 -800 G>A				
GG	198 (82.5)	64 (78.1)	1	
GA	39 (16.3)	15 (18.3)	1.19	0.846
AA	3 (1.2)	1 (1.2)	1.03	1
dominant (GA+AA vs GG)			1.18	0.846
recessive (AA vs GG+GA)			1.00	1
Missing	*0*	*2 (2.4)*		

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Table 3 *(continued)*

	All patients			
	No RT-induced nocturia	RT-induced nocturia	OR	BH adjusted p-value
TGFB1 -509 C>T				
CC	134 (55.9)	29 (35.4)	1	
CT	86 (35.8)	44 (53.7)	2.36	**0.008**
TT	20 (8.3)	7 (8.5)	1.62	0.568
dominant (CT+TT vs CC)			2.22	**0.010**
recessive (TT vs CC+CT)			1.06	1
Missing	*0*	*2 (2.4)*		
TGFB1 codon 10 T>C				
TT	106 (44.1)	18 (21.9)	1	
TC	100 (41.7)	54 (65.9)	3.18	**0.001**
CC	34 (14.2)	9 (11.0)	1.56	0.568
dominant (TC+CC vs TT)			2.77	**0.005**
recessive (CC vs TT+TC)			0.78	0.802
Missing	*0*	*1 (1.2)*		
TGFB1 codon 25 G>C				
GG	196 (81.7)	67 (81.7)	1	
GC	44 (18.3)	13 (15.9)	0.86	0.854
CC	0	0	-	
dominant (GC+CC vs GG)			0.86	0.854
recessive (CC vs GG+GC)	-	-	-	
Missing	*0*	*2 (2.4)*		
TGFB1 g.10780 T>G				
TT	92 (38.3)	18 (21.9)	1	
TG	107 (44.6)	50 (61.0)	2.39	**0.015**
GG	41 (17.1)	13 (15.9)	1.62	0.491
dominant (TG+GG vs TT)			2.18	**0.025**
recessive (GG vs TT+TG)			0.93	1
Missing	*0*	*1 (1.2)*		

Abbreviations: RT = radiotherapy; OR = odds ratio; BH = Benjamini-Hochberg procedure; *TGFB1* = transforming growth factor β1.
Data in parentheses are percentages.

Next, multivariate analysis was performed with following variables: pretreatment nocturia symptoms, radical prostatectomy, TURP, *TGFB1* -509, codon 10, and g.10780 polymorphisms. Because the -509 and the codon 10 SNP are not independent (VIF -509 = 34.1; VIF codon 10 = 38.7), only 1 of these SNPs was included in the analysis. Moreover, because the dose differences for RT-induced nocturia are clinically irrelevant, these were not included. Because of its clinical importance, TURP was also entered in the

analysis. This multivariate analysis revealed that the development of acute radiation-induced nocturia is significantly associated with radical prostatectomy, presence of pretreatment nocturia symptoms, previous TURP, and the *TGFB1* codon 10 and -509 polymorphisms (Table 4). The *TGFB1* g.10780 SNP does not remain significantly associated with the development of radiation-induced nocturia in multivariate analysis.

Table 4: Effect of clinical and genetic factors on the risk of radiation-induced nocturia from multivariate logistic regression.

Analysis 1				Analysis 2			
Clinical/Genetic Factor		OR	P-value	Clinical/Genetic Factor		OR	P-value
Nocturia pre-treatment (n)		**4.64**	**<0.001**	Nocturia pre-treatment (n)		**4.01**	**<0.001**
Radical prostatectomy (n)		**0.14**	**<0.001**	Radical prostatectomy (n)		**0.15**	**<0.001**
TURP (n)		**0.31**	**0.010**	TURP (n)		**0.37**	**0.016**
TGFB1 codon 10 T>C				*TGFB1* -509 C>T			
	TT	1			CC	1	
	TC	**3.45**	**0.001**		CT	**2.50**	**0.030**
	CC	0.95	0.927		TT	1.93	0.377
TGFB1 g.10780 T>G				*TGFB1* g.10780 T>G			
	TT	1.00			TT		
	TG	1.84	0.107		TG	1.42	0.441
	GG	1.45	0.473		GG	0.83	0.777

Analysis 1: multivariate analysis with factors nocturia pre-treatment, radical prostatectomy, TURP, *TGFB1* g.10780 T>G, *TGFB1* codon 10 T>C.
Analysis 2: multivariate analysis with factors nocturia pre-treatment, radical prostatectomy, TURP, *TGFB1* g.10780 T>G, *TGFB1* -509 C>T.

Linkage analysis and haplotype determination

All polymorphisms are located in the 5' region of the *TGFB1* gene. LD was measured among the SNPs using the allele frequency data of all patients. There was LD between the -509 and the codon 10 polymorphism (r^2=.66) and between the -509 polymorphism and the intronic g.10780 (r^2=.55). There was no LD between the other polymorphisms ($r^2 \leq .32$). The polymorphisms have been used to reconstruct five *TGFB1* haplotypes (H1-H5) (Table 5). The most frequent haplotype (H1) composes all wild-type alleles (GCTGT) and was considered as reference haplotype. To correlate haplotypes with clinical radiosensitivity, haplotype frequencies were calculated for the group with and without RT-induced acute nocturia. This analysis showed that the H2 haplotype

was more frequent in the acute nocturia group; however, this was not statistically significant (OR = 1.68; P=.124).

Table 5: Associations between *TGFB1* haplotypes and RT-induced acute nocturia.

						Estimated frequency (%)				
	-800 G>A	-509 C>T	codon 10 T>C	codon 25 G>C	g.10780 T>G	All (n=322)	No RT-induced nocturia (n=240)	RT-induced nocturia (n=82)	OR	P-value
H1	G	C	T	G	T	40.3	42.3	34.4	1	
H2	G	T*	C*	G	G*	28.4	26.0	35.6	1.68	0.124
H3	G	C	T	G	G*	12.5	13.1	10.6	0.99	0.836
H4	A*	C	T	G	T	9.5	9.4	10.0	1.31	0.730
H5	G	C	C*	C*	T	8.6	8.8	8.1	1.13	0.999

Abbreviations: RT = radiotherapy; OR = odds ratio. *alleles differing from the reference haplotype H1.

DISCUSSION

This study was performed to analyze the influence of nongenetic and genetic factors on the development of acute radiation-induced nocturia. Significant associations were found between the *TGFB1* -509 C>T and codon 10 T>C variant alleles and the development of RT-induced acute nocturia in prostate cancer patients. Also, primary IMRT (as opposed to postoperative IMRT) and the presence of pretreatment nocturia contribute to the variability in radiation toxicity.

This study is the first that examines genetic markers in *TGFB1* and their association with acute nocturia following IMRT for prostate cancer patients. The polymorphisms in *TGFB1* have been extensively examined in normal tissue radiobiology. Much research has been performed to find associations between SNPs in the *TGFB1* gene and several RT-induced late adverse events (13). These results show no consistency, however. Currently, the radiogenomics consortium is working on a meta-analysis of published and unpublished data to confirm or refute the relationship between *TGFB1* SNPs and late radiotoxicity (20). Only 2 studies have reported on the correlation of SNPs in the *TGFB1* gene and acute radiation toxicity. Whereas Suga et al (16) could not find an association between -509 C>T and early skin reactions in Japanese breast cancer patients, Zhang et al (17) were able to demonstrate an association of the variant allele of -509 C>T with an increased risk of acute radiation-induced

oesophageal toxicity in Chinese lung cancer patients. Our study demonstrates a significant association between the variant alleles of *TGFB1* -509 C>T and codon 10 T>C and the occurrence of radiation-induced acute nocturia in prostate cancer patients.

In this study, 1 specific radiation-induced side effect –nocturia- was considered. Former studies (13) used a single grade that resulted of combining multiple symptoms such as haematuria, dysuria, urgency, nocturia, and increased frequency. Using this approach in our study, 105 instead of 82 patients would have been categorized as having RT-induced acute GU toxicity. Subdividing the patients in this manner leads to the loss of the significant associations between the *TGFB1* polymorphisms (-509 C>T and codon 10 T>C) and the general radiation-induced GU injury (data not shown). This illustrates that combining multiple symptoms can mask significant effects and confound the analysis.

In this study, pretreatment nocturia was strongly linked to the development of acute RT-induced nocturia. This was also found in the study of Peeters et al (6). Registering pretreatment symptoms data is mandatory, but omitting the registration implicates that the pretreatment symptom cannot be included as a confounding factor, leading to an inaccurate quantification of RT-induced toxicity. Jereczek-Fossa et al (7) showed that the development of acute genitourinary side effects is strongly correlated with the development of late events. This is also the case for nocturia in our study. Of the patients developing acute grade ≥2 nocturia, 51% developed at least late grade 1 nocturia (ie, 2-3 times/night), and 38% developed late grade ≥2 nocturia (>4 times/night or medication needing).

Our study shows that the incidence of acute nocturia was 3 times lower in patients treated with postoperative IMRT compared with patients treated with definitive IMRT. This is in accordance with Zelefsky et al (11). Therefore, we hypothesize that edema of the prostatic urethra is the predominant factor contributing to acute urinary complications. This might also explain the excellent response to α-blocker therapy (1). The lack of a significant response of nonsteroidal anti-inflammatory drugs (NSAIDs) suggests that the inflammatory component is clinically less important (11). Nevertheless, the current study indicates that TGFB1, a mediator of inflammation, is one of the many factors

involved in the pathogenesis of RT-induced nocturia. Because evidence exists that acute GU toxicity originates from damage to the urethra, we introduced a surrogate for the urethral dose, the CTV_{median}. The difference between patients with and without acute nocturia was found to be clinically irrelevant. This raises the question of whether the parameter chosen is an adequate surrogate for the urethral dose. Nevertheless, in future work, it will be necessary to study the complete dose-volume histogram of the urethra. This will be difficult because it is not easy to delineate the urethra.

A strength of our investigation is the relatively large number of patients enrolled. We were also able to build a nearly complete data set with few missing values (with the exception of hypercholesterolemia status). Furthermore, patients were from an ethnically homogeneous population, and patient recruitment as well as clinical outcome data collection were carried out prospectively. We investigated 1 endpoint of RT-induced GU injury and were able to include a large number of clinical and treatment variables, including the presence of pretreatment symptoms. Nevertheless, a major issue in genetic association studies is the increasing risk for false-positive findings. We anticipated this problem by controlling the false discovery rate by means of the Benjamini-Hochberg procedure. Although the associations hold after correcting for multiple testing, the results of this study should be validated in an independent study.

In conclusion, radical prostatectomy and the presence of mild nocturia before therapy and the variant alleles of *TGFB1* -509 C>T and codon 10 T>C are identified as factors involved in the development of acute radiation-induced nocturia. Because a specific symptom was investigated, this study is unique and is also a call for standardization of radiation toxicity assessment in RT treatment of prostate cancer. The results of this study may be useful in research focusing on prediction of late severe nocturia after IMRT for prostate cancer.

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CONFLICTS OF INTEREST NOTIFICATION

The author(s) indicated no potential conflicts of interest.

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SUPPLEMENTARY MATERIAL

Table e1: Polymerase chain reaction-restriction fragment length polymorphism (RFLP) methods.

db SNP-ID	Genotype	T_a	Restriction Enzyme	Primers
rs1800469	-800 G>A	60°C	HpyCh4IV	5'-GCA GTT GGC GAG AAC AGT TG-3' 5'-TGG GTC ACC AGA GAA AGA GG-3'
rs1800469	-509 C>T	60°C	Bsu36I	5'-GCA GTT GGC GAG AAC AGT TG-3' 5'-TGG GTC ACC AGA GAA AGA GG-3'
rs1800471	Arg25Pro	58°C	Bgl I	5'-TGT TCG CGC TCT CGG CAG-3' 5'-GAC CTC CTT GGC GTA GTA G-3'

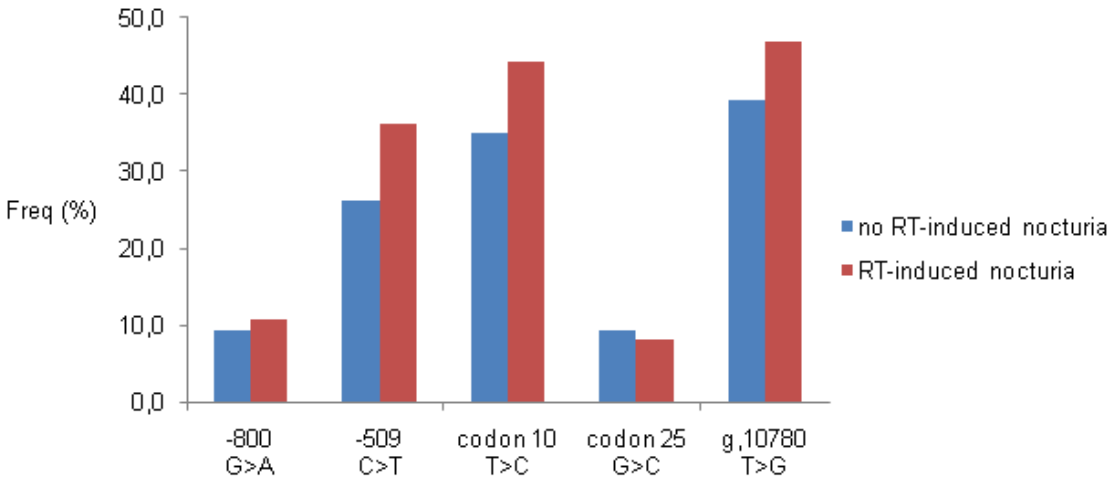
Abbreviations: T_a: annealing temperature of PCR reaction.

Table e2: High Resolution Melting curve Analysis (HRMA) methods.

db SNP-ID	Genotype	T_a	Primers
rs1800470	Leu10Pro	64°C	5'-ACC ACA CCA GCC CTG TTC-3' 5'-AGC ACC AGT AGC CAC AGC AG-3'
rs22241717	g.10780 T>G	60°C	5'-GTC GGC TGG TTA CAA GGT C-3' 5'-GCT TGG CAA CAG AGT GAG AC-3'

Abbreviations: T_a: annealing temperature of HRMA reaction.

Figure e1: Minor allele frequencies of the *TGFβ1* polymorphisms.



7 Paper II

Integrated models for the prediction of late genitourinary complaints after high-dose intensity modulated radiotherapy for prostate cancer: Making informed decisions

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ABSTRACT

Background and purpose: To develop predictive models for late radiation-induced haematuria and nocturia allowing a patient individualized estimation of pre-treatment risk.

Materials and methods: We studied 262 prostate cancer patients treated with curative intensity modulated radiotherapy to the intact prostate or prostate bed. A total of 372 variables were used for prediction modeling, among which 343 genetic variations. Toxicity was scored using an in-house developed toxicity scale. Predictor selection is achieved by the EMLasso procedure, a penalized logistic regression method with an EM algorithm handling missing data and crossvalidation avoiding overfit. Model performance was expressed by the area under the curve (AUC) and by sensitivity and specificity.

Results: Variables of the model predicting late haematuria (36/262) are bladder volume receiving ≥75 Gy, prostatic transurethral resection and four polymorphisms. (AUC = 0.80, sensitivity = 83.3%, specificity = 61.5%). The AUC drops to 0.67 when the genetic markers are left out. The model that predicts for late nocturia (29/262) contains the minimal clinical target volume (CTV) dose, the CTV volume and three polymorphisms (AUC = 0.76, sensitivity = 75.9%, specificity = 67.4%). This model is a better predictor for nocturia compared to the nongenetic model (AUC of 0.60).

Conclusions: We were able to develop models that predict for the occurrence of late radiation-induced haematuria and nocturia, including genetic factors which might improve the prediction of late urinary toxicity.

INTRODUCTION

Long-term toxicity after radiotherapy may cause substantial morbidity (1). Quality of life outcomes are of particular importance as most patients survive early-stage prostate cancer after treatment and should therefore be part of the treatment decisions (2). Prediction models of radiotherapy-induced side effects impairing patients' quality of life, can help this medical decision making.

Recent refinements of intensity-modulated radiotherapy (IMRT) and image-guided techniques allow for a better sparing of the rectum, which results in an

acceptable level of severe late gastrointestinal (GI) complications, such as rectal bleeding (1-3). In contrast, due to the full inclusion of the bladder neck and, in case of postoperative RT, the vesicourethral anastomosis in the high-dose region, the risk of developing severe genitourinary (GU) toxicity has remained rather unchanged (1, 4). GU symptoms occur with a 10-year cumulative incidence of approximately 20% in patients treated with high-dose IMRT.

Moreover, the incidence does not seem to plateau, as it is the case for GI toxicity (1, 2). Factors already known to predict for chronic GU toxicity are pre-treatment urinary complaints, prior transurethral resection of the prostate (TURP) and the presence of acute toxicity (5-7). Although bladder dose-volume effects are demonstrated (8-10), methodological differences have obscured the link between lower urinary tract dose and toxicity (11). Intrinsic factors as genetic polymorphisms, which are mainly responsible for the interpatient variability, can add predictive value to the pre-treatment risk-estimation of an individual patient. Therefore, the purpose of this study was to develop integrated predictive models containing clinical, dosimetric and genetic data for the prediction of late GU sequelae in prostate cancer patients. This would enable us to individualize patient treatment.

MATERIALS AND METHODS.

Study population

This study enrolled 262 patients treated with IMRT as primary (n = 180) or post-operative treatment (n = 82) for prostate cancer at the Ghent University Hospital between 1999 and 2011. All patients had at least 12 months of follow-up (range: 1–13 yr).

The dose was prescribed as the median dose to the planning target volume. Prescription doses were delivered with 18 MV photons of an Elekta linear accelerator (Crawley, UK) as described in detail in (7, 12, 13). Patients in the postoperative setting were treated with 74 Gy (adjuvant) or with 76Gy (salvage). Three dose levels (74, 76 and 80 Gy) were given to patients treated with primary IMRT. Patient setup, verification, target volume description and normal tissue constraints are reported previously (7, 12, 13).

Adjuvant androgen deprivation consisting of a luteinizing hormone-releasing hormone analog, was administered in 157 patients. Patient characteristics are listed in Table 1.

Table 1: Patient and tumour characteristics.

Characteristics	All patients
Age, yr	
Median	65
Range	49-82
Follow-up, yr	
Median	4
Range	1-13
Prescribed radiotherapy dose, Gy	
Median	80
Range	74-80
Prescription protocol	
74 Gy, 36 fractions	54 (20.6)
76 Gy, 37 fractions	63 (24.0)
80 Gy, 38 fractions	145 (55.3)
Radical Prostatectomy	
Yes	82 (31.3)
No	180 (68.7)
Tumor stage	
T1	78 (29.8)
T2	110 (42.0)
T3	70 (26.7)
T4	4 (1.5)
Gleason score	
≤6	131 (50.0)
7	83 (31.7)
8-10	45 (17.2)
Unknown	3 (1.1)
PSA before RT, ng/ml	
Median	6.7
Range	0.0-150.0
≤10 ng/ml	187 (71.4)
>10 ng/ml	75 (28.6)
Lymph node dissection	
Yes	93 (35.5)
No	168 (64.1)
Unknown	1 (0.4)
Adjuvant AD	
Yes	156 (59.5)
No	106 (40.5)

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Table 1 *(continued)*

Characteristics	All patients
Prior TURP	
Yes	39 (14.9)
No	223 (85.1)
Pre-treatment symptoms	
Yes	108 (41.2)
No	154 (58.8)

Abbreviations: PSA = prostate-specific antigen; AD = androgen deprivation; TURP = transurethral resection of the prostate.
Data are given as no. (%) unless otherwise indicated.

In order to have a comfortably filled bladder (>200 mL) patients were instructed to drink ±750 cc prior to their therapy. Bladder filling was checked to match the volume on planning computed tomography (CT) to avoid under/overfilling of the bladder. Before 2010, this was checked by a portable bladder ultrasound, thereafter, daily cone-beam CT was applied (7, 14). The maximum bladder dose was set at 80 Gy. Dose-volume histograms (DVH) were calculated using an in-house-developed planning system, with a final dose computation using a commercial radiotherapy planning system (Pinnacle; Philips Medical Systems, Best, The Netherlands). The bladder was delineated as a solid organ. The minimal, mean and maximal dose to the clinical target volume (CTV), the CTV volume (cc), the mean and maximal bladder dose (Gy), the bladder volume (B_{vol} (cc)) and the percentage of the bladder volume receiving more than 10, 20, 30, 40, 50, 60, 65, 70 and 75 Gy (termed B10 (%) to B75 (%)) were considered as predictors.

All patients completed a pre-IMRT questionnaire, registering baseline GU symptoms and patients' medical history (diabetes, hypertension, previous surgery, and smoking). These factors were also considered as predictors. Toxicity was registered following an in-house developed grading system (see Supplementary Table e1) based on the Radiation Therapy Oncology Group (RTOG), the Common Toxicity Criteria for Adverse Events (CTCAE v.3.0) and SOMA/LENT toxicity scoring systems (12, 13). It was defined as an increase in toxicity symptoms, taking the pre-RT score into consideration, and was recorded as the maximal score of radiation-induced toxicity. If symptom scores

improved (fewer symptoms after than before RT), these were recorded as a zero score. Acute toxicity was defined as toxicity occurring during or within 3 months after the end of RT. Late toxicity was defined as toxicity occurring for the first time >3 months after the end of IMRT or as acute toxicity lasting longer than 3 months. Severe late GU symptoms were defined as grade 2 or 3 toxicity. Prediction models were only generated for symptoms with an incidence >10%.

Genomic DNA was obtained from fresh blood using the Gentra Puregene Blood kit. The study was approved by the local ethics committee (UZG2007/560) and all study patients provided written informed consent.

Single nucleotide polymorphism (SNP) selection

Genes encoding proteins involved in the early response, DNA damage response, DNA repair, oxidative metabolism and steroid hormonal metabolism, were chosen after comprehensive literature search (more details in Supplementary File 1). SNPs in these genes were selected using ECR browser (15). This was based on functional tagging of multispecies evolutionary conserved regions, which is an indication for biological function. Details can be found in (16). The selection was expanded with a number of SNPs previously reported to be associated with radiation-induced injury or cancer. Finally, 384 SNPs were retained.

Genotyping

Genotyping was performed using the Illumina GoldenGate assay (DNAVision, Charleroi, Belgium), genotyping data and clustering of SNP genotypes were managed in GenomeStudio (Illumina). Upon processing, quality control processes were run to guarantee the accuracy of the genotyped dataset. Genotypes were excluded based on call rate (<75%), GenCall score (<0.4) which is a measure of quality and reliability, minor allele frequency (<0.05) and deviation from Hardy-Weinberg equilibrium ($p<0.001$). This resulted in the elimination of approximately 9% of the SNPs.

Statistical analysis

In a first step, model selection is achieved by the EMLasso procedure. The Lasso is a penalized regression method that shrinks down to zero the coefficient of the markers that have little apparent effect on the trait and retains those variables that have the strongest collective impact (17). Missing data are handled by a stochastic expectation-maximization (EM) algorithm that is imposed to the predictors. To control for overfit tenfold cross-validation is used. As such, a limited set of variables is selected (adding more variables does not increase the predictive stability). Details of the method are published by Sabbe et al. (18) and discussed by De Ruyck et al (19). Secondly, a simple logistic regression model is fit to the data with only the selected variables to obtain the model coefficients (β). The individual probabilities (*p*) were calculated through ln *p*/(1-*p*) = β0+β1*x1+β2*x2+β3*x3… with x = predictor. Subsequently, the prediction performance is measured by the area under the curve (AUC) of the receiver operating curve (ROC) and by sensitivity and specificity in the optimal operator point of the ROC curve. This point is determined with a higher penalty (1.5) for false negatives. To check for calibration the Hosmer-Lemeshow goodness-to-fit (GOF) test was applied. P-values >0.05 indicate that the fitted model adequately describes the observed outcomes in the data (20). Estimates of late GU probabilities were calculated using the Kaplan-Meier method. For all analyses, the statistical platform R was used (packages addendum, NumDfr, GLoMo, snowfall, EMLasso, Survival) (21).

RESULTS

Late GU toxicity

The most frequently observed late grade 2+ GU symptoms were haematuria and nocturia present in 36 (14%) and 29 (11%) of the patients, respectively, with grade 3 toxicity present only in 1 and 4 patients. Together, they encompass more than 50% of the late urinary symptoms. Fig. 1 shows the cumulative incidence of grade 2–3 haematuria and nocturia. Other radiation-induced urinary toxicity included dysuria (n = 4), urgency (n = 9), increased daily frequency (n = 18) and incontinence (n = 20); the latter predominantly occurred in patients treated in the postoperative setting. The occurrence of late radiation-

induced toxicity was not significantly different between the three prescription regimens.

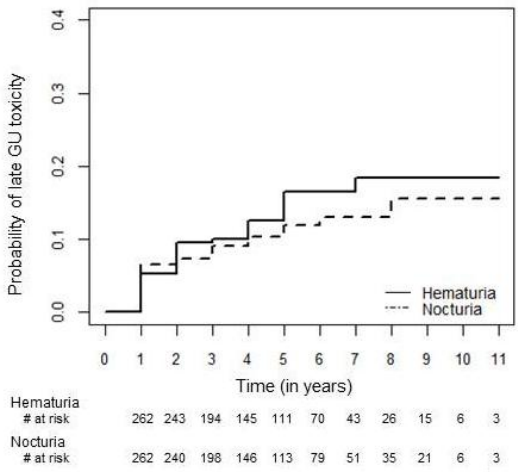

Fig 1: Cumulative incidence of late radiation-induced haematuria and nocturia.

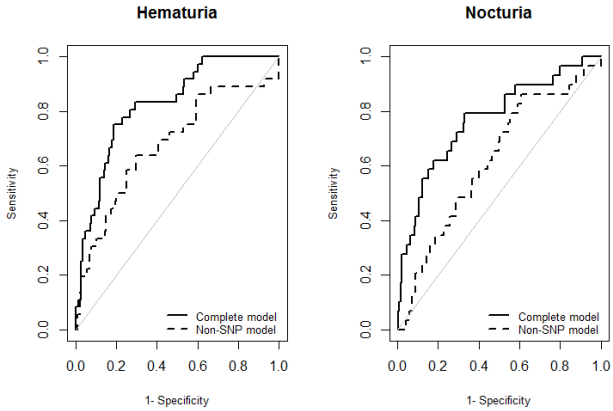

Fig 2: ROC curves for the different endpoints.

Prediction modeling

Patient and clinical characteristics are depicted in Table 1. The 343 genetic polymorphisms and the 29 patient- and treatment-related parameters under consideration are available in Supplementary Tables e2 and e3. In total, 372 variables were available for model selection.

Late haematuria. The prediction model for late haematuria has an AUC of 0.80 and consists of six parameters: B75, prior TURP and the genotypes rs3931914CG, rs2293054AG, rs708498GG and rs845552AG (GOF χ^2 = 10.5; p = 0.234). Sensitivity and specificity are 83.3% and 61.5%, respectively, at the threshold of 8%. The AUC drops to 0.67 when the 4 genetic markers are left out (Figure 2). Data for the variables in the model are shown in Table 2.

Table 2: Data for factors included in the model for late haematuria.

	No RT-induced haematuria (n=226)	RT-induced haematuria (n=36)
B75 (%)		
Median	5.0	8.0
Range	0-22	1-22
Unknown	5	1

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Table 2 (*continued*)

	No RT-induced haematuria (n=226)	RT-induced haematuria (n=36)
Prior TURP		
No	200 (89.7)	23 (10.3)
Yes	26 (66.7)	13 (33.3)
HMGRC rs3931914		
CC	145 (92.4)	12 (7.6)
CG	67 (75.3)	22 (24.7)
GG	14 (87.5)	2 (12.5)
NOS1 rs2293054		
GG	105 (82.0)	23 (18.0)
GA	102 (94.4)	6 (5.6)
AA	19 (73.1)	7 (26.9)
PTGER2 rs708498		
GG	167 (90.8)	17 (9.2)
GA	51 (75.0)	17 (25.0)
AA	8 (80.0)	2 (20.0)
EGFR rs845552		
AA	75 (92.6)	6 (7.4)
AG	101 (80.2)	25 (19.8)
GG	49 (90.7)	5 (9.8)
Unknown	1	0

Abbreviations: RT = radiotherapy; B75 = percentage of the bladder volume receiving 75 Gy or more; TURP = transurethral resection of the prostate; *HMGRC* = hydroxy-methyl-glutaryl CoA reductase gene; *NOS1* = nitric oxide synthase 1 gene; *EGFR* = epidermal growth factor receptor gene; *PTGER2* = prostaglandin E receptor 2 gene.
Data are given as no. (%) unless otherwise indicated.

Late nocturia. Variable selection procedure revealed a model comprising the following variables: the CTV min, the CTV volume and the rs1799983GT, rs1045485GG and rs4808611TC genotypes (GOF χ^2 = 3.8; p = 0.878). This model has an AUC of 0.76 with sensitivity of 75.9% and specificity of 67.4% at the threshold of 9%. Data for the variables in the model are shown in Table 3. The model including the SNPs was a better predictor for nocturia compared to the non-genetic model (AUC of 0.60) (Figure 2). The presence of acute grade 2 toxicity also adds predictive value to the model (AUC = 0.78). This factor can, however, not be included in the model as the occurrence of acute toxicity is unknown before the start of RT (data not shown).

The coefficients for the calculation of the probability are available in Supplementary Tables e4 and e5.

Table 3: Data for factors included in the model for late nocturia.

	No RT-induced nocturia (n=235)	RT-induced nocturia (n=29)
CTV min (Gy)		
Median	72	73
Range	64-79	67-78
Unknown	9	1
CTV volume (cc)		
Median	41	54
Range	7-129	17-127
Unknown	7	0
NOS3 rs1799983		
GG	83 (80.6)	20 (19.4)
GT	122 (96.1)	5 (3.9)
TT	27 (87.1)	4 (12.9)
Unknown	1	0
CASP8 rs1045485		
GG	178 (93.2)	13 (6.7)
GC	49 (77.8)	14 (22.2)
CC	6 (75.0)	2 (25.0)
NR2F6 rs4808611		
CC	157 (94.0)	10 (6.0)
CT	69 (80.2)	17 (19.8)
TT	5 (71.4)	2 (28.6)
Unknown	2	0

Abbreviations: RT = radiotherapy; CTV = clinical target volume; *NOS3* = nitric oxid synthase 3 gene; *CASP8* = caspase 8 gene; *NR2F6* = nuclear receptor subfamily 2, group F, member 6 gene.
Data are given as no. (%) unless otherwise indicated.

DISCUSSION

The main purpose of the current study was to design predictive models suitable in clinical practice to identify patients at risk for developing severe urinary symptoms. Late radiation toxicity is related to both dosimetric and clinical risk factors, as well as, the patients' genetic make-up. We were able to construct well-calibrated models for late radiation-induced haematuria and nocturia.

This study shows that genetic factors have the potential to improve prediction of late toxicity. There are several advantages using genotypes as

biomarkers: easy to analyze, stable and affordable. Four genetic markers are included in the model predicting late radiation-induced haematuria. The potentially functional 5'UTR SNP, rs3931914, is situated in the *HMGCR* gene encoding hydroxy-methylglutaryl-coenzyme A reductase. This enzyme is rate-limiting in the novo synthesis of cholesterol. The SNP can generate a binding motif and can induce the loss of several other motifs for transcription factors (22). In the current study, heterozygotes predict for the development of late haematuria. The other variants rs2293054, rs708498 and rs845552 in the *NOS1* gene, the *PTGER2* gene and the *EGFR* gene, respectively, were selected based on their evolutionary conservation. In case of nocturia, three genetic variants provide additional predictive value. The variant rs1799983 (Asp298Glu) is located in the *NOS3* gene, which protein is responsible for nitric oxide (NO) production. The functional variant has been shown to result in a reduction of the NO production which results in lower levels of oxidative stress (23). We show that carriers of the heterozygote genotype have a lower risk to develop late nocturia. Previous studies could demonstrate a similar effect for this SNP with a decreased risk for radiation-induced telangiectasia and pneumonitis (23, 24). Two SNPs included in our model were previously shown to be associated with breast cancer risk, rs4808611 and rs1045485 (Asp302His) (25, 26). This is plausible as it is hypothesized that there is an overlap between polymorphisms associated with breast cancer and radiosensitivity (27). In the current study, rs4808611 heterozygotes and carriers of the variant allele of rs1045485 have a higher risk to develop late nocturia. Radiosensitivity is considered to be an inherited complex, polygenic trait dependent on many SNPs each with small effect sizes (28). Although, in this study, we notice that the contribution of SNPs is more than expected. This can be due to sample size.

Only few studies were able to develop predictive models of late urinary toxicity including dose-volume information (8, 10). This is due to the highly variable bladder filling during radiotherapy which leads to difficulties in calculating the actual dose to the bladder (5). Specific instructions to ensure a stable bladder volume have been shown to minimize the discrepancy between the planning DVH and the actual dose (11). In the current study, daily medical imaging was applied to verify bladder filling and the discrepancy is expected to

be low. The percentage of the bladder volume receiving ≥75 Gy is shown to be predictive for late GU toxicity. Previous studies (8-10) also indicated that small high-dose regions contribute to the development of late GU injury, which confirms the serial behavior of the bladder for chronic urinary toxicity. The minimal dose to the CTV and the CTV volume indicate the involvement of the prostatic urethra for late nocturia as the prostatic urethra was not considered as an organ at risk in the planning system.

We found prior TURP to be predictive for a higher occurrence of late haematuria. Previous studies have shown that TURP before RT was associated with less acute toxicity (7, 29). This procedure was probably performed to alleviate baseline urinary symptoms, as those increase the risk for acute toxicity. Nevertheless, this procedure might deteriorate the late urinary symptoms (30). We and many others found that the presence of acute radiation-induced grade 2 GU symptoms is predictive for the development of late toxicity (4-6). This factor is, however, unknown at the start of RT and is therefore no genuine predictor. The presence of baseline urinary symptoms was not found to be predictive for late toxicity.

The prediction of radiation morbidity has been valued by others (6). Predictive models can guide the allocation of patients to treatment groups based on their probability of severe radiotherapy toxicity and simultaneously improve the therapeutic ratio. Patients at high risk may be offered an alternative treatment, such as, radical prostatectomy or active surveillance. Or, for patients who receive radiotherapy, advanced planning corrections can be introduced to better individualize radiotherapy treatment. It can enable us to define acceptance criteria for future use in RT treatment for prostate cancer. Current analyses are suitable for practice. Nevertheless, validation and assessment of clinical usefulness are needed before implementing these models in the clinic for routine use. Clinical usefulness can be quantified by decision-analytic methods, such as net benefit and decision curve analysis (20, 31). Therefore, an optimal clinical decision threshold should be determined taking into account the harms and benefits of the alternative treatment. In this context, adopting an alternative treatment without impairing local tumour control and survival, is a matter of further research and debate. In the current study, the threshold for late haematuria and late nocturia penalizes the number of false negatives. From a

clinical point of view, this is rather arbitrary as the alternative treatment is not yet known.

This study is susceptible to the shortcomings of every retrospective analysis. We did not include the time-course of the events, which may restrict the applicability of the resulting models at different time points, especially when the events are reversible or transient. We welcome other centers to join in, testing the predictive power in independent cohorts and upgrading the number of events.

In conclusion, late radiation-induced haematuria and nocturia can be predicted by combining clinical, dosimetric and genetic data. Inclusion of genetic data can refine prediction of late urinary toxicity. Validation and assessment of the clinical usefulness are necessary before implementing these models in the clinic.

CONTRIBUTORS

SDL, KDR, WDN and HT participated in the design, coordination and supervision of the study. GDM, PO and VF were involved in patient follow-up and the acquisition of the data. All authors critically revised the manuscript and approved final manuscript.

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CONFLICT OF INTEREST.

The authors declare that they have no conflicts of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2014.04.005.

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SUPPLEMENTARY MATERIAL

Supplementary Table e1: The in-house developed Genitourinary (GU) toxicity scale.

GU	Grade 1	Grade 2	Grade 3	Grade 4
Nocturia	Increase in frequency ≤2 normal	Increase >2 normal but less than every hour	Once or more per hour	Not defined
Frequency	Increase in frequency ≤2 normal	Increase >2 normal but less than every hour	Once or more per hour	Not defined
Haematuria	Microscopic	Gross	Gross with cloths	Requiring transfusion
Dysuria	No therapy	Oral treatment (no narcotic analgesics)	Narcotic analgesics	Not defined
Urgency	No therapy	Urgency requiring therapy	Narcotic analgesics	Not defined
Incontinence	No therapy	Therapy or using two or fewer pads per day	Using more than two pads per day	Surgery

Supplementary file 1 – References of the selection of SNPs

SNPs selected for the current study were based on the reported association of several genes identified in the early response to ionizing radiation, DNA checkpoint control and repair of DNA damage upon exposure of cells to ionizing radiation [1-7]. We also included SNPs from genes encoding proteins involved in the metabolism of side products of the radiolysis of water [8]. In addition, genes in the hormonal metabolism were selected [9-12], complemented with SNPs in a variety of genes shown to be involved in radiation response [13-18]. Several of the selected SNPs are shown to be associated with cancer predisposition in case-control studies [19-28].

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Table e1: The selection of 343 genetic polymorphisms suitable for analysis.

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
DNA damage response						
ATM	Ataxia Telangiectasia Mutated	rs1801516	0.14	G>A	Coding	Asn1853Asp
ATR	Ataxia telangiectasia and Rad3 related	rs1802904	0.17	A>G	Coding	Gln=
		rs2229032	0.17	G>A	Coding	Arg2425Gln
		rs2227929	0.37	T>C	Coding	Asp=
		rs2227928	0.44	C>T	Coding	Met211Thr
MRE11	Meiotic recombination 11 homolog A	rs2509943	0.45	G>C	Coding	Leu=
RAD50	DNA repair protein RAD50	rs17166050	0.22	C>T	Intronic	
H2AFX	H2A histone family, member X	rs643788	0.42	T>C	5'-flanking	
MDC1	Mediator of DNA-damage checkpoint1	rs28986317	0.07	G>C	Coding	Ala=
CHK1	Checkpoint kinase 1 (Ser/Thr kinase)	rs491528	0.30	G>T	Intronic	
CHK2	Checkpoint kinase 2 (Ser/Thr kinase)	rs2236141	0.13	C>T	5'-flanking	
BRCA1	Breast cancer 1, early onset	rs12516	0.32	C>T	3'-UTR	
		rs4986850	0.07	G>A	Coding	Asp693Asn
BRCA2	Breast cancer 2, early onset	rs9534262	0.49	C>T	Intronic	
TP53	Tumor protein p53	rs2287498	0.07	G>A	5'-flanking	
TP53BP1	Tumor protein p53 binding protein 1	rs560191	0.33	G>C	Coding	Asp=
TP53BP2	Tumor protein p53 binding protein 2	rs17739	0.16	C>T	3'-UTR	
		rs1153933	0.07	C>T	Coding	Ser=
		rs34683843	0.09	C>A	Coding	Gln229Lys
MDM2	MDM2 oncogene, E3 ubiquitin protein ligase	rs769412	0.07	A>G	Coding	Glu=
CDKN2B	Cyclin-dependent kinase inhibitor 2B	rs2069426	0.12	C>A		
CDKN2C	Cyclin-dependent kinase inhibitor 2C	rs12855	0.09	C>T	3'-UTR	
WRN	Werner syndrome, RecQ helicase-like	rs2230009	0.07	G>A	Coding	Val114Ile
CDKN3	Cyclin-dependent kinase inhibitor 3	rs2179896	0.28	G>A	Intronic	
PLK2	Polo-like kinase 2	rs32613	0.07	G>T	3'-UTR	
		rs15009	0.35	C>G	3'-UTR	
		rs1042994	0.12	T>C	Coding	Phe=
		rs702723	0.44	A>G	Intronic	
		rs702722	0.13	T>C	Coding	Ile=
		rs15915	0.21	G>A	5'-UTR	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
cdc6	Cell division cycle 6	rs13706	0.12	G>A	Coding	Val441Ile
		rs4134994	0.14	A>G	5'-flanking	
CCND1	Cyclin D1	rs1944129	0.49	G>A	5'-flanking	
		rs9344	0.48	G>A	Coding	Pro=
		rs7177	0.46	A>C	3'-UTR	
CCND2	Cyclin D2	rs1049612	0.41	A>G	3'-UTR	
DNA repair						
Non-homologous end-joining						
KU70	X-ray repair cross-complementing protein 6	rs2267437	0.38	C>G	5'-flanking	
KU80	X-ray repair cross-complementing protein 5	rs207906	0.14	G>A	Coding	Thr=
		rs7581055	0.11	A>G	Intronic	
		rs1438162	0.39	A>G	Intronic	
XRCC4	X-ray repair cross-complementing protein 4	rs17284218	0.43	A>T	Intronic	
		rs11951257	0.48	T>C	Intronic	
		rs6872787	0.07	G>C	Intronic	
		rs1056503	0.13	T>G	Coding	Ser=
PRKDC	Protein kinase, DNA activated, catalytic polypeptide	rs10109984	0.38	T>C	Intronic	
		rs8178071	0.17	G>A	Intronic	
LIG4	DNA ligase IV	rs1805388	0.13	C>T	Coding	Thr9Ile
		rs9520823	0.31	T>G	Intronic	
XLF	Non-homologous end-joining factor 1	rs1378641	0.32	T>C	Intronic	
		rs17608747	0.21	C>T	Intronic	
POLM	DNA polymerase mu	rs3218655	0.16	G>T	Coding	Leu=
POLL	DNA polymerase lambda	rs3730477	0.23	C>T	Coding	Arg438Trp
		rs3730475	0.31	T>C	Intronic	
RPA1	Replication protein A1	rs3786136	0.23	C>T	Intronic	
		rs12150513	0.47	T>G	Intronic	
		rs2230930	0.17	C>T	Coding	Ser=
		rs3744766	0.15	G>C	3'-UTR	
		rs12727	0.20	G>C	3'-UTR	
		rs17734	0.34	A>G	3'-UTR	
Homologous recombination						
Rad51	DNA repair protein RAD51 homolog 1	rs1801320	0.06	G>C	5'-UTR	
XRCC2	X-ray repair cross-complementing protein 2	rs3218536	0.09	G>A	Coding	Arg188His
XRCC3	X-ray repair cross-complementing protein 3	rs1799796	0.35	A>G	Intronic	
		rs861539	0.36	C>T	Coding	Thr241Met
		rs1799794	0.20	A>G	5'-UTR	
RAD52	DNA repair protein RAD52 homolog	rs7303748	0.44	C>T	Intronic	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
Base-excision repair						
PARP-1	Poly-(ADP-ribose)-polymerase	rs732284	0.18	G>C	Intronic	
		rs1805405	0.19	C>A	Intronic	
OGG1	8-oxoguanine DNA glycosylase	rs2269112	0.17	C>T	Intronic	
		rs2072668	0.23	C>G	Intronic	
NUDT1	Nucleoside diphosphate linked moiety X-type motif 1	rs1799832	0.21	C>T	Coding	Asp=
APEX1	Apurinic-apyrimidinic endonuclease 1	rs2275007	0.41	G>A	Coding	Gln=
		rs1130409	0.49	T>G	Coding	Asp148Glu
XRCC1	X-ray repair cross-complementing protein 1	rs25487	0.37	G>A	Coding	Gln399Arg
		rs1799782	0.08	C>T	Coding	Arg194Trp
		rs3213245	0.41	T>C	Coding	Gly59Ser
		rs2682587	0.17	C>A	Intronic	
LIG3	DNA ligase 3	rs2066505	0.11	G>A	Intronic	
PCNA	Proliferating cell nuclear antigen	rs17349	0.11	C>T	Intronic	
FEN1	Flap structure-specific endonuclease1	rs174538	0.30	G>A	5'-flanking	
Nucleotide-excision repair						
XPC	Xeroderma pigmentosum, complementation group C	rs2279017	0.40	G>T	Intronic	
		rs2227998	0.28	G>A	Coding	Arg=
		rs2228000	0.25	C>T	Coding	Ala499Val
		rs2228001	0.42	A>C	Coding	Lys939Gln
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1	rs3212961	0.16	C>A	Intronic	
		rs11615	0.39	T>C	Coding	Asn=
		rs3212986	0.24	G>T	3'-UTR	
ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	rs13181	0.37	T>G	Coding	Lys751Gln
		rs1052555	0.33	C>T	Coding	Asp=
ERCC4	Excision repair cross-complementing rodent repair deficiency, complementation group 4	rs762521	0.23	G>A	Intronic	
		rs1799801	0.31	T>C	Coding	Ser=
RAD23B	RAD23 homolog B	rs1805330	0.11	C>T	Intronic	
		rs1805329	0.17	C>T	Coding	Ala249Val
		rs10868	0.13	C>T	3'-UTR	
POLK	DNA polymerase kappa	rs3213801	0.23	G>A	Coding	Ala=

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
Oxidative metabolism						
SOD2	Superoxide dismutase 2, mitochondrial	rs2842960	0.47	T>C	Intronic	
CAT	Catalase	rs1049982	0.31	C>T	5'-UTR	
GPx2	Glutathione peroxidase 2	rs3759681	0.27	C>T	5'-flanking	
GPx3	Glutathione peroxidase 3	rs4958872	0.23	T>C	Intronic	
		rs8177447	0.16	C>T	Intronic	
GPx4	Glutathione peroxidase 4	rs4807542	0.19	G>A	Coding	Pro=
PRDX1	Peroxiredoxin 1	rs2356559	0.33	G>A	Intronic	
TXNRD2	Thioredoxin reductase 2	rs1139795	0.17	G>A	Coding	Pro=
		rs5993853	0.33	C>T	Intronic	
GSTA4	Glutathione S-transferase alpha 4	rs16883343	0.25	C>T	5'-flanking	
GSTA5	Glutathione S-transferase alpha 5	rs2397118	0.05	T>C	Coding	Val55Ile
GSTP1	Glutathione S-transferase pi 1	rs1138272	0.09	C>T	Coding	Ala114Val
		rs1871042	0.35	C>T	Intronic	
GSTM2	Glutathione S-transferase mu 2	rs592792	0.14	G>A	Coding	Asn=
GSTM5	Glutathione S-transferase mu 5	rs2229059	0.09	T>C	Coding	Leu=
p22phox	Cytochrome b-245, alpha polypeptide	rs4673	0.34	C>T	Coding	Tyr72His
NOS1	Nitric oxide synthase 1	rs2291908	0.29	A>G	Intronic	
		rs1093329	0.43	G>A	Intronic	
		rs2293054	0.30	G>A	Coding	Ile=
		rs11612772	0.27	G>C	Intronic	
		rs561712	0.41	G>A	Intronic	
		rs3782218	0.15	C>T	Intronic	
NOS2	Nitric oxide synthase 2	rs1137933	0.27	C>T	Coding	Asp=
		rs3794763	0.21	G>A	Intronic	
		rs3794766	0.26	C>T	Intronic	
NOS3	Nitric oxide synthase 3	rs3918226	0.09	C>T	5'-flanking	
		rs1799983	0.36	G>T	Coding	Asp298Glu
		rs3918227	0.11	C>A	3'-UTR	
		rs3730305	0.09	C>A	Intronic	
		rs753482	0.23	T>G	Intronic	
		rs7830	0.32	C>A	Intronic	
MT1X	Metallothionein 1	rs4783950	0.38	T>C	ncRNA	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
Hormonal metabolism						
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	rs11742194	0.09	C>T	Intronic	
		rs3931914	0.24	C>G	5'-flanking	
		rs3846662	0.44	T>C	Intronic	
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	rs6163	0.38	C>A	Coding	Ser=
Cyp19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	rs17601241	0.09	G>A	Intronic	
		rs4324076	0.43	C>A	Intronic	
		rs4646	0.24	C>A	3'-UTR	
		rs6493497	0.12	G>A	5'-flanking	
HSD17B3	Hydroxysteroid (17-beta) dehydrogenase 3	rs7022250	0.39	G>C	Intronic	
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2	rs523349	0.31	C>G	Coding	Leu89Val
GnRH1	Gonadotropin releasing hormone 1	rs6185	0.24	G>C	Coding	Trp16Ser
CGA	Glycoprotein hormones, alpha polypeptide	rs932742	0.30	A>G	Intronic	
FSHB	Follicle stimulating hormone, beta polypeptide	rs609896	0.46	A>G	Intronic	
FSHR	Follicle stimulating hormone receptor	rs2072488	0.26	T>C	Intronic	
		rs12473181	0.13	C>T	Intronic	
		rs3788981	0.44	G>T	Intronic	
		rs3913668	0.38	T>C	Intronic	
		rs3788982	0.13	G>A	Intronic	
		rs3913665	0.43	C>T	Intronic	
		rs1504190	0.48	C>T	Intronic	
		rs11894971	0.06	C>T	Intronic	
		rs13033004	0.25	T>C	Intronic	
		rs1394205	0.28	G>A	5'-UTR	
		rs2268363	0.17	T>C	Intronic	
ESR1	Estrogen receptor 1	rs3020331	0.44	C>T		
		rs6899458	0.26	G>A	Intronic	
		rs7761133	0.18	T>C	Intronic	
		rs12204714	0.38	T>C	Intronic	
		rs3020328	0.27	T>C	Intronic	
		rs3020432	0.32	A>G	Intronic	
		rs9322351	0.12	T>C	Intronic	
		rs1062577	0.08	T>A	3'-UTR	
		rs2228480	0.22	G>A	Coding	Thr=
		rs2234693	0.43	T>C	Intronic	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
ESR2	Estrogen receptor 2	rs2987983	0.32	T>C	Intronic	
PGR	Progesterone receptor	rs1042838	0.19	G>T	Coding	Val660Ala
SHBG	Sex hormone-binding globulin	rs13894	0.09	C>T	Coding	Arg126Cys
		rs858521	0.42	C>G	Intronic	
		rs6259	0.13	G>A	Coding	Asp356Asn
COMT	Catechol-O-methyltransferase	rs4633	0.47	T>C	Coding	His=
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	rs1056836	0.45	C>G	Coding	Leu432Val
NR5A2	Nuclear receptor subfamily 5, group A, member 2	rs2821361	0.49	G>A	Intronic	
		rs17722672	0.05	A>C	Intronic	
		rs2821367	0.33	T>C	Intronic	
		rs2821368	0.17	C>G	Coding	Pro=
		rs3828112	0.29	A>G	Intronic	
		rs2247328	0.35	A>G	Intronic	
		rs2737673	0.46	T>C	Intronic	
		rs12041297	0.14	A>C	Intronic	
		rs2737679	0.13	A>T	Intronic	
		rs2816969	0.13	T>C	Intronic	
		rs3790800	0.30	C>T	Intronic	
		rs16846145	0.06	A>G	Intronic	
		rs7556049	0.11	T>C	Intronic	
		rs2247019	0.19	C>T	Intronic	
		rs1060060	0.33	G>A	Coding	Asn=
		rs1060061	0.48	C>T	3'-UTR	
		rs2816912	0.34	C>A	3'-UTR	
NCOA1	Nuclear receptor coactivator 1	rs11125744	0.10	C>G	Coding	Thr=
		rs2289394	0.44	G>A	Intronic	
		rs3731628	0.09	A>T	Coding	Pro=
		rs17737058	0.24	C>G	3'-UTR	
NCOA3	Nuclear receptor coactivator 3	rs1537306	0.11	G>C	Intronic	
		rs2230782	0.09	G>C	Coding	Gln586His
		rs4810648	0.12	T>C	Intronic	
		rs11699879	0.27	A>G	3'-UTR	
		rs11547289	0.25	G>A	3'-UTR	
Early response						
EGR1	Early growth response 1	rs11743810	0.41	T>C	Intronic	
FOS	FBJ murine osteosarcoma viral oncogene homolog	rs2239615	0.26	A>T	5'-UTR	
		rs7101	0.26	T>C	5'-UTR	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	rs2282695	0.31	C>G	Coding	Ala=
		rs1049739	0.41	A>G	3'-UTR	
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	rs3774932	0.45	G>A	Intronic	
		rs230492	0.34	G>A	Intronic	
		rs3774964	0.34	A>G	Intronic	
ATF2	Activating transcription factor 2	rs212348	0.20	T>A	Intronic	
		rs212347	0.15	T>C	Intronic	
		rs12693057	0.08	G>A	Intronic	
MAPK1	Mitogen-activated protein kinase 1	rs7286558	0.06	C>T	Intronic	
		rs2986657	0.45	A>G	Intronic	
		rs4233292	0.10	A>G	Intronic	
		rs1982227	0.18	C>G	Intronic	
MAPK11	Mitogen-activated protein kinase 11	rs2076139	0.22	C>T	Coding	Ser=
		rs742185	0.23	G>A	Intronic	
MAPK12	Mitogen-activated protein kinase 12	rs1129880	0.32	C>T	Coding	Ser=
MAPK13	Mitogen-activated protein kinase 13	rs1059227	0.31	C>A	Coding	Thr=
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	rs8904	0.41	C>T	3'-UTR	
		rs2233419	0.19	C>T	Intronic	
		rs1050851	0.21	C>T	Coding	Ala=
		rs1957106	0.35	G>A	Coding	Asp=
		rs2233409	0.23	C>T	5'-flanking	
CHUK	Conserved helix-loop-helix ubiquitous kinase	rs11597086	0.44	A>C	Intronic	
		rs2230804	0.48	A>G	Coding	Ile268Val
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	rs2294100	0.06	T>A	Intronic	
Varia						
DDX17	DEAD (Asp-Glu-Ala-Asp) box helicase 17	rs1043402	0.29	G>A	3'-UTR	
		rs5750609	0.12	C>T	Intronic	
POLA2	Polymerase (DNA directed), alpha 2, accessory subunit	rs487989	0.11	G>A	Coding	Gly583Arg
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	rs2302599	0.15	G>T	Intronic	
PPP6C	Protein phosphatase 6, catalytic subunit	rs6744	0.49	G>A	3'-UTR	
		rs4838249	0.35	A>G	3'-UTR	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
PPARγ	Peroxisome proliferator-activated receptor gamma	rs13433696	0.25	G>A	Intronic	
		rs1801282	0.12	C>G	Coding	Pro12Ala
		rs2972162	0.47	T>C	Intronic	
		rs709158	0.35	A>G	Intronic	
		rs1175543	0.35	A>G	Intronic	
		rs3856806	0.12	C>T	Coding	His=
HSPA1A	Heat shock 70kDa protein 1A	rs1043618	0.47	G>C	5'-UTR	
HSPA1L	Heat shock 70kDa protein 1-like	rs2227956	0.18	T>C	Coding	Thr493Met
COX1	Prostaglandin-endoperoxide synthase 1	rs1213266	0.07	G>A	Intronic	
		rs5788	0.15	C>A	Coding	Gly=
COX2	Prostaglandin-endoperoxide synthase 2	rs689466	0.20	A>G	5'-flanking	
		rs20417	0.17	G>C	5'-flanking	
		rs5275	0.36	T>C	3'-UTR	
		rs5277	0.15	G>C	Coding	Val=
PTGES	Prostaglandin E synthase	rs2302821	0.08	A>C	3'-UTR	
PTGER2	Prostaglandin E receptor 2 (subtype EP2)	rs708498	0.17	G>A	Intronic	
		rs708494	0.37	T>C	5'-flanking	
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	rs493489	0.12	C>A	Intronic	
PTGER4	Prostaglandin E receptor 3 (subtype EP4)	rs11957406	0.45	A>G	Intronic	
mTOR	Mechanistic target of rapamycin	rs1135172	0.26	C>T	Coding	Asp=
		rs2295080	0.30	T>G	5'-flanking	
AKT	v-akt murine thymoma viral oncogene homolog 1	rs1130233	0.28	G>A	Coding	Glu=
		rs2494748	0.35	T>C	Intronic	
IL1A	Interleukin 1, alpha	rs2856836	0.30	T>C	3'-UTR	
		rs3783550	0.29	A>C	Intronic	
		rs3783546	0.29	G>C	Intronic	
		rs17561	0.29	G>T	Coding	Ala114Ser
		rs3783539	0.28	G>A	Intronic	
EGFR	Epidermal growth factor receptor	rs4947963	0.37	T>C	Intronic	
		rs11766798	0.29	G>A	Intronic	
		rs2302535	0.33	A>C	Intronic	
		rs11238350	0.16	T>C	Intronic	
		rs845552	0.45	A>G	Intronic	
		rs1050171	0.43	A>G	Coding	Gln=
		rs1140475	0.11	C>T	Coding	Thr=
		rs2293348	0.30	G>A	Intronic	
		rs2293347	0.11	G>A	Coding	Asp=
		rs2227983	0.27	G>A	Coding	Lys521Arg

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
ENG	Endoglin	rs16930129	0.09	C>T	Coding	Leu=
		rs10987759	0.07	T>C	5'-flanking	
ITGB2	Integrin, beta 2	rs235326	0.35	C>T	Coding	Val=
		rs2230528	0.23	G>A	Coding	Gly=
TGFB1	Transforming growth factor, beta 1	rs1800468	0.10	G>A	5'-flanking	
		rs1800469	0.28	C>T	5'-flanking	
		rs1800470	0.37	T>C	Coding	Pro10Leu
		rs1800471	0.09	G>C	Coding	Arg25Pro
		rs2241717	0.42	T>G	Intronic	
SNPs previously found associated with cancer susceptibility through GWAS analysis						
		rs3888929	0.30	G>A	Unknown	
		rs4867592	0.39	C>A	Unknown	
HAL	Histidine ammonia-lyase	rs7970524	0.25	T>C	5'-flanking	
		rs12003093	0.27	A>G	Unknown	
VDR	1,25-dihydroxyvitamin D3 receptor	rs4760658	0.34	A>G	Intronic	
EPDR1	Ependymin related protein 1	rs1376264	0.35	C>T	5'-flanking	
MRE11A	Meiotic recombination 11 homolog A	rs2155209	0.33	T>C	3'-UTR	
MRE11A		rs569143	0.44	C>G	Intronic	
GWAS Breast cancer						
CASP8	Caspase 8	rs1045485	0.14	G>C	Coding	Asp302His
IL1B	Interleukin 1, beta	rs1143634	0.24	C>T	Coding	Phe=
IL4	Interleukin 4	rs2070874	0.15	C>T	5'-UTR	
FGFR2	Fibroblast growth factor receptor 2	rs2981582	0.38	C>T	Intronic	
FGFR2		rs2981579	0.39	C>T	Intronic	
CASC16	Long intergenic non-protein coding RNA 918	rs3803662	0.23	C>T	ncRNA	
		rs889312	0.27	A>C	Unknown	
		rs13281615	0.39	A>G	Unknown	
LSP1	Lymphocyte-specific protein 1	rs3817198	0.32	T>C	Intronic	
EMBP1	Embigin pseudogene 1	rs11249433	0.40	T>C	Intronic	
RAD51B	RAD51 homolog B	rs999737	0.25	C>T	Intronic	
		rs2067980	0.14	A>G	Unknown	
STXBP4	Syntaxin binding protein 4	rs6504950	0.29	G>A	Intronic	
CCDC170	Coiled-coil domain containing 170	rs3757318	0.06	G>A	Intronic	
		rs1562430	0.43	A>G	Unknown	
TNNT3	Troponin T type 3	rs909116	0.49	T>C	Intronic	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
		rs13387042	0.46	A>G	Unknown	
CDKN2B-AS1	CDKN2B antisense RNA 1	rs1011970	0.17	G>T	Intronic	
ZMIZ1	Zinc finger, MIZ-type containing 1	rs704010	0.42	G>A	Intronic	
		rs614367	0.14	C>T	Unknown	
BABAM1	BRISC and BRCA1 A complex member 1	rs8170	0.20	C>T	Coding	Lys=
BABAM1		rs3745185	0.41	G>A	Intronic	
NR2F6	Nuclear receptor subfamily 2, group F, member 6	rs4808611	0.18	C>T	Intronic	
ANKLE1	Ankyrin repeat and LEM domain containing 1	rs2363956	0.48	T>G	Coding	Leu184Trp
GWAS Prostate cancer						
		rs2660753	0.10	C>T	Unknown	
SLC22A3	Solute carrier family 22, member 3	rs9364554	0.28	C>T	Intronic	
LMTK2	lemur tyrosine kinase 2	rs6465657	0.44	T>C	Intronic	
		rs6983267	0.42	G>T	Unknown	
LOC727677	uncharacterized LOC727677	rs1447295	0.14	C>A	Intronic	
MSMB	microseminoprotein, beta-	rs10993994	0.47	C>T	5'-flanking	
CTBP2	C-terminal binding protein 2	rs4962416	0.28	T>C	Intronic	
HNF1B	HNF1 homeobox B	rs4430796	0.46	A>G	Intronic	
HNF1B		rs11649743	0.20	G>A	Intronic	
		rs1859962	0.46	G>T	Unknown	
		rs2735839	0.15	G>A	Unknown	
CLPTM1L	CLPTM1-like	rs401681	0.47	C>T	Intronic	
JAZF1	JAZF zinc finger 1	rs10486567	0.23	G>A	Intronic	
DAB2IP	DAB2 interacting protein	rs1571801	0.25	C>A	Intronic	
EEFSEC	Eukaryotic elongation factor, selenocysteine-tRNA-specific	rs10934853	0.34	C>A	Intronic	
		rs16902094	0.17	A>G	Unknown	
		rs445114	0.31	T>C	Unknown	
		rs8102476	0.40	C>T	Unknown	
		rs11228565	0.24	G>A	Unknown	
EHBP1	EH domain binding protein 1	rs2710647	0.42	C>T	Intronic	
EHBP1		rs721048	0.20	G>A	Intronic	
		rs6545977	0.48	G>A	Unknown	
THADA	thyroid adenoma associated	rs1465618	0.25	G>A	Intronic	
PDLIM5	PDZ and LIM domain 5	rs17021918	0.35	C>T	Intronic	
PDLIM5		rs12500426	0.47	C>A	Intronic	

Gene	Description	rs number	MAF	Allele substitution	Genomic location	Amino acid substitution
		rs7679673	0.35	C>A	Unknown	
		rs1512268	0.48	G>A	Unknown	
		rs5759167	0.48	G>T	Unknown	
		rs10086908	0.32	T>C	Unknown	
		rs620861	0.30	C>T	Unknown	
		rs4242382	0.14	G>A	Unknown	
		rs7841060	0.24	T>G	Unknown	
		rs12543663	0.30	A>C	Unknown	
		rs1016343	0.25	C>T	Unknown	
		rs13252298	0.27	A>G	Unknown	
		rs4871008	0.40	C>T	Unknown	
		rs6470494	0.31	C>T	Unknown	
		rs10090154	0.14	C>T	Unknown	

Table e3: Overview of the 29 patient and treatment related parameters.

Patient data (n=9)	**Treatment parameters (n=20)**
Pre-therapy symptoms	Lymph node dissection
(RT-induced acute symptoms)	Radical prostatectomy
Age	Gleason score
Diabetes	Adjuvant androgen deprivation
Hypertension	B10 (%)
Smoking	B20 (%)
Haemorrhoids	B30 (%)
Previous surgery	B40 (%)
Crohn disease or irritable bowel disease	B50 (%)
Transurethral resection of the prostate	B60 (%)
	B65 (%)
	B70 (%)
	B75 (%)
	Bladder Mean (Gy)
	Bladder Max (Gy)
	Bladder Volume (cc)
	CTV Min (Gy)
	CTV Mean (Gy)
	CTV Max (Gy)
	CTV Volume (cc)

Abbreviations: RT = radiotherapy; PSA = prostate-specific antigen; BX = percentage of the bladder volume receiving X Gy or more; CTV = clinical target volume.

Table e4: Outcome of modeling analysis for late haematuria.

	Complete model	**Non-SNP model**
Model Predictors	Coefficients	
(Intercept)	-3.32	-3.67
Bladder Vol 75 (cc)	0.44	0.40
*HMGRC*_rs3931914_CG	1.67	-
*NOS1*_rs2293054_GA	-1.46	-
*PTGER2*_rs708498_GG	-1.42	-
*EGFR*_rs845552_AG	0.95	-
TUR	1.20	1.43

Table e5: Outcome of modeling analysis for late nocturia.

	Complete model	**Non-SNP model**
Model Predictors	Coefficients	
(Intercept)	-1.34	-2.21
CTV Volume (cc)	0.36	0.31
CTV Min (Gy)	0.27	0.36
*NOS3*_rs1799983_GT	-1.67	-
*CASP8*_rs1045485_GG	-1.30	-
*NR2F6*_rs4808611_CT	1.14	-

For both models: Model expression: $\ln p/(1-p) = \beta_0 + \beta_1*x_1 + \beta_2*x_2 + \beta_3*x_3 + \beta_4*x_4 + \beta_5*x_5 + \beta_6*x_6 + \ldots$ with p=probability to develop severe late haematuria/nocturia, β=coefficient, x=variable/parameter/predictor and $e^{\beta 1}$=increase in odds for predictor 1 (in the case of continuous variables: for an increase of the predictor by 1) when the other predictors remain unchanged.

8 Paper III

Factors modifying the risk for developing acute skin toxicity after whole-breast intensity modulated radiotherapy

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ABSTRACT

Background: After breast-conserving radiation therapy most patients experience acute skin toxicity to some degree. This may impair patients' quality of life, cause pain and discomfort. In this study, we investigated treatment and patient-related factors, including genetic polymorphisms, that can modify the risk for severe radiation-induced skin toxicity in breast cancer patients.

Methods: We studied 377 patients treated at Ghent University Hospital and at Clinic and Maternity Sainte-Elisabeth in Namur, with adjuvant intensity modulated radiotherapy (IMRT) after breast-conserving surgery for breast cancer. Women were treated in a prone or supine position with normofractionated (25x2 Gy) or hypofractionated (15x2.67 Gy) IMRT alone or in combination with other adjuvant therapies. Patient- and treatment-related factors and genetic markers in regulatory regions of radioresponsive genes and in *LIG3*, *MLH1* and *XRCC3* genes were considered as variables. Acute dermatitis was scored using the CTCAEv3.0 scoring system. Desquamation was scored separately on a 3-point scale (0-none, 1-dry, 2-moist).

Results: Two-hundred and twenty patients (58%) developed G2+ dermatitis whereas moist desquamation occurred in 56 patients (15%). Normofractionation (both $p<0.001$), high body mass index (BMI) ($p=0.003$ and $p<0.001$), bra cup size ≥D ($p=0.001$ and $p=0.043$) and concurrent hormone therapy ($p=0.001$ and $p=0.037$) were significantly associated with occurrence of acute dermatitis and moist desquamation, respectively. Additional factors associated with an increased risk of acute dermatitis were the genetic variation in *MLH1* rs1800734, smoking during RT ($p=0.008$) and supine IMRT ($p=0.004$). Patients receiving trastuzumab showed decreased risk of acute dermatitis ($p<0.001$).

Conclusions: The normofractionation schedule, supine IMRT, concomitant hormone treatment and patient related factors (high BMI, large breast, smoking during treatment and the genetic variation in *MLH1* rs1800734) were associated with increased acute skin toxicity in patients receiving radiation therapy after breast-conserving surgery. Trastuzumab seemed to be protective.

BACKGROUND

Breast-conserving therapy with the adjuvant use of radiotherapy (RT) has gained an established role in the treatment for early-stage breast cancer with excellent long-term local control and survival (1). During or shortly after the course of breast cancer RT, a large portion of the patients will experience acute radiation dermatitis to some degree, varying from mild to brisk erythema with or without moist desquamation and occasionally ulceration of the skin (2). There is accumulating clinical evidence that acute reactions are associated with the development of late toxicity: Lilla et al. showed that telangiectasia are in fact late sequelae of moist desquamation and acute erythema is shown to be a risk factor for poor cosmetic outcome (3-5). Though the skin is not a dose-limiting tissue, skin toxicity is associated with impairment of patients' quality of life, causes pain and discomfort and limits activities (2, 6). The challenge is to minimize these side effects without losing efficacy of the treatment.

Over the years, many attempts have been made to reduce the number of patients experiencing acute skin toxicity and inferior cosmetic outcome by introducing improved radiation techniques, such as intensity-modulated radiotherapy (IMRT). This technique has been shown to be superior over conventional wedge-based whole breast irradiation by delivering a more homogenous dose through the breast and removing the radiation hot spots; it results in an approximately 20% reduction of the frequency of moist desquamation (6, 7). Large breast size significantly contributes to dose inhomogeneity, hot spots and toxicity (7, 8). The variation in clinical response is, however, only partly explained by treatment factors such as radiation dose, fractionation scheme, and concomitant therapies. Patient-related features (e.g. bra cup size and body mass index (BMI)) also play a role together with an unknown contribution from genetic factors. Up to now there are no data available to estimate directly the heritability of clinical radiosensitivity based upon family history of radiotherapy toxicity, but it is likely to be somewhat lower than for chromosomal and cellular radiosensitivity, which have been calculated to be 58-78% (9).

Acute toxicity is initiated by depletion of acutely responding epithelial tissues and damage to microvessels (10). Numerous studies have reported on genetic

variations modifying the clinical radiosensitivity risk, predominantly in pathways based on mechanistic understanding of the radiation pathogenesis (reviewed in (11)). In the present study, single nucleotide polymorphisms (SNPs) in genes involved in major DNA repair pathways (*LIG3*, *XRCC3, MLH1*) and in regulatory regions that influence the expression levels of radioresponsive genes are considered (12-16).

To gain a better insight into the development of radiation-induced dermatitis and moist desquamation, we evaluated the association between patient and treatment features with these endpoints. The association between SNPs and the different clinical endpoints was also studied.

METHODS

The study population consists of 377 breast cancer patients treated with adjuvant IMRT with curative intent after breast-conserving surgery (stage T1-3, N0-1, M0). Of them, 282 breast cancer patients were treated at the Ghent University Hospital (GUH) and 95 patients were treated at Clinique and Maternité Sainte-Elisabeth (CMSE) in Namur. All patients had a follow-up of at least 3 months after RT.

At GUH, patients were treated in prone or supine position using a multi-beam IMRT technique in supine position and a tangential 2-beam field-in-field IMRT technique in prone position as described previously (17). The whole breast was treated with hypofractionated radiotherapy (40.05 Gy in 15 fractions (18)) with 6-MV photons of an Elekta Synergy linear accelerator (Crawley, United Kingdom). An additional photon boost of 10 Gy in 4 fractions to the tumour bed was given to 75% of the patients. For the prone patient setup, a unilateral breast holder (Van De Velde, Schellebelle, Belgium) and a prone breast board (Orfit Industries) were used (19). Twenty-two patients were treated in prone position with voluntary moderate deep inspiration breath hold. At CMSE Namur, a sliding window tangential field-IMRT technique was used associated with moderate deep inspiration breath hold whenever the primary beam intersected the heart as previously described by Remouchamps et al (20). Patients with bra cup size ≥D received normofractionated radiotherapy (50.00 Gy in 25 fractions), women with bra cup size <D received hypofractionation or normofractionation according to the preference of the

radiation oncologist (n=28). More than 90% received an additional boost of 10 Gy in 4 fractions with electron beams.

Adjuvant systemic therapy

Adjuvant hormone therapy, consisting of tamoxifen or aromatase inhibitors, was administered in most patients concomitantly with IMRT. The others received hormone therapy sequentially after IMRT. Patients who received adjuvant chemotherapy, combination of antracyclines and taxanes, completed chemotherapy before IMRT, while trastuzumab was allowed concomitantly with IMRT.

Data collection

Data on patients' medical history, tumor and treatment characteristics were collected prospectively. Table 1 gives an overview of the patient characteristics for patients treated at GUH and CMSE Namur.

Acute toxicity was assessed weekly during treatment and at 1-2 weeks after treatment. The reported toxicity represents the maximal reported acute toxicity, either during or after completion of IMRT. Acute dermatitis was documented according to a standard protocol using the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 scoring system. This grades patients with mild erythema or dry desquamation as 1, moderate to brisk erythema or patchy moist desquamation mostly confined to the skin folds as 2 and confluent moist desquamation as 3. Desquamation was scored separately on a 3-point scale (0-none, 1-dry, 2-moist). Grade 2-3 toxicity was considered clinically relevant and was included in the analysis. Genomic DNA was isolated from a fresh blood sample taken before start of radiotherapy, using the Puregene genomic DNA purification kit (Gentra Systems, Minneapolis, MN). The study was approved by the local ethics committees and all study patients provided written informed consent.

Table 1: Patient characteristics for patients treated at GUH and CMSE Namur.

Patient/clinical factor		GUH (n=282)	CMSE Namur (n=95)
Age (years)			
Median		57.5	59,0
Range		30-82	35-82
Bra cup size			
Small	A	13 (4.6)	3 (3.2)
	B	85 (30.2)	33 (34.7)
	C	101 (35.8)	34 (35.8)
Large	D	53 (18.8)	16 (16.8)
	E	16 (5.7)	5 (5.3)
	F	7 (2.5)	3 (3.2)
	G + H	2 (0.6)	1 (1.0)
	Missing	*5*	*0*
BMI			
Median		25.5	26
Range		16-50	16-38
Missing		2	0
Menstruation			
No		235 (83.3)	76 (80.0)
Yes		45 (16.0)	18 (18.9)
Missing		*2*	*1*
Smoking during RT			
No		244 (86.5)	79 (83.2)
Yes		35 (12.4)	16 (16.8)
Missing		*3*	*0*
Diabetes			
No		254 (90.1)	84 (88.4)
Yes		22 (7.8)	11 (11.6)
Missing		*6*	*0*
Hypertension			
No		196 (69.5)	66 (69.5)
Yes		81 (28.7)	29 (30.5)
Missing		*5*	*0*
Fractionation			
Normo		0	45 (47.4)
Hypo		282	50 (52.6)
Missing		*0*	*0*
Treatment position			
Supine		195 (69.1)	95 (100.0)
Prone		87 (30.9)	0
Missing		*0*	*0*

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Table 1 *(continued)*

Patient/clinical factor	GUH (n=282)	CMSE Namur (n=95)
Boost		
No	64 (22.7)	7 (7.4)
Yes	218 (77.3)	88 (92.6)
Missing	*0*	*0*
Nodal irradiation		
No	241 (85.5)	87 (80.6)
Yes	41 (14.5)	21 (19.4)
Missing	*0*	*0*
Hormonal therapy		
No	46 (16.3)	25 (26.3)
Concomitant	236 (83.7)	7 (7.4)
Sequential (after IMRT)	0	63 (66.3)
Missing	*0*	*0*
Chemotherapy		
No	188 (66.7)	55 (57.9)
Yes	94 (33.3)	40 (42.1)
Missing	*0*	*0*
Trastuzumab		
No	257 (91.1)	83 (87.4)
Yes	25 (8.9)	12 (12.6)
Missing	*0*	*0*

Abbreviations: GUH = Ghent University Hospital; CMSE = Clinic Maternity Sainte-Elisabeth; BMI = Body Mass Index.
Data are given as no. (%) unless otherwise indicated.

Selection of candidate genes/polymorphisms and genotyping

Eight candidate polymorphisms were selected for genotyping (Table 2). Of these, five SNPs (rs3888929, rs4867592, rs7970524, rs12003093, rs4760658) were chosen as they putatively affect the expression levels of radiation-responsive genes directly, or by *trans* effects, based on genetic linkage and association analysis as described previously by Smirnov et al.. The authors suggested that those regulatory variants might be able to contribute to the development of genetic tools for radiosensitivity (16). The other SNPs were chosen based on their previous association with toxicity induced by radiotherapy or methylating agents (*XRCC3* rs861539, *LIG3* rs3744355, *MLH1* rs1800734) (12-15). Genotyping was performed using restriction fragment length polymorphism analyses, high resolution melting curve analyses, single

base extension techniques or direct sequencing. For reproducibility control, 15% of all samples were duplicated. The concordance rate between duplicate samples was 100%. Primers details are available on request. Tests for deviation from Hardy-Weinberg equilibrium, for the entire sample showed that the rs4867592 SNP had a p-value <0.0001 and was excluded from further analyses.

Table 2: Characteristics of the SNPs.

Gene or gene regulator	rs number	MAF*	Nucleotide substitution	Genomic location	Amino acid substitution	Ref.
LIG3	rs3744355	9.1	G>C	5'-flanking	-	[12, 13]
MLH1	rs1800734	22.6	G>A	5'-UTR	-	[14]
XRCC3	rs861539	39.0	C>T	Coding	Thr241Met	[15]
PHLDA3	rs3888929	30.3	G>A	Unknown	-	
LCP2	rs4867592	19.1	C>A	Unknown	-	
LTHA4	rs7970524	25.1	T>C	5'-flanking	-	[16]
NDUFB6	rs12003093	23.4	A>G	Unknown	-	
VDR	rs4760658	36.6	A>G	Intronic	-	

Abbreviations: Minor allele frequency in Caucasian population.

Statistical analysis

The studied endpoints were development of acute radiation-induced dermatitis (CTCAE G2+) and moist desquamation. For the clinical association analysis, univariate analysis was initially carried out to assess the relationship between patient- (age, bra cup size (A+B+C vs. ≥D), BMI, menstruation, smoking during RT, diabetes, hypertension) and treatment-related factors (fractionation scheme, treatment position, boost dose to tumour bed, nodal irradiation, hormone therapy, chemotherapy and trastuzumab) and the endpoints. Patients with and without G2+ acute skin toxicity were compared by means of the Mann-Whitney test for continuous variables and the χ^2-test for categorical variables. Power calculations were performed with Power for Genetic Association analyses (21). For these we took into account: the incidence of dermatitis (58%) or moist desquamation (15%) observed in our cohort, the lowest minor allele frequency (9%) of the considered SNPs, a probability adjusted by the number of SNPs ($\alpha=6.25 \times 10^{-3}$) under a dominant genotypic test, and a genotype relative risk of ≥1.5. This resulted in a power of 94.3% for acute dermatitis and 60.9%

for moist desquamation. To assess the independent effect of each polymorphism, unconditional logistic regression analyses were performed to calculate crude ORs. The Benjamini-Hochberg (BH) procedure was used to control for multiple testing (i.e. 43 tests per endpoint: 28 genetic and 15 clinical parameter tests) to reduce the risk of finding false-positive associations. Variables with $p<0.05$ were tested in a multivariate logistic regression analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). R library *multtest* (http://www.r-project.org/) was used to perform the multiple testing analyses.

RESULTS

Acute radiation-induced skin toxicity data were available for all 377 patients. Two-hundred twenty patients (58%) developed G2+ dermatitis. The occurrence of dermatitis did not differ between both centres (GUH: 57% (162/282), CSME: 61% (58/95)). Moist desquamation (patchy or confluent) occurred in 56 patients (15%) and differed between both centres: 10% of the patients treated at GUH and 30% of the patients treated at CMSE ($p<0.001$).

Table 3: Associations between patient- and therapy-related characteristics and acute G2+ dermatitis.

Patient/clinical factor	All (n=377)	G0-1 (n=157)	G2+ (n=220)	P-value	P_{BH}-value
Bra cup size					
A+B+C	269 (71.4)	130 (48.3)	139 (51.7)		
≥D	103 (27.3)	26 (25.2)	77 (74.8)	<0.001	0.001
BMI					
Median	26	24	26		
Range	16-50	16-37	16-50	<0.001	0.001
Smoking during RT					
No	323 (85.7)	141 (43.7)	182 (56.3)		
Yes	51 (13.5)	14 (27.5)	37 (72.5)	0.029	0.156
Fractionation					
Normo	45 (11.9)	6 (13.3)	39 (86.7)		
Hypo	332 (88.1)	151 (45.5)	181 (54.5)	<0.001	<0.001
Treatment position					
Supine	290 (76.9)	108 (37.2)	182 (62.8)		
Prone	87 (23.1)	49 (56.3)	38 (43.7)	0.002	0.019

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Table 3 *(continued)*

Patient/clinical factor	All (n=377)	G0-1 (n=157)	G2+ (n=220)	P-value	P_{BH}-value
Nodal irradiation					
No	315 (83.6)	141 (44.8)	174 (55.2)		
Yes	62 (16.4)	16 (25.8)	46 (74.2)	0.006	0.037
Hormonal therapy					
No	71 (18.8)	39 (54.9)	32 (45.1)		
Concomitant	243 (64.5)	94 (38.7)	149 (61.3)		
Sequential (after IMRT)	63 (16.7)	24 (38.1)	39 (61.9)	0.041	0.207
Hormones (concomitant)					
Tamoxifen	*155*	*62 (40.0)*	*93 (60.0)*		
Aromatase inhibitor	*85*	*32 (37.6)*	*53 (62.4)*		
Chemotherapy					
No	243 (64.5)	92 (37.9)	151 (62.1)		
Yes	134 (35.5)	65 (48.5)	69 (51.5)	0.045	0.215
Trastuzumab					
No	340 (90.2)	133 (39.1)	207 (60.9)		
Yes	37 (9.8)	24 (64.9)	13 (35.1)	0.003	0.026

Abbreviations: G = CTCAEv.3 grade; BMI = Body Mass Index; p_{BH} = corrected p-value by Benjamini-Hochberg procedure.
Data are given as no. (%) unless otherwise indicated.

Acute radiation-induced skin toxicity

Table 3 depicts the parameters associated with acute G2+ dermatitis, in univariate analysis. Bra cup size ≥D (p<0.001), BMI (p<0.001) and smoking during RT (p=0.029) were associated with the development of G2+ dermatitis. Irradiation of the nodal region (p=0.006) and the use of concomitant hormone therapy (p=0.041) were also associated with an increased risk of acute dermatitis, with no difference in incidence between aromatase-inhibitors and tamoxifen. In contrast, patients receiving trastuzumab or having received chemotherapy seem to be less prone to the development of RT-induced acute dermatitis (p=0.003 and p=0.045, respectively). Furthermore, patients treated with hypofractionated radiotherapy develop less dermatitis when compared to patients treated in the normofractionated regimen (p<0.001). And, patients treated in prone position developed less dermatitis than patients treated supine (p=0.002). In multivariate analysis, chemotherapy and nodal irradiation were no longer significant (Table 4).

Table 4: Multivariate analysis for G2+ dermatitis and moist desquamation.

Clinical/genetic factor	Acute G2+ dermatitis		Moist desquamation	
	OR	P-value	OR	P-value
Center (CMSE vs. GUH)	-	-	3.206	0.158
BMI	1.088	0.003	1.170	<0.001
Bra cup size (cup ≥D vs. cup A+B+C)	2.833	0.001	2.146	0.043
Smoking (yes vs. no)	2.711	0.010	-	-
Fractionation (hypo vs. normo)	0.083	<0.001	0.096	<0.001
Treatment position (prone vs. supine)	0.399	0.004	0.373	0.074
Hormone therapy				
No	1		1	
Concomitant	3.207	0.001	4.770	0.037
Sequential (after IMRT)	1.003	0.994	1.078	0.901
Nodal irradiation (yes vs. no)	1.975	0.100	-	-
Chemotherapy (yes vs. no)	0.954	0.877	-	-
Trastuzumab (yes vs. no)	0.177	<0.001	-	-
MLH1 rs1800734 G>A				
GG	1		-	
GA	0.492	0.008	-	-
AA	0.537	0.232	-	-

Abbreviations: GUH = Ghent University Hospital; CMSE = Clinic Maternity Sainte-Elisabeth; BMI = Body Mass Index; MLH1 = MutL protein homolog 1

For moist desquamation, univariate significant associations were found with bra cup size ≥D (p<0.001), BMI (p<0.001), normofractionation (p<0.001), supine positioning (p=0.002), concurrent hormone therapy (p=0.004) and CSME center (p<0.001) (Table 5). In multivariate analysis (Table 6), bra cup size ≥D, BMI, fractionation and hormone therapy remained statistically significant. Treatment center was no longer significantly associated with moist desquamation due to the fact that the normofractionated schedule was only prescribed at CMSE.

Table 5: Associations between patient- and therapy-related characteristics and moist desquamation.

Patient/clinical factor	All (n=377)	No (n=321)	Yes (n=56)	P-value	P_{BH}-value
Bra cup size					
A+B+C	269 (71.4)	242 (90.0)	27 (10.0)		
≥D	103 (27.3)	76 (73.8)	27 (26.2)	<0.001	0.001
BMI					
Median	26	25	29		
Range	16-50	16-40	21-50	<0.001	<0.001
Fractionation					
Normo	45 (11.9)	22 (48.9)	23 (51.1)		
Hypo	332 (88.1)	299 (90.1)	33 (9.9)	<0.001	<0.001

continued on next page

Table 5 (*continued*)

Patient/clinical factor	All (n=377)	No (n=321)	Yes (n=56)	P-value	P_{BH}-value
Treatment position					
Supine	290 (76.9)	239 (82.4)	51 (17.6)		
Prone	87 (23.1)	82 (94.3)	5 (5.7)	0.002	0.019
Hormonal therapy					
No	71 (18.8)	62 (87.3)	9 (12.7)		
Concomitant	243 (64.5)	214 (88.1)	29 (11.9)		
Sequential (after IMRT)	63 (16.7)	45 (71.4)	18 (28.6)	0.004	0.029
Hormones (concomitant)					
Tamoxifen	*155*	*139 (89.7)*	*16 (10.3)*		
Aromatase inhibitor	*85*	*74 (87.1)*	*11 (12.9)*		

Abbreviations: GUH = Ghent University Hospital; CMSE = Clinic Maternity Sainte-Elisabeth; BMI = Body Mass Index; p_{BH} = corrected p-value by Benjamini-Hochberg procedure.
Data are given as no. (%) unless otherwise indicated.

Genetic analysis

The only significant p-value, in univariate analysis, was for acute radiation-induced dermatitis with the GA genotype of rs1800734 in the *MLH1* gene with a BH-adjusted p-value of 0.029 (Table 6). Adjusting for above mentioned factors by multivariate regression analysis had no effect on the statistically significant association. None of the other SNPs had any effect on the risk of acute skin toxicity.

Table 6: Effect of *MLH1* rs1800734 on radiotherapy acute skin reactions.

	Acute G2+ dermatitis					Moist desquamation				
	G0-1 (n=157)	G2+ (n=220)	OR	P-value	P_{BH}-value	No (n=321)	*Yes* (n=95)	OR	P-value	P_{BH}-value
MLH1 rs1800734 G>A										
GG	81 (51.6)	146 (66.4)				189 (58.9)	38 (67.9)			
GA	64 (40.8)	60 (27.3)	0.52	0.004	0.029	110 (34.3)	14 (25.0)	0.63	0.172	0.477
AA	9 (5.7)	12 (5.5)	0.74	0.514	0.804	17 (5.3)	4 (7.1)	1.17	0.788	0.915
Missing	*3 (1.9)*	*2 (0.9)*				*5 (1.6)*	*0*			
GG vs. GA+AA (dominant)			0.55	0.005	0.033			0.71	0.257	0.575
GG+GA vs. AA (recessive)			0.94	0.889	0.936			1.35	0.600	0.860

Abbreviations: MLH1 = MutL protein homolog 1, p_{BH} = corrected p-value by Benjamini-Hochberg procedure.
Data are given as no. (%) unless otherwise indicated.

DISCUSSION

This study was performed to analyze the influence of treatment and patient-related factors on the development of acute radiation-induced skin toxicity. Bra cup size, BMI, smoking, treatment position, choice of RT schedule and the administration of adjuvant therapies seem to contribute to the variability in radiation skin toxicity. Also, the *MLH1* rs1800734 SNP was found to be significantly associated with the development of acute dermatitis.

Our data support the hypothesis that acute toxicity does not increase with moderate hypofractionation (22). In fact, the occurrence of acute skin toxicity was significantly higher among patients treated with normofractionation compared to the hypofractionated schedule. There are only few reports studying hypofractionation in overweighed or large-breasted patients (23, 24). We observe a 20% decrease in dermatitis and an even larger decrease (70%) in moist desquamation in large-breasted patients treated in supine position with hypofractionation compared to normofractionation (see supplementary table e1). Bra cup size and BMI were also confirmed as significant risk factors for the development of acute skin toxicity, in accordance with the majority of published reports (7, 8, 25-27). Both are measures of breast volume as BMI was previously found to be strongly correlated with breast volume (27). The association between larger breast volume and toxicity is thought to be due to dose inhomogeneity, high dose regions, and the bolus effect in the inframammary and axillary regions (8). Due to the unavailability of dose homogeneity and hot spot data for the complete dataset, we were unable to test this for the total patient population, but the hypothesis is confirmed in a subset of the population (19). Goldsmith et al. show that dose inhomogeneity is insufficient to explain the association and other factors like the presence of more adipose tissue might also play a role (25). In prone position, the skin creases disappear, dose homogeneity is improved and hot spots are reduced leading to a reduction in acute skin toxicity (17). In this study, we found a decrease in radiodermatitis and moist desquamation in patients treated with prone-IMRT. Especially patients with large breast sizes are expected to have a great benefit from prone-IMRT as shown by Mulliez et al. (19).

In this study, two types of adjuvant hormone therapy, tamoxifen or aromatase-inhibitors, were concurrently administered with radiotherapy to hormone receptor positive breast cancer patients. Present data show that use of hormone therapy is, regardless the type, associated with an increase in radiation-induced dermatitis. This is in accordance with a previous study investigating the effect of tamoxifen on acute skin reactions (26). But in contrary with the COHORT randomized trial, that shows no difference between concurrent and sequential administration of letrozole; the latter was administered 3 weeks after RT when it is supposed that the radiosensitising effect of endocrine therapy is minimal (28). Concurrent administration of trastuzumab and IMRT was found to be associated with lower rates of acute dermatitis in the present study. This finding needs to be put in perspective as it is in contradiction with the observation of a large randomized study that could not find a difference in acute toxicity (29). Longer follow-up will be necessary to observe the effect of concurrent administration on cardiac toxicity.

Our study shows an association between the *MLH1* rs1800734 SNP and lower rates of acute radiation-induced dermatitis: heterozygotes are less present in the G2+ dermatitis group. The SNP maps 93 base pairs upstream of the *MLH1* transcription site in the core promoter, a region essential for maximum transcriptional activity (30). The SNP was previously shown to be associated with acute myeloid leukemia after methylating chemotherapy for Hodgkin disease (15). *MLH1* gene encodes MutL protein homolog 1 which is involved in DNA mismatch repair. Suga et al. found statistically significant associations with rs3744355 in the 5' flanking region of the *LIG3* gene and acute radiation-induced skin reactions in the Japanese population and Murray et al. provided replicated evidence for this association in a European Caucasian population (12, 13). We, however, could not confirm this association. Smirnov et al. hypothesized that regulatory variants might be able to contribute to the development of genetic tools to predict for radiosensitivity (16). This could not be demonstrated in our study population.

Radiation-induced dermatitis includes erythema, edema, dry and moist desquamation as symptoms of inflammation probably triggered by cell death (31). One of the shortcomings in this study is the fact that erythema was not

measured objectively with a colorimeter. As the CTCAE criteria are based on subjective scoring, the difference between mild, moderate and brisk erythema is observer-dependent. This probably explains the large number of patients developing G2+ acute dermatitis when compared to other reports. A strength of our investigation is the nearly complete data set for a relatively large number of patients enrolled. Furthermore, patient recruitment as well as clinical outcome data collection were carried out prospectively. Although the associations hold after correcting for multiple testing, the results of this study should be validated in an independent study.

CONCLUSION

A number of treatment and patient related factors are identified that modify the risk for the development of acute skin toxicity after whole-breast IMRT. Large bra cup, BMI, normofractionation and concomitant hormone therapy contribute to the development of moist desquamation. Patient related factors (high BMI, large breast, smoking during treatment and the genetic variation *MLH1* rs1800734), choice of RT schedule and the administration of adjuvant therapies affect the development of radiodermatitis.

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COMPETING INTERESTS

The author(s) declare that they have no competing interests

AUTHORS' CONTRIBUTIONS

SDL participated in conception and design, the acquisition, performed the statistical analysis and interpretation of the data and drafted the manuscript. TM, LV, VR, AVG, MG and WDN participated in acquisition of the data. EDS carried out the genotyping work and helped in the statistical analysis. KDR,

WDN and HT participated in the conception and design. All authors critically revised the manuscript and approved the final manuscript

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SUPPLEMENTARY MATERIAL

Table e1: Toxicity in large-breasted patients (cup ≥D) treated in supine position for the different fractionation schedules.

	Acute G2+ dermatitis			Moist desquamation		
	G0-1 (n=10)	G2+ (n=59)	P-value	No (n=46)	*Yes* (n=23)	P-value
Fractionation						
Normo	0	17 (100.0)		5	12 (70.6)	
Hypo	10	42 (79.2)	0.058	41	11 (20.8)	<0.001

Part III

General discussion

9 Challenges in radiation toxicity research

In this section, different issues will be discussed based on the hurdles that we got over during current PhD project and these will need to be taken into consideration in future research in the field.

Predicting the risk to develop normal tissue toxicity has been referred to as ‘the Holy Grail of radiobiology’ (1). Two approaches can be applied for risk stratification: the individual and the population approach (2). The individual approach identifies individuals at high risk for whom an alternative to the conventional treatment can prevent the development of toxicity or, conversely, identifies the patients at low risk for whom intensification of treatment is a possibility. The population approach focuses on identifying the underlying cause of treatment-related toxicity and provides a generalized intervention that shifts the whole risk distribution at the population level (3). The different characteristics of prediction (individual approach) and association (population approach) are described in the first section.

Because of the lack of standardising the assessment of radiation-induced toxicity, multiple symptoms are recorded by multiple scoring systems. In the second section, possible approaches are described that handle this kind of information. Adverse events are usually scored as a graded response but analysed as a binary endpoint, with potential loss of information. In this section, special interest goes to the prediction of an ordinal response which is illustrated by the prediction of acute dermatitis.

The first generation of GWASs provided valuable insights into the genetic basis of human traits and diseases with common variants conferring small increments in risk (4). In the third section, the predictive value of these variants under the form of polygenic risk profiles is illustrated in other traits and diseases with a longer history of genetic research, like height and cancer susceptibility. Then, an overview is given of the progress made in the radiogenomics field.

9.1 Association versus prediction

This PhD dissertation includes association models for severe acute radiation-induced nocturia in prostate cancer patients (chapter 6) and for severe acute radiodermatitis and moist desquamation in breast cancer patients (chapter 8). It also includes prediction models for late radiation-induced nocturia and haematuria in prostate cancer patients (chapter 7). Such association and prediction models are different in their objectives, their measurements, and their applicability in clinical context. Association studies aim to identify aetiological associations of factors with a disease and may be an indication for potential interventions for preventing or treating the disease (5). Prediction studies, on the other hand, are applied to evaluate factors in making individual clinical decisions. The performance measures of association studies, odds ratio (OR), relative risk or correlation coefficient, are related to statements made at population level, but do not apply in decision making; a strong association between a factor and disease is usually not sufficient to adequately discriminate individuals between different outcomes (5, 6). The performance of prediction models should be assessed on three fundamental levels: (i) discrimination, reflecting the ability to discriminate between different outcomes, can be quantified by measures as sensitivity, specificity and area under the receiver operating characteristic curve (AUC) (or concordance statistic), (ii) calibration, reflects how close the predictions are similar to the actual risk and (iii) clinical usefulness, by quantifying the harms and benefits of the alternative leading to an optimal decision threshold (as discussed in chapter 7) (7, 8). A good prediction model is a model that includes the smallest number of factors while preserving predictive value.

Many studies (9-12) use the standard back- or forward stepwise procedure for predictor selection. This method sequentially introduces predictors into the model and makes a judgement solely based on p-values. This is in contrast to the method applied in this PhD dissertation. Here, we performed the Lasso method that is based on effect sizes by imposing penalties to the regression coefficients. The Lasso procedure is particularly useful when a large amount of predictors is considered. It is a logistic regression method that includes only a

subset of predictors into the model, setting the coefficients of the variables with negligible effects to zero (13). In this PhD research, models are developed to predict urinary toxicity in prostate cancer patients. The models contained dosimetric and clinical variables and genetic markers and resulted in acceptable predictive performance (results see chapter 7). A comparable approach was previously performed to predict esophagitis in lung cancer patients (including chemotherapy treatment, lymph node stage, mean esophageal dose, gender, overall treatment time, RT technique and four polymorphisms), dysphagia (including concurrent chemotherapy, dose delivered to 2% of the superior pharyngeal constrictor muscle and one polymorphism) and xerostomia (including age, mean dose to contralateral parotid glands, to ipsilateral parotid gland and to contralateral submandibular gland, volume of contralateral submandibular gland and baseline xerostomia score) in head-neck cancer patients (14-16). The Lasso method is the recommended approach for normal tissue complication modelling over stepwise selection and Bayesian model averaging due to its better predictive power (16).

Other modelling methods to obtain an individualised estimation of the risk of toxicity including dosimetric and clinical factors, involve machine learning techniques like artificial neural networks, support vector machines or random forest model (17, 18).

A totally different approach to predict normal tissue toxicity includes the radiobiological NTCP models; they calculate the probability that a certain percentage of patients will experience adverse reactions. These models convert dose inhomogeneity within an organ at risk to the equivalent uniform dose (EUD), incorporating information from the entire dose-volume histogram (DVH) (19). In the previous approach single dose-volume points derived from the DVHs are included. Recently, NTCP models with inclusion of known clinical risk factors and genetic data have been developed. Two studies investigated late rectal toxicity in prostate cancer patients: DeFraene *et al.* published results on NTCP models on rectal bleeding (including abdominal surgery and cardiovascular disease), late faecal incontinence (including abdominal surgery and diabetes) and on high stool frequency (including baseline stool frequency) (20) and Rancati *et al.* developed models for late rectal bleeding (including

abdominal surgery and acute toxicity), severe chronic faecal incontinence and mean faecal incontinence (both including disease of the colon or abdominal surgery) (21). One study developed an NTCP model for radiation pneumonitis in lung cancer patients including mean lung dose, smoking status and four polymorphisms (22). For GU toxicity, clinically useful prediction tools have not yet been developed. Several studies have, however, defined dose constraints to limit late GU toxicity (23-26). As these models can be used as a guidance tool in clinical practice, they are more indicative for average trends rather than outcomes on individual patients (19).

9.2 Normal tissue toxicity phenotype

In radiation oncology, the search for (bio)markers associated with or predictive for radiation-induced toxicity is impeded by the use of multiple and diverse endpoints from a variety of scoring systems (27). First, multiple individual symptoms of a specific organ, are merged into a single grade (28, 29). For this PhD research, we chose to study the individual urinary symptoms, with nocturia being the most prevalent, as the aetiology of these different symptoms is unknown and probably different from each other (30). We showed that aggregation of multiple symptoms is associated with a loss of specificity and statistical resolution (chapter 6). This approach is supported by Bentzen *et al.* who demonstrate that there is no association between the late reactions fibrosis and telangiectasia in breast cancer patients and, because of their difference in aetiology, they recommend to analyse them as two separate endpoints (31). Second, miscellaneous scoring systems impair between-study comparisons and pooling of data. These problems can be anticipated by the development of a novel metric like the Standardized Total Average Toxicity (STAT) score, measuring the overall clinical radiosensitivity (27). Another possible direction is the investigation of one type of normal tissue reaction, like fibrosis, assessed in different patient cohorts, instead of examining different normal tissue reactions in patients treated for the same disease (32).

Normal tissue reactions can be ordered into a spectrum ranging from simple biological endpoints to complex functional endpoints as shown in Figure 9.1 (32). Depending on the nature of the research, a pragmatic patient-centred or a

mechanistic biology-centred approach can be applied. If the aim of the study is to establish predictive signatures suitable for clinical decision making, patient-oriented endpoints are of importance. Conversely, biological endpoints will be more appropriate for the analysis of the pathogenic mechanisms (28, 32). This is of particular importance in studies using genome-wide approaches, where robust phenotyping is essential (33).

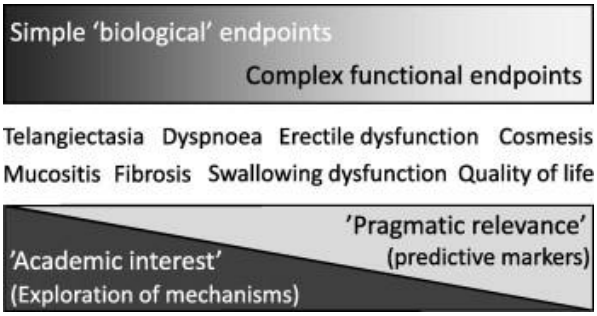


Figure 9.1: Spectrum of normal tissue reactions. From Andreassen et al. (32).

The available toxicity scoring systems allocate dynamic continuous symptoms into different grades, ranging from 0 to 5 according to the severity of the symptoms. These graded endpoints are in many studies dichotomised into no or mild (G0-1) and moderate or severe toxicity (G2+). Although this approach is associated with a loss of information, it is often applied for statistical reasons. Moreover, logistic regression modelling of binary endpoints has advantages in terms of interpretation of the findings (28, 34). Considering outcome as an ordinal variable is another possibility and is illustrated in 9.2.1 for the prediction of acute dermatitis in breast cancer patients.

Nonetheless, grading of continuous symptoms is prone to subjectivity which can be related to differences in toxicity scales between research groups and, when medical intervention is included in the scoring system, to the physician's clinical practice and perception of the severity of the event (34, 35). Therefore, it would be of great interest to define radiation-induced injury by objective, quantitative measurements on a continuous scale (35). Kelsey et al. uses the dose-dependent changes in single photon emission computed tomography (SPECT) lung perfusion as an objective measure of radiosensitivity (36). De Ruysscher et al. studied the potential of CT density changes to quantify radiation-induced lung damage (35). Moist desquamation could be predicted in

hairless mice by measuring the thermal effusivity of the skin by three-dimensional thermal tomography (37). Tissue compliance meter is recently shown to be a reproducible method to quantify radiation-induced fibrosis (38). Furthermore, bladder and rectal function could be objectively measured by anal sphincter pressures and rectal capacity or by an urodynamic examination including flowmetry and cystometry (39, 40).

Such objective measurements together with laboratory tests can, in their turn, serve as surrogate endpoints which might be useful as early indicators of a subclinical effect since some symptoms appear after a long latency period. Evidently, surrogacy requires an association between changes in the clinical endpoint and changes in surrogate endpoints (28). This is a matter of further investigation.

9.2.1 Prediction of acute dermatitis as ordinal endpoint

In paper III of this PhD dissertation, the association of patient and treatment factors with acute dermatitis after irradiation for breast cancer was investigated. It was recorded by the CTCAEv3.0 scoring system that grades symptoms like mild erythema or dry desquamation as grade 1 (G1), moderate to brisk erythema or patchy moist desquamation mostly confined to the skin folds is classified as grade 2 (G2) and confluent moist desquamation as grade 3 (G3). Further details of treatment and patient characteristics can be found in paper III. As the dataset has very few missing values, a complete case analysis was performed omitting the patients with incomplete data.

To predict acute dermatitis, two approaches were studied. For the binary approach, patients with moderate-severe (G2+) versus no-mild (G0-G1) toxicity were considered; the ordinal approach handles all separate grades. Binary and ordinal logistic regression, respectively, were applied for prediction modeling. Variable selection was performed by the LASSO procedure for both approaches. The final binary model was based on the AUC, the final ordinal model was based on the VUS (Volume under the ROC surface). For both models maximisation of the Youden Index (J) was used to define the optimal cut-off values. A J=0 is complete overlap and J=1 is complete separation of the

different classes, prediction models with J≥0.20 have acceptable prediction performance (41). All steps are carried out with cross validation to control for overfit, 10-fold cross validation for binary outcome, 3-fold for ordinal approach which is preferred over 5 and 10-fold cross validation as the analysis deals with all classes.

Individual risk scores are calculated according to the formula, with β the coefficient of its corresponding predictor X:

$$\text{Risk score} = \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p$$

The higher the risk score, the higher the risk for developing toxicity. Based on these risk scores, the probability to develop a certain grade of toxicity is calculated for each patient. In case of ordinal analysis, this risk score will give each patient its probability to develop G0, G1, G2 or G3 toxicity.

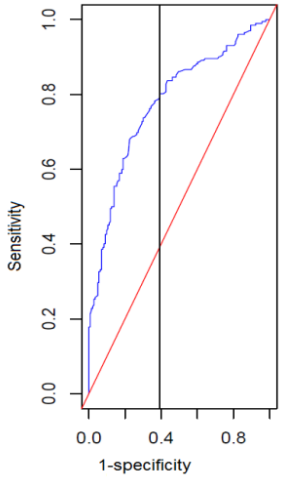
In total, 345 breast cancer patients can be included in the analysis. Of them, 3.2% develop no toxicity (G0), 38.3% develop G1 dermatitis, G2 toxicity is present in 48.1% and 10.4% develop G3 toxicity; one hundred forty-three patients develop no toxicity and two hundred and two patients develop G2+ dermatitis.

The final prediction model for the *binary* outcome is shown in Table 9.1. This model has an AUC of 0.77 with sensitivity of 83% and specificity of 61% at cutoff of 20% with J=0.20 (Figure 9.2). The model contains, apart from smoking, all the variables that remained statistically significant in multivariate modeling. Nodal irradiation, on the other hand, was picked up in the prediction model, while it did not reach statistical significance in the association study.

Table 9.1: Predictors and their coefficients for acute dermatitis as a binary endpoint.

Predictors (X)	Coefficients (β)
Intercept	1.342
BMI	0.022
Hypofractionation schedule	-0.748
Prone treatment position	-0.406
Nodal irradiation	0.119
Trastuzumab	-0.559
Bra cup size cup ≥D	0.539
Concomitant hormone therapy	0.106
MLH1 rs1800734 GG	0.104

Figure 9.2: ROC curves for acute dermatitis as binary endpoint.



The model to predict acute dermatitis as an *ordinal* endpoint is shown in Table 9.2. Nodal irradiation, hormone therapy and the *MLH1* SNP are no longer selected compared to the binary prediction model. This is a consequence of selecting variables for optimal prediction of four classes instead of two classes. The model has a VUS of 0.72 with J=0.28.

Table 9.2: Predictors and their coefficients for acute dermatitis as an ordinal endpoint.

Predictors (X)	Coefficients (β)
BMI	0.098
Hypofractionation schedule	-1.792
Prone treatment position	-1.112
Trastuzumab	-1.431
Bra cup size cup ≥D	0.662

Based on the coefficients, risk scores and probabilities are calculated; they are plotted against each other in Figure 9.3. The risk score cut-offs separate the different classes, optimizing the number of true positives in each class.

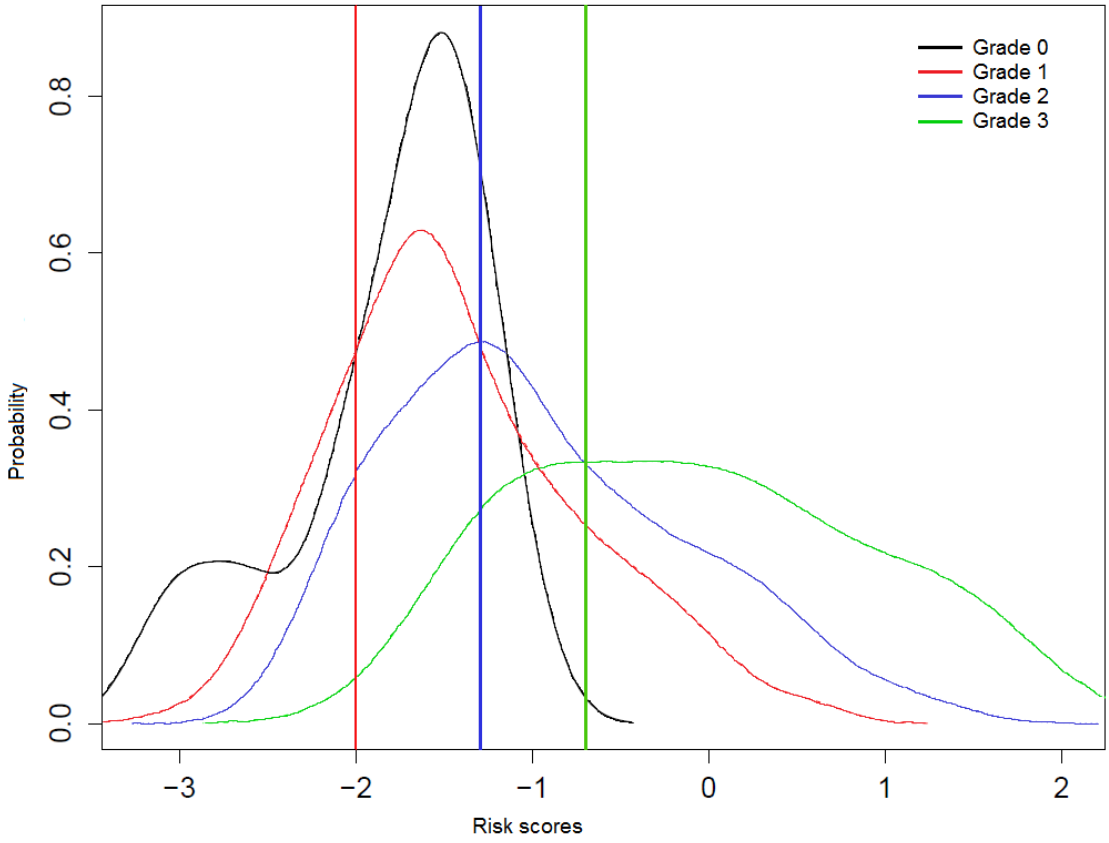


Figure 9.3: Risk scores vs. the probability to develop a certain grade of toxicity for the study population. The latter is represented by the coloured lines. The vertical lines represent the risk score cut-off values to classify patients into different classes. In a model with perfect classification, the highest probabilities to develop a certain grade of toxicity would correspond to the category the patient is classified to. For the current model, there is moderate overlap as represented by the VUS of 0.72.

For this preliminary analysis, it was chosen to calculate cut-off values based on the maximisation of J; this is comparable with the calculation of sensitivity and specificity by taking the minimal distance to the perfect point of the ROC curve when considering a binary endpoint. Dependent on the harms and benefits of the alternative treatment (clinical usefulness), this trade-off between false negatives and false positives can be penalized differently, for example as applied in paper II (chapter 7). In the prediction analysis of an ordinal endpoint, this trade-off is more difficult; the grades in-between are bounded by two cut-off values which makes it difficult to optimize sensitivity and specificity for each grade. This means that more choices need to be made in relation to the costs and benefits of the alternative treatments. Uniformity of scoring by different

clinicians becomes a more critical issue in ordinal approach. And, another criticism to the ordinal approach is that there is more chance to have classes that are not well-balanced from statistical point of view, especially for the extremes. This can add more uncertainty into the model and can lead to an underestimation of the coefficients. Nonetheless, the ordinal prediction approach goes together with gain in information compared to the binary approach, it also enters a new level of complexity. For these reasons it is recommended to implement an ordinal approach model only when different alternative treatments are available and adjust the number of classes to the number of available alternatives.

Hypofractionated IMRT in prone position would be a valid alternative to offer patients at high risk for developing G2 or G3 toxicity. With the right guidance and expertise, it would be feasible to implement this technique and it does not affect tumour control. Therefore, the binary approach would be the best solution to reduce severe acute skin toxicity after breast irradiation.

These results warrant some caution as all the measurements are performed on the same patient population and should be validated in an independent cohort before clinical implementation.

9.3 Added value of genomics

Entering the era of GWASs, the search for genetic variants associated with common traits and diseases was accelerated with impressive results in finding genetic factors involved in many conditions (a comprehensive list of studies can be found at www.genome.gov/gwastudies/ (42, 43). In order to make this possible, large consortia were established to facilitate and promote multi-centre collaboration of researchers. The GIANT consortium, for example, identified in 183.727 individuals more than 180 loci influencing height, the most heritable human trait (44). These loci, which explain ~10% of the phenotypic variation, are non-randomly clustered within biologically relevant pathways and probably underlie relevant functional and biological information to the study of human growth (44).

Furthermore, GWASs have identified common genetic variants that confer susceptibility to different types of cancer. Although, the low effect size of each

sequence variant implies a poor predictive utility of a genetic test based on one risk allele. The combination of multiple susceptibility alleles, assuming a multiplicative model, may result in a polygenic risk profile that can be used for risk prediction (45). To date, a total of 76 susceptibility loci for breast cancer and 77 for prostate cancer have been identified in approximately 87.000 and 50.000 individuals, respectively, performed by the COGS consortium (46-48). The estimated proportion of familial risk explained by those loci is ~15% for breast cancer and ~30% for prostate cancer (47, 48). The polygenic risk profile for breast and prostate cancer based on these susceptibility loci shows an AUC of 0.63 and 0.68, respectively (49) and can identify a small portion of the population at a clinically meaningful level of risk; the top 1% of the individuals in the highest risk stratum has a 3.2-fold greater risk for breast cancer and a 4.7-fold greater risk for prostate cancer, relative to the population average (3, 48, 49). For comparison, the latter risk estimate is similar to that conferred by deleterious mutations in *BRCA2* (48, 50). These findings suggest that polygenic profiling is promising for risk stratification which can be further improved as more susceptibility loci are identified and by adding more information (like lifestyle factors) into the risk model.

In radiogenomics research, the radiogenomics consortium (RGC) was established in 2009 and has about 150 members from >80 institutions in 19 countries, among them are major collaborative groups from the United Kingdom (RAPPER) (51), the United States (GenePARE) (52) and Japan (RadGenomics) (53). The RGC acts as a framework to pursue grant applications, share data and samples and conduct meta-analyses (54, 55). It also develops guidelines to encourage best practices for data collection and for reporting radiogenomics studies (56).

Many, mostly small, studies have reported associations between SNPs in candidate genes involved in DNA repair, inflammation and radiation response pathways, and radiation toxicity in multiple types of cancer (reviewed in (57)). The most intensively studied genetic variant in radiogenomics is the -509 (rs1800469) SNP in the *TGFB1* gene. Large studies or meta-analyses testing this association with radiation-induced toxicity could, however, not confirm the previous associations. Two literature based meta-analyses (58, 59), a large

RAPPER study comprising 778 participants (60) and a meta-analysis conducted through a joint RGC effort, including published and unpublished data from 2782 patients (61), did not detect any significant association between the *TGFB1* SNP and late toxicity. The latter two studies were well-powered to detect small differences (60, 61). All other previously reported associations were tested in a large (n=1613) independent validation study and could also not confirm the associations (62). Recently, the first well-replicated report identified a SNP near the *TNFα* gene to be associated with late RT adverse reactions in breast cancer. This candidate gene study was carried out on a test set containing 340 women, followed by replication of these results in three additional cohorts, two German cohorts containing 748 patients and a RAPPER cohort including 948 women (63).

Several GWASs in radiogenomics have been performed (64-68). The first GWAS was published by Kerns *et al..* Radiation-induced ED was studied in African-American men (n=79) after prostate cancer treatment. The rs2268363 SNP in the *FSHR* gene was found to be associated with the endpoint at the genome-wide significance level ($p=5.5 \times 10^{-8}$) (64). The RadGenomics project performed a genome-wide screen of microsatellites in 360 patients with diverse cancer types. They identified a marker in the *SEMA3A* promoter region associated with acute adverse reactions (65). Furthermore, three 2-stage GWASs, consisting of a discovery and replication cohort, were performed in prostate cancer patients treated with RT to identify SNPs with the development of late urinary symptoms, defined as the change in AUA Symptom Score relative to baseline, late rectal bleeding and late ED (66-68). Late urinary symptoms are associated with the 9p21.2 region containing 8 SNPs, with combined p-values ranging from 8.8×10^{-6} to 6.5×10^{-7}. These variants reside in a haplotype block encompassing the *IFNK* gene which is involved in inflammation (66). Two SNPs that tag the 11q14.3 locus have combined p-values reaching genome-wide significance (5.4×10^{-8} and 6.9×10^{-7}) for association with late rectal bleeding. A polygenic risk score including the top 17 SNPs resulted in an OR of 1.7 and an AUC of 0.74 in the replication cohort (67). For ED, 12 SNPs were identified in both cohorts. Combining these SNPs in a cumulative score, a one-allele increase in the cumulative SNP score increased the OR of developing ED with 2.2, taking into account the nongenetic factors age, androgen deprivation,

treatment and ancestry. This model including SNPs and the nongenetic factors, resulted in an AUC of 0.89 which drops to 0.75 when the genetic markers are left out. The model was validated in two small external cohorts (68). In our study, we did not first perform association tests to select the SNPs for the prediction model. We relied on the Lasso method to select those SNPs (and other variables) with the largest effect size. We noticed the same trend that including genetic information into a model already containing patient or treatment-related data, increases the predictive performance. The AUC of the models for haematuria or nocturia decreases more than 0.10 points of AUC when the genetic data was left out (see paper II). However, the rather large effect of the SNPs on the predictive performance can be an overestimation due to sample size. The findings need to be confirmed in additional validation studies with larger sample sizes.

We noticed in this doctoral research, that for some cases the heterozygous genotypes but not the homozygotes for the minor allele are associated with toxicity. A possible explanation is that these results are consistent with the over-dominant model in which the heterozygotes confer an advantageous effect over both homozygotes. However, in large GWASs most SNPs are consistent with the additive (allele-dose) model (69), with only a minority of SNPs deviating from this and follow the common dominant or recessive models (47, 48). This means that our results might be biased by insufficient numbers of patients carrying the homozygous variant genotype.

In this PhD research, the SNP selection was predominantly based on the candidate gene approach. We notice that genome-wide significance ($p \leq 5x10^{-8}$) (70) is difficult to achieve and that the GWAS approach is the way to go. Progress is already made by studies performed by Kerns *et al.* (66-68) but larger cohorts and independent validation sets are needed to identify true susceptibility loci. Currently, the OncoArray project is ongoing. OncoArray is a custom-genotyping chip containing, besides 300,000 GWAS backbone SNPs, 1,000 prostate SNPs and 1,000 breast SNPs specifically chosen by the RGC (71). In total, 5,450 RGC prostate samples will be genotyped from five RGC groups, including the Ghent group.

10 Alternative strategies to predict radiosensivity

Other than genetic variations, cellular assays can be developed to predict clinical radiosensitivity. Among them are DNA damage assays, radiation-induced apoptosis and gene expression profiles. They involve the analysis of changes before and after the *ex vivo* irradiation of a blood or cell sample.

A number of *in vitro* methods have been examined to determine the intrinsic radiation sensitivity of patients. These include clonogenic cell survival assays, chromosomal damage (dicentrics, micronuclei) and chromatid breaks (G2 assay) but have shown to be of limited use (72-75).

One of the earliest steps in the cellular repair of DNA DSB is phosphorylation of thousands of H2AX molecules, presented as γH2AX, in the chromatin flanking the DSB site (76). γH2AX foci, each representing one DSB, can be measured by flow cytometry or counted in cell nuclei by immunofluorescence microscopy (77). The kinetics of foci loss is a measure of DNA repair capacity and can be exploited as a measure of cellular radiosensitivity (78-80). Several studies evaluated whether patients with severe toxicity have impaired DNA repair mechanisms, with the intention to develop a diagnostic test predicting these responses. In the study of Rübe *et al.*, the highly sensitive γH2AX foci assay was applied to identify patients with impaired DSB repair capacity. They could distinguish ATM homozygote, heterozygote and normal individuals. They were also able to detect DSB repair deficiencies in three of 23 children with solid tumours. Of them, two children manifested unexpected serious adverse events, like life-threatening radiation pneumonitis and lethal spinal cord necrosis (81). Chua *et al.* assessed the number of γH2AX/53BP1 foci in lymphocytes of breast cancer patients exhibiting severe late clinical radiation-induced photographic changes compared to women (n=7) with mild or no changes (n=7). Higher levels of residual DSB were expressed in women with clinical changes (82). γH2AX/53BP1 foci were stained as 53BP1 co-localizes with γH2AX in order to ensure the enumeration of genuine radiation-induced foci (82). Residual γH2AX expression in head-neck cancer patients increased with the severity of acute mucositis and skin reactions (83). The findings of Burton *et al.* and Li *et al.* are in line with these results; persistent

γ-H2AX expression, measured by flow cytometry, is higher in lymphocytes from overreactors compared to lymphocytes of non-overreactors (84, 85). Li *et al.* confirmed the predictive value of γ-H2AX expression for severe mucositis. A relative fluorescence intensity of γ-H2AX cut-off value was determined at 24 h post-irradiation, with an AUC value of approximately 0.80 and sensitivity and specificity being 100% and 53.8%, respectively (85). Other studies could, however, not verify this correlation between γ-H2AX foci and acute or late tissue damage (86-89). These studies are performed in rather small study populations and large prospective cohorts are necessary to validate these findings.

A French research group proposed radiation-induced lymphocyte apoptosis (RILA) as a useful tool for the prediction of radiation-induced toxicity. RILA is assessed by flow cytometry on fresh blood samples exposed to 8 Gy x-rays. A decreased apoptotic response of CD4+ or CD8+ T-lymphocytes to irradiation has been observed in AT patients and in patients suffering from late radiation-induced adverse events when compared to healthy controls (90-95). This assay was prospectively investigated in 399 patients with miscellaneous cancers treated with RT. The AUC for the development of grade ≥2 at 2 years was for CD8 0.83 and 0.71 for CD4, reflecting a greater effect for the CD8 than the CD4 apoptosis assay. Sensitivity and specificity for the cut-off values of the percentage apoptotic CD4 >15% and ≤10%, were ~80% and for the cut-off values of CD8 >24% and ≤16% were ~90% (94). The discriminative power of the test was confirmed in a recent study of the research group (96). CD4+ and CD8+ T-lymphocytes were chosen because of their better flow cytometrical separation compared with other types of lymphocytes (90, 93). There is still no biological explanation for this relationship. Nowadays, other research groups have replicated this finding (97-99) but not always with success (89, 100-102). Although, the ones that refute the predictive potential of RILA, deviated from the protocol proposed by Oszahin *et al.* (94).

Gene expression profiling is another approach for the prediction of normal tissue toxicity. Its potential is already proven in other disciplines, for example the development of Mammaprint® for the identification of women at risk of

breast cancer metastasis (103, 104). A number of studies investigated gene expression profiles to predict normal tissue toxicity after RT (105-114). However, only one study has been validated (113, 115). A classifier was developed containing 13 genes that show differential expression in fibroblast cell lines irradiated *in vitro* with the fractionated scheme of 3x3.5 Gy in intervals of 24h. The cell lines were derived from 14 breast cancer patients (113, 114). This classifier was reduced to 9 genes for technical reasons in the validation study. It was tested in fibroblast cultures derived from 160 head-neck cancer patients. The classifier showed a sensitivity of 100% and a very low specificity of 19.5% for the prediction of grade 3 subcutaneous fibrosis (115).

11 Final conclusions

Knowledge of the factors that modify the risk or models calculating the risk for toxicity are useful in clinical practice to prevent the development of radiation-induced toxicity. In this PhD research, factors related to RT dosimetry, adjuvant cancer treatments, acquired co-morbid conditions or factors inherent to the patient are investigated for their association or their predictive value for the endpoint under investigation.

Acute and late radiation-induced nocturia in prostate cancer patients was a frequently recorded urinary symptom in our patient cohort. The presence of mild pre-treatment complaints and treatment with primary RT were confirmed as risk factors. Additionally, an association between acute nocturia and the -509 and codon 10 *TGFB1* SNPs were found and remain statistically significant after multiple testing correction. Acute radiation-induced nocturia improves the predictive performance of the prediction model developed for late nocturia. Although, as it is no genuine predictor (unknown at start RT) it cannot be included in the model to calculate the individualized pre-treatment risk. Other factors in the model are the minimal clinical target volume (CTV) dose, the CTV volume and the *NOS3* rs1799983GT, *CASP8* rs1045485GG and *NR2F6* rs4808611TC genotypes. They had an acceptable level of discriminating ability (AUC=0.76) with sensitivity of 75.9% and specificity of 67.4%. Another urinary symptom, predominantly expressed in late phase, is haematuria. A prediction model was constructed and included the bladder volume receiving ≥75 Gy, prostatic transurethral resection and the *HMGRC* rs3931914CG, *NOS1* rs2293054AG, *PTGER2* rs708498GG and *EGFR* rs845552AG polymorphisms. The model also shows a good discrimination with AUC=0.80 and with sensitivity of 83.3% and specificity of 61.5%. The AUC drops to 0.60 and 0.67 for nocturia and haematuria, respectively, when leaving the genetic markers out of the model. This research implies a valuable role for genetic polymorphisms in prediction, albeit with a smaller contribution. Radiation-induced moist desquamation and radiation-induced dermatitis, an aggregation of inflammatory symptoms like erythema, edema and desquamation, were studied in breast cancer patients. The factors influencing the development of both these symptoms are BMI, large bra cup size, fractionation schedule and concurrent

hormone therapy. Additional factors modifying the risk of acute dermatitis were supine IMRT, the administration of trastuzumab and the genetic variation *MLH1* rs1800734. The latter association holds after correction for multiple testing.

This PhD research shows that the success of predicting normal tissue toxicity after RT will depend on our efforts to collaborate. On the one hand, this research field connects experts from different disciplines like radiobiologists, radiation oncologists and geneticists but also radiation physics and statisticians. On the other hand, large patient groups with a standardized collection of radiation dosimetric, clinical and biological data will be necessary to perform the genotyping studies. Recently, through the RGC, a EU-funded project called REQUITE is started. This project aims at validating the existing prediction models, with or without genetics, in 5,300 prostate, breast and lung cancer patients undergoing RT using identical treatment and toxicity data collection forms.

12 Future perspectives

The aim of developing a prediction model is to find a combination of factors that accurately predicts an individual patient's outcome. Validating such a model should demonstrate that the combination of these factors is reliable and suitable in independent external datasets. Then, the clinical usefulness should be determined by comparing the tailored treatment with standard treatments in the clinic (116). It should, however, be noticed that alleviating normal tissue toxicity may not be at the expense of local tumour control or survival. Therefore large integrated predictive systems will have to be developed incorporating factors simultaneously predicting for local control, survival, treatment toxicity, quality of life and costs (116).

Furthermore, in highly technological, innovative and rapidly evolving fields such as radiotherapy, predictive models will need continuous re-evaluation (116, 117). As evidence-based medicine and consecutive guidelines always lag somewhat behind practice, data mining of historical data from routine clinical practice could be used for decisions concerning new patients, also known as Rapid Learning, to speed-up this process (118-121). An additional advantage is the large number of readily available patients with unbiased selection compared to clinical trials (only 3% of cancer patients are included in clinical trials). A drawback of this approach is the low quality of the data (117).

Even after large GWASs of tens of thousands of people, much of the heritability remains unexplained. This is also referred to as the 'missing heritability' (4, 122). Some of this can be attributed to imperfect tagging of a strongly associated SNP leading to underestimation of the true effect size (123) or to the presence of rare variants with relatively large effects that are not tagged by the typical markers used in GWAS (4, 33). These variations can be identified by extending the reach of GWAS through fine mapping, imputation and denser single-nucleotide polymorphism (SNP) arrays (122). An additional drawback of GWAS is the ineffective capturing of structural variations such as insertions, deletions, inversions and copy number variants, which commonly occur in the human genome (124). Such variants have already been shown to have strong associations with several conditions (125, 126). Another possibility

is the application of next-generation sequencing technologies, which enable identification of rare and private (unique to an individual or family) variants through whole-exome or whole-genome sequencing (33, 127). Subsequently, acquiring such an enormous amount of data will require the development and optimisation of available software to handle data storage and data analysis (128). Other sources of phenotypic variation to explore are through transcriptome and proteome profiling, miRNAs, epigenomics and protein modification studies.

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Part IV:

Curriculum Vitae – De Langhe Sofie

Curriculum Vitae – De Langhe Sofie

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A1 PUBLICATIONS

De Langhe S, De Ruyck K, Ost P, Fonteyne V, Werbrouck J, De Meerleer G, De Neve W, Thierens H. Acute Radiation-Induced Nocturia in Prostate Cancer Patients Is Associated With Pretreatment Symptoms, Radical Prostatectomy, and Genetic Markers in the *TGFB1* Gene. *International Journal of Radiation Oncology Biology Physics*. 2013;85:393-399.

De Langhe S, De Meerleer G, De Ruyck K, Ost P, Fonteyne V, De Neve W, Thierens H. Integrated models for the prediction of late genitourinary complaints after high-dose intensity modulated radiotherapy for prostate cancer: making informed decisions. *Radiotherapy and Oncology*. Accepted.

De Langhe S, Mulliez T, Veldeman L, Remouchamps V, van Greveling A, Gilsoul M, De Schepper E, De Ruyck K, De Neve W, Thierens H. Factors modifying the risk for developing acute skin toxicity after whole-breast intensity modulated radiotherapy. *BMC Cancer*. Submitted.

De Ruyck K, Duprez F, Werbrouck J, Sabbe N, **De Langhe S**, Boterberg T, Madani I, Thas O, De Neve W, Thierens H. A predictive model for dysphagia following IMRT for head and neck cancer: Introduction of the EMLasso technique. *Radiotherapy and Oncology. 2013;107:295–299*.

CONFERENCES

International conferences

ESTRO 31
9-13 May 2012 – Barcelona, Spain
Poster discussion: Pre-treatment nocturia, radical prostatectomy and *TGFB1* SNPs are associated with radiation-induced nocturia

2nd ESTRO forum

19-23 April 2013 – Geneva, Switzerland

Poster communication: Prediction of radiation-induced toxicity in prostate cancer patients: biomarker models for nocturia and haematuria

Radiogenomics Consortium 5th annual meeting

16-17 October 2013 – Cambrigde, United Kingdom

Attendance

National conferences

5th PhD meeting of the Department of Basic Medical Sciences

8 February 2012 – Ghent, Belgium

Oral communication: Radiogenomics in prostate cancer patients

OncoPoint 2012

23 May 2012 – Ghent University Hospital, Belgium

Poster storm session: Pre-treatment nocturia, radical prostatectomy and *TGFB1* SNPs are associated with radiation-induced nocturia