Radiation-induced collateral damage: impact on metastasis

Lynn Feys
Experimental conditions show that radiotherapy-induced damage of normal tissue induces a pro-metastatic environment. Our research revealed that irradiation of lung epithelial cells promotes secretion of chemokines CXCL12 and MIF. These chemokines are responsible for activation of the CXCR4 pathway in triple-negative breast cancer cells, resulting in a pro-metastatic character. Partial irradiation of mice lungs showed an increase in metastasis compared to unirradiated lungs. These findings allow us to explore the effects of radiotherapy on metastasis formation and contribute to the search for anti-metastatic strategies.
Radiation-induced collateral damage: impact on metastasis

Lynn Feys
2016

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<tbody>
<tr>
<td><strong>A</strong></td>
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<tr>
<td>Abl</td>
<td>Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte cell adhesion molecule</td>
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<tr>
<td>ALK</td>
<td>Acute leukemia kinase</td>
</tr>
<tr>
<td>AMD3100</td>
<td>1,1’-[1,4 phenylenebis (methylene)]bis[1,4,8,11-tetraazaazacyclotetra decane]</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>α-SMA</td>
<td>Alfa smooth muscle actin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>Aur-A</td>
<td>Aurokinase-A</td>
</tr>
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<td><strong>B</strong></td>
<td></td>
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<tr>
<td>BALB</td>
<td>Brag albino</td>
</tr>
<tr>
<td>BALF</td>
<td>Broncho-alveolar lavage fluid</td>
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<tr>
<td>BC</td>
<td>Breast cancer</td>
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<tr>
<td>BCC</td>
<td>Breast cancer cell</td>
</tr>
<tr>
<td>BCT</td>
<td>Breast-conserving therapy</td>
</tr>
<tr>
<td>BL</td>
<td>Basal-like</td>
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<tr>
<td>BLBC</td>
<td>Basal-like breast cancer</td>
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<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
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<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine C-C motif ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>CE</td>
<td>Continuous exposure</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>c-Met</td>
<td>Cellular mesenchymal-to-epithelial transition factor</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide + methotrexate + fluorouracil</td>
</tr>
<tr>
<td>CMLE</td>
<td>Conditioned medium lung epithelial cells</td>
</tr>
<tr>
<td>CMLE-IR</td>
<td>Conditioned medium irradiated lung epithelial cells</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>CXCL</td>
<td>Chemokine C-X-C motif ligand</td>
</tr>
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<td>CXCR</td>
<td>Chemokine C-X-C motif receptor</td>
</tr>
<tr>
<td>CYR61</td>
<td>Cysteine-rich angiogenic inducer 61</td>
</tr>
<tr>
<td><strong>D</strong></td>
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<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine tetrahydrochloride hydrate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2 phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ddPCR</td>
<td>Droplet digital PCR</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3,3'3' tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DM</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>E</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>eV</td>
<td>Electron volt</td>
</tr>
<tr>
<td>FAC</td>
<td>Fluorouracil + anthracycline + cyclophosphamide</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>H</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>H2AX</td>
<td>Histone 2A, member X</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HR</td>
<td>Homologue recombination</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICE</td>
<td>Incontinuous exposure</td>
</tr>
<tr>
<td>IDC-NOS</td>
<td>Invasive ductal carcinoma of no special type</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Immunomodulatory</td>
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<tr>
<td>INPP4B</td>
<td>Inositol polyphosphate-4 phosphatase type II B</td>
</tr>
<tr>
<td>IR</td>
<td>Irradiation</td>
</tr>
<tr>
<td>IVIS</td>
<td>In vivo imaging system</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
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</table>
K

kVp  Kilovoltage peak

L

LAR  Luminal androgen receptor
LBP  Lipopolysaccharide binding protein
LET  Linear energy transfer
LRFS Local recurrence-free survival
LRR  Locoregional recurrence

M

MAPK  Mitogen-activated protein kinase
MEK 1/2  Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2
MHC  Major histocompatibility complex
MIF  Macrophage migration inhibitory factor
miR  MicroRNA
ML  Mesenchymal-like
MMP  Matrix metalloproteinase
MRI  Magnetic resonance imaging
MRM  Modified radical mastectomy
MSL  Mesenchymal stem-like
mTOR  Mechanistic target of rapamycin
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

MYC  Myelocytomatosis viral oncogene

N

NBLC  Normal-like breast cancer
NFκB  Nuclear factor kappa B
NGF  Nerve growth factor
NHEJ  Non-homologous end joining
NK  Natural killer cell
NOD/SCID  Nonobese diabetic/severe immunodeficiency
NRAS  Neuroblastoma rat sarcoma viral oncogene homolog

O

OS  Overall survival

P

PARP  Poly-adenosine-diphosphate-ribose-polymerase
PBS D⁻  Phosphate-buffered saline without calcium and magnesium
PBS D⁺  Phosphate-buffered saline supplemented with calcium and magnesium
PCR  Polymerase chain reaction
pCR  Pathologic complete response rate
PDGF  Platelet-derived growth factor
PET  Positron emission tomography
PGE2: Prostaglandin E2
PLK: Polo-like kinase
PMRT: Postmastectomy radiation therapy
PORT: Postoperative radiation therapy
PPi: Pyrophosphate
PR: Progesterone receptor
PRL: Partial right lung
PTEN: Phosphatase and tensin homolog

SPSS: Statistical package for the social science
SRB: Sulforhobamine B
Src: Sarcoma oncogene
STAT3: Signal transducer and activator of transcription 3
STR: Short tandem repeat

Rb: Retinoblastoma
RBE: Relative biological effectiveness
RFU: Relative fluorescence unit
Rho: Rhodopsin
RLU: Relative luminescence unit
RNA: Ribonucleic acid
RNS: Reactive nitrogen species
ROI: Region of interest
ROS: Reactive oxygen species
RT: Radiation treatment/Radiotherapy

TAM: Tumor-associated macrophages
TBE: Tumor bed effect
TGF-β: Transforming growth factor beta
TH-cell: Helper T cell
TME: Tumor microenvironment
TNBC: Triple-negative breast cancer
TNC: Tenascin C
TNF: Tumor necrosis factor
TP: Tumor protein

UNS: Unclassified
uPA: Urokinase-type plasminogen-activator

SARRP: Small animal radiation research platform
SCP: Single cell-derived population
SD: Standard deviation
SDF-1: Stromal cell-derived factor 1
SM: Simple mastectomy
SPECT: Single photon emission CT

VCAM: Vascular cell adhesion molecule
VEGF: Vascular endothelial growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>WT</td>
<td>Whole thorax</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV integration site family member</td>
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Summary

Experimental conditions show that radiotherapy-induced damage of normal tissues induces a pro-metastatic environment. This research focussed on the search for factors associated with these pro-metastatic effects, which can be used as potential therapeutic targets.

Recent studies show that radiation treatment of cancer inhibits primary tumor growth but has disturbing side effects which can contribute to invasion and metastasis of the tumor.\(^1\)\(^2\) Both cancer and host cells may contribute to these effects.\(^3\)\(^4\) For instance, in breast cancer, the lung is an important organ that can suffer from collateral damage. The potential impact of this damage on invasion and metastasis needs to be further investigated to improve patient outcome. Currently there are no adequate in vivo models which study the metastatic effect of irradiation in healthy tissue. Von Essen et al.\(^5\) described multiple in vivo models but those are dated.\(^5\)

Therefore, our first aim was to develop an in vivo model where follow-up of tumor progression was possible, taking the 3R’s (replacement, reduction and refinement) of animal welfare into account. We found that a bioluminescent cancer cell model had the same characteristic as a parental cancer cell model, making it an ideal tool for further use in in vitro and in vivo studies.

The main aim of this research was to develop an in vivo model to investigate the effects of radiotherapy on host cells and their contribution to pro-invasive and pro-metastatic side effects. We irradiated healthy lung cells/tissue and studied its effects on triple-negative breast cancer cells both in vitro and in vivo. As illustrated (Fig. 1), our research revealed that irradiation of lung epithelial cells promotes secretion of chemokines CXCL12 and MIF. These chemokines are responsible for activation of the CXCR4 pathway in triple-negative breast cancer cells, resulting in a pro-metastatic character. Partial irradiation of mice lungs showed an increase in amount of
metastases and metastatic growth compared to non-irradiated lungs. Further analysis revealed that disruption of the CXCL12/CXCR4 and MIF/CXCR4 interaction is essential for the breast cancer cells to return to their “normal” phenotype.

These findings allow us to explore the effects of radiotherapy on metastasis formation and contribute to the search for anti-metastatic strategies.

Figure 1: Summary figure. Schematic figure showing the multiple processes occurring after irradiation of healthy lung cells/tissue. CXCL12 (chemokine C-X-C motif ligand 12), MIF (macrophage migration inhibitory factor) and CXCR4 (chemokine C-X-C motif receptor 4).
Samenvatting

Experimentele condities tonen aan dat beschadiging van normaal weefsel door radiotherapie een pro-metastatische omgeving creëert. Dit onderzoek spitst zich toe op het vinden van metastase-stimulerende factoren die gebruikt kunnen worden als potentiële therapeutische kandidaten.

Recente studies tonen aan dat radiotherapie de groei van de primaire tumor afremt maar verontrustende bijwerkingen vertoont die kunnen bijdragen tot invasie en uitzaaïngen van de tumor.¹ ² Zowel kanker- als gastheercellen kunnen bijdragen tot deze effecten.³ ⁴ Bij borstkanker bijvoorbeeld, is de long een belangrijk orgaan dat te lijden heeft onder collaterale beschadiging door radiotherapie. De potentiële impact van deze schade op invasie en metastasering moet nader onderzocht worden om de kans op genezing voor de patiënt te verbeteren. Op dit moment zijn er onvoldoende in vivo modellen voor de studie van pro-metastatische effecten van bestraling in gezond weefsel te bestuderen. Von Essen et al.⁵ beschreven reeds meerdere in vivo modellen maar die zijn inmiddels gedateerd.⁵

Daarom was ons eerste doel het ontwikkelen van een in vivo model waar opvolging van tumorprogressie mogelijk was conform de 3V's (vervanging, vermindering en verfijning) voor het dierenwelzijn. Bioluminescente kankercellen vertoonden dezelfde kenmerken als de oorspronkelijke kankercellen, waardoor deze cellen een ideaal hulpmiddel zijn voor verdere in vitro en in vivo studies.

Het belangrijkste doel van dit onderzoek was het ontwikkelen van een in vivo model om de effecten van radiotherapie op gastheercellen en hun bijdrage aan de pro-invasieve en pro-metastatische bijwerkingen te kunnen onderzoeken. Gezond lungenlongcellen/lungweefsel werden bestraald en de effecten ervan op triple-negatieve borst kankercellen in vitro en in vivo werden bestudeerd. Zoals geïllustreerd (Fig. 1), blijkt uit ons onderzoek dat bestraling van pulmonaire epitheelcellen de secretie van de
chemokinen CXCL12 en MIF stimuleert. Deze chemokinen zijn verantwoordelijk voor de activering van het CXCR4-traject in triple-negatieve borstkankercellen, wat resulteert in een metastase-stimulerend karakter. Gedeeltelijke bestraling van longen bij muizen leidde tot een toename van het aantal metastasen in die long en een toename in grootte van de metastasen, in vergelijking met niet-bestraalde proefdieren. Uit verdere analyse bleek dat verstoring van de CXCL12/CXCR4- en MIF/CXCR4-interacties essentieel is voor omvorming van de borstkankercellen naar hun "normale" phenotype.

Deze bevindingen laten toe de effecten van radiotherapie op metastasevorming te onderzoeken en kunnen ons helpen in de zoektocht naar anti-metastatische strategieën.
Figuur 1: Overzichtsfiguur. Schematische figuur die de verschillende processen weergeeft die plaatsvinden na bestraling van gezonde long cellen/weefsel. CXCL12 (chemokine C-X-C motief ligand 12), MIF (macrofaag migratie inhiberende factor) en CXCR4 (chemokine C-X-C motief receptor 4).

References


Chapter 1  Research Outline

Chapter 2  Epidemiology, diagnosis and treatment of TNBC

Chapter 3  Radiobiology

Chapter 4  Research Objectives

Introduction
Chapter 1

Research Outline
This thesis aims to understand the impact of radiotherapy (RT) on normal lung tissue and its collateral impact on metastasis of triple-negative breast cancer (TNBC). The thesis is subdivided in 4 parts. The first part (PART I) provides general background information about TNBC and radiation, outlining our research problem in its bigger context. PART II focusses on the design of an ideal *in vivo* model and the use of this model in TNBC radiation research, and in PART III research results and their usefulness in practice are critically discussed.

**PART I: Introduction**

In Chapter 2, an overview of literature focussing on the epidemiology, diagnosis and different treatment possibilities of TNBC is given. Since several clinical studies describe conflicting results about RT and TNBC, this chapter also describes the pro and cons of RT found in literature. Chapter 3 is a literature study about the effects of irradiation on either cancer cells, on cells of the microenvironment or on cells in distant tissues. Both tumor promoting and tumor suppressing effects are described. Special emphasis is given to the tumor promoting effects of RT. The first part ends with a presentation of the research objectives (Chapter 4).

**PART II: Original research**

This part contains the original research presented in two research articles, which are accepted in international peer-reviewed journals. Chapter 5 shows the results of the first paper. In this paper we described a series of quantification techniques to assess *luc2* expression. Results showed that for *in vitro* and *in vivo* use, there is no significant difference as long as copy numbers are not significantly different. The results demonstrated that *luc2* transfection had no effect on the “cancer”-characteristics of the cells. The second paper, presented in Chapter 6, shows that the *in vitro* and *in vivo* bioluminescent models can be used to investigate the pro-metastatic effect of radiotherapy. Since the lung is an important organ that suffers
from collateral radiation damage, we irradiated normal lung epithelial cells. Direct and indirect effects of irradiated normal lung epithelial cells were studied on triple-negative breast cancer cells. The results revealed stimulation of the pro-metastatic characteristics of breast cancer cells upon contact with irradiated epithelial cells. This was confirmed by in vivo model experiments. Targeted, image guided radiation of the mouse lung was possible on the SARRP (small animal radiation research platform). This in vivo model revealed a higher bioluminescent signal of metastatic cancer cells in the lungs of partial lung irradiated mice. In vitro results showed an important contribution of the CXCR4 pathway to the pro-metastatic effects. Interestingly, selective inhibition of CXCR4 on the cancer cells makes them insensitive for the pro-metastatic signals secreted by irradiated epithelial cells. Together with the in vivo results, this research suggests that radiotherapy has the potential to stimulate metastasis in breast cancer patients.

PART III: Discussion

This part represents a general discussion of the research results. Chapter 7 addresses the feasibility of the in vivo model in the clinic. Differences between our model and the patient situation are discussed and improvements to our model are proposed. In Chapter 8 the main observations of our research were compared with studies of other groups that focussed on the effect of radiotherapy and upregulation of CXCR4 pathway in breast cancer. Since our results indicated that inhibition of CXCR4 on the cancer cells counteracts the negative effects of radiotherapy, in Chapter 9 the possibilities of the CXCR4 pathway as a therapeutic target are discussed. In this chapter we also discussed the importance of CXCL12 and MIF as potential biomarkers in TNBC patients. The eventual prognostic and diagnostic usability of CXCR4 is briefly discussed. Future research is needed (I) on the clinical features of CXCL12-CXCR4 pathway and radiotherapy and (II) on improvements of the in vivo model, making it more patient-related. This is extensively discussed in Chapter 10.
Chapter 2

Epidemiology, diagnosis and treatment of TNBC
2.1 Definition and epidemiology of TNBC

2.1.1 Definition

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012 worldwide.\(^1\)

Breast tumor gene expression profiling according to the PAM50 classification revealed five intrinsic breast cancer subtypes: luminal A, luminal B, HER2-enriched (HER2-E), basal-like (BLBC) and normal breast-like breast cancer (NBLC).\(^2\)\(^-\)\(^4\) 80% of clinical triple-negative breast cancers (TNBC) are basal-like tumors, based on PAM50 intrinsic subtype classification. These tumors are characterized by the expression of genes found in normal basal/myoepithelial breast cells, including basal cytokeratin (CK5/6, CK14 and CK17), smooth muscle actin, vimentine, EGFR, P-cadherin and caveolin 1 and 2.\(^2\)\(^\)\(^-\)\(^5\)\(^-\)\(^12\) They lack the expression of ER, PR and HER2. Tumor protein 53 (TP53) gene and retinoblastoma 1 (Rb1) mutations or deletions are commonly seen in this tumor subtype.\(^2\)\(^,\)\(^5\)\(^,\)\(^7\)\(^,\)\(^11\) TNBC comprises a heterogeneous collection of breast cancer types, representing approximately 10-20% of all breast cancers.\(^13\)\(^-\)\(^15\) Based on immunohistochemical staining, TNBC is lacking the expression of hormonal receptors for progesterone and estrogen, and lacking HER2 amplification.\(^14\) The American Society of Clinical Oncology/College of American Pathologists described it as: <1% ER, <1% PR staining based on immunohistochemistry staining (IHC), and HER2 negative by IHC or fluorescent in situ hybridization (FISH).

Based on gene expression profile analysis from over 587 publically available TNBC samples, also called Lehmann’s classification, 6 subtypes were identified and labelled as basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal-like (ML), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR) (Fig.
Each displays a unique pathological and molecular signature. The BL1 subtype is characterized by elevated cell cycle (MYC, NRAS and PLK1) and DNA damage response gene expression (ATR/BRCA pathway). High Ki-67 expression supports the highly proliferative nature of this subtype. BL2 subtype is associated with gene expression patterns enriched for genes related to growth factor signaling (EGF, NGF, MET, Wnt/β-catenin - and IGF-1R pathway) as well for glycolysis and glucogenesis. It has high levels of myoepithelial markers (TP63 and CD10) and is characterized by PTEN mutations (Fig. 2). IM subtype is dominated by the expression of genes involved in cytokine pathways (IL-12 and IL-7), immune cell signaling pathways (TH1/TH2 pathway, NK cell pathway, B- and T cell receptor pathway and DC pathway), core immune signal transduction pathways (NFκB, TNF and JAK/STAT pathway) and antigen processing and presentation (Fig. 2). Both ML and MSL subtype share elevated expression of genes involved in motility (Rho pathway), epithelial-to-mesenchymal transition (EMT) and cell differentiation pathways (Wnt/β-catenin, ALK and TGF-β pathway). However, the MSL subtype expresses low levels of proliferation genes and has high expression of genes important for the growth factor signaling pathways (inositol phosphate metabolism, calcium signaling, G-protein coupled receptor and ERK1/2 signaling, adipocytokine signaling, EGFR, FGF2 and PDGF), angiogenesis pathway (VEGFR2) and genes associated with stem- and mesenchymal cell-specific markers. The MSL subtype is also known as the claudin-low cancer type (Fig. 2). Claudin-low cancer cells have lower proliferation rate and enriched expression in genes involved in cancer stem cell-like phenotype displaying low expression of claudin 3, 4 and 7 compared to other TNBC subtypes. LAR subtype TNBC shows enriched gene expression in hormonal pathways (steroid synthesis, porphyrin metabolism and androgen/estrogen metabolism). It is characterized by luminal gene expression and driven by androgen receptor signaling (Fig. 2). A “seventh” subtype is described as unclassified (UNS) and covers the TNBCs that do not fit in the other 6 subtypes.
Figure 1: Scheme of different TNBC subtypes. The different gene expression subtypes are associated with distinct molecular features. BL1 (basal-like 1), BL2 (basal-like 2), IM (immunomodulatory), ML (mesenchymal-like), MSL (mesenchymal stem-like) and LAR (luminal androgen receptor). Adapted from Turner NC et al. 2013.17

When we correlate the triple-negative subtypes defined by Lehmann to PAM50 “intrinsic” breast cancer subtype classification, the UNS and BL1 TNBC subtypes correlated strongly to the basal-like intrinsic subtype (BL), respectively 76% and 85%. Moderate correlation between BL2, IM and M TNBC subtypes and BL intrinsic subtype was seen. No correlation was seen between LAR subtype and BL intrinsic subtype. However a strong correlation was seen between LAR subtype with either luminal A and luminal B subtype (82%) and HER2 enriched intrinsic subtypes.19

TNBC also share features with BRCA1 mutated breast cancers. Approximately 80% of the breast cancer patients with a hereditary BRCA1 mutation, are diagnosed with
TNBC.\textsuperscript{20} Sporadic TNBC are also known for their BRCA\textsubscript{1}ness, i.e. they show the same histological characteristics and clinical outcome as BRCA1-mutation carriers.\textsuperscript{21-23}

### 2.1.2 Clinical features & risk factors

TNBC is associated with aggressive clinicopathologic features including young age at diagnosis (mean age < 50 years), higher mean tumor size, poor or undifferentiated and typically high-grade tumors, elevated mitotic count, central zone of fibrosis and/or necrosis, pushing margin of invasion, poor or lacking tubule formation, brisk lymphocytic infiltration and high nuclear-cytoplasmic ratio.\textsuperscript{14, 24-29} They are known as interval cancers (cancer that appear between two organized mammography detection rounds), because of the high breast density in younger people and the high proliferation rate of TNBC in relation with the detection interval this cancer can stay undetected at first screening.\textsuperscript{30} Radiological features of TNBC are variable, although several reports describe TNBC as a well-circumscribed mass, with absence of spiculated margins, absence of microcalcifications, and absence or disruption of an echogenic halo.\textsuperscript{31, 32}

The majority of TNBCs are representing the invasive ductal carcinoma of no special type (IDC-NOS).\textsuperscript{26, 33, 34} Morphologically however, TNBC may represent a high prevalence of rare histological cancer subtype such as atypical or typical medullary carcinomas, apocrine carcinomas, pleomorphic lobular carcinomas, metaplastic carcinomas, adenoid cystic carcinomas and secretory carcinomas.\textsuperscript{25, 28, 29, 33-35} Medullary carcinoma, adenoid cystic carcinoma and secretory carcinoma have a better prognosis than the other morphologic subtypes, with a minimal regional recurrence rate.\textsuperscript{24, 36-38}

TNBC more frequently affects premenopausal women and is more prevalent in women of African-American or Hispanic origin compared with other breast cancer
types. A clinical study that compared women with breast cancer in the United States and Ghana showed the progressively increasing frequency of TNBC among white American, African-American, and Ghanaian/Africans. These disparities in incidence among different racial groups strongly suggest that there must be genes or mutations that predispose women, particularly premenopausal African-American women and African women, to TNBC.

Few studies showed that there is an increased risk of TNBC in women with high parity and young age at first term full pregnancy. A reduced risk is seen for women with a longer duration of breast-feeding and later age at first birth. The role of oral contraceptive in TNBC is controversial. Dolle et al. reported that oral contraceptive usage for more than 1 year was associated with a 2.5-fold increased risk of TNBC compared to women who had used oral contraceptives for less than 1 year or never. However, Phipps et al. found that there was no association with TNBC risk and oral contraceptive use.

Obesity is strongly associated with poor prognosis in patients with TNBC. Premenopausal women with TNBC are more likely to be overweight or obese. Not only obesity, also insulin resistance, impaired glucose tolerance, dyslipidaemia and hypertension are linked with increased risk of TNBC.

The relative survival for women with TNBC is poorer than for women with other types of breast cancer, with an increased mortality in the first 5 years after treatment. TNBC patients are more likely to develop a recurrence during the first 3 years after therapy followed by a decrease in relapse 8 to 10 years after treatment. Metastases are rarely preceded by local recurrence and vice versa local recurrence is not a predictive marker for metastatic disease. TNBC is an aggressive disease associated with more visceral and soft-tissue relapse, like the spinal cord and meninges, brain, liver and lung metastasis, where bone recurrence is less common compared to other breast cancer subtypes. When distant metastases occur in
TNBC, the progression to death is more rapid than in other breast cancer subtypes (median survival time TNBC: 9 months, versus other BC types: 20 months).\textsuperscript{13, 33}

### 2.2 Treatment possibilities

Treatment options are limited as these tumors are naturally resistant to existing targeted hormonal therapies by absence of ER, PR and lack of overexpression of HER2, which logically leads to the use of locoregional treatments.\textsuperscript{15} Locoregional management of breast cancer can be done in 2 ways: breast-conserving therapy (BCT) involving a lumpectomy and adjuvant radiation therapy (RT) or simple or radical mastectomy (MT) with or without RT.\textsuperscript{47} Minami et al.\textsuperscript{48} considered that when TNBC is diagnosed in women with young age, African-American roots, Jewish descent or women with a high-risk family history of breast and ovarian cancer, BRCA-testing should be included as a part of pretreatment assessment. In those cases it is advised to undergo bilateral mastectomy.\textsuperscript{47, 48}

Standard treatment possibilities for patients with TNBC are surgery followed by chemotherapy or neoadjuvant chemotherapy followed by surgery whether or not followed by radiotherapy. Most studies have indeed administered chemotherapy followed by radiotherapy in postmastectomy and postBCT treated patients. This conventional sequence is based on the ability of chemotherapy to reduce metastatic spread in women by inhibiting cancer cell proliferation and spreading, radiotherapy presents an effect of local–regional control by killing any cancer cells remaining after surgery. Interestingly, TNBC does appear to be more responsive to chemotherapy than other breast cancer types, and improvements in chemotherapy are likely to benefit patients with this subtype of breast cancer, because of rapid proliferation rates and defects in DNA repair. The lack of targeted therapeutic options emphasizes the urgent need to optimize the current treatment management of patients with
TNBC and reduce their risk of locoregional recurrence (LRR) and distant metastases (DM).

### 2.2.1 Surgery

Lowery et al. performed a comprehensive search for studies examining outcomes after BCT and/or mastectomy according to breast cancer subtype. Out of a total of 12,592 breast cancer patients from 15 different studies, they concluded that patients with TNBC have an increased risk of developing locoregional relapses compared to non-TNBC, irrespective of breast conservation therapy or mastectomy. The aggressive nature of TNBC is more likely the cause of the poor outcome in these patients (Fig. 2).
Figure 2: Forest plots showing locoregional outcomes with breast conserving treatment and mastectomy. For non-TNBC relative to TNBC. Adapted from Moran et al.\textsuperscript{50} Each study is shown by the point estimate of the relative risk (RR; square proportional to the weight of each study) and 95% CI for the RR (extending lines); the combined RR and 95% CI by random effects calculations are shown by red diamonds. A. non-TNBC patients were less likely to develop LRR than TNBC patients after either BCT (RR 0.49; 95% CI 0.33–0.73) or mastectomy (RR 0.66; CI 0.53–0.83).

The aggressive nature of TNBC leads to the logical assumption of superiority of mastectomy over BCT. However, Bayoumi et al.\textsuperscript{51} showed that the surgical type did not affect the local recurrence or overall survival (OS) significantly in TNBC patients.\textsuperscript{51} This was confirmed by a large retrospective study by Zumsteg et al.\textsuperscript{52} who found no difference in locoregional control, distant relapse, overall recurrences or overall survival between BCT and mastectomy treated T1-2N0 TNBC patients. Bhoo et al.\textsuperscript{53} found the same results in T1-2N0 patients but for T3-4, N2-3 tumor staged patients BCT had significantly increased 5y-relative survival rate compared to mastectomy with adjuvant radiotherapy (Fig. 3).
### Classification | Definition
--- | ---
**Primary tumor** (T) | 
TX | Primary tumor cannot be assessed  
T0 | No evidence of primary tumor  
Tis | Carcinoma *in situ*  
T1 | Tumor ≤ 2 cm in greatest dimension  
T2 | Tumor > 2 cm but ≤ 5 cm in greatest dimension  
T3 | Tumor > 5 cm in greatest dimension  
T4 | Tumor of any size with direct extension to the chest wall and/or to the skin  
**Regional lymph nodes (N)** | 
NX | Regional lymph nodes cannot be assessed  
N0 | No regional lymph node metastasis  
N1 | Metastasis in movable ipsilateral level I, II axillary lymph node(s)  
N2 | Metastasis in ipsilateral level I, II axillary lymph nodes fixed or matted, or in clinically detected* ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis  
N3 | Metastasis in ipsilateral infraclavicular lymph node(s) (level III axillary) with or without level I, II axillary lymph node involvement; or in clinically detected* ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement  
**Distant metastasis (M)** | 
MX | Distant metastasis cannot be assessed  
M0 | No distant metastasis  
M1 | Distant metastasis  

*Figure 3: Clinical TNM classification.* Adapted from ACJJ Cancer staging manual 7th edition; Edge SB *et al.*54 * Clinically apparent is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination.

However, conflicting results are seen in the retrospective study of Abdulkarim *et al.*55 They reported that for all TNBC cases, BCT + RT significantly increase 5y LRR and 5y OS compared to MRM (modified radical mastectomy) + RT (94% vs. 87% and 87% vs. 68%). Also BCT lead to less DM than MRM+RT (9.4% vs. 27.8%). The results of early diagnosed TNBC cases (T1–T2N0), only included mastectomy
patients without postoperative radiation therapy (PORT) versus BCT with PORT. They never compared patients who underwent mastectomy with PORT versus BCT with PORT. This makes it difficult to assess the true impact of surgical treatment type in early diagnosed TNBC. The study of Parker et al. showed a significant difference in local recurrence rate, 0% and 10.6% respectively for BCT and MRM ($p=0.02$). The 5y disease-free survival rates for the BCT and mastectomy groups were 68% and 57%, respectively ($p=0.14$), and 5y OS was increased for the BCT group compared with the MRM group (89% vs. 69%; $p=0.01$). They concluded that BCT is not contraindicated in TNBC, however, in this study the mastectomy groups had a larger neoplasm size and advanced stage of disease.

Studies could be biased by a heterogeneous population including all clinical stages and imbalance between BCT and mastectomy groups in terms of tumor stage, nodal stage, and lymphovascular invasion. Not all T1-2N0 patients received adjuvant chemotherapy, e.g. in Zumsteg et al. 81% versus Abdulharim et al. 35%. Statistical analysis differs between different studies, which can also lead to conflicting results as Zumsteg et al. proposed.

Although TNBC is associated with worse locoregional relapse outcomes compared to other breast cancer subtypes, there is no need for more radical surgery. Research indicates that TNBC rather develops an expanding growth pattern instead of an extensive intraductal spread, so mastectomy has no advantage in this type of breast cancer.
2.2.2  Chemotherapy

The kind of chemotherapy treatment used in patients not only relies on age, axillary lymph node status, and tumor grade and size, but also on specific toxicities, patient preferences, and cost effectiveness.\textsuperscript{58}

TNBC, especially BL1 subtype, are known for their superior sensitivity and responsiveness to agents of chemotherapy compared to other breast cancer subtypes due to their highly proliferative character.\textsuperscript{48, 59} Shorter OS and disease-free intervals have been seen in TNBC patients who did not receive adjuvant chemotherapy.\textsuperscript{60} In the past, first generation alkylating-based chemotherapy was used (cyclophosphamide, methotrexate and fluorouracil; CMF) to treat stage I and II breast cancer (Fig. 4). This was rapidly replaced by an anthracycline-based therapy (fluorouracil, anthracycline and cyclophosphamide; FAC).\textsuperscript{61, 62} Recently, a third generation taxane-based chemotherapy, a classic combination of anthracyclines and taxanes is encouraged as breast cancer treatment.\textsuperscript{58, 63} Unfortunately, a perfect combination for TNBC is still not found.

Neoadjuvant chemotherapy is proven to be beneficial in TNBC patients; it increases TNBC pathologic complete response rate (pCR) compared to non-TNBC patients.\textsuperscript{64-66} Patient with pCR had a better overall survival.\textsuperscript{64, 65, 67} However, some trials claim that there is no significant difference in local recurrence-free survival (LRFS) and OS between neoadjuvant and adjuvant chemotherapy.\textsuperscript{51}
### Stage grouping

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**Figure 4: Anatomical staging breast cancer.**

Adapted from ACJJ Cancer staging manual 7th edition; Edge SB et al. \(^{54}\)

* N1mic = micrometastases greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm.

A subgroup of TNBC patients manifests BRCA1 mutations, characterized by problematic homologue recombinant DNA repair. Surprisingly similar rearrangements are seen in sporadic TNBC patients. Preclinical studies show that BRCA1 mutations increased the sensitivity of cancer cells to platinum salts (cisplatin) and other alkylating agents (cyclophosphamide). \(^{66, 68, 69}\) Platinum salts are DNA damaging agents which, by cross-linking the cancer DNA, causes irreversible collapse of DNA repair leading to cancer cell death. \(^{69}\) Petrelli *et al.* \(^{70}\) showed that neoadjuvant platinum-based therapy significantly increased pCR of TNBC compared to non-TNBC patients. Sensitivity of the patient to this therapy is correlated with p63/p73 protein co-expression in breast cancer cells. \(^{71, 72}\)
Not only the type of chemotherapeutical agent, also time of administration and dose regimens are important. Dose-dense schedules (same agents and total dose of conventional chemotherapy but with shorter intervals) should be considered in tumors with high proliferation rate.\textsuperscript{73, 74} In TNBC patients, dose-dense therapy showed an improved outcome compared to the normal dose regime.\textsuperscript{73, 74}

To conclude, in stage I and II TNBC, third generation chemotherapy regimens utilizing polychemotherapy administered in a dose-dense fashion seem to be the most effective tools available for treatment of TNBC patients.\textsuperscript{67, 74}

The problem with TNBC is the heterogeneous character, whereby different subtypes have different specific mutations that lead to variable sensitivity to chemotherapy.\textsuperscript{72} Therefore, identification of specific subtype biomarkers is necessary to predict the response to the given chemotherapy regime and to find appropriate targeted therapies. Masuda \textit{et al.}\textsuperscript{75} investigated the clinical relevance and pCR of the different TNBC subtypes. There results showed that TNBC subtype and pCR status were significantly associated (p<0.05) with the highest pCR rate for BL1 subtype (52%).

\subsection*{2.2.3 Radiotherapy}

Conform recent ESO-ESMO consensus guidelines, radiotherapy is indicated for all patients with invasive carcinoma of the breast under the following conditions: I) received BCT; II) underwent mastectomy with tumors of 5 cm or larger or with signs of persistent involved margins; or III) underwent mastectomy with more than 4 axillary nodal metastases ; or IV) in women with 1-3 positive axillary nodes with additional risk factors like young age, vessel invasion and low number of examined axillary lymph nodes.\textsuperscript{47, 48}
There has been much debate about whether TNBC could benefit from PORT regardless of surgical intervention. Therefore, several retrospective studies have analyzed the role of radiation therapy in TNBC, but their findings are conflicting.

Bayoumi et al.\textsuperscript{51} could identify that the PORT treated patients had worse prognostic factors, as for 75\% of PORT treated patients had stage III disease, compared with non-PORT treated patients (56\%). Despite this, the patients in the non-PORT group experienced more local recurrences (19.5\%) compared with the PORT group (7\%). Three-year LRFS for PORT patients was higher than for non-PORT patients (80\% vs. 58\%, respectively, \( p=0.049 \)) and 5y-LRFS for PORT was also significantly higher (94\% vs. 82\%, \( p<0.001 \)), independent on type of surgery.\textsuperscript{51} This was confirmed by Steward et al.\textsuperscript{76} were a significant increase in 4y OS was observed in patients who received PORT (77.34\% vs. 59.8\%, \( p<0.0001 \)).

For postmastectomy radiotherapy (PMRT), Bhoo et al.\textsuperscript{53} found that radiotherapy after mastectomy, in T1-2N0-1 tumors; significantly decreased the 5y relative survival in PMRT (94.7\% vs. 82.3\%), while no differences were seen in T3-4N2-3 patients. The absence of beneficial effect of PMRT observed by Bhoo et al.\textsuperscript{53} was confirmed by Abdulkarim et al.\textsuperscript{55} where PMRT had no effect on 5y LRR free survival (85\% vs. 87\%) and LRR (9.8\% vs. 11.7\%). Even worse, the comparison of mastectomy vs. PMRT revealed that patients without PMRT showed increased 5y OS (82\% vs. 62\%) and decreased DM (9.8\% vs. 27.8\%).\textsuperscript{55} Kyndi et al.\textsuperscript{77} showed there was no significant difference in OS after PMRT compared to no PMRT. Despite a significant reduction in LRR after PMRT was reported (15\% vs. 32\%, \( p<0.001 \)).\textsuperscript{77} The absence of a positive effect on the OS can be due to micrometastases formation, since there was no increase of visible distant metastasis or due to radioresistance of the tumor.\textsuperscript{77} Controversially, Jagsi et al.\textsuperscript{78} promote PMRT in node negative tumors with high risk of LRR. Relevant factors that should be considered in addition to
axillary involvement include tumor size, vessel invasion, margin status, and menopausal status.\(^{78, 79}\)

Other groups report that the survival benefit of adjuvant radiation in TNBC patients is only attributed to those who received BCS.\(^{76}\) Steward \textit{et al.}\(^{76}\) showed a beneficial effect of PORT in BCS (Hazard ratio: 1 vs. 0.3; BCS and BCS+PORT respectively, \(p=0.0004\)) but no benefit was observed in either simple mastectomy (SM) or modified radical mastectomy (MRM). Dragun \textit{et al.}\(^{80}\) investigated BCS and influence of PORT and could conclude that PORT treated patients had a higher 3y LRFS (80\% vs. 58\%, \(p=0.049\)), but no difference was seen in OS (39\% in PORT and 32\% in non-POR; \(p=0.4\)).

Radiotherapy is not only important in local cancer control, it also enhances the anticancer effect of chemotherapy on cancer cells.\(^{81}\) Wang \textit{et al.}\(^{81}\) found improved recurrence-free and overall survival in patients who received combined therapy in comparison to those who only received chemotherapy.

This result suggests that breast conservation therapy, which routinely incorporates radiation, might be more appropriate than mastectomy for patients with early stage TNBC but further investigation is warranted. It would be interesting to investigate the effect of radiotherapy on the different TNBC subtypes. Like BRCA-deficient cancers have an increased radiosensitivity because of deficient repair of the induced lethal DNA breaks and these patients could have a better outcome with radiotherapy.\(^{55}\) Most likely, BL1 and BL2 subtypes would have a higher benefit of radiotherapy because of their highly proliferative character. IM subtype may represent most medullary TNBC, a subtype that has a relatively good radiosensitivity.\(^{16, 82}\)

Radiosensitivity remains a problem in successful treatment of TNBC.\(^{77}\) Several proteins, lipids, genes and microRNAs that are involved in radioresistance are
identified and can be used as biomarker to select patient more likely to benefit from radiotherapy.\textsuperscript{83-85} Recently Ren \textit{et al.}\textsuperscript{86} discovered that the microRNA-27a(miR-27a) axis plays an important role in modulating response to radiotherapy in TNBC cells. CDC27 is a direct target of miR-27a and downregulation increased radioresistance of the TNBC cells.\textsuperscript{86} Gasparini \textit{et al.}\textsuperscript{87} found a positive correlation between miR-155 levels and positive response to radiotherapy. High levels of miR-155 are associated with lower RAD51 levels and better overall survival in TNBC patients.\textsuperscript{87} Detection of these markers in patients might be useful to identify the subgroup of patients who could benefit from radiation treatment and help the development of novel therapeutic strategies to overcome radioresistance and improve radiosensitivity.

\textbf{2.2.4 \textit{Targeted therapy}}

Primary triple-negative breast cancers are still treated as if they were a single disease entity. However as described above, it is clear they do not behave as a single entity in response to current therapies. With the classification of TNBC, by Lehmann \textit{et al.}\textsuperscript{16} in different subgroups with minimal molecular differences, molecular targeted drugs have been developed and tested for treatment of specific TNBC subgroups. It needs to be mentioned that targeted therapies are only used in locally advanced and/or metastatic breast cancer set-up or clinical trials.

\textbf{2.2.4.1.1 Poly-adenosine-diphosphate-ribose-polymerase (PARP) inhibitors}

PARPs are involved in the detection and repair of DNA damage and are important in cell cycle progression and apoptosis.\textsuperscript{88} PARP levels are elevated in TNBCs, especially in BL1, BL2 subtypes and BRCA1-associated tumors (Fig. 5).\textsuperscript{16, 59} Therefore, PARP inhibitors enhance the effect of DNA damaging agents, such as radiotherapy and chemotherapy in BRCA1 mutated cells or cells with impaired
homologous recombination (HR) complex or in carcinomas with elevated PARP levels.\textsuperscript{89}

The use of PARP inhibitors is currently under clinical development and are targeted to PARP-1 and PARP-2 protein.\textsuperscript{90} Unfortunately, until now no success was seen for PARP inhibitors in TNBC but clinical trials are ongoing.\textsuperscript{91-93}

2.2.4.2 **Anti-angiogenic agents**

TNBC are known as highly proliferative tumors. To sustain this rapid tumor growth, development of new blood vessels is necessary to provide the tumor with nutrients and unfortunately forms a gate for metastasis.\textsuperscript{94, 95} Vascular endothelial growth factor (VEGF) is a key angiogenic mediator that stimulates endothelial cell proliferation and regulates vascular permeability.\textsuperscript{94, 96} TNBC has enhanced vascular proliferation that supports rapid growth, early metastasis and has been found to express high levels of VEGF.\textsuperscript{97, 98} The available VEGF targeting treatments work in two different ways. The first one prevents VEGF to interact with its receptor on endothelial cells by using a monoclonal antibody that binds to VEGF (bevacizumab/avastin). The second uses small molecule angiogenesis inhibitors (sunitinitib) blocking the VEGF receptor.

Despite the increased pathologic complete response rate observed in neoadjuvant bevacizumab it did not translate into a survival benefit.\textsuperscript{99, 100} Ranpura \textit{et al.}\textsuperscript{101} showed that the addition of bevacizumab was associated with an increased risk of fatal adverse events, especially when bevacizumab was used in combination therapy with taxanes or platinum drugs.

2.2.4.3 **EGFR inhibitor (HER1 inhibitor)**

With approximately 60\% of the TNBC expressing EGFR, targeting EGFR could be a promising therapy for TNBC.\textsuperscript{102} Unfortunately, Carey \textit{et al.}\textsuperscript{103} found in the TBCRC 001 trial, that treatment with a monoclonal antibody against EGFR (cetuximab) alone or in combination with carboplatin had very low response rate in TNBC
patients (6% and 17% respectively). EGFR amplifications are reported in 5% of the TNBC patients.\textsuperscript{102} Especially BL2 subtype shows an unique genetic mutations involving growth factor signaling, resulting in upregulation of EGFR (Fig. 5).\textsuperscript{16} Identifying patients with these mutations could be beneficial for further trials with EGFR targeting therapies.

The other classes of EGFR inhibitors are agents who target the EGFR tyrosine kinase domain, but erlotinib and gefitinib are not very effective in the treatment of BC.\textsuperscript{59}

\subsection*{2.2.4.4 \textbf{Aurora kinase inhibitors}}

Aurorakinase A (Aur-A), is a member of the mitotic serine/threonine kinase family and plays a crucial role in the shaping of cell mitotic spindles, centrosome duplication and cytokinesis.\textsuperscript{104} Xu \textit{et al}.\textsuperscript{105} showed that TNBC cells overexpress Aur-A and inhibition of this kinase decreased cell proliferation and prevented cell migration \textit{in vitro}.\textsuperscript{105} Overexpression of Aur-A was associated with high Ki-67 level, which corresponds to a highly proliferative TNBC subtype. Aur-A kinase inhibitors could be promising for BL1 subtype because of their high proliferation rate (Fig. 5).\textsuperscript{59, 105} Recently described Aur-A kinase inhibitors that are used in preclinical settings are alisertib (MLN 8237) and AMG 900.\textsuperscript{106}

\subsection*{2.2.4.5 \textbf{IGF-1R inhibitors}}

The insulin-like growth factor 1 receptor (IGF-1R) signaling pathway is an important regulator of growth, survival, migration, angiogenesis and invasion in cancer.\textsuperscript{107} IGF-1R is amplified in basal breast cancer, and high levels of IGF-1R protein are seen in TNBC patients (Fig. 5).\textsuperscript{59, 108-110} Available treatments targeting the IGF-1R signaling pathway work in two different ways: monoclonal antibodies against IGF-1R (cixutumumab) and tyrosine kinase inhibitor (BMS-754807).\textsuperscript{48, 111} Litzenburger \textit{et al}.\textsuperscript{111} showed that in mouse models, treatment of TNBC with BMS-754807 resulted in tumor regression when combined with doxetaxel.
2.2.4.6 **PI3K/Akt/mTOR inhibitors**

15-20% of TNBCs have genetic mutations in PI3K pathway-associated genes. The PI3K pathway is involved in regulation of cell proliferation, survival and migration. Activation of the PI3K pathway can occur by at least 2 ways. First, through mutations that activates PIK3CA, which are also the most frequent genetic event in breast cancer. The other way of PI3K pathway activation is by loss of PTEN tumor suppressor gene function and/or INPP4B gene function loss, causing the increase of PI3K activation and downstream Akt and/or mTOR activation. Because of the high mutation rate of PI3K pathway-associated genes in TNBC, targeting this pathway could be a good alternative treatment. Inhibitors of this pathway have been tested in preclinical and clinical trials. ML and LAR subtypes have expression profiles with the most frequent PI3K pathway-associated gene mutations; therefore they are more sensitive to PI3K pathway inhibitors compared with other TNBC subtypes (Fig. 5).

The PI3K pathway is important in the stabilization of the BRCA1/2 protein levels. Inhibiting PI3K makes BRCA-proficient patients sensitive to PARP inhibition.

2.2.4.7 **Src inhibitors**

Compared to other breast cancer types, TNBC showed higher frequency of aberrant sarcoma oncogene (Src). ML subtype expresses genes that are associated with EMT and cell motility. Non-receptor tyrosine kinase-src plays a critical role in cell migration, proliferation, differentiation and cell survival. Therefore targeting Src could be useful for ML subtype TNBC (Fig. 5).

Src inhibitor dasatinib, a small molecule tyrosine kinase inhibitor which blocks Src and Abl kinase, selectively inhibits growth of TNBC cell lines but showed modest results when given as monotherapy.
Androgen receptor (AR) inhibition
The LAR subtype expresses high levels of AR which are driven by AR signaling and which represents a therapeutic target for this subtype (Fig. 5).\textsuperscript{17, 19}

AR antagonist bicalutamide, used as monotherapy, showed a clinical beneficial rate of 19\% in patients with metastatic, AR positive and hormone receptor negative breast cancer.\textsuperscript{119}

Heat shock protein (Hsp) 90 inhibition
AR requires Hsp90 chaperone for proper protein folding and stability of several proteins that play an important role in proliferation, survival and angiogenesis.\textsuperscript{120} Distinct modes of Hsp90 inhibition cause destabilization and degradation of Hsp90 target proteins in a proteasome-dependent mechanism.\textsuperscript{121}

Hsp90 inhibitor ganetespib, reduced viability of TNBC cell lines and suppressed lung metastasis in experimental mouse models.\textsuperscript{122} In a phase II clinical trial, clinical response was seen in a breast cancer population with metastatic disease.\textsuperscript{123} Further trials with Hsp90 inhibitors as monotherapy or in combination with radio-or chemotherapy are justified in more targeted patients harboring specific Hsp90 oncoproteins.\textsuperscript{123} LAR TNBC cell lines are the most sensitive to Hsp90 inhibitor (Fig. 5).\textsuperscript{16}
Figure 5: Scheme of different TNBC subtypes and there specific targeted therapies. BL1 (basal-like 1), BL2 (basal-like 2), IM (immunomodulatory), ML (mesenchymal-like), MSL (mesenchymal stem-like) and LAR (luminal androgen receptor). *Adapted from Turner NC et al.* and *Mancini P et al.*
2.3 References


Chapter 3

Radiobiology
3.1 Ionizing radiation: X-rays

3.1.1 Definition

X-rays are high-energy electromagnetic waves without electric charge, i.e. photon beams. X-rays cause excitations and indirect ionisation along their way through the body. The process of ionization of an atom occurs when the energy of the absorbed X-ray is bigger than the binding energy of the orbital electron of an atom in the medium. The orbital electron excites the atom, resulting in a free electron (Compton electron) and the X-ray photon loses energy due to the interaction but continues to travel through the medium (Compton scattering). The ionized atom returns to its steady state by emission of a characteristic X-ray (fluorescent radiation) or an Auger-electron. In X-ray radiation, ionization and excitation are caused by the electrons of the medium and are called indirect ionizing radiation. Ionizing radiation deposits a relatively large amount of energy into a small volume of medium, resulting in damage to the surrounding cellular components. Ionization properties depend on the energy of the photon (eV) and the chemical composition of the medium. Electromagnetic radiation is ionizing when it has a photon energy level (eV) >124 eV, corresponding with a $\lambda < 10^{-6}$ cm.$^1$ (Based on Radiation Dosimetry and Radioprotection / Prof. dr. Hubert Thierens – UGent: Department of Basic Medical Sciences, 2010. Non published)

3.1.2 Biological effects

To compare the biological effectiveness of different kinds of radiation in a quantitative way, relative biological effectiveness (RBE) was introduced. RBE is the ratio of the absorbed dose for a reference radiation (250 kVp X-ray or Co$^{60}$ γ-ray) to the absorbed dose from a test radiation to give the same level of biological effect in a given tissue. RBE is an experimentally measured quantity dependent on the radiation linear energy transfer (LET), dose, conditions (dose tempo, dose fractions), but also environmental factors (O$_2$), type of biological system (cell lines) and end-point of
3.1.2.1 Direct and indirect actions of radiation on DNA

Ionizing events disrupt DNA molecules by knocking out an intramolecular bonding electron. The resulting molecule is now called a radical and will react with surrounding water or other DNA molecules. When the absorbed energy is too low for ionization, excitation of the DNA molecules takes place. The DNA molecules fall apart and form 2 radicals that will react with the surrounding molecules (Fig. 6).\(^1\)

Indirect damaging effects are caused by interaction of ionizing radiation with H\(_2\)O molecules. The complex reaction where H\(_2\)O molecules are ionized is called radiolysis. In this process 2 radicals are formed that will interact and damage DNA molecules. In the presence of O\(_2\) disrupted molecules and radicals recombine to form reactive oxygen species (ROS). These consist of peroxyl radicals, strong oxidant, which will further react and will form superoxide and H\(_2\)O\(_2\) (Fig. 6).\(^1\) (Based on Radiobiology and Radiopathology / Prof. dr. Anne Vral – UGent: Department of Basic Medical Sciences, 2010. Non published)
3.2 Radiation and breast cancer

3.2.1 Effect on breast cancer cells

3.2.1.1 Tumor inhibiting effects

Ionizing radiation holds the potential to kill cancer cells by transferring sufficient quantities of energy to the cancer cell which will break down all kinds of chemical bonds (DNA, RNA, proteins and lipids). However, indirect damaging effects appear more frequent as cells consist mainly of water. (Based on Radiobiology and Radiopathology / Prof. dr. Anne Vral – UGent: Department of Basic Medical Sciences, 2010. Non published)

DNA

DNA breakdown causes different kinds of biochemical changes in DNA molecules: (I) single strand (SSB) and (II) double strand DNA breaks (DSB), (III) base damage or loss, (IV) formation of cross links (DNA-DNA or DNA-protein) or (V) sugar damage. Normal cells have two kinds of double strand DNA DSB repair mechanisms: (I) ‘error free’ repair, i.e. homologous recombination and (II) ‘error prone’ repair, i.e. non-homologous end joining are the most important pathways. The latter is the most used and can result in misrepair, causing mutations and chromosomal aberrations. Chromosomal aberrations are variations in chromosomal number (e.g. hyperdiploïdie > 46 chromosome) or structure due to fusion of genes or rearranged genes (e.g Philadelphia chromosome; specific translocation of DNA between chromosome 9 and 22 which results in a shortened chromosome 22 and lengthening of chromosome 9). Other DNA damages are mostly repaired by base-excision repair (base modification e.g methylated bases), nucleotide-excision repair (bulky lesions as pyrimidine dimers) or mismatch repair (single-base mismatches). (Based on Radiobiology and Radiopathology / Prof. dr. Anne Vral – UGent: Department of Basic Medical Sciences, 2010. Non published)

A critical component in the homologous recombination repair pathway is BRCA1. Mutations in the BRCA1-gene give rise to genetic instability and impaired DNA
repair, which results in an increased risk for breast and ovarian cancer.\textsuperscript{5} The positive aspect of this impaired DNA repair is seen in BRCA1-mutated cancer cells that are highly sensitive to DNA damaging agents (e.g. radiotherapy, chemotherapy).\textsuperscript{5-7} Cell cycle checkpoints are responsible for the correct completion of the cell cycle. They represent intracellular signal transduction pathways that stop the cell cycle upon DNA damage or replicative stress. This allows the cell to repair its damage, or when there is too much damage, to undergo cell death.\textsuperscript{8} The mechanism of cell death response to ionizing radiation is very complex and depends on different factors.\textsuperscript{9} Different radiation-induced cell death processes are: (I) apoptosis, (II) mitotic cell death, (III) necrosis, (IV) senescence and (V) autophagy.\textsuperscript{9,10}

More specifically, cells in the G2/M-phase of the cell cycle are the most radiosensitive.\textsuperscript{11} Cells in G0-phase (out of cell cycle) or slowly proliferating cells (long G1-phase) are less radiosensitive.\textsuperscript{12} During G0-phase gene regions are covered with proteins that protect the DNA from radiation damage. So highly proliferative cells have more cells in the radiosensitive phase and are the main target of ionizing radiation.\textsuperscript{12} Radiosensitivity is also related to the DNA-repair pathway that is utilized.\textsuperscript{13} When cells are irradiated during S-phase of the cell cycle, homologues recombination is used to repair ds DNA breaks (during M-phase NHEJ is used) and this error-free DNA repair helps the cell to survive and correlates with an increase in radioresistance.\textsuperscript{13}

**Cytoplasm**

In the cytoplasm, ionizing radiation causes formation of ROS, reactive nitrogen species (RNS) and upregulation of NO which contribute to the toxic effect in irradiated and non-irradiated cells.\textsuperscript{14} ROS contributes to the generation of hydroxyl radicals.
**Plasma membrane**

Ionizing radiation and free radicals can cause damage to the plasma membrane, resulting in activation of the sphingomyelin pathway. Activation of sphingomyelinase results in breakdown of sphingomyelin, thus generating ceramide which is a potent mediator of apoptosis.\textsuperscript{15} This results in p53-independent cell death.\textsuperscript{15}

**Mitochondria**

Ionizing agents cause changes in mitochondria. Loss of mitochondrial membrane potential and release of cytochrome C activates the caspase cascade that causes apoptosis.\textsuperscript{16, 17} It also leads to enhanced release of ROS/RNS by the mitochondria, that in turn will activate p53-dependent cell death.\textsuperscript{18} Upon ionizing radiation, ceramide can be directly released by the mitochondria through direct activation of mitochondrial ceramide synthase.\textsuperscript{15}

Important for all off these events is that they induce the expression of genes whose products activate multiple signal transduction cascades, which in their turn sustain the tumor inhibiting effect or promote cell survival.\textsuperscript{19} The balance between the two determines the fate of the irradiated cell. When cancer cells are led into the cell death pathway, these early biochemical events will evolve in time and manifest as morphological changes off the tumor, resulting in tumor regression.

**3.2.1.2 Tumor promoting effects**

Unfortunately, under certain conditions a population of cancer cells manages to survive radiotherapy, this either because the tumor received a sublethal radiation dose and/or because the tumor manages to survive by making use of repair mechanisms. This leads to activation of signal transduction pathways and release of soluble factors by the cancer cell. These factors will have an autocrine or paracrine effect on the cancer cells and surrounding tissue. Surviving cells are more resistant to therapy and have more metastatic potential, which results in more aggressive and
malignant recurrent tumors. Dying cancer cells can also contribute to tumor-promoting events. They provide signals to promote tumor repopulation.\(^{20}\) Different mechanisms contribute to this radiation-induced tumor-promoting event and are described below.

**Upregulation of proteases, bioactive lipids, cytokines and other growth factors**

**Proteases**

Matrix metalloproteinases (MMP) cleave extracellular matrix (ECM) and basement membrane components, promoting invasion of cancer cells into surrounding tissue. Current research is mostly focused on the upregulation of MMP-2 and MMP-9 after radiation exposure. It is described that irradiation of pancreatic cancer cells increases invasion of the cancer cells through transition of latent MMP-2 to the active form and increases the expression and activity of MMP-9 in irradiated cancer cells.\(^{21-24}\) Enhanced MMP-2 secretion contributes to invasion of cancer cells through Src-dependent EGFR activation, triggering p38/Akt and the PI3K/Akt-signaling pathway.\(^{25}\) Sublethal radiation enhances cancer cell invasion by MMP-9 expression through the PI3K/Akt/NF-\(\kappa\)B pathway.\(^{23}\) Correspondingly, investigations of *in vivo* models of prostate cancer found a correlation between the appearance of radiation-induced metastasis and activation of MMP-9.\(^{26}\)

Urokinase-type plasminogen-activator (uPA) is a protease with cytokine-like activity. Irradiation of cancer cells results in secretion of uPA which promotes cancer cell migration, invasion and angiogenesis *in vitro* and *in vivo*.\(^{24, 27}\)

Huang *et al.*\(^{20}\) showed that irradiated, dying 4T1 breast cancer cells promote cell growth of non-irradiated, healthy 4T1 *in vitro* and *in vivo*, in a caspase 3-dependent manner.
Proteases also contribute to the release of growth factors via their proteolytic activities, indirectly activating signaling pathways that play a role in tumor invasion and metastasis.\textsuperscript{28}

**Bioactive lipids**

Dying cells can also stimulate tumor growth by releasing growth-promoting signals, the surviving cancer cells are stimulated to proliferate. Huang et al.\textsuperscript{20} found that caspase 3 regulates the release of growth-promoting prostaglandin E2 (PGE2) by dying cancer cells. Within hours after radiation increased secretion of prostaglandins and thromboxanes is seen which contributes to the radioprotective effect of the cancer cells.\textsuperscript{29} Inhibition of prostaglandin synthesis, making use of cyclooxygenase-2 inhibitor, was associated with improved irradiation response.\textsuperscript{29}

**Cytokines and other growth factors**

Ionizing radiation triggers rapid activation of multiple cytokine cascades, with a peak production 4-24 hours after irradiation.\textsuperscript{30} Cytokines can be divided into groups with related functional properties. Pro-inflammatory cytokine such as TNF-\(\alpha\), IL-1\(\alpha\) and \(\beta\), IL-17; angiogenic/vascular VEGF, TNF-\(\alpha\) and FGF; anti-inflammatory IL-4, IL-10 and TGF-\(\beta\); pro-fibrotic IL-6 and TGF-\(\beta\); immune-stimulatory IL-2, IL-4 and IL-7, and hematopoietic CSF1, GM-CSF, IL-3, EPO.\textsuperscript{31} Radiation enhances the secretion of many cytokines. Particularly, upregulation of pro-inflammatory, angiogenic and fibrotic cytokines contribute to the radiation-induced pro-invasive and metastatic character of the cancer cells.

For example, upregulation of IL-6 secretion contributes to cancer cell invasion. By activation of IL-6/JAK/STAT3 signaling in BCC it promotes tumorigenesis and metastasis.\textsuperscript{32} After radiation exposure TGF-\(\beta\) secretion is upregulated in different kinds of cancer cell lines. Irradiation of cancer cells enhances migration and invasion through TGF-\(\beta\)-mediated EMT. Inhibition of TGF-\(\beta\) signaling reverses radiation-
induced EMT and decreases migration and invasion of the cancer cells.\textsuperscript{33} In neuroblastoma it is described that radiation triggers TNF-\( \alpha \) transactivation and secretion in a NF-\( \kappa B \)-dependent manner which favors survival.\textsuperscript{34} Increased VEGF concentration is detected in conditioned medium of irradiated glioblastoma cells. Autocrine activation of VEGFR receptor enhances glioblastoma cell motility.\textsuperscript{35} This increased VEGF secretion also stimulates angiogenesis by influencing endothelial cells present outside the radiation field.\textsuperscript{24}

**Activation of signaling pathways**

Irradiation of cancer cells might promote invasion and metastasis, but the molecular mechanisms involved have not been fully elucidated. The signal transduction pathways, that sustain anti-tumor effects can also be used to promote tumor survival, proliferation, migration and invasion.\textsuperscript{19} By upregulated receptor activation and/or secretion of soluble factors, these pathways are stimulated.\textsuperscript{36} The most important radiation-induced types of signaling work through activation of EGFR and IGF-1R and other growth factor receptors, activating the PI3K, MAPK, JAK/STAT, and p38 pathways, finally resulting in activation of pro-caspases and NF-\( \kappa B \).\textsuperscript{19, 37, 38}

For example, activation of EGFR after irradiation has been reported by several groups. They conclude that radiation-induced EGFR stimulation leads to activation of the MAPK pathway and causes a growth stimulatory effect in human carcinoma cells (Fig. 7).\textsuperscript{19, 39} Invasive potential of glioma cell is enhanced after irradiation by activation of EGFR and IGF-1R, through Rho signaling via PI3K pathways (Fig. 7).\textsuperscript{40} Osuka et al.\textsuperscript{41} showed that radiation exposure caused an upregulation of both IGF-1 secretion and IGF-1R expression, leading to downstream Akt survival signaling in glioma stem cells. Furthermore, treating radioresistant cells with an IGF-1R inhibitor markedly increased sensitivity to radiation (Fig. 7).\textsuperscript{41}
In non-small lung cancer, the PI3K/Akt pathway is associated with the intrinsic radiosensitivity mechanism, hypoxia and tumor cell proliferation.\textsuperscript{42} All these effects contribute to radioresistance of non-small lung cancer (Fig. 7).\textsuperscript{42}

The radiation-induced increase in migration and metastatic potential of cervical cancer cells operates via the K-Ras pathway. Ras plays a prometastatic role via the activation of c-Raf/p38, whereas interference of those mediators via either RNA interference-mediated knockdown or the use of chemical inhibitors substantially reverses the radioresistance-associated increase in cell migration (Fig. 7).\textsuperscript{43}

Another important signaling pathway is activated upon c-Met stimulation. c-Met overexpression was seen in breast cancer cells after irradiation, resulting in ligand-independent receptor phosphorylation and also making cancer cells more sensitive to HGF. This promotes radiation-induced invasion, cell survival and proliferation. Treatment with c-Met inhibitor enhanced radiotherapy efficacy and caused xenograft tumor regression.\textsuperscript{44}

Recent studies showed an induced expression of HIF-1\(\alpha\) and ROS in irradiated lung cancer cells, leading to transcriptional activation of CXCR4 and increased invasiveness via the SDF-1/CXCR4 pathway.\textsuperscript{36}
Figure 7: Pathways involved in radiation-induced tumor-promoting effects. Radiation stimulates different intracellular pathways. Each color corresponds to a receptor specific signaling cascade described in a study. References are indicated above the receptors. Studies about EGFR signal are indicated in blue. Studies about IGFR are indicated in green and studies involving other growth factor receptors (GFR) are indicated in yellow. Arrow represents an activating interaction. The results of these receptor stimulations, described in all of the referred articles, contribute to the tumor promoting effect of radiotherapy. Adapted from Moncharmont C et al.\textsuperscript{37}
Radiation-induced EMT

The effects of radiation-induced increased secretion of growth factors and increased activation of multiple signal pathways lead to genetic changes, causing disruption of the genetic epithelial balance (Fig. 8). This leads to molecular changes in the cancer cells: (I) loss or decrease of epithelial markers and (II) gain or increase of mesenchymal markers (Fig. 8). Eventually, it leads to phenotypic changes of the cancer cells: (I) spindle-cell shape, (II) loss of polarity, (III) loss of cell-cell contact (IV) gain of cell-matrix contact and (V) pseudopodia formation. Researchers showed that ionizing radiation of cancer cells induces EMT. Radiation-induced EMT results in increased motility, migration, invasion and even metastasis of the irradiated cancer cells. In cancer cells with higher metastatic potential, radiation exposure can induce EMT more easily. Martin et al. showed that during radiotherapy, mobilization of viable cancer cells into the circulation promotes metastasis formation.

Figure 8: Overview EMT. EMT, a process where cells progressively differentiate from epithelial phenotype to mesenchymal phenotype. The epithelial and mesenchymal cell markers and EMT regulators are listed. Adapted from Kalluri R and Weinberg RA.
3.2.2  Effect on local tumor microenvironment

In the last decade radiation techniques have improved, in a way that the radiation dose to the tumor has increased while minimizing radiation dose delivered to surrounding “healthy” tissue. Nevertheless, some surrounding tissue is still damaged by the effects of ionizing radiation, both directly and indirectly. Irradiated cells send signals, through soluble factors or direct intercellular communication, to the neighbouring cells. These can disrupt normal cell function causing either cell death or eliciting a response through activation of several signal transduction pathways, causing a signalling cascade. This effect is the so-called ‘radiation induced bystander effect’ (RIBE). This effect can lead to repair of the damaged cells or may trigger them to die. Together with the direct targeted effect, these non-targeted effects increase the damage caused by radiotherapy (Fig. 9).\(^5^2\) This causes changes to the tumor microenvironment in a way that it could promote cancer initiation and progression. These effect have only been described in \textit{in vitro} and \textit{in vivo} experiments and tissue models.\(^5^3\)

![Figure 9: Radiation damage dose response curve.](image)
In patients, long-range effects between or within tissues are reported, as so called “abscopal effect”. This non-targeted effect, actually an out-of-field effect, can better be described as low dose radiation effects, caused by (I) head leakage, (II) scattering at the beam collimator and flattening filter, and (III) internal scatter of the radiation beam. But the abscopal effect can also be caused indirectly, by systemic secretion of cytokines after radiation or by tumor-adaptive immune response.

3.2.2.1 CAF-myofibroblast-stroma

Cancer-associated fibroblasts (CAF) and myofibroblasts are important components of the tumor stroma. Moreover, CAF activation into myofibroblasts is another key step in radiation-induced fibrosis and radiation-induced cancer cell invasion, where these cells play an active role in the synthesis and remodeling of extracellular matrix (ECM) components. Upregulation of ECM proteins (such as tenascin C (TNC) and collagen I), cytokines (such as HGF, TGF-β and CXCL12), and matrix remodeling enzymes secretion are seen in CAF upon irradiation. HGF secretion is increased by irradiated fibroblasts. This increases invasion of cancer cells through upregulated c-Met phosphorylation and MAPK activity. Increased secretion of IL-1β by irradiated fibroblasts stimulates the expression of COX-2 in breast cancer cells, which leads to PGE2 induced stimulation of MMP-2 production. Huang et al. revealed that irradiated mouse fibroblasts stimulate cancer cell repopulation by enhanced release of PGE2 into the supernatant in a caspase 3-dependent way. Barcellos-Hoff et al. investigated the effect of irradiation on the microenvironment, thereby focusing on the role of TGF-β in this process. They conclude that irradiation induces a rapid and persistent TGF-β activation. Further, TGF-β induces the proliferation of certain types of cancer cells (glioma, breast carcinoma and osteosarcoma), activation and differentiation of epithelial cells and myofibroblasts. It also contributes to the local creation of a immunosuppressive environment. All of the above effects contribute to the transformation of tumor stroma from a defensive to offensive player in tumorigenesis. Inhibition of TGF-β prior to
irradiation increases breast cancer cell radiosensitivity \textit{in vitro} and promotes tumor radiation control \textit{in vivo}. \cite{66}

Research showed that irradiated fibroblasts trigger responses in non-irradiated neighboring cells, contributing to the radiation-induced-bystander effect. Irradiated mouse fibroblasts stimulated growth of squamous cell carcinoma cells and enhanced their expression of growth-, invasion- and motility-related molecules (c-Met, Ras, MAPK cascade, MMP-1 and -9) through TGF-\(\beta\)-mediated bystander mechanisms. \cite{67} TGF-\(\beta\) and SDF-1, secreted by fibroblasts exposed to radiation, stimulate tumor progression through activation of JAK/STAT, Rho/Rock, PI3K/Akt and MAPK pathways. \cite{36, 68}

Primary tumors recurring in the irradiated field are characterized by a long latency period and a reduced volumetric growth rate with high metastatic rate, poor oxygen supply and resistance to treatment. This is the so-called “tumor-bed-effect” (TBE). \cite{59} TBE is a dose dependent and local effect, only observed in tumors arising inside the irradiated stroma. \cite{59} Irradiation of tumor stroma is one of the components contributing to this effect; the other component is the effect of radiation on the tumor vascularity. \cite{69} TBE promotes invasion and metastasis by upregulation of matricellular proteins (CYR61), pro-metastatic cytokines and proteases and by cooperation with integrins (\(\alpha V\beta 5\)). \cite{60, 70, 71}

These observations not only occur in experimental set-ups. Adjuvant radiotherapy improves local tumor control but failure of this control gives rise to tumor recurrence in pre-irradiated stroma with these tumors being associated with increased metastasis risk and poor prognosis. \cite{72, 73}

On the long term, early fibroblast activation and increased inflammation response will lead to increased ECM production and remodeling, giving rise to radiation-induced fibrosis and irreparable normal tissue damage. \cite{60}
3.2.2.2 **Endothelial cells-tumor vasculature**

Irradiation of primary tumors causes damage to the vasculature of both tumor and normal surrounding tissue. This leads to endothelial cell dysfunction, characterized by increased permeability, detachment from basement membrane and cell death.\(^{60}\) Inadequate vascular supply in the irradiated stroma contributes to the tumor-bed-effect by potentiating tumor hypoxia and triggering the immune response by increased secretion of cytokines and growth factors.\(^{36},^{60}\) Radiation not only causes hypoxia in one part of the tumor, but also causes re-oxygenation other parts of the tumor. This leads to cell death and cell shrinkage, therefore causing decreased oxygen consumption. This contributes to the sustained hypoxia state.\(^{28}\) Radiation-induced hypoxia reduces the production of ROS, decreases DNA damage in cancer cells, thus preventing cell death.\(^{60}\) Hypoxia induces rapid increase of hypoxia-inducible factor 1α (HIF-1α) in irradiated tumors as a result of increase in oxidative stress and improvement in glucose and oxygen availabilities.\(^{74}\) This leads to stimulation of transcription of many genes encoding for proteins regulating cancer cell growth, invasion, metastasis, angiogenesis, lymphangiogenesis and radioresistance pathways.\(^{28},^{36},^{75},^{76}\)

Irradiation of melanoma xenografts increased the fraction of hypoxic cells in a tumor mass *in vivo*. Experimentally induced melanoma tumors that regrew in the pre-irradiated bed, had hypoxia-mediated upregulation of metastasis-promoting genes that contributed to metastasis formation by inducing neoangiogenesis, facilitating hematogenous spread and upregulation of uPAR.\(^{69}\) Other *in vivo* models showed that KitL/c-Kit signaling is critically involved in metastasis formation of breast tumors growing in a pre-irradiated bed.\(^{77}\)

Upon irradiation, pro-survival signaling pathways are activated in endothelial cells. This promotes angiogenesis and is associated with radiotherapy failure and enhances radiation-induced metastasis.\(^{28},^{78}\) Even low-dose radiation (lower or equal to 0.8 Gy) led to ligand-independent phosphorylation of the VEGFR-2 receptor on endothelial cells, promoting endothelial cell migration and preventing endothelial cell death.
through PI3K/Akt and MEK/ERK signaling pathways.\textsuperscript{78} Under hypoxic conditions, low-dose radiation induces VEGF expression in endothelial cells.\textsuperscript{78} Irradiation of endothelial cells causes activation of surface receptors ($\alpha_v\beta_3$ integrin), protecting them from apoptosis and increases their ability to trigger angiogenesis.\textsuperscript{79} Radiation with a single dose 3 Gy promotes endothelial cell survival and capillary formation, \textit{in vitro} and \textit{in vivo}, through activation of Akt/GSK-3$\beta$ signaling.\textsuperscript{80} 5 Gy radiation causes HIF-1$\alpha$ up-regulation in endothelial cells and up-regulation of a HIF-1$\alpha$-independent SDF-1, which mediates endothelial migration.\textsuperscript{81} However, high-dose radiation (8-20 Gy) locally suppressed FGF-2 and VEGF-induced angiogenesis in quiescent blood vessels. Inhibited sprouting, migration, proliferation and premature senescence and enhanced matrix adhesion of endothelial, without increasing cell death, were described \textit{in vitro}, making quiescent endothelial cells resistant to radiation-induced cell death.\textsuperscript{75, 82} Cytokines are released into the systemic circulation and trigger receptors located on the surface of e.g. bone marrow cells, which are released into the circulation. These mobilized bone marrow-derived angiogenic cells home to the tumor and stimulate vascularization and lymphangiogenesis.\textsuperscript{75, 76}

Radiation-related EGFR activation or radiation-related ROS stimulation of cancer cells triggers angiogenesis by the secretion of growth factors (VEGF, bFGF), proteases (MMP-2) or by inhibiting endogenous angiogenic inhibitor (angiostatin).\textsuperscript{28, 83-86} In mice, MMP-inhibitors interfered with the anti-tumor action of radiation, leading to inhibition of tumor growth and angiogenesis.\textsuperscript{87} Exogenous administration of angiogenesis inhibitors, could be beneficial for a subset of patients. In mouse models, administration of recombinant angiostatin prevented the growth of metastases after radiation treatment of primary Lewis lung carcinoma.\textsuperscript{84}

Radiation-induced vascular damage facilitates tumor cell intravasation. Secretion of VEGF by cancer cells increased permeability of tumor blood vessels, promoting intravasation of cancer cells.\textsuperscript{76} Hypoxia-induced angiopoietin 2 expression in
endothelial cells, which results in reduced coverage of endothelial cells by pericytes and enhances cancer cell leakage into the blood stream. 76

3.2.2.3 Immune cells
Radiation has an anti-inflammatory and immunosuppressive effect through the destruction of tumor associated immune cells.55 It triggers immune reaction and inflammation by causing release of “danger signals” as well as tumor-derived antigens. The first in line are cells of the innate immune system, like dendritic cell, macrophages and neutrophils that produce cytokines and chemokine to attract antigen presenting cells.88, 89 These cells will migrate to draining lymph nodes and present the tumor associated antigens to nearby T-cells. These will migrate toward the tumor and can cause tumor cell death.89 But in the tumor microenvironment (TME), immune cells can either promote or inhibit cancer development after radiation exposure, dependent on maturation state and their position in TME.89, 90 Only local events will be described in this paragraph. Systemic events will be described in the effects on distant tissues. The type of cancer cell death determines the type of elicited immune response. Radiation-induced cell death generally occurs through apoptosis or necrosis but in some cases, due to DNA damage in the TME, it can lead to mitotic catastrophe.55, 90

Radiation-induced DNA damage, leads to activation of NF-κB in an ATM-dependent way or, alternatively, radiation exposure activates NF-κB independent from DNA damage through activation of TNFR-associated factors. NF-κB activation causes the expression of “pro-inflammatory” molecules like TNFα, IL-1, IL-6.90 Leukocyte detection of these molecules activates both innate and adaptive immune responses.90 The first one to respond are innate leukocytes: (I) dendritic cells (DC), (II) macrophages, (III) natural killer cells (NK) and (IV) mast cells.90 DCs present at the time of irradiation are less effective antigen presenting cells and yield induced immune responsiveness, but the bystander effect enhances non-irradiated DC
activation and stimulates specific anti-tumor immunity.\textsuperscript{90} Tumor cell death-associated-release of danger signals, like ATP release, acts on purinergic receptor of DCs, leading to activation of the inflamasome that sustains immune response by secretion of pro-inflammatory cytokines.\textsuperscript{90} During radiation exposure, calreticulin and HMGB1 become exposed on the surface of dying cells and elicit an anti-tumor reaction through toll-like receptor 4 on DCs and macrophages.\textsuperscript{91} Radiation activates proteolytic activities of tumor-associated macrophages (TAM)\textsuperscript{92} directly through activation of NF-\(\kappa\)B by high-dose radiation, or indirectly through activation of adaptive antitumor immunity at low-doses radiation.\textsuperscript{86, 93} Exposure of macrophages to Th1 cytokines (TNF-\(\alpha\), IFN-\(\gamma\) and GM-CSF) leads to enhanced cytotoxic effect and production of pro-inflammatory cytokines (IL-1\(\alpha/\beta\) and IL-6).\textsuperscript{90, 94} Exposure to Th2 cytokines (IL-4 and IL-13) on the other hand blocks cytotoxic T lymphocyte activity and promotes angiogenesis and tissue remodeling by secretion of TGF-\(\beta\), VEGF and MMP-9.\textsuperscript{90, 94} Dying cancer cells release Hsp70 and other danger molecules targeting them for cell lysis by NK cells. Low-dose radiation results in stimulation of the antitumor reaction stimulated by NK cells \textit{in vivo}.\textsuperscript{95} Radiation promotes MMP-9 dependent VEGF production by mast cells.\textsuperscript{90}

Some tumor cells can escape these events by downregulation of MHC class I molecules but, remarkably, most of the irradiated cancer cell lines have an upregulation of MHC class I molecules and/or other surface molecules (like ICAM and Fas/CD95), promoting recognition or killing by cytotoxic T-cells.\textsuperscript{55, 89, 96} Also, upregulation of adhesion molecules ICAM-1, VCAM-1, E-selectin and P-selectin increases extravasation of lymphocytes into the tumor environment, enhancing immune cell trafficking in TME.\textsuperscript{55, 89}

The role of immunity in radiation response can be regarded as a double-edged sword. Identical mediators can either cause pro- or anti-tumor effects depending on the context. Inhibition of pro-tumor immune pathways in combination with radiotherapy
could be effective for tumor suppression and improve anti-tumor outcome of radiotherapy.

3.2.2.4 Epithelial cells

In breast cancer, the irradiated stroma promotes tumorigenic potential of non-irradiated epithelial cells. When normal mouse mammary epithelial cells (COMMA-D cells) were transplanted in an irradiated host, more quickly a higher tumor incidence with larger tumors was found than when implanted in a non-irradiated host. Andarawewa et al. found that ionizing radiation induces persistent ERK/MAPK activation in nonmalignant mammary cells and stimulates them to undergo TGF-β-mediated (secreted by irradiated stroma) EMT. This can lead, in the long term, to carcinogenesis.

These findings sustain the idea that the irradiated tumor and corresponding microenvironment contribute to the radiation-enhanced invasion and metastasis of cancer cells. Pathological effects of the above events are summarized chronological in Fig. 10
Figure 10: Overview radiation-induced effects. Early biological events cause acute tissue effects, which are usually transient and normally resolve within 3 months of completing treatment. These events also result in more protracted biological effects that can manifest in tissues as late biological effects and secondary malignancies. Higher radiation dose per fraction seems to increase the severity of late adverse effects. dsDNA, double-stranded DNA; ECM, extracellular matrix; ROS, reactive oxygen species; RNS, reactive nitrogen species; ssDNA, single-stranded DNA; TGF-β, transforming growth factor-β; TH, T helper cell. Adapted from Barker H et al. 2015.60
3.2.3 **Effect on distant tissues**

3.2.3.1 **Abscopal effect/out of the radiation-field**

Local irradiation of primary tumor can cause regression of metastatic lesions that are not even in the irradiated field. This phenomenon is called “abscopal effect”. Induction of endogenous anti-tumor adaptive and innate immunity is an important player in this effect.\(^{55}\) Diverse contributing mechanisms are known, such as trafficking of lymphocytes into TME and toward metastatic lesions. Radiation stimulates anti-tumor T-cell based immunity by increasing the number of activated CD8\(^+\) T cells in TME. CXCR6\(^+\) CD8\(^+\) T cells are attracted to radiation-induced CXCL16 upregulation in 4T1 breast cancer cells.\(^{99}\)

Furthermore, the systemic effect of the stress response to ionizing radiation, i.e. the release of cytokines, contribute to the abscopal effect.\(^{93}\) The abscopal effect is dose dependent with a key role for p53 and its downstream pathways.\(^{100}\) Radiation-activated p53 results in the production of growth inhibitors that could contribute to the abscopal effect.\(^{101}\)

Not only metastasis-inhibiting effects are seen, abscopal effects also include induction of genomic instability, cell death, and oncogenic transformation in normal tissues.\(^{56}\) Camphausen *et al.*\(^{100}\) reported the acceleration of metastatic growth in immunocompetent mice after irradiation of the primary tumor. Regression in urine MMP-2 levels was consistent with the regression of the primary tumor as well with the decreased production of angiostatin. They conclude that eradication of the primary tumor results in the growth of previously dormant lung metastases because of the decrease in systemic angiostatin levels.\(^{84}\) Radiation increased TGF-\(\beta\) serum levels in a metastatic breast cancer transgenic mouse model, resulting in increased growth of distant metastasis.\(^{102}\)
The abscopal effect is accompanied by waves of macrophage activation and production of cytokines. When tissues are damaged by radiation, inflammatory monocytes are recruited and differentiate into macrophages which will migrate to the damaged tissue. These activated macrophages produce inflammatory cytokines, including IL-1\(\alpha\), IL-1\(\beta\), IL-6, TNF-\(\alpha\) and TGF-\(\beta\), lasting up to 16 weeks after irradiation. Inflammation-generated oxidative stress leads to formation of reactive molecular species that can damage DNA, lead to mutagenesis and cancer in distant tissues.

This shows that local irradiation not only leads to TME responses but also triggers tissue responses that have systemic effects on tumor metastasis formation, dependent on radiation doses, fractionation and tissue type. The kind of signals generated by irradiated and non-irradiated cells, as well as the activation of different innate immune cells, influences radiation-induced immune-modulating effects. The abscopal effect is extremely rare in patients but in combination with immunotherapy the positive effect (anti-metastatic effects) can be enhanced.

### 3.2.3.2 Direct collateral damage

Despite very accurate radiation techniques, healthy distant tissue still gets irradiated. This happens through internal scatter, leakage of collimated sources and scattering at beam collimator or filters, or just because the healthy tissue is inevitably located in the irradiation field.

The same events as described in local effects happen at non-targeted sites: direct and indirect DNA damage effects, cell death, cytokine storms and inflammation, all leading to healing tissue or dying tissue resulting into fibrosis formation. Normal healthy tissues, which receive clinically relevant dose-radiation, will regenerate early and almost completely. Bergner and colleagues mimicked the lung situation by irradiation of cell culture monolayer, laboratory human organ culture and Goettinger
minipig assay. They concluded that lung epithelial cells have a remarkably high radioresistance.\textsuperscript{107}

Radiation damage to normal tissue can be chronologically categorized in: (I) acute (early) effects, (II) consequential effects and (III) late effects.\textsuperscript{108} The acute effects are observed during treatment or shortly after treatment. They affect mostly fast-proliferating tissues, like skin and mucosa. It also affects the stem-cell compartment, which causes loss of normal tissue turn-over.\textsuperscript{108} Consequential effects appear after persistent acute damage and when acute reactions fail to heal.\textsuperscript{108} Late effects appear months/years after radiation exposure and tend to occur in tissues with slow turnover rate. They manifest as fibrosis, necrosis, atrophy, vascular damage and tumor formation.\textsuperscript{108} Late radiation effect could lead to impaired normal function of the healthy tissue.\textsuperscript{106}

Like in breast cancer for example, a mean lung dose of $\geq 18\text{–}20$ Gy or irradiated lung volume of $\geq 25\%$ lung exposed to 20 Gy results in steeply rising probability of pneumonitis formation and reduction of lung function.\textsuperscript{109, 110} Pneumonitis is a lung’s response to radiation damage, appearing 6-16 weeks after radiation. This early phase is characterized by epithelial cell loss, microvascular permeability and increased expression of inflammatory cytokines.\textsuperscript{110} Laboratory animal studies showed that radiation induces a biphasic pro-inflammatory cytokine expression. The initial radiation response occurred within hours followed by a second more persistent secretion of pro-inflammatory cytokines, causing histopathologic alterations.\textsuperscript{111} This recruits inflammatory cells, not only to the irradiated part of the lung, which in turn will produce ROS and more cytokines.\textsuperscript{112} Laboratory animal studies revealed an important role or TNF-\textit{\textalpha} in the induction of post-radiation lung inflammation.\textsuperscript{113} Also early radiation-induced changes in IL-6 plasma levels were seen in non-small cell lung cancer patients.\textsuperscript{114} When this inflammation becomes chronic, fibrosis and vascular sclerosis will appear after months to years.\textsuperscript{106, 110} Incidental exposure of the
cardiopulmonary region to radiation also increases the rate of ischemic heart disease and secondary lung cancer.\cite{115,116} One of the important pro-fibrotic cytokines in this process is TGF-β; it has a central role in fibroblast activation and immune response modulation.\cite{117,118} Elevated TGF-β plasma levels were observed after lung irradiation but could not be used as a reliable and validated predictive assay.\cite{110} More prospective studies are required.

Secondary malignancies are of some concern in a patient population with a long life expectancy. As described by Barcellos-Hoff et al.\cite{119} the carcinogenic action of radiation exposure on normal healthy tissue is caused by two coexisting events: (I) radiation causes DNA damage and alters genomic sequences in a way that normal cells transform to malignant cells or (II) radiation induces signals that alter multicellular interactions and phenotypes, which trigger malignant cell formation. Healthy tissue is less radiosensitive than tumor tissue and investigators showed that there is an important role for genetic susceptibility in the risk of carcinogenesis after radiation exposure.\cite{93,120} The pathophysiological and functional expression of radiation-induced damage depends on the tissue type and its radiosensitivity, and is a stochastic effect that depends on radiation dose rate, total dose, dose fractions and quality of radiation (high or low LET).\cite{118,121}

Direct collateral damage on distant tissues leads to activation of pathways that not only stimulate wound healing, fibrosis or secondary malignancies, but also could stimulate metastasis formation of the primary tumor. Although mechanisms contributing to this effect are not clear, upregulation of cytokines, bioactive lipids, and other growth factors contributing to this effect are currently widely discussed.\cite{122-124} Cytokines secreted in the initial irradiation response could contribute to this metastatic process. In this thesis we will discuss a potential mechanism that stimulates metastasis formation after radiation exposure.
3.3 References


Chapter 4

Research Objectives
Recent studies show that radiation treatment of cancer inhibits primary tumor growth but has disturbing side effects which can contribute to invasion and metastasis of the tumor.\textsuperscript{1, 2} The majority of researchers use \textit{in vivo} models to study the effect of radiation of the primary tumor or tumor microenvironment on tumor progression. A point of discussion concerning these models is that either cancer \textsuperscript{3, 4} or stromal cells \textsuperscript{5, 6} were irradiated in culture conditions and subsequently injected into the animal. Available \textit{in vivo} models that should mimic radiotherapeutic strategies used in breast cancer patients; do not represent the patient tumor position. In these studies, they irradiate subcutaneous located primary tumors \textsuperscript{7, 8}, tumors in the fourth mammary fat pad \textsuperscript{2, 9} or tumors at the dorsal site of the mouse.\textsuperscript{10, 11} These models are not suitable to study the effect of \textbf{radiation-induced collateral damage} to distant tissues. There are \textit{in vivo} studies about the effects of healthy tissue radiation and \textbf{metastasis} formation, but the controversy about these models is the use of whole body irradiation \textsuperscript{12} or partial irradiation by shielding of the non-irradiated parts.\textsuperscript{11, 13} Therefore, our first aim is to develop an \textbf{in vivo model} with accurate and precise irradiation of tissues, which are potentially implicated in radiation-induced collateral damage of breast cancer progression.

Conform the 3R principle of animal welfare we aim to perform longitudinal monitoring in the same mouse and therefore we make use of bioluminescent cancer cells. Conflicting studies exist about the effect of luciferase transfection and its function on cancer cell behaviour. Our second aim is to determine minimal quantitative and functional requirements for bioluminescent \textit{in vitro} and \textit{in vivo} techniques, before implementation in a cancer model. Optimization of animal handling on the \textbf{small animal radiation research platform (SARRP)} is necessary.

With this \textit{in vivo} model we want to study whether there is an effect of radiotherapy-induced collateral tissue damage on metastasis formation, and if so, our third aim is
to identify **pathways** that drive these effects. Therefore, appropriate *in vitro* simulation is warranted. Studies of cancer cell lines exposed to irradiated lung epithelial cells or supernatant of these cells allow us to study the **indirect effects** of radiation on metastasis-associated cellular activities.

Our ultimate aim is to find molecular pathways that drive the metastasis-associated consequences of radiation-induced collateral damage.

### 4.1 References


Chapter 5  Quantitative and functional requirements for bioluminescent cancer models

Chapter 6  Radiation-induced lung damage promotes breast cancer lung-metastasis through CXCR4 signalling

Original Research
Chapter 5

Quantitative and functional requirements for bioluminescent cancer models.

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5.1 Abstract

Background

Bioluminescent cancer models are widely used but detailed quantification of the luciferase signal and functional comparison with non-transfected control cell lines are generally lacking. In this study we provide quantitative and functional tests for luciferase transfected cells.

Materials and Methods

We quantified the luciferase expression in BLM and HCT8/E11 transfected cancer cells, and examined the effect of long-term luciferin exposure. We also investigated functional differences between parental and transfected cancer cells.

Results

Our results showed that quantification of different single cell-derived populations are superior with droplet digital polymerase chain reaction. Quantification of luciferase protein level and luciferase bioluminescent activity is only useful when there is a significant difference in copy numbers. Continuous exposure of cell cultures to luciferin leads to inhibitory effects on mitochondrial activity, cell growth and bioluminescence. These inhibitory effects correlate with luciferase copy number. Cell culture and mouse xenograft assays showed no significant functional differences between luciferase-transfected and parental cells.

Conclusions

Luciferase-transfected cells should be validated by quantitative and functional assays before starting large-scale experiments.

Keywords: Bioluminescence, BLM, mouse xenograft, cancer model, in vivo imaging
5.2 Introduction

Luciferase transfected cancer cells are widely used in biomedical research applications. Bioluminescence has the advantage of longitudinal monitoring of tumor growth, metastasis formation or therapeutic responses.\textsuperscript{1, 2} The use of bioluminescence in animal experiments can significantly reduce the number of animals that need to be sacrificed.\textsuperscript{3, 4} Luciferase bioluminescence is based on the oxidation of luciferin, in the presence of oxygen, adenosine triphosphate (ATP) and magnesium and results in the production of CO\textsubscript{2}, inorganic pyrophosphate (PPi), adenosine monophosphate (AMP) and oxyluciferin. Oxyluciferin, in excited state, falls back to its steady state by emitting light.\textsuperscript{5, 6} The emitted light can be detected by a sensitive charged-coupled device (CCD) camera.\textsuperscript{5, 6}

Despite the global biomedical use of luciferase transfected cell lines, there are conflicting data about the effect of the luciferin-luciferase reaction on functional characteristics of the transfected cells. One group of researchers found that a high expression level of luciferase alters the transfected cells, causing inhibition of tumor growth.\textsuperscript{7} Others claim that there are no effects on cell growth, metabolism and immunological properties.\textsuperscript{8-10}

In the present study, we used different methods to quantify the amount of luciferase in transfected single-cell-derived populations. Furthermore, a minimal required number of functional tests were used to examine the impact of luciferase transfection and luciferin addition to cancer cells.
5.3 Materials and Methods

5.3.1 Cell lines

The human colon cancer cell line HCT-8/E11 and the BLM melanoma cell line (American type culture collection (ATCC), Manassas, VA, USA) were maintained as described elsewhere\textsuperscript{11, 12} The authenticity of ATCC cell lines was confirmed by short tandem repeat profiling in the last 6 months before use.

5.3.2 Transfection and selection of cells

Cancer cells were transfected with the pGL4.50 vector (Promega, Madison, WI, USA) containing the firefly luciferase gene (\textit{lux}2) and the antibiotic resistance gene (Hygro) by using FuGene (Promega). Luciferase-transfected cells were cultured in the presence of hygromycin B (400 \textmu g/ml; Life Technologies, Waltham MA, USA). The surviving colonies were screened for bioluminescence positivity by adding culture medium supplemented with 150 \textmu g/ml D-luciferin and using the \textit{in vivo} imaging system (IVIS) as detector (Xenogene, Alamede, CA, USA). In a second selection round, bioluminescent and antibiotic-resistant clones were seeded in a dilution of one cell/well in a 96-cell culture well/plate under continuous hygromycin selection. Different single-cell-derived subcloned populations were selected and named BLM\_luc SCP 1, 15 and 16, and a HCT8/E11\_luc SCP 3, 8 and 16.

5.3.3 Antibodies and reagents

Antibodies used were goat polyclonal anti-luciferase (G745A, 1:1000, Promega) and mouse monoclonal anti-\textit{\alpha}-tubulin (T5168, 1:5000, Sigma-Aldrich, St.Louis, MO, USA). D-Luciferin, potassium salt (PerkinElmer, Waltham, MA, USA) was used in bioluminescent imaging (BLI) measurements and OneGlo (Promega) was used for
relative luminescence unit (RLU) measurements. Hygromycin B (Life Technologies) antibiotics were used to select luciferase positive single cell-derived populations.

5.3.4 Short tandem repeat profiling

DNA was extracted by using DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). The PowerPlex® 16 System (Promega) was used for human identification applications, according to the manufacturer’s protocol.

5.3.5 DNA extraction and restriction digest

DNA concentration was measured by spectrophotometric measurement on an Ultrospec Plus Spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Each DNA sample (200 ng) was digested with 2 units of HaeIII in a total volume of 40 µl for 1 h at 37°C. The digestion reaction mixture was diluted 2-fold to a final concentration of 2.5 ng/µl. Two microliters (5 ng) were assayed per 20 µl droplet digital polymerase chain reaction (ddPCR).

5.3.6 Primer and probe design

The GenBank sequence EU921840.1, encompassing the luciferase reporter vector pGL4.50[luc2/CMV/Hygro], was used for the design of the luc2 primers. A primer-BLAST was run from the start codon at bp 859 until the end of the coding region of luc2, namely bp 2511 (www.ncbi.nlm.nih.gov/tools/primer-blast). The following parameters were adapted: PCR product size: 70-120 bp; Max Tm difference: 2°C; primer length: min 16 – opt 20 – max 30; GC clamp: 2; Max GC in primer 3’ end: 2; Primer GC content (%): min 30 – opt 50 – max 80. All primers were analyzed in silico using DINAMelt for homo- or hetero-dimer or formation (unafold.rna.albany.edu). The amplicon was then analyzed using mfold for investigating secondary structures. Primer pairs were selected with the least homo- or
hetero-dimer and secondary structures. The resulting \texttt{luc2} assay sequences were (forward primer) 5'-CCCGCACACCGCTATCC-3' and (reverse primer) 5'-TGACACGCACCCGAAAGC-3'.

5.3.7 \textit{ddPCR workflow}

The PCR reaction mixture resulted from a 2× ddPCR Mastermix (Bio-Rad, Hercules, CA, USA), 20× primer and probes solution (final concentrations of 250 and 100 nM, respectively), 2 µl template (2.5 ng/µl) and water (variable volume) in a final volume of 20 µl. Each ddPCR reaction mixture was then loaded into the sample well of an eight-channel disposable droplet generator cartridge (Bio-Rad). A volume of 70 µl of oil containing emulsion-stabilizing, biocompatible surfactant was loaded into adjacent oil wells, and the microfluidic chip was loaded into the droplet generator. The droplet generator simultaneously partitions the sample into \textasciitilde20,000 monodispersed droplets of known volume. After removing the cartridge from the droplet generator, the droplets in the droplet well were then transferred with a multichannel pipette to a 96-well PCR plate, heat-sealed with foil and then DNA was amplified to endpoint using a T100 Thermal Cycler (Bio-Rad) and the cycling protocol: 95°C for 10 min then 40 cycles of 95°C for 15 s and 59°C for 1 min (2.5°C/s ramp rate) with a final step at 98°C for 10 minutes and 12°C hold. Plates containing amplified droplets were loaded into a QX100 droplet reader (Bio-Rad), which streams droplets single-file (\textasciitilde1,500 droplets/s) past a two-colour FAM/HEX detector. Discrimination between droplets that did not contain target (negatives) and those that did (positives) was achieved by applying a global fluorescence amplitude threshold. The fluorescence threshold was set depending on the assay: BLM\_\texttt{luc} copy number analysis had a threshold of 809 relative fluorescence units (RFUs) and HCT8/E11\_\texttt{luc} copy number analysis had a threshold of 2395 RFUs. Concentration estimates were based on the fraction of droplets where amplification is modelled as a
Poisson distribution. Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.3.2.0 (Bio-Rad). The experiment was carried out in triplicate and analyzed data were also merged.

5.3.8 Luciferase reporter assay luminometer

To quantify the relative luminescence per cell, 4000 luciferase-transfected cancer cells were seeded in a black/clear bottom 96 cell culture well plate. Six hours after seeding, firefly luciferase activity was monitored using OneGlo luciferase assay kits (Promega) according to the manufacturer's instructions. Values are reported as relative luminescence units (RLU).

5.3.9 Continuous/intermittent exposure assay

To test the effect of continuous exposure of luciferin on cell lines with different luciferase expression, $10^3$ BLM_luc SCP 1 and 16 were seeded in a black/clear bottom 96-well cell culture plate. Cells were exposed to medium supplemented with luciferin, daily refreshment (intermittent exposure) versus not (continuous exposure). Luminescence signal, mitochondrial activity and total protein concentration were compared at different time points. Luminescence signal was measured using OneGlo luciferase assay kit (Promega). Values are reported as RLU.

5.3.10 Mouse strain and animal care

Animals were treated according to the European guidelines on animal experiments (2010/63/EU). Animal studies were approved by the Animal Ethics Committee of Ghent University, Belgium (ECD 10/36). Mice used in these studies were 4-week-old female NOD/SCID mice (Harlan, Indianapolis, IN, USA). One million BLM as a control or BLM_luc cells, suspended in 100 μl serum-free culture medium, were
subcutaneously injected at the lower right flank. Each group consisted of three mice. In vivo images were made every week after inoculation. The primary tumor volume was quantified weekly as by caliper measurements of the longest and the shortest tumor diameter \((V = 0.4 \times \text{longest axis} \times \text{shortest axis}^2)\).\(^{13}\) After 50 days, mice were sacrificed and tumor and lungs were resected.\(^{13}\)

5.3.11 **Bioluminescent imaging and quantification**

In total, 4000 luciferase-transfected cancer cells were seeded in a black/clear bottom 96-well cell culture well plate. Six hours after seeding firefly luciferase activity was monitored. Bioluminescent imaging of cancer cells, primary and metastatic tumor growth and *ex vivo* imaging of the lungs was performed as described previously.\(^{14}\)

5.3.12 **Polyacrylamide gel electrophoresis and Western blotting**

Samples of parental and luc2-transfected cancer cells for western blot analysis of luciferase expression were prepared, the proteins were separated, and immunostained as described by Hendrix *et al.*\(^{15}\) Bands were quantified by ImageJ software (Wayne Rasband, Bethesda, MD, USA). Parental cells were used as negative control, a commercial luciferase-positive cell line was used a positive reference control.

5.3.13 **3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay**

MTT assay was used to investigate mitochondrial activity changes in BLM luc2-transfected cells after continuous/intermittent luciferin exposure. The assay was performed as described elsewhere.\(^{16}\) Data are reported as optical density (OD).
5.3.14  **Sulforhodamine B (SRB) assay**

SRB assay was used to investigate changes in total protein in BLM luc2-transfected cells after continuous/intermittent luciferin exposure as previously described.\textsuperscript{16} Data are reported as OD.

5.3.15  **Type I collagen invasion assay**

To test invasion through extracellular matrix, the collagen type I invasion assay was used. The assay was performed according to De Wever et al.\textsuperscript{17} Briefly, $5 \times 10^4$ BLM, BLM\_luc, HCT8/E11 and HCT8/E11\_luc cells were seeded as a single-cell suspension on 0.1\% type I collagen gel (Santa Cruz Biotechnology, Dallas, TX, USA). After 24-h incubation at 37°C and 10\% CO\textsubscript{2}, invasiveness was scored.

5.3.16  **Statistical analysis**

Statistical analysis was performed using GraphPad Prism and confirmed by IBM SPSS Statistics 21.0 software. D’Agostino-Pearson was used for testing normal distribution. Normal distributed data were analyzed using unpaired t-test, adjusted with Welch’s correction when variance was statistically different. All nonparametric data were analyzed using Mann-Whitney U test. All values are expressed as the mean ± SD. P value of < 0.05 was considered statistically significant. Statistical tests were two sided. All data are representative of at least three independent experiments.
5.4 Results

5.4.1  Luciferase transfection has no effect on cell specific short tandem repeats (STR).

To verify that luciferase transfection did not change the stability of the fingerprinting profiles of the parental cancer cells, STR analysis was performed. STR profiles were in line with absence of differences between the transfected cancer cells and the parental cancer cells (Fig. 1).
Figure 1. Short tandem repeat (STR) sequence comparison between parental and luc2-transfected BLM and HCT8/E11. A. Comparison between BLM and BLM_luc cell line shows no difference in SRT sequence. B. Comparison between HCT8/E11 and HCT8/E11_luc cell line shows no difference in SRT sequence.

5.4.2 Droplet digital polymerase chain reaction (ddPCR) surpasses all other quantification techniques.

Luciferase copy numbers, protein levels and activities were assessed between different SCPs by different quantification techniques. ddPCR provided absolute quantification of the luc2 DNA molecules. Analysis of the transfected BLM_luc SCPs revealed a significant difference in copy number of luc2 between SCP 1 and SCP 16 and SCP 15 and SCP 16 (p<0.05) (Fig. 2A). Analysis of transfected HCT8/E11_luc SCPs revealed no significant difference in copy numbers of luc2 (Fig. 3A). Luciferase protein quantification was achieved by western blot. Quantification showed no clear
differences in relative protein expression between different SCPs (Fig. 2B and 3B). Results showed that BLM_luc SCP 16, with the lowest copy number, also had the lowest protein expression but no significant difference was seen with BLM_luc high copy number clones. For BLM_luc SCP 1 and 15, copy numbers were slightly different; the protein expression had the same trend (Fig. 2B). We used two techniques to measure the bioluminescent intensity (BLI). The first was measured on a cellular lysate followed by the addition of luciferase substrate. Quantification of BLI showed only significant difference in RLU/cell between BLM_luc SCP 1 and 16 ($p<0.001$) and between BLM_luc SCP 15 and 16 ($p<0.001$) (Fig. 2C). The second technique to quantify bioluminescent signals was by addition of luciferin to the living cancer cells and detection of BLI by a CCD camera. Quantification of these results also revealed a significant difference in BLI between BLM_luc SCP 1 and 16 and between BLM_luc SCP 15 and 16, but IVIS measurements revealed that BLM_luc SCP 15 had more BLI than BLM_luc SCP 1 despite having a lower copy number. No signal was seen in parental BLM cells (Fig. 2D). Serial dilution of different BLM_luc SCP’s revealed that a higher luc2 copy number not only resulted in higher BLI, but also detection of lower cell numbers. As expected, no signal was observed in parental BLM cells (Fig. 2E). In HCT8/E11 transfected cells no correlation is seen between increased copy number and increased bioluminescent signal (Fig. 3).
Figure 2: Droplet digital polymerase chain reaction (ddPCR) surpasses all other quantification techniques in a distinctive character of luc2 quantification. A. Bar charts illustrating copy number quantification by ddPCR of luc2-transfected BLM cells (SCP 1, 15 and 16) and parental cells as negative control (-). B. Bar charts illustrating luciferase protein expression quantification by Western blot of luc2-transfected BLM cells (SCP 1, 15 and 16) and parental cells as negative control (-) and a commercially available luciferase positive cell line was used as positive control (+). Data are represented as relative fold change compared with the corresponding positive control (+). C. Bar charts illustrating bioluminescence quantification by emitted light detection with luminometer of transfected BLM cells (SCP 1, 15 and 16) and parental cells as negative control (-). Results are presented from three wells per assay from three independent experiments. D. Bar charts illustrating bioluminescence quantification by emitted light detection with CCD camera of luc2-transfected BLM cells (SCP 1, 15 and 16) and parental cells as negative control (-). Results are presented from three wells per assay from three independent experiments. E. In vitro bioluminescence of BLM_luc SCPs and parental cell (-). Cells from each SCP were serially diluted (in triplicate) from 10,000 to 20 cells/well. Dilution of parental cells was used as negative control (-). Values are represented as the mean ± SD; * p<0.05, ** p<0.01 and *** p<0.001 (Mann-Whitney-U test).
5.4.3  Continuously luciferin exposure causes cell fatigue.

*In vitro* and *in vivo* experiments with luciferase-positive cells require the addition of luciferin. We tested if there was a difference in mitochondrial activity, total protein and RLU between transfected cells continuously exposed to luciferin and transfected cells where the luciferin was washed off after every measurement. This was performed for cells with a high (BLM_luc SCP 1) and a low (BLM_luc SCP 16) *luc2* copy number. MTT results showed that after 4 days of continuous exposure to luciferin, the mitochondrial activity decreased compared to cells under intermittent exposure.
(Fig. 4A). The total amount of protein also decreased under continuous exposure, resembling slower cell growth (Fig. 4B). After 1 day's continuous exposure, a decrease in RLU was seen compared to intermittent exposure (Fig. 4C). MTT and SRB results showed that after 3 days (BLM_luc SCP 1) or 4 days (BLM_luc SCP 16) only, there was an inhibitory effect on cell growth. These effects were more pronounced in cells with high luc2 copy number.
Figure 4: Continuously luciferin exposure causes cell fatigue. Effect of luciferin exposure on mitochondrial activity as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (A), total protein concentration as measured by sulforhodamine B (SRB) assay (B) and luciferase activity measured by luminometry (C) in cancer cells with a high luc2 copy number (SCP 1) and low luc2 copy number (SCP 16). Closed bars represent intermittent exposure to luciferin; open bars represent continuous exposure to luciferin. Results are presented from three wells per assay from three independent experiments. Values are represented as the means ± SD. *p<0.05, **p<0.01 and ***p<0.001 (Mann-Whitney-U test).

5.4.4 Functional comparison between luciferase transfected cancer cells and parental cells

SCP s should have identical functional characteristics compared not only to each other but also to the parental cells. Morphologically, we did not detect a difference between the transfected SCPs and the parental cell line (Fig. 5A). Collagen invasion revealed no difference in invasive phenotype or invasion index (Fig. 5B). To confirm that the luc2-transfected BLM_luc SCP’s grew in vivo in a manner comparable to the parental cells, both were injected subcutaneously (n=3 per cell line) and tumor volume was monitored using calliper measurements. Tumor growth pattern in mice injected with BLM_luc was similar to that of the mice injected with the parental BLM (Fig. 6A). In general, the tumor BLI resembled the calliper-measured tumor volume (Fig. 6B and C). At day 50, mice were sacrificed and ex vivo imaging was carried out to confirm lung metastasis (Fig. 6D). Immunohistological comparison of primary tumor and lung metastases showed no difference between parental and luciferase-transfected BLM cells. There was a necrotic centre, and high vascular density was observed at the periphery of the primary tumor. Both tumor types from BLM parental and BLM_luc cells lacked the presence of inflammatory cells and fibroblasts minimally infiltrated. The topographical localization, size and number of metastases in the lung were similar for both cell lines (Fig. 6E and F).
Figure 5. Luciferase-transfected cancer cells and parental cells have similar characteristics in vitro. 
A: Representative phase-contrast micrographs of luciferase-transfected and parental cells. B: Comparison of type I collagen invasion assay. Luciferase-transfected cells and parental cells were seeded as single cells. After 24 hours, invasive and non-invasive cells were counted and an invasion percentage was quantified. Results are presented from three wells per assay from three independent experiments. Values are represented as the mean percentages ± SD.
Figure 6. Luciferase-transfected cancer cells and parental cells have similar characteristics in vivo.
A: Four-week-old NOD/SCID female mice were injected subcutaneous with $1 \times 10^6$ BLM_luc or parental BLM cells in 0.1 ml of serum-free culture medium. Tumor volume of three mice was monitored for 50 days by caliper measurements.

B: Quantification of bioluminescent signal of BLM_luc cancer cell growth in vivo.

C: Imaging of one representative animal at different time points after injection of BLM_luc.

D: Image of a representative pair of lungs of a mouse 50 days after
subcutaneous implantation of BLM_luc cells; mice were sacrificed and total lung metastasis was quantified by bioluminescent imaging. Immunohistochemical staining was used for conventional morphological analysis of primary tumor and lung metastases. *Necrotic tissue; arrowhead, blood vessels (E); arrows, metastatic lesion (F).

5.5 Discussion

Many researchers already studied factors that may have an influence on BLI signaling including: type of luciferase\(^\text{18, 19}\), level of luciferase expression\(^\text{7}\), concentration of luciferin injected\(^\text{20}\), way of luciferin injection\(^\text{21, 22}\), time of imaging\(^\text{23}\), metabolism of cell/tissue\(^\text{24}\), anaesthetics\(^\text{25, 26}\) and plasma proteins\(^\text{27}\).

Different complementary luciferase quantification methods were performed here on multiple SCPs of a melanoma and a colorectal cancer cell line transfected with a luciferase-expressing plasmid. Superior sensitivity of ddPCR was observed compared to the bioluminescence assays and western blot in discriminating quantitative luciferase differences between the SCPs. To our knowledge, we are the first to report the comparison of luciferase activity with luciferase copy numbers and conclude that high protein expression and high BLI or RLU does not always mean that these cells have a higher luc2 copy number. HCT8/E11_luc SCPs had only a small difference in copy number but significant differences in BLI. The difference in RLU and BLI can be explained by the difference in sensitivity of the quantification technique. RLU quantification is based on single-point measurements, while BLI quantification is based on the signal of the entire well.

Removal of luciferin-containing medium after measuring BLI is a necessary precaution because continuous exposure to luciferin reduces mitochondrial activity and total protein after prolonged incubation. Both these findings suggest a decrease in growth because of continuous luciferin exposure. A high level of luciferase continuously fuelled by luciferin and cellular ATP consumes a significant proportion
of the ATP pool necessary for maintaining cellular metabolism and growth. Moreover, luciferase-expressing cells consume oxygen during the luciferase–luciferin reaction, which leads to a hypoxic state. Hypoxia is known to reduce cell proliferation in a hypoxia-inducible factor-dependent manner and continuous exposure to luciferin leads to excessive oxygen consumption, resulting in growth stasis. In addition, a build-up of oxyluciferin or oxidative damage occurring during the luciferase–luciferin reaction may also play a role in growth stasis. However, the latter hypothesis was rejected by Tiffen et al. who claimed that limiting cofactor (i.e. oxygen and ATP) cannot cause excessive production of oxyluciferin. That these effects are more pronounced in SCPs with a high luc2 copy number is probably due to higher consumption of ATP and oxygen than in those with a low copy number. Therefore it can be concluded that replacement of luciferin-containing culture medium by regular culture medium needs to become the golden standard after luciferase quantification in in vitro experiments.

The important question of whether manipulation by luciferase transfection initiates functional differences between the transfected and the parental cell line needs to be answered. Our research provides basic knowledge essential for working with luciferase-transfected cell lines. Bolin and co-workers studied the difference in orthotopic breast tumor growth and metastasis formation between SCPs with high and low BLI in vivo. Similar tumor growth and metastasis profiles were observed between the transfected SCP cells and parental cells. In our research, we only tested the SCP with highest BLI and compared it with the parental cell line. With this high intensity SCP, lower numbers of cancer cells can be detected, suggesting a more sensitive detection of early stages of metastasis or tumor responses to therapy. According to Bratkiewicz et al. a high luciferase expression may affect tumor growth in vivo if animals are exposed to luciferin continuously and serial re-imaging. We showed that our high expressing luc2 SCP had a similar primary tumor growth
profile and lung metastasis rate compared to the parental line. Jenkins and co-workers are one of the few who are described a comparison in tumor growth between parental and transfected cell lines, similar tumor progression was seen in both cell lines, but no further functional test were performed \(^{32}\). Other examples are described by Thalheimer \textit{et al.}\(^4\) and Clark \textit{et al.}\(^8\) Talheimer \textit{et al.}\(^4\) first investigated \textit{in vitro} luciferase activity in a serial dilution before use \textit{in vivo} but never compared with the parental cells \textit{in vivo}. Clark \textit{et al.}\(^8\) investigated the effect of luciferase transcription on cell characteristics. No significant difference was observed in cell growth/migration and invasion but a significant difference in gene expression of cytokine gene was observed. From this research, we can conclude that investigators should test their transfected cells on a small number of mice to guarantee that luciferase transfection does not change tumor behavior \textit{in vivo}.

\textbf{Author’s contribution}

LF conceived the idea, designed, researched for information, approved and drafted the manuscript. BD and CV: bioluminescent set-up, SV and JV: ddPCR, ODW and MB: supervised the study and helped writing the manuscript. All authors read and approved the final manuscript.

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5.6 References


Chapter 6

Radiation-induced lung damage promotes breast cancer lung-metastasis through CXCR4 signaling.

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6.1 Abstract

Radiotherapy is a mainstay in the postoperative treatment of breast cancer as it reduces the risks of local recurrence and mortality after both conservative surgery and mastectomy. Despite recent efforts to decrease irradiation volumes through accelerated partial irradiation techniques, late cardiac and pulmonary toxicity still occurs after breast irradiation. The importance of this pulmonary injury towards lung metastasis is unclear. Preirradiation of lung epithelial cells induces DNA damage, p53 activation and a secretome enriched in the chemokines SDF-1/CXCL12 and MIF. Irradiated lung epithelial cells stimulate adhesion, spreading, growth, and (transendothelial) migration of human MDA-MB-231 and murine 4T1 breast cancer cells. These metastasis-associated cellular activities were largely mimicked by recombinant CXCL12 and MIF. Moreover, an allosteric inhibitor of the CXCR4 receptor prevented the metastasis-associated cellular activities stimulated by the secretome of irradiated lung epithelial cells. Furthermore, partial (10%) irradiation of the right lung significantly stimulated breast cancer lung-specific metastasis in the syngeneic, orthotopic 4T1 breast cancer model.

Our results warrant further investigation of the potential pro-metastatic effects of radiation and indicate the need to develop efficient drugs that will be successful in combination with radiotherapy to prevent therapy-induced spread of cancer cells.

6.2 Introduction

Postoperative radiotherapy reduces the risk of both recurrence and mortality of breast cancer, and is nowadays standard treatment in the management of breast cancer after conservative surgery and after mastectomy to anticipate the high risk of relapse.\textsuperscript{1,2} Despite this progress, locoregional postradiotherapy relapses still occur in about 7-12.6\% of the patients within the 5 years after treatment.\textsuperscript{3-5} Relapses
occurring within a preirradiated area are associated with an increased risk of local invasion, metastasis formation and poor prognosis compared to relapses occurring outside of the irradiated area.\textsuperscript{6} Recent experimental evidence supports these clinical observations. In murine xenograft models, tumors developing within preirradiated beds are more invasive and metastatic compared to tumors growing outside irradiated beds, a condition also referred to as “tumor bed effect”. Kuonen \textit{et al.}\textsuperscript{7} investigated cellular and molecular mechanisms underlying the tumor bed effect in breast cancer by using the 4T1 triple-negative murine model mimicking local relapse after radiotherapy and identified the role of cancer cells and mobilized myeloid cells as a metastasis promoting mechanism in breast. Also, radiation-induced stemness of residual breast cancer cells increased spontaneous lung metastasis.\textsuperscript{8}

Although these experimental models adequately address the impact of the local tumor bed effect, these \textit{in vivo} models do not consider the incidental exposure of the cardiopulmonary region to ionizing radiation after postoperative radiotherapy. Incidental cardiopulmonary irradiation is clinically important since it increases the subsequent rate of ischemic heart disease and secondary lung cancer risk.\textsuperscript{9, 10} Radiotherapy regimens for breast cancer have changed since these trials; the doses of up to 15 Gy to which the cardiopulmonary region was exposed are now generally lower.\textsuperscript{9, 10} Nevertheless, in most women receiving contemporary radiotherapy protocols, the cardiopulmonary region receives doses of 1 to 10.9 Gy.\textsuperscript{11} The estimated percentage of total irradiated lung volume may range from 2.7 to 17.6\% in a study population receiving tangential radiation beams.\textsuperscript{12}

Lungs are a prime target organ for breast cancer metastasis but the impact of incidental radiation exposure on lung metastasis is unknown. In this paper, we experimentally and molecularly addressed whether preirradiation of lung epithelial cells impacts metastasis-associated cellular activities of well-characterized triple-negative human MDA-MB-231 and murine 4T1 breast cancer cells. Using a murine
xenograft model, lung metastasis formation was evaluated after exposure of 10% volume of the right lung to clinically relevant doses of radiation.

6.3 Material and Methods

6.3.1 Cell lines

EA.hy926, a human endothelial cell line was obtained from ATCC (Manassas, VA, USA). MDA-MB-231GFP_luc, a human triple-negative breast cancer cell line.\(^{13}\) 4T1_luc, a mouse triple-negative breast cancer cell was obtained from Sibtech (Brookfield, CT, USA). Beas-2B cell line, a human normal lung epithelial cell line, was kindly provided by Prof. K. De Bosscher (Cytokine Receptor Lab, VIB, Ghent University). EA.hy926, MDA-MB-231GFP_luc, and 4T1_luc cells were maintained in DMEM culture medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Waltham, MA, USA), and 2.5 μg/mL fungizone (Bristol-Myers, Squibb, Belgium). Beas-2B cells were maintained in MEM culture medium supplemented with 0.05% L-glutamine (w/v), 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 2.5 μg/mL fungizone. EA.hy926, 4T1_luc and Beas-2B were incubated with 5% CO\(_2\). MDA-MB-231GFP_luc cells were incubated with 10% CO\(_2\). Authenticity of ATCC cell lines was confirmed by short tandem repeat profiling in the last 6 months before use. Cell cultures were tested for mycoplasma contamination monthly by using MycoAlert Plus Kit (Lonza, Basel, Switzerland). MDA-MB-231GFP_luc, GFP expression was continuously induced with doxycycline (500 ng/mL, Sigma-Aldrich, St.Louis, MO, USA). 4T1_luc were selected with Zeocine (500 μg/mL, Invitrogen).
6.3.2 Antibodies and reagents

Primary and secondary antibodies and reagents are described in Supplementary Materials and Methods.

6.3.3 Conditioned medium of irradiated and non-irradiated bronchial epithelial cells

Normal lung epithelial cells were cultured in a 25 cm$^2$ culture flask. Cells were irradiated using a Small Animal Radiation Research Platform, SARRP system (X-ray tube: ISOVOLT 225 M2 X-ray source; SARRP system, XStrahl®, Surrey, UK) at a constant rate of 3.45 Gy/min, for 174 seconds, thus receiving a single-fraction of 10.0 Gy (220 kV and 13.0 mA, using a 0.15 mm copper filter and a 10 x 10 cm collimator). Cells were positioned at a source-to-surface distance (SSD) of 34 cm. Control “sham” samples (0 Gy) received similar handlings except for the irradiation. Conditioned medium containing soluble factors derived from irradiated epithelial cells (CM$^{LE-IR}$) and medium of the sham epithelial cells (CM$^{LE}$) was prepared as described in Supplementary Materials and Methods.

6.3.4 Quantification DNA double-strand breaks (DSB)

Quantification of $\gamma$-H2Ax is used to quantify DSB. For the \textit{in vitro} experiments on human Beas-2B lung epithelial cells a mouse monoclonal anti-$\gamma$-H2AX primary antibody was used in combination with an Alexa488-conjugated rabbit anti-mouse secondary antibody and DAPI nuclear counterstain. The protocol as described in Depuydt \textit{et al.}\textsuperscript{14} was followed. For $\gamma$-H2AX foci analysis in the mouse lung tissue a rabbit polyclonal anti-$\gamma$-H2AX primary antibody was used in combination with a biotinylated goat anti-rabbit secondary antibody. To visualize the foci an immunoenzymatic staining using horse radish peroxidase-conjugated streptavidin and DAB was applied followed by haematoxylin counterstaining. The protocol as
described in Bolcaen et al.\textsuperscript{15} was followed. Mice were euthanized 15 minutes after receiving thoracic sham or 10 Gy irradiation.

### 6.3.5 Viability assay

The viability was analysed with the LIVE/DEAD kit for mammalian cells (Invitrogen), as described in Supplementary Materials and Methods.

### 6.3.6 Functional assays with direct cell-cell contacts

**Cell growth assay.** Beas-2B cells, cultured in DMEM with 10\% FBS until 70\% confluency, received a sham or 10 Gy radiation. After 24 hours $1 \times 10^3$ MDA-MB-231GFP\_luc or 4T1\_luc cells were added to suspended and seeded together with $2 \times 10^5$ sham or 10 Gy irradiated Beas-2B cells. After 4 days of co-culture, medium was changed and luciferine containing medium was added (150 \(\mu\)g/mL) 2 minutes before imaging. Imaging time was 2 min/cell plate. Light emitted from the breast cancer cells was detected by a highly sensitive CCD camera in the In Vivo Imaging System Lumina II (IVIS\textsuperscript{\textregistered}, Caliper Life Science, Hopkinton, MA, USA). Analysis was achieved with Living Image\textsuperscript{\textregistered} software (Caliper Life Science). There was a correlation between cell number and bioluminescence in vitro, using the In Vivo Imaging System (data not shown).\textsuperscript{16}

**Cell adhesion assay.** To study the difference in adhesion of breast cancer cells to irradiated versus non-irradiated epithelial cells, Beas-2B were cultured in a 6-well plate until confluence. The monolayer was sham or 10 Gy. 24 hours after treatment $2 \times 10^4$ MDA-MB-231GFP\_luc or 4T1\_luc cells were added to the monolayers. To study the effect of AMD3100, a CXCR4 antagonist, cancer cells were pretreated for 30 minutes with 10 \(\mu\)M AMD3100. In AMD3100 condition, pretreated cancer cells were added to the monolayer with a total AMD3100 concentration of 10 \(\mu\)M. Adhesion was analyzed after 24 hours using IVIS as described above.
6.3.7 Functional assays with conditioned media

Cell growth assay. MDA-MB-231GFP _ luc and 4T1 _ luc cells (2 x 10⁴) were seeded in 24-well plates and treated either with CM^{LE−IR} or CM^{LE}. To study the effect of recombinant CXCL12 or MIF on cancer cell growth, cells were treated with CM^{LE} supplemented with MIF or CXCL12 (50 ng/mL). The effect of an inhibitor was studied by pretreatment of the cancer cells with 10 μM AMD3100 for 30 minutes and a total AMD3100 concentration of 10 μM in CM^{LE−IR}. After 4 days cell numbers were analyzed by bioluminescent signal detection as described above.

Migration assay. MDA-MB-231GFP _ luc cells (1 x 10⁵ cells) were plated in the upper compartment of a Transwell chamber (24-well insert, pore size 8 μm, Corning Incorporated, New York, NY), while in the lower compartment CM^{LE−IR}, CM^{LE} or CM^{LE} supplemented with CXCL12 (50 ng/mL) was used as a chemoattractant. To study the effect of AMD3100, cancer cells were pretreated 30 minutes with 10 μM AMD3100, and AMD3100 was added in both compartments in CM^{LE−IR} with a final 10 μM concentration. Migration was stopped after 8 hours incubation and the insert was washed with PBS^{D}. Cells from the apical side were removed using a cotton swab before fixation with ice-cold methanol and DAPI staining. After 4 washing steps with PBS^{D−}, the filter was mounted onto glass using glycergel mounting medium (Dako, Carpinteria, CA, USA). Cell nuclei were analyzed by counting 6 different, randomly chosen fields with a 10x objective on a Zeiss Axiovert 200M fluorescent microscope.

Transendothelial migration assay. Formation and analysis of endothelial monolayer is described in Supplementary Materials and Methods and Supplementary Figure S7. Doxycycline-induced MDA-MB-231GFP _ luc cells (1 x 10⁵) were added on top of the endothelial cells. CM^{LE−IR}, CM^{LE} or CM^{LE−IR} + AMD3100 were used as a chemoattractant. After incubation for 24 hours the apical side of the chamber was washed twice with PBS^{D−} and scraped gently with a cotton swap. Migrated cancer cells, green fluorescent, were counted from 6 different, randomly chosen fields with a
10x objective on a Zeiss Axiovert 200M fluorescent microscope (Carl Zeiss, Micro-imaging, Heidelberg, Germany).

**Morphology analysis.** For quantification of morphological changes, $2 \times 10^5$ MDA-MB-231GFP_luc single cells were seeded on glass cover slips in the presence of control medium or CM$^{LE-IR}_U$. After 4 days of incubation, cells were fixed with 3.7% formaldehyde for 20 minutes. Permeabilization with 0.1% Triton-X100 was done for 5 minutes and cells were blocked for 30 minutes, while shaking, with 1% BSA. Next, the cell cultures were stained with F-actin stain phalloidin-Alexa Fluor 594 and DAPI and imaged with a Zeiss Axiovert 200M fluorescent microscope. Of each condition, 20 cells from 4 different glasses were used to score factor shape with the formula: $(\text{perimeter})^2/(4 \times \pi \times \text{area})$.

### 6.3.8 Protein analysis

**SDS_PAGE and Western blot analysis.** Lysate preparation, SDS-PAGE and Western blot analysis are described in Supplementary Materials and Methods.

**Cytokine array.** RayBio®Label-Based human antibody array 507 (L-507, RayBiotech Inc., Norcross, GA, USA) was used to identify the source of the cytokines playing a key role in the effect of irradiated bronchial epithelial cells. Cytokine array analysis is described in Supplementary Materials and Methods.

**ELISA analysis.** CXCL12 and MIF secretion levels were measured using quantitative immunometric sandwich enzyme immunoassays (ELISA=enzyme-linked immunosorbent assay), following the manufacturer’s recommended procedures (R & D Systems, Minneapolis, MN, USA). Optical density was measured at 450 nm of wavelength, with correction set to 570 nm, on a PARADIGM™ Microplate Detection Platform (Beckman Coulter, Brea, CA, USA). Triplicate cultures of cells were tested for each experimental condition.
6.3.9 **Animal studies**

*Radiotherapy treatment planning.* In this study, two different dose plans were set up and executed on the SARRP. One delivered 10Gy to the entire thorax, the other delivered 10 Gy to 10% volume of the right lung. First, dose distributions were calculated using the on-board CT as described earlier.17 4-week-old BALB/c female mice (Charles River, L’Abresle, France), were anesthetized, fixed on a plastic bed and placed on a holder secured onto the robotic positioning table. Cone-beam (CB) CT imaging is achieved by rotating the stage that supports the animal, horizontally between the stationary X-ray source and a flat-panel detector. The uncollimated primary beam, 20 cm × 20 cm at isocenter, is used for imaging. X-rays of 70 kV emitted from the 0.4 mm focal spot and filtered by 1 mm thick aluminum were employed. Images were acquired at a current of 1 mA, with “continuous” beam-on as well as “continuous” stage rotation. Three hundred and sixty (360) projections were acquired over 360°. Second, the treatment isocenter was placed in the lower part of the right hemisphere of the lung. One posterior-anterior beam with a size of 3 x 3 mm was selected to irradiate 18 mm³ of the right lung with 10 Gy, which resembles approximately 10% volume of the right lung (8.57% calculation based on Knuts *et al.* 18). For whole thorax treatment, two posterior-anterior beams, with a size of 9 x 3 mm and 10 x 10 mm at isocenter, were used to cover the entire thorax (Supplementary Fig. S5B). Treatment isocenters were set for both beams in the middle of the lung (Supplementary Fig. S5A, S5C). The CT scans are imported into Muriplan software (Xstrahl®). Next, image intensity-based tissue segmentation was performed to allow correct dose calculation throughout the different tissue densities. The voltage of the X-ray source is fixed at 220 kV with a tube current of 13 mA, emitted from the 3.0 mm focal spot and filtered by a copper filter of 0.15 mm. Mice with sham treatment only underwent the imaging part.
4T1_luc triple-negative mouse breast cancer model. All mice were orthotopically injected 24 hours after radiation treatment. One million 4T1_luc cells, suspended in a 100 µL mixture of serum-free DMEM and Matrigel (1:1), were injected into the mammary fat pad. The primary tumor volume was quantified as the product of caliper measurements of the longest and the shortest tumor diameter \( V = 0.4 \times \) (longest axis) \( \times \) (shortest axis)\(^2\). Primary and metastatic tumor growths were monitored by bioluminescence. First, mice were giving an intraperitoneal injection of 250 µL D-luciferin in PBS (150 µg/g mouse). Then, animals were anesthetized with 5% isoflurane in oxygen for induction and 1.5% isoflurane in oxygen for maintenance. Next, bioluminescent imaging was initiated 10 minutes after injection by a cooled CCD camera in the IVIS with a 15-cm field of view, binning factor of 8, 1/f stop and open filter. Exposure times were set automatically, depending on the luciferase signaling activity. ROIs were drawn for primary tumor and metastatic lesions and were calculated by the IVIS software, expressed in total flux (photon/s). Background photon flux was defined, for primary growth on a blanc mouse and for metastatic lesions on a normal lung, and extracted from all animal values. Images were initiated every 4 days after inoculation. After 4 weeks, mice were sacrificed and tumor and lungs were resected. Lungs were placed into 6-well plates and ex vivo bioluminescent imaging was performed by adding 300 µg/mL D-luciferin in excess. After imaging, all tissues were fixed in 4% buffered formalin. H&E staining and immunohistochemistry were performed using a NexES automated slide staining system (Ventana Medical Systems, Tucson, AZ) on paraffin sections. Animals were treated according to the European guidelines on animal experiments (2010/63/EU). They were kept in 12h light- dark cycles, with ad libitum access to food and water. Animal studies were approved by the Animal Ethics Committee of Ghent University, Belgium (ECD 10/36).
6.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism and confirmed by IBM SPSS Statistics 21.0 software. D’Agostino-Pearson was used for testing normal distribution. Normal distributed data were analyzed using unpaired t-test, adjusted with Welch’s correction when variance was statistically different. All nonparametric data were analyzed using Mann-Whitney U test. All values in box- and whisker blots are expressed as the mean and 95% confidence interval. All other values are expressed as the mean ± SD. A P value of < 0.05 was considered statistically significant. Statistical tests were two-sided. All data are representative of at least three independent experiments.

6.4 Results

6.4.1 Radiation effects on damage response and senescence markers in normal lung micro-environments.

To assess treatment-induced damage response in normal cells of the lung microenvironment, we examined mouse lung tissue that was excised 15 minutes after receiving thoracic sham or 10 Gy irradiation. We found evidence of DNA damage in lung epithelial cells as determined by the phosphorylation of histone H2AX on Ser139 (γ-H2AX) within 15 minutes after 10 Gy irradiation (Fig. 1A). To further ascertain the consequence of DNA damage in benign cells, we established an in vitro model treating Beas-2B epithelial cells of the lung microenvironment with a 10 Gy single radiation dose which substantially increased the number of γ-H2AX foci (Fig. 1B). Irradiated cells showed no increase in cell death (Fig. 1C, lower panel), but showed a more spread morphology with enlarged nuclei and increased cytoplasmic surface area (Fig. 1C, upper panel). Furthermore, activation of p53 and increased expression of
the p21 cell cycle arrest protein were observed (Fig. 1D, Supplementary Fig. S1). An indicator of cellular senescence, p21, was maintained up to 4 days after irradiation, which explains the lower number of cells (Fig. 1B, C and D).
Figure 1: Lung epithelial cells radiation response and senescence markers. A. Immunohistochemical (IHC) staining of γ-H2Ax foci using an immunoenzymatic DAB staining method (brown color) combined with a haematoxylin counterstaining in sham or 10 Gy irradiated mouse lung tissue. B. Immunocytochemical (ICC) staining of γ-H2AX foci (Alexa488 labeled secondary antibody, green color) combined with a DAPI nuclear counterstaining (blue color) in sham or 10 Gy irradiated Beas-2B lung epithelial cells. C. Upper 4 panels, phase contrast micrographs of Beas-2B lung epithelial cells two or four days post sham or 10 Gy irradiation. The 10 Gy condition shows less dense cell culture, a more spread cell morphology with enlarged nuclei and increased cytoplasmic surface area. Lower 2 panels, live/dead - viability/cytotoxicity test. Assay shows live cells as green and dead cells as red. Four days after single irradiation dose of 10 Gy shows no increase of Beas-2B cell death. D. Western blot (WB) analysis of p53 and p21 on total cell lysates from Beas-2B cells treated with single-fraction 10 Gy or sham. Total p53 expression is unchanged after irradiation but increase in p53 phosphorylation is observed at day 1 after treatment and normalizes at day 4. Total expression of p21 is increased until day 4. GAPDH and tubulin are used as loading control.

6.4.2 Impact of irradiated lung epithelial cells breast cancer cell growth and adhesion

Irradiated or sham-irradiated Beas-2B cells were grown in co-culture with 4T1_luc or MDA-MB-231GFP_luc triple-negative breast cancer cells and cancer cell growth was monitored by measuring luciferase activities after 4 days of co-culture. Co-culture with irradiated Beas-2B cells significantly enhanced the relative cancer cell growth 1.7-and 2.8-fold respectively compared to co-culture with non-irradiated Beas-2B cells (4T1_luc: sham vs. 10 Gy: 1.000 ± 0.030 vs. 1.740 ± 0.172; p<0.001; MDA-MB-231GFP_luc: sham vs. 10 Gy: 1.000 ± 0.067 vs. 2.806 ± 0.203; p<0.001) (Supplementary Fig. S2A, Fig. 2A).

To study the effect on cancer cell adhesion, we seeded breast cancer cells on a monolayer of Beas-2B epithelial cells 24 hours after irradiation or sham-irradiation. Co-culture with irradiated Beas-2B cell monolayer significantly increased adhesion of both cancer cells 1.7-and 1.3-fold respectively compared to co-culture with non-irradiated Beas-2B cell monolayer (4T1_luc: sham vs. 10 Gy: 1.000 ± 0.068 vs. 1.66 ± 0.321; p<0.001; MDA-MB-231GFP_luc: sham vs. 10 Gy: 1.000 ± 0.182 vs. 1.328 ± 0.210; p<0.001) (Supplementary Fig. S2B, Fig. 2B).
6.4.3 Impact of soluble factors derived from irradiated lung epithelial cells on breast cancer cell morphology, growth, migration and extravasation

To further investigate these effects we collected conditioned medium of non-irradiated (CM$^{LE}$) and irradiated Beas-2B cells (CM$^{LE-IR}$). Incubation of 4T1_luc and MDA-MB-231GFP_luc cells with CM$^{LE-IR}$ significantly increased relative cell growth 1.8- and 1.9-fold respectively compared to incubation with CM$^{LE}$ (Supplementary Fig. S2C, Fig. 2C) (CM$^{LE}$ vs. CM$^{LE-IR}$; 4T1_luc: 1.000 ± 0.314 vs. 1.800 ± 0.730; p<0.001; MDA-MB-231GFP_luc: 1.001 ± 0.159 vs. 1.891 ± 0.569; p<0.001).

F-Actin staining of single cells revealed a more elongated morphology upon CM$^{LE-IR}$ compared to CM$^{LE}$ (Fig. 2D, lower panel). This is shown by an increase in mean factor shape of MDA-MB-231GFP_luc cells incubated with CM$^{LE-IR}$, this was 2.2-fold higher compared to the CM$^{LE}$ condition (CM$^{LE}$ vs. CM$^{LE-IR}$; 3.742 ± 1.686 vs. 8.240 ± 2.197; p<0.001) (Fig. 2D, upper panel).

Morphological changes suggest an impact on the migratory potential of cells. To investigate the effect of irradiated epithelial cells on breast cancer cell migration we employed TransWell® culture chambers separated into two compartments by microporous filters. In the lower compartment CM$^{LE}$ or CM$^{LE-IR}$ was added, while on top MDA_MB-231GFP_luc cells were seeded. As shown in Fig. 2E, the directed migration of MDA-MB-231GFP_luc cells was significantly increased by 3.4-fold in the cells in presence of CM$^{LE-IR}$ compared to those of CM$^{LE}$ (CM$^{LE}$ vs. CM$^{LE-IR}$; 49.46 ± 18.52 ells vs. 166.70 ± 60.75 cells; p<0.001).

Extravasation, the migration of cancer cells through the endothelial wall into the target parenchyma, is another critical step in metastasis. This functional activity was biomimicked by studying directed breast cancer cell migration to CM$^{LE}$ or CM$^{LE-IR}$.
through a monolayer of endothelial cells. Under CM\textsuperscript{LE–IR} conditions 4.7-fold more MDA-MB-231GFP\_luc migrated through the endothelial layer compared to CM\textsuperscript{LE} conditions (CM\textsuperscript{LE} vs. CM\textsuperscript{LE–IR}: 52.00 ± 39.69 cells vs. 243.70 ± 57.25 cells; \(p=0.002\)) (Fig. 2F, Arrowheads in CM\textsuperscript{LE} point to migrated cancer cells.).

Summarized, we have shown that the secretome of irradiated Beas-2B lung epithelial cells contains factors that reorganize breast cancer cells to a more elongated shape and increase growth, migration and extravasation of the cancer cells.
Figure 2. Impact of irradiated lung epithelial cells on breast cancer cell growth and adhesion. A. Box plots illustrating the relative cell growth of MDA-MB-231GFP_luc cells. Co-culture of breast cancer with irradiated Beas-2B cells increases relative cell growth compared to co-culture with sham treated Beas-2B cells. Quantification by bioluminescent imaging after 4 days incubation. Data is represented
as relative fold change compared with the corresponding control value. MDA-MB-231GFP_luc: n=6; ***, $p<0.001$ (Unpaired t-test with Welch’s correction). 

B. Box plots illustrating the relative cell adhesion of MDA-MB-231GFP_luc cells. Relative Adhesion of breast cancer cells to irradiated Beas-2B cell monolayer is increased compared to sham treated Beas-2B cell monolayer. Quantification by bioluminescent imaging after 24 hours incubation. Data is represented as relative fold change compared with the corresponding control value. MDA-MB-231GFP_luc: n=15; ***, $p<0.0001$; circle = outlier (Unpaired t-test with Welch’s correction).

Impact of CM\textsuperscript{LE,IR} on breast cancer cell morphology, growth, migration and extravasation. 

C. Box plots illustrating the relative cell growth of MDA-MB-231GFP_luc cells. Treatment of breast cancer cells with CM\textsuperscript{LE,IR} increases relative cell growth compared to cells treated with CM\textsuperscript{LE}. Quantification by bioluminescent imaging after 4 days incubation. Data is represented as relative fold change compared with the corresponding control value (CM\textsuperscript{LE}). MDA-MB-231GFP_luc: n=21; ***, $p<0.001$ (Mann-Whitney-U test).

D. Box plots illustrating the extent of cell spreading of MDA-MB-231GFP_luc cells in CM\textsuperscript{LE,IR} versus CM\textsuperscript{LE} conditions, as quantified by factor shape (upper panel). Treatment with CM\textsuperscript{LE,IR} showed enhanced cell spreading, corresponding with the significantly altered cell shapes (lower panel). Fluorescence microscopy images of cells double stained with phalloidin for actin filaments (red) and DAPI counterstaining for nuclei (blue) after 4 days of incubation with CM\textsuperscript{LE,IR} versus CM\textsuperscript{LE}. n=20; ***, $p<0.001$ (Mann-Whitney-U test).

E. Box plots illustrating total migrated cell number of MDA-MB-231GFP_luc cells in CM\textsuperscript{LE,IR} versus CM\textsuperscript{LE} conditions (upper panel). Nuclei of migrated cells were stained blue by DAPI (lower panel). n=12; ***, $p<0.001$ (Unpaired t-test with Welch’s correction).

F. Box plots illustrating total migrated cell number of MDA-MB-231GFP_luc cells through endothelial monolayer in CM\textsuperscript{LE,IR} versus CM\textsuperscript{LE} conditions (upper panel). CM\textsuperscript{LE,IR} enhances breast cancer cell extravasation significantly. Endothelial cells were stained red with Vibrant DiI. Extravasated MDA-MB-231GFP_luc cells are green (lower panel). n=6; **, $p=0.0022$ (Mann-Whitney-U test). Arrowheads indicating GFP positive migrated breast cancer cells.

**6.4.4 Increased secretion of CXCL12 and MIF by irradiated lung epithelial cells**

The composition of CM\textsuperscript{LE} and CM\textsuperscript{LE,IR} was assessed to determine which cytokines were secreted by non-irradiated versus irradiated lung epithelial cell. Semi-quantitative results from a cytokine array showed that CM\textsuperscript{LE,IR} contained a total of 52 cytokines with a signal that exceeded that of CM\textsuperscript{LE} condition (Fig. 3A; Supplementary Table S1). We selected the CXCL12 and MIF cytokines for further study because of a high fold change (more than 80-fold) and a known role in breast cancer metastasis.\textsuperscript{19} CXCL12 and MIF, which had an 83.88-and 86.46-fold higher presence, respectively, in CM\textsuperscript{LE,IR} compared to CM\textsuperscript{LE} (Supplementary Table
1). Responding to fold changes (relative changes) rather than absolute change is intrinsically important in chemokine attraction and consequently regulation of metastasis.\textsuperscript{20, 21} Quantitative ELISA data showed that irradiated lung epithelial cells had a secretion of CXCL12 and MIF that is 5.8- and 7.9-fold higher, respectively, than npn-irradiated lung epithelial cells (Fig. 3B) (CM\textsuperscript{LE} vs. CM\textsuperscript{LE-IR}; CXCL12: 61.07 ± 12.59 vs. 353.70 ± 126.00 pg/mL/10\textsuperscript{6} cells/24h; \( p=0.010 \); MIF: 1.072 ± 0.390 vs. 8.495 ± 0.695 ng/mL/10\textsuperscript{6} cells/24h; \( p=0.004 \)). According to previous literature, both cytokines may affect metastasis through activation of the CXCR4 receptor on cancer cells.\textsuperscript{19} Western blot analysis confirmed that cancer cell lines, that are known to have an invasive phenotype, have a higher expression of CXCR4 (45-47 kDa) (Fig. 3C-D).\textsuperscript{22}
Figure 3: Increased secretion of CXCL12 and MIF by irradiated lung epithelial cells. A. Cytokine array identifies enhanced presence of CXCL12 and MIF in CM\textsuperscript{LE,IR} compared to CM\textsuperscript{LE}. B. ELISA analysis of CXCL12 (upper panel) and MIF (lower panel) on CM\textsuperscript{LE,IR} versus CM\textsuperscript{LE}. For each condition 2 different samples were used in triplicate. Values are presented as the mean ± SD. CXCL12: **, \textit{p}=0.010. MIF: **, \textit{p}=0.004 (Mann-Whitney U test). C. Western blot analysis of CXCR4 expression on total lysate of breast cancer cells. Lane 1-5, represents MDA-MB-231GFP\_luc (invasive cell line) in different conditions (MDA 231: parental MDA-MB-231GFP\_luc; MDA 231 I4 D4: MDA 231 cells exposed to CM\textsuperscript{LE,IR} for 4 days; MDA 231 C4 D4: MDA 231 cells exposed to CM\textsuperscript{LE} for 4 days; MDA 231 RL 0 Gy: MDA 231 isolated from non-irradiated mouse lung; MDA 231 RL 10 Gy: MDA 231 isolated from irradiated...
mouse lung. Lane 6: SKBR3 (non-invasive cell line). Lane 7: MDA-MB-231H2N (invasive cell line). Lane 8: MDA-MB-453 (invasive cell line). Lane 9: MCF 10A (non-invasive cell line). Lane 10: MCF7/6 (non-invasive cell line). D. Quantitative analysis CXCR4 protein expression level. Western blot analysis of CXCR4 protein expression in different breast cancer cell lines after tubulin normalization. Protein levels relative to control, MDA-MB-231GFP_luc.

6.4.5 Effect of recombinant CXCL12 and MIF on breast cancer cell growth and migration

To verify if CXCL12 and MIF may contribute to the effects observed with the secretome of irradiated cells, the recombinant forms were supplemented to the secretome of non-irradiated cells and used to assess the functional impact on cancer cells. CM^{LE} supplemented with recombinant CXCL12 or MIF induced an increase in cell growth as demonstrated for MDA-MB-231GFP_luc cells (Fig. 4A) (CM^{LE} vs. CM^{LE} + CXCL12: 1.002 ± 0.157 vs. 1.807 ± 0.241; p<0.001; CM^{LE} vs. CM^{LE} + MIF: 1.002 ± 0.157 vs. 2.010 ± 0.259; p<0.001) and induced an increase in cell migration after treatment with CXCL12 (Fig. 4B) (CM^{LE} vs. CM^{LE} + CXCL12: 48.830 ± 18.640 vs. 82.830 ± 29.360; p=0.038). Experiments with MIF did not show significant differences in cancer cell migration (data not shown).
Figure 4: Effect of recombinant CXCL12 and MIF on breast cancer cell growth and migration. 

A. Box plots illustrating the relative cell growth of MDA-MB-231GFP_luc cells. Treatment of breast cancer cells with CXCL12 (50 ng/mL) or MIF (50 ng/mL) increases relative cell growth compared to control. Quantification by bioluminescent imaging after 4 days incubation. Data is represented as relative fold change compared with the corresponding control value (CM15). n=6; ***, p<0.001 (Unpaired t-test).

B. Box plots illustrating difference in total migrated cell number of MDA-MB-231GFP_luc cells treated with CXCL12 (50 ng/mL) (upper panel). Nuclei of migrated cells were stained blue with DAPI (lower panel) n=6; *, p=0.038 (Unpaired t-test).
6.4.6 Allosteric targeting of CXCR4 receptor reversed paracrine effect induced by irradiated epithelial cells

To investigate whether the observed effects depend on activation of the CXCR4 receptor, AMD3100, an allosteric inhibitor of CXCR4 receptor, was used. Addition of AMD3100 reversed the pro-metastasis associated effects of CMLE-IR such as relative cell growth (Fig. 5A) (CMLE-IR vs. CMLE-IR + AMD3100: 1.000 ± 0.107 vs. 0.717 ± 0.144; P < 0.001), relative adhesion (Fig. 5B) (CMLE-IR vs. CMLE-IR + AMD3100: 1.000 ± 0.060 vs. 0.665 ± 0.166; p<0.001), migration and extravasation of breast cancer cells (Fig. 5C &D) (Migration - CMLE-IR vs. CMLE-IR + AMD3100: 162.40 ± 74.62 cells vs. 75.33 ± 33.32 cells; p=0.009; extravasation - CMLE-IR vs. CMLE-IR + AMD3100: 243.70 ± 57.25 cells vs. 100.40 ± 57.11 cells; p=0.017). Furthermore, AMD3100 treatment partially reversed CMLE-IR-induced morphological changes, as measured by factor shape (Fig. 5E) (CMLE-IR vs. CMLE-IR + AMD3100: 8.240 ± 2.197 vs. 5.243 ± 2.772; p=0.004).
Figure 5: Effect of an allosteric CXCR4 inhibitor on breast cancer cell growth and migration. A. Box plots illustrating the relative cell adhesion of MDA-MB-231GFP_luc cells. Treatment of breast cancer cells with AMD3100 (10 μM) decreases relative cell adhesion to irradiated Beas-2B cell monolayer.
Box plots illustrating the relative cell growth of MDA-MB-231GFP_luc cells. Treatment of breast cancer cells with AMD3100 (10 μM) decreases relative cell growth in presence of CM LE-IR. (Unpaired t-test with Welch’s correction). n=15; ***, p<0.001 (Unpaired t-test). In C. and D., quantification by bioluminescent imaging after 4 days incubation. Data is represented as relative fold change compared with the corresponding control value. C. Box plots illustrating impact of AMD3100 (10 μM) on total migrated cell number of MDA-MB-231GFP_luc cells stimulated by CM LE-IR. Nuclei were stained blue with DAPI (lower panel). n=9; **, p=0.009 (Unpaired t-test with Welch’s correction). D. Box plots illustrating total transendothelial migrated cell number of MDA-MB-231GFP_luc cells stimulated by CM LE-IR in presence of AMD3100 (10 μM) or control. n=6; *, p=0.017 (Mann-Whitney U test). E. Box plots illustrating the extent of CM LE-IR–induced cell spreading of MDA-MB-231GFP_luc cells treated with AMD3100 (10 μM) versus control, as quantified by factor shape (upper panel). Fluorescence microscopy images of cells double stained with phalloidin for actin filaments (red) and DAPI counterstaining for nuclei (blue) (lower panel). n=20; **, p=0.004 (Mann-Whitney U test).

6.4.7 Paracrine activation of ERK, Akt and STAT3 in breast cancer cells by CM LE-IR and recombinant CXCL12

CM LE-IR activated multiple CXCR4-dependent downstream cascades, like ERK, Akt and STAT3, in MDA-MB-231GFP_luc cells (Fig. 6A). These pathways are involved in mediating cellular proliferation, survival, migration, transformation and differentiation. In agreement, addition of recombinant CXCL12 or MIF stimulated Akt, ERK and STAT3 activation, although an equal concentration of MIF showed a smaller increase than CXCL12 (Fig. 6A, Supplementary Fig. S3A).

Next, we examined the impact of Trametinib and AMD3100 on CM LE-IR-induced ERK, Akt and STAT3 activation. Previous literature showed that triple-negative breast cancer cells are the most sensitive for treatment with MEK inhibitor. Nanomolar concentrations of Trametinib, an allosteric MEK1/2 inhibitor, completely blocked CM LE-IR–induced ERK activation (Fig. 6B, Supplementary Fig. S3B) with no impact on Akt or STAT3 activation. Similar effects were observed when combining recombinant CXCL12 or MIF with Trametinib (Fig. 6C-D, Supplementary Fig. S3C-D). In accordance with the functional experiments, AMD3100 decreased CM LE-IR–induced Akt and STAT3 pathway activation; a minor impact on pERK was
observed in the cancer cells exposed with $\text{CM}^{\text{LE}_{-\text{IR}}}$ + AMD3100 compared to $\text{CM}^{\text{LE}_{-\text{IR}}}$ alone (Fig. 6B). These effects with AMD3100 are less pronounced when $\text{CM}^{\text{LE}}$ is combined with recombinant CXCL12 and MIF (Fig. 6C-D, Supplementary Fig. S3C-D).
Figure 6: Paracrine activation of ERK, Akt and STAT3 in breast cancer cells by CM\textsuperscript{LE,IR} and recombinant CXCL12. A-D. Western blot analysis of pERK, pAkt and pSTAT3 in MDA-MB-231GFP\_luc cells treated with recombinant CXCL12 (50 ng/mL) or MIF (50 ng/mL) A. CM\textsuperscript{LE}, CM\textsuperscript{LE,IR}, in the presence of AMD3100 (10 \mu M) or Trametinib (50 nM) B. Recombinant CXCL12 (50 ng/mL) C. or MIF (50 ng/mL) D. in presence of AMD3100 (10 \mu M) or Trametinib
(50 nM) Tubulin is used as loading control. All data are representative of at least three independent experiments.

6.4.8 Impact of partial lung irradiation on lung-specific breast cancer metastasis in a syngeneic mouse model.

A syngeneic, orthotopic triple-negative breast cancer model 4T1_luc was developed to study the impact of irradiation of the lung on the formation of lung metastasis. Patient studies showed that approximately 10% of the total lung volume is irradiated during breast cancer RT, with a mean lung dose of 10 Gy.$^{11, 12}$ All groups of mice received CT scan radiation for the localization of lung tissue; in the sham treatment group, no further irradiation was performed; the WT group received a 10 Gy irradiation to the whole thorax; the PRL group (partial right lung) received a 10 Gy irradiation to approximately 18mm$^3$ part (10% of total volume) of the right lung (Supplementary Figure S4-S5). 4T1_luc cells (1 x 10$^6$) were orthotopically injected 24 hours after irradiation. Neither WT nor PRL irradiation did significantly impact primary tumor growth (Fig. 7A) (sham vs. WT vs. PRL: 446 ± 111 mm$^3$ vs. 471 ± 63 mm$^3$ vs. 526 ± 109 mm$^3$). Four weeks after orthotopic 4T1_luc breast cancer inoculation, all lungs were prelevated and bioluminescent activity in the separate lungs was compared between the three groups. Mice which received a 10 Gy PRL irradiation showed more bioluminescent signal, and thus more metastasis, in each lung compared to WT irradiated or sham treated mice (Fig. 7B). In addition, when only bioluminescent signal of those lungs with activity were included we observed significantly higher signals in the right lung of PRL irradiated mice compared to the right lung of sham treated mice, suggesting more metastatic growth ($p=0.043$) (Fig. 7C, Supplementary Fig. S6); a similar trend was observed for the left lung but no significant value was reached. Semi-quantitative histological examination of metastatic lung tissue demonstrated more and larger metastasis in mice receiving
PRL irradiation compared to sham or WT (Fig. 7D, as illustration. Fig 7E, quantification). This was confirmed by quantitatively analyzing the total metastatic area between the groups. In sham groups an average metastatic area of 1.2% was reached, comparable to the WT irradiated group with an average area of 1.3%. Both groups are remarkably, but not significantly, lower than the PRL irradiated group, with a three-fold increase of metastatic area (5.0%) (Fig. 7E). Moreover, comparing total lung bioluminescent signal (signal right and left lung as one), sham treated mice still had the lowest signal but WT irradiated mice and PRL irradiated mice showed a 2.1- and 7.1-fold increase, respectively, in signal (Fig. 7F).
Figure 7: Impact of partial lung irradiation on lung-specific breast cancer metastasis in a syngeneic mouse model. A. 4 week old BALB/c female mice were injected orthotopically with $1 \times 10^6$ 4T1_luc cells in 0.1 mL of serum free DMEM with 50% Matrigel. Tumor formation was monitored for 28 days by caliper measurement. Tumor volumes were measured as indicated. Sham and WT IR: n=6 and PRL:
B-C & F. 28 days after implantation of the cells, mice were sacrificed and total lung metastasis was quantified by bioluminescent imaging. Lung were quantified separately (B & C) or as one (F). Sham and WT: LL and RL, \( n=6 \) and PRL: LL and RL, \( n=5 \) (B). Only lungs containing metastasis were quantified, showing significant increase in signal in right lung of PRL mice. Sham: LL, \( n=5 \); RL, \( n=4 \); WT: LL, \( n=4 \); RL, \( n=5 \); PRL: LL, \( n=4 \); RL, \( n=4 \); \( *; p=0.043 \) (Mann-Whitney U test) (C). D. H&E staining of lungs from indicated groups. The metastatic areas are encircled (black). E. Quantification of the percentage of lung metastatic area calculated per mouse. Sham and WT: \( n=6 \) and PRL: \( n=5 \).

6.5 Discussion

Because of the absence of oestrogen-, progesterone- and HER2 receptor\(^{25} \), triple-negative breast cancers are not curable with hormonal treatment and HER2 targeted therapies. Standard mastectomy or breast conserving surgery with adjuvant radio- and chemotherapy is used as a standard to treat these patients\(^{26} \). Postoperative radiotherapy of breast cancer reduces the risk of local recurrence and mortality after both conservative surgery and mastectomy.\(^1 \)\(^-\)\(^2 \) Despite recent efforts to decrease irradiation volumes and improved irradiation techniques, late cardiac and pulmonary toxicity does still occur after breast irradiation.\(^6 \)\(^-\)\(^11 \) The implications of this pulmonary injury for lung metastasis are unclear. A randomized trial in high-risk postmastectomy patients showed that the long-term probabilities of lung metastases are significantly lower in the irradiated (RT) patients compared to non-irradiated (no-RT) patients. Interestingly, distant metastasis as first failure (independent from local relapse) was 47% in the RT group compared to 37% in the no-RT group, suggesting that lung metastases do not necessarily occur after local relapse.\(^{27} \)

Although it is generally assumed in this case that micrometastasis in the lungs were present before breast cancer was treated, and alternatively, admittedly provocative, hypothesis is that radiation-induced collateral damage of the lung influences lung-specific metastasis. Metastasis can be an early event and stay undetected at the time of diagnosis.\(^{28} \)\(^-\)\(^{29} \) Patients, presenting with micrometastasis, receive radiotherapy which can stimulate metastatic growth.\(^{28} \) The difference in our \textit{in vivo} model is that we first irradiated the lung and waited for metastasis to grow. Our model is not
relevant for the effect of ionizing radiation on already established micrometastasis because we graft syngeneic breast cancer cells after ionizing radiation. We irradiate part of the right lung, graft syngeneic breast cancer cells and study the impact on spontaneous lung metastasis formation. Lung irradiation has no impact on orthotopic tumor growth. However, we observe more and bigger metastatic foci in the lungs of PRL mice which can be caused by increased attraction of circulating cancer cells and/or stimulation of cancer cell release from the primary tumor and/or preparation of the metastatic niche (promoting adhesion and colonization of breast cancer cells). Breast cancer patients who did receive postmastectomy radiotherapy showed most often distant metastasis as first site of failure in contrast to patients who did not receive postmastectomy radiotherapy, here locoregional relapse and distant metastasis as first site of failure were equally common.\textsuperscript{27} Studies (using post-mortem samplings) should be designed to investigate a potential relationship between collateral lung radiation-damage and lung metastasis in breast cancer patients. The literature provides no level-one evidence because no randomized trials have been done correlating the dose indices of irradiated lung volumes with lung-specific metastasis in breast cancer. A clinical study with breast cancer patients undergoing postoperative radiation therapy of 50 Gy at 2 Gy/fraction, 5 days/week showed that the pulmonary region received a mean lung dose of approximately 10 Gy. If more than 3 axillary lymph nodes were affected, a supraclavicular field was added with equal dose and fractionation resulting in a higher mean pulmonary dose of 15.8 Gy.\textsuperscript{11} This collateral irradiation to the lungs resulted in reduced pulmonary function in the first two years after the postoperative radiotherapy.

Histone H2AX phosphorylation is a recognized marker of DNA damage, i.e. double strand breaks.\textsuperscript{30} The manner how DNA responds to radiation damage, indicated by $\gamma$-H2AX foci, has been investigated in mouse models receiving thoracic radiation and shown to be correlative with fibrosis in distressed mice.\textsuperscript{31} In our experiments, sham-irradiated lung tissue sections of BALB/c mice were almost completely negative for
γ-H2AX foci, whereas 10 Gy thoracic irradiation resulted in a massive increase in the number of pulmonary nuclei with γ-H2AX foci throughout the tissue sections, irrespective of cell type. *In vitro* experimentation on lung epithelial cells, the major pulmonary cell type, revealed potent H2AX and p53 responses to radiation which coincided with morphological changes, induction of cellular senescence markers and an enhanced secretion of multiple chemokines and growth factors. Gunjal *et al.*\(^{32}\) demonstrated that ovarian cancer cells responding to heat-sensitive chemoattractants released from irradiated organs, including the lung, are more migratory and metastatic. Our *in vitro* irradiation experiment confirmed the increased release of CXCL12 and MIF upon irradiation. The heat-sensitive chemokines CXCL12 and MIF, both known to promote breast cancer metastasis, showed the highest fold change in secretion by irradiated lung epithelial cells compared to control, which was confirmed in our study.\(^{19, 33}\) The irradiation caused induction of a metastasis-receptive microenvironment that promotes trafficking and homing of cancer cells to the lung.\(^{23, 33-36}\) Once the cells arrive in the lung, an increased level of CXCL12 and MIF retain the breast cancer cells in lung and provides it with survival and growth factors, so metastatic growth is enhanced.\(^{37-41}\) It is not known whether CXCL12 and MIF are increased in lungs of irradiated breast cancer patients but future research could investigate the sputum, serum or urine of breast cancer patients in the acute or long-term response to radiation. Müller and co-workers were the first to demonstrate CXCR4-mediated metastasis of breast cancer cells to CXCL12-rich environments, like bone marrow, brain, lungs and liver.\(^{19}\) Elevated CXCR4 expression in breast cancer cells negatively correlates with overall survival and disease-free survival in breast cancer patients and is correlated with malignant breast stem cell activity.\(^{42, 43}\) Targeting this pathway could thus be a promising therapeutic addition to radiotherapy. To investigate this, we used an allosteric inhibitor of CXCR4, AMD3100 or Plerixafor®. AMD3100 was originally developed for HIV treatment and is nowadays used in combination with G-CSF as a stem cell mobilizer in patients
with multiple myeloma and lymphoma.\textsuperscript{19, 44-46} Our co-culture findings showed that treatment of breast cancer cells with AMD3100 blocks the functional and biochemical effects induced by the secretome of irradiated epithelial cells. Nevertheless, the toxic effects of AMD3100 in long-term treatment limits its clinical potential.\textsuperscript{47} However, Peng \textit{et al.}\textsuperscript{48} identified a small cyclic peptide (LY2510924) that inhibits CXCL12 and CXCR4 interaction and downstream signaling and function. Currently, this peptide is included in clinical trials on patients with advanced cancer.\textsuperscript{49} The CXCL12 peptide analog CTCE-9908 inhibits lung metastasis in mouse models\textsuperscript{50, 51} and tests in phase I/II clinical trials in cancer patients showed no major adverse effects.\textsuperscript{52} Furthermore, the anti-CXCL12 aptamer NOX-A12 inhibits brain tumor recurrences after irradiation in rats.\textsuperscript{53} Potentially favorable collateral inhibition of the CXCR4-CXCL12 axis may prevent lung fibrosis, improving the quality of life in breast cancer patients.\textsuperscript{54}

We are not the first to report radiotherapy-stimulated relapse in preclinical models. Ohuchida \textit{et al.}\textsuperscript{55} reported that irradiation of stromal pancreatic fibroblast increased invasiveness of pancreatic cancer cell by upregulating c-Met phosphorylation and MAPK activity. Irradiation of mouse embryo fibroblasts stimulates cancer cell repopulation in cell culture and xenograft models.\textsuperscript{56} This effect is lost when the fibroblasts are deficient for caspase 3, a key executioner of programmed cell death. Moreover, radiotherapy is not unique in inducing pro-metastatic effects in preclinical models. Comparative analysis of matched colorectal cancer specimens shows that neoadjuvant chemotherapy results in increased presence of pro-invasive $\alpha$-SMA-positive CAFs (cancer associated fibroblast).\textsuperscript{57} Monnier \textit{et al.}\textsuperscript{58} demonstrated that after irradiation of the stromal bed, oral squamous cell carcinomas showed increased invasion and metastasis through the matricellular protein CYR61. Irradiation also has an effect on VEGF production and local angiogenesis, which finally contributes to metastasis formation.\textsuperscript{59}
Our results warrant further investigation of the potential pro-metastatic effects of radiation and indicate the need to develop efficient drugs which can be combined efficiently with radiotherapy in order to prevent therapy-induced spread of cancer cells. Nevertheless, the most efficient and simple solution is to prevent normal tissue irradiation. In breast cancer, the cardiopulmonary region can be spared of high doses by using multi-beam intensity-modulated radiotherapy or arc techniques, but often at the cost of a low dose spread.\textsuperscript{60} Irradiating the breast when patients are in prone instead of supine position has been shown to spectacularly improve all lung dose-volume indices.\textsuperscript{61, 62}

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6.6 References


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6.7 Supplementary data

6.7.1 Supplementary figures

Supplementary Figure S1: Lung epithelial cells radiation response and senescence markers. A. Quantification of p21 protein expression level in irradiated and non-irradiated Beas-2B lysates made 1 and 4 days after treatment. Quantification normalized according the tubulin protein expression level. Protein levels relative to control, Beas-2B sham day 1. B. Quantification of Pp53 and p53 protein expression level in irradiated and non-irradiated Beas-2B of lysates made 1 and 4 days after treatment. The ratio Pp53/p53 is shown in the graph. Quantification is normalized according the GAPDH protein expression level. Protein levels relative to control, Beas-2B sham day 1.
Supplementary Figure S2: Impact of irradiated lung epithelial cells on 4T1_luc breast cancer cell adhesion and growth. A. Box plots illustrating the relative cell growth of 4T1_luc cells. Co-culture of breast cancer cells with irradiated Beas-2B cells increases relative cell growth compared to co-culture with sham treated Beas-2B cells. Quantification by bioluminescent imaging after 4 days of incubation. Data is represented as relative fold change compared with the corresponding control value. 4T1_luc: n=6; ***, p<0.001 (Unpaired t-test with Welch’s correction). B. Box plots illustrating the relative cell adhesion of 4T1_luc cells. Relative adhesion of breast cancer cells to irradiated Beas-2B cell monolayer is increased compared to sham treated Beas-2B cell monolayer. Quantification by bioluminescent imaging after 24 hours incubation. Data is represented as relative fold change compared with the corresponding control value. 4T1_luc: n=10; ***, p<0.001. (Unpaired t-test with Welch’s correction). C. Box plots illustrating the relative cell growth of 4T1_luc cells. Treatment of breast cancer with CM1E_IR increases relative cell growth compared to cells treated with CM1E. Quantification by bioluminescent imaging after 4 days incubation. Data is represented as relative fold change compared with the corresponding control value (CM1E). 4T1_luc: n=18; ***, p<0.001 (Mann-Whitney-U test).
Supplementary Figure S4: Quantification pAkt/Akt, pSTAT3/STAT3 and pERK/ERK. Western blot analysis of the ratio pAkt/Akt, pSTAT3/STAT3 and pERK/ERK protein expression after tubulin normalization Protein levels relative to control MDA-MB-231GFP_luc cells exposed to CMLE. All data are representative of at least three independent experiments. For Western blot visualisation see Figure 6.
Supplementary Fig. S4

Supplementary Figure S4. *In vivo* experiment set up
Supplementary Figure S5: SARRP protocol. A. SARRP protocol partial right lung irradiation. Accurate irradiation of the mouse was achieved by making an image of the lung by cone beam CT. Treatment isocenter was indicated on CT. Next, we mark the different kinds of tissue (segmentation) and the SARRP software calculates the mouse-specific dose. B. SARRP protocol whole thorax irradiation. Accurate irradiation of the mouse was achieved by making an image of the lung by cone beam CT. Treatment isocenters were indicated on CT. Next, we mark the different kinds of tissue (segmentation) and the SARRP software calculates the mouse-specific dose. C. Visualization isocenter partial lung irradiation. Mice were anesthetized, fixed on a plastic bed and placed on a holder secured onto the robotic positioning table. Laser beam indicates treatment isocenter.
Supplementary Figure S6: *Ex vivo* set-up. Red square indicating lungs without bioluminescent signal, without metastasis.
Supplementary Fig. S7: *In vitro* functional assay: Transendothelial migration
### Table 1: Cytokine expression levels. Image quantification of cytokine array. CMLE values were used as control and compared with CMLEIR.

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**Legend:**
- **equal expression**
- **25-fold overexpression**
- **50-fold overexpression**
- **>50-fold overexpression**
6.7.3 Supplementary Materials and Methods

6.7.3.1 Antibodies and reagents
The following primary antibodies were used for Western blot analysis, IHC and ICC: rabbit polyclonal anti-Akt (9272, 1:1000), rabbit polyclonal anti-ERK1/2 (p44/42 MAPK, 9102, 1:1000), rabbit monoclonal anti-phospho-Akt (Ser473, 4058, 1:1000), rabbit polyclonal anti-phospho-ERK1/2 (p44/42 MAPK; Thr202/Tyr204, 9101, 1:1000), rabbit polyclonal anti-phospho-p53 (Ser15, 9284, 1:500), rabbit polyclonal anti-phospho-STAT3 (Tyr705, 9131, 1:1000), rabbit polyclonal anti-stat3 (9132; 1:1000) (Cell Signalling Technology, Danvers, MA, USA), rabbit polyclonal anti-CXCR4 (NB100-56437, 1:500) (Novus Biologicals, Cambridge, UK), rabbit polyclonal anti-p21 (H-164, sc-756, 1:500), mouse monoclonal anti-p53 (DO-1, sc-126, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-A-tubulin (T5168, 1:5000), mouse monoclonal anti-GAPDH (G8795, 1:5000) (Sigma-Aldrich, St. Louis, MO, USA) mouse monoclonal anti-γ-H2AX (613402, 1:500) (Biolegend, Sant Diego, CA, USA) rabbit polyclonal anti-γ-H2AX (00059, 1:3000) (Bethyl Laboratories, Montgomery, TX, USA). Secondary antibodies coupled to horseradish peroxidase, Alexa-488 or Alexa-594 was obtained from Amersham Pharmacia Biotech (Diegem, Belgium) or Sigma-Aldrich. Nuclear staining DAPI stain, DAB (D5637) and F-actin stain phalloidin-Alexa Fluor 594 were from Sigma-Aldrich. Following reagents were used: AMD3100 octahydrochloride (3299) (Tocris Bioscience,Bristol, UK), Trametinib (S2673) (Selleckchem, Houston, TX, USA), recombinant MIF (289-MF-0002), recombinant CXCL12 (350-NS-010) (R&D Systems, Minneapolis, MN, USA), D-luciferin, Firefly, potassium salt (122796) (PerkinElmer, Waltham, MA, USA), Zeocine (E6110) (Promega, Madison, WI, USA) Doxycycline, FITC-Dextran (46945) (Sigma-Aldrich), Vybrant®DiI cell-labelling solution (V-22885) (Molecular Probes, Waltham, MMA, USA). In the in vitro experiments, AMD3100 was used at 10 μM, Trametinib at 50 nM, recombinant
CXCL12 and MIF at 50 ng/mL, D-luciferin at 150 µg/mL, Zeocine at 500 µg/mL, Doxycycline at 500 ng/mL, FITC-Dextran at 1 mg/mL and Vybrant®DiI 5µL/mL/10⁶ cells. In vivo D-luciferin was used at 150 mg/kg mice and ex vivo at 300 µg/mL.

6.7.3.2 Viability Assay

Beas_2B cells were seeded in a 24-well plate. Plates were treated either with 10 Gy or 0 Gy. After 4 days cells were rinsed 3 times with PBS²⁺ and 200 µL of LIVE/DEAD mixture was added to the cells for 30 minutes at 37°C in 5% CO₂. Mixture contained 5 µM calcein-AM (living cells, green fluorescent 496/517nm) and 20 µM ethidium homodimer-1 (dead cells, red fluorescent 528/617 nm). Cells were washed with PBS²⁺ and analysed by fluorescence microscopy (Zeiss 510 META confocal laser scanning microscope, Carl Zeiss, Micro-imaging, Heidelberg, Germany).

6.7.3.3 Conditioned medium of irradiated and non-irradiated bronchial epithelial cells

Three days after treatment, cells were washed, three times, and put on serum-free DMEM culture medium with 100 U/mL penicillin, 100 µg/mL streptomycin, 2,5 µg/mL fungizone and put in an incubator at 37°C and 5% CO₂. After 24 hours the medium of both groups was harvested separately and centrifuged for 5 minutes at 1000 rpm on 4°C. The supernatants were collected and filtered through a 0.2 µM Whatmann filter. All conditioned media were normalized to an equal number of 6,25 x 10⁵ cells. Both conditioned media were stored at -20°C until further use for experiments.

6.7.3.4 Protein analysis

Lysate preparation, SDS PAGE and Western blot analysis

Lysates were made of 70% confluent cell cultures by PBS containing 1% Triton X-100, 1% NP-40 (Sigma), and the Halt® Protease and Phosphatase inhibitor cocktail (1:100, Thermo Scientific). After sonication and centrifugation of lysate (14000 rpm
for 10 minutes at 4°C), supernatant was used for measuring protein concentration by using RC DC™ protein assay kit (Bio-Rad Laboratories S.A.-N.V.). Samples were prepared with an equal amount of protein in Laemmli sample buffer with 5% β-mercaptoethanol and 0.005% bromophenol blue. Next, samples were boiled for 5 minutes at 95°C. Finally, proteins were separated by electrophoresis. Proteins from the gel were blotted onto a nitrocellulose membrane and blocked in 5% nonfat milk in PBSD or 4% BSA in PBSD for phosphorylated proteins, both supplemented with 0.5% Tween-20 and immunostained. Next, a chemiluminescent substrate for horseradish peroxidase (ECL western blotting detection reagent; GE Healthcare, Belgium) was added and signal was measured and visualized by ProXima 2850 (Isogen Lifescience, De Meern, Netherlands). Quantification was done by ImageJ software.

_Cytokine array._

Human antibody assay was performed as per manufacturer’s instructions. Briefly, array membranes were blocked with 5% BSA/TBS (0.01 M Tris HCl pH 7.6/0.15 M NaCl) for 1 hour. Membranes were then incubated with about 2 ml of CMLE or CMLE-IR after normalization with equal amounts of protein. After washing 3 times with PBSD supplemented with 0.1% Tween 20, the membranes were then incubated with a cocktail of biotin-labelled antibodies against different individual cytokines. The membranes were then washed and incubated with HRP-conjugated streptavidin (2,5 pg/ml) for 1 hour at room temperature. After 2 times wishing with PBSD supplemented with 0.1% Tween 20, the signals were detected by ProXima 2850. Densitometric values of spots were quantified using ImageJ Software (Scion Corp., Frederick, MD, USA).
6.7.3.5 Functional assays with conditioned media

Transendothelial migration assay

Endothelial monolayer formation and confirmation. EA.hy926 endothelial cells were transiently red-fluorescent labeled with Vybrant® DiI cell-labelling solution according to the manufacturer’s protocol. Endothelial cells were seeded in the upper compartment of a Matrigel (100 µg/mL) coated 8 µm pore size Transwell culture system and cultured in normal cell conditions at 37°C in 5% CO₂ for 5 days until a monolayer was formed. Monolayer permeability was tested by fluorescent measuring of 70 kDa FITC-dextran flux across the endothelial cell monolayer. Briefly, the endothelial monolayer was washed 2 times with PBS⁺ followed by addition of 150 µL of FITC-Dextran (1 mg/mL) to the upper chamber and 700 µL PBS⁺ in the lower chamber. After 1 hour incubation at 37°C in 5% CO₂, FITC-Dextran concentration from each lower chamber was calculated using a fluorescent multi-well plate reader with excitation and emission wavelength of 485 nm and 530 nm, respectively (SpectraMax® Paradigm® Multi-Mode Microplate Detection Platform reader, Molecular Devices, Sunnyvale, California, USA). When no fluorescent intensity was detected, EA.hy926 reached confluence.
Chapter 7  Clinical relevance

Chapter 8  Main observations

Chapter 9  Role of CXCR4 pathway as therapeutic target or prognostic/preventive marker in TNBC

Chapter 10  Future perspectives

General discussion & conclusion
Chapter 7

Clinical relevance
7.1 *In vivo* model

Our first aim was to develop an *in vivo* model to study metastasis progression. We managed to fulfil this goal with the use of bioluminescent imaging. The proposed model meets 2 of the 3R criteria (Reduction and refinement, not replacement). The use of bioluminescent cancer cells enables longitudinal monitoring of cancer progression in the same animal, so fewer animals need to be sacrificed. Moreover, metastatic disease can be detected more rapidly, thus avoiding unnecessary animal suffering. Bioluminescent imaging also has more advantages compared to other visualisation techniques like magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and single photon emission CT (SPECT). All advantages and disadvantages are pointed in Table 1.

**Table 1. Advantages and disadvantages BLI**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>low cost</td>
<td>no deep tissue penetration</td>
</tr>
<tr>
<td>high throughput</td>
<td>low spatial resolution</td>
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<tr>
<td>non-invasive</td>
<td>ATP, O2 Mg2+ dependent</td>
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<tr>
<td>high sensitivity</td>
<td>requires exogenous luciferin</td>
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<tr>
<td>lack of toxicity</td>
<td>not practical for large animals/humans</td>
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<tr>
<td>low background luminescence</td>
<td>absorption/scattering by animal tissue</td>
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<tr>
<td>no endogenous luciferase expression</td>
<td>requires genetically encoded luciferase</td>
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<tr>
<td>no need for external light source</td>
<td>dependent on substrate</td>
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Although visualisation of animal anatomy and monitoring organ functions remains important in the use of animal cancer models. Bioluminescent imaging is emerging as an increasingly useful and low-cost technique to understand the basic tumor processes, tumor progression and monitoring of therapeutic interventions.² ⁴
Fortunately, mouse models have a lung radiosensitivity equivalent to that of humans and have similar timelines for the development of pneumonitis and fibrosis.\textsuperscript{5} Results found in our research can thus be extrapolated to the situation in the patient.\textsuperscript{5} The simulation of human radiotherapy trials in mice is not 100\% relevant but has much potential to provide radiobiological understandings that can improve the design of human trials.

### 7.2 SARRP vs. IMRT patients

To investigate the effects of radiotherapy, we needed to set-up a radiation schedule for mice. In the past, researchers used whole body irradiation or partial body irradiation (using led shielding).\textsuperscript{6-9} This still resulted in unwanted irradiation of healthy tissue. To study irradiation effects on small parts of the body, adapted image-guided radiation equipment is needed. In our model we make use of the SARRP to achieve accurate irradiation of only 10\% of the right lung. To ensure the accuracy of the dose positioning, a cone beam CT of the animal was made and the isocenter was indicated (Fig. 1). Then we marked the different kinds of tissue so that mouse-specific dose calculation could be achieved. To end with accurate irradiation of indicated tissue, SARRP beam diameter was adjusted to 3 x 3 mm.
SARRP was chosen over human radiation equipment because it is capable to deliver a dose at sufficient precision. Similar to human equipment, radiation beams from different directions can reach the target by rotating the source, limited to an arc (Fig. 1). SARRP uses kilovoltage (kV) X-rays, which have the advantage to have a steeper build-up (air-tissue interface) compared to the megavoltage (MV) X-rays produced by linear accelerators. The latter can be extended up to several centimeters, which would cause a dose build-up /-down gradient of the order of the animal size (Fig. 2). Also the re-build-up phenomenon in the lung-tissue interfaces is negligible with kV X-rays. MV X-rays are precise up to a few mm, for irradiation of small animals this needs to be sub-millimeter precision (Fig. 2). Another problem with MV X-rays is the lateral beam penumbra, which can extend several mm beyond estimated radiation field. The use of kV X-rays diminishes this effect well below of 1 mm (Fig. 2).
Figure 2: Left panel: Monte Carlo calculations of depth dose profiles for a water/air/water/air slab phantom, modeling a low density cavity embedded in tissue. The beam is a 1 mm diameter pencil beam and dose scoring was performed over the whole width of the beam. For the 6 MV beam and the cobalt photons, the buildup can be clearly seen behind both air/water interfaces. The kV beam exhibits only a minimal buildup effect. Missing backscatter is a more subtle effect but is discernable at the water/air interfaces for the cobalt and 6 MV beam, but is minimal for the kV beam. Right panel: calculated dose profiles in water at the depth of maximum dose clearly showing the more extensive beam penumbra for the higher energy photon beams. This calculation separates the effect of the beam spreading in water from other beam widening effects (collimator scatter, focal spot broadening).\textsuperscript{10}

Disadvantages of kV X-rays is that the radiation beams are not equally absorbed in the different kinds of tissue. Low energy kV X-rays are predominantly absorbed by photo-electric processes. This effect is largely dependent on the composition of the tissue, what results in differential absorption in bone. MV X-ray has a more similar absorption of radiation beams in all kinds of tissue because their effects are mostly Compton scatter related, and less dependent on tissue composition. This is important for dose calculations, where for MV X-rays these are far less sensitive to tissue composition than for kV X-ray.\textsuperscript{10} This leads us to another problem of differences in relative biological effectiveness (RBE). kV X-ray have higher LET values than MV X-ray (250 kV = 3 keV/\(\mu m\) and 3MV = 0.3 keV/\(\mu m\)) meaning that the dose required for kV X-ray causing the same damage as MV X-ray is lower.\textsuperscript{11}

Another difference between our model and patient treatment is the use of single dose radiation. We used a single fraction of 10 Gy conform the data of Darby \textit{et al.}\textsuperscript{12} and an irradiation target of 10\% of the right lung conform the data of Kimsey \textit{et al.}\textsuperscript{13}
Using fractionated radiation in the *in vivo* model would be the ideal set-up, but entails technical complications. Positioning of the mouse and the position of the lung need to be the same in every session. This is hard to accomplish because of strong respiratory movements and relocation of the lungs inside the mice. The effect of fractionated versus acute irradiation doses on cytokine profile was studied by Desai *et al.*\(^\text{14}\) Their results showed that after radiation exposure the levels of cytokines increased in a dose dependent manner and that the fold-change in cytokine level was lower in conditioned medium of fractionated irradiated cells than after acute dose radiation. However, upregulation was still seen compared with non-irradiated cells, proving that our result can also be representative for dose fractionation radiation. Cytokine production may not only promote metastasis but it may also have an effect on the immune response. Lee *et al.*\(^\text{15}\) showed that acute dose irradiation produces a more potent immune response than fractionated irradiation, promoting eradication of cancer cells.\(^\text{15}\) Further research elucidating the mechanisms behind these discrepancies would be interesting.

### 7.3 Patient relevance

Results from a prospective study in 41 irradiated breast cancer patients revealed that postoperative radiation therapy of 50 Gy at 2 Gy/fraction, 5 days/week resulted in a mean pulmonary dose of approximately 10 Gy.\(^\text{16}\) Only when more than 3 axillary lymph nodes were affected, a supraclavicular field was added with equal dose and fractionation resulting in a higher mean pulmonary dose of 15.8 Gy.\(^\text{16}\) Results from a second prospective study in 34 breast cancer patients showed that the estimated percentage of total irradiated lung volume may range from 2.7 to 17.6% in a study population receiving tangential radiation beams.\(^\text{13}\) These studies show that the 10% right lung irradiation and the 10 Gy dose is a representative set-up for the patient situation.
Many clinical trials prove that collateral radiation to the lungs causes a late damaging effect. Irradiation results in reduced pulmonary function in the first two years after the postoperative radiotherapy and increases the subsequent rate of ischemic heart disease and secondary lung cancer risk.\textsuperscript{12, 16-18}

Clinical trials with TNBC where adjuvant radiotherapy versus no-radiotherapy were compared, have a general conclusion that radiotherapy has an important role in local tumor control but has no significant positive impact on OS and DM formation.\textsuperscript{19-24} Wang \textit{et al.}\textsuperscript{24} even revealed that the percentage of patients with 1 or 2 metastases increases after adjuvant radiotherapy. Remarkably increased distant relapse is more pronounced in patients treated with mastectomy adjuvant radiotherapy compared to patients treated with mastectomy or BCS without adjuvant radiotherapy.\textsuperscript{22} Although there is no significant difference in the location of TNBC relapse between patients receiving adjuvant radiotherapy and patients who did not, the lung is still the most frequent site of initial recurrence.\textsuperscript{20}

It is generally assumed that in this case the micrometastases were already present in the lungs before the breast cancer was treated. This can be explained by the fact that metastasis can be an early event in tumor progression and stays undetected at time of diagnosis.\textsuperscript{25, 26} The difference with our hypothesis is that we assume that radiation-induced collateral damage of the lung has promoting influence on lung-specific metastasis. We first irradiated the lung then initiated a primary tumor and allowed metastasis formation. Our model is not relevant for the effect of ionizing radiation on already established micrometastases because we graft syngeneic breast cancer cells after ionizing radiation. We irradiate a small part of the right lung, graft syngeneic breast cancer cells and study the impact on spontaneous lung metastases formation. Lung irradiation has no impact on orthotopic tumor growth. However, we
observe more and bigger metastatic foci in the lungs of PRL mice which can be caused by increased attraction of circulating cancer cells, and/or stimulation of cancer cell release from the primary tumor, and/or preparation of the metastatic niche (promoting adhesion and colonization of breast cancer cells).

Our findings highlight not only the importance of preventing metastasis but also the importance of intensive post-irradiation follow-up. So that early metastasis formation in TNBC patients can be detected and treated early. Studies (using post-mortem samplings) should be designed to investigate a potential relationship between collateral lung radiation-damage and lung metastasis in breast cancer patients. The literature provides no level-one evidence because no randomized trials have been done correlating the dose indices of irradiated lung volumes with lung-specific metastasis in breast cancer.
7.4 References


Chapter 8

Main Observations
It has been shown that radiation can cause late secondary malignancies and that irradiation of a tumor bed can promote metastasis, but experimental set-ups showing the impact of radiation on the formation of a metastasis-receptive microenvironment are lacking. We developed a syngeneic breast cancer model where partial irradiation of healthy mouse lung increases metastatic load and size in both lungs.

The potential mechanisms are: (I) Stimulation of micrometastases. Thoen et al.\textsuperscript{1} suggested that most patients with distant failure of locally advanced esophageal cancer treatment already have subclinical metastatic disease at time of diagnosis, and local radiation-induced effects can stimulate further progression.\textsuperscript{1} The stimulation of already present metastases can be induced by events similar to TME effects after radiation exposure. The balance between pro- and anti-metastasis promoting events in this case is shifted to the metastasis promoting events. (II) Attraction and stimulation of circulating tumor cells. Irradiation of endothelial cells stimulates cancer cell extravasation, which is an important event in affecting metastasis.\textsuperscript{2} Local inflammation and cytokine production initiated by radiation exposure, not only attracts normal stem cells for tissue regeneration, but also for angiogenesis. It also stimulates attraction of circulating cancer cells, extravasation and growth of the present cancer cells. Accidental radiation turns normal tissue into a metastasis receptive environment.\textsuperscript{3}

In this research we make use of \textit{in vitro} models to mimic the metastatic cascade and to reveal underlying molecular pathways.

We showed that irradiation of normal lung epithelial cells causes DNA double-strand breaks, which lead to upregulation of proteins involved in cell cycle arrest and senescence. This activates cell signaling cascades which promote secretion of cytokines and chemokines. The \textit{in vitro} results showed that co-culture of irradiated epithelial cells with breast cancer cells increased cellular adhesion and cell growth. They also showed that, when mimicking the \textit{in vivo} situation, there is not only a
Local, target-related effect, but also a distant- and off-target related increase in breast cancer cell growth (Fig. 3). This means that the irradiated lung epithelial cells secrete factors that also promote cancer cell growth outside the irradiated area. When supernatant of irradiated lung epithelial cells was added to breast cancer cells increased cell migration, growth and cellular dedifferentiation into a more mesenchymal phenotype was observed.

**Figure 3: Partial irradiation in vitro effect**

**A.** Co-culture set-up: Irradiation of 10% lung epithelial monolayer with single dose 10 Gy. 24 hours after IR 4T1_luc breast cancer cells were added, adhesion was determined after 1 day co-culture, cell growth after 4 days co-culture. **B.** Box plots illustrating the relative attachment and cell growth of 4T1_luc cells. Co-culture of breast cancer with irradiated Beas-2B cells significantly increases the relative attachment locally compared to co-culture with sham treated Beas-2B cells. Co-culture of breast cancer with irradiated Beas-2B cells significantly increases the relative cell growth locally and globally compared to co-culture with sham treated Beas-2B cells. Quantification by bioluminescent imaging was performed after 1 and 4 days incubation. Data is represented as relative fold change compared with the corresponding control value. 4T1_luc: n=6; *= p<0.05 and **= p< 0.01 (Mann-Whitney U test).

Human protein array study of 570 human proteins in the supernatant revealed that there was an increased secretion of more than 50 proteins (6.7.2 Suppl. Table). FunRich analysis revealed that more than 50% of the upregulated cytokines play an important role in cellular communication and signal transduction but also 20% of the
cytokines are important members of the immune response (LBP, CSF-1, CXCL1, CXCL2, CCL2, IL-6, ALCAM, IL-7, IL-13, CXCL8 and CSF-3). This shows that irradiation of healthy cells is accompanied by an immune reaction of these cells. This can play an important role in the stimulation of lung metastasis formation.\textsuperscript{4, 5} In the top-10 of most upregulated cytokines, two important chemokines were observed: CXCL12 and MIF. Shu \textit{et al.}\textsuperscript{6} revealed an upregulation of CXCL12 in both serum and bronchoalveolar lavage fluid (BALF) of the mouse, up to 28 days after partial thorax irradiation. \textit{In vitro} studies with recombinant CXCL12 and MIF revealed similar results as with the supernatant. Both are stimulators of the CXCR4 receptor. Müller \textit{et al.}\textsuperscript{7} were the first to describe CXCL12-mediated chemotaxis of CXCR4 expressing breast cancer cells towards normal human tissues which express high amounts of CXCL12 like lung, liver, brain, bone marrow and lymph nodes.

This research concludes that local irradiation of healthy lung tissue causes DNA damage in the epithelial cells, which leads to activation of signal cascades that trigger secretion of chemokines and cytokines. Notably, an upregulated secretion of CXCL12 and MIF is seen. Both activate the CXCR4 receptor on the breast cancer cells, resulting in activation of signal transduction pathways. In its turn this stimulates chemotaxis and homing of breast cancer cells to the lungs. Breast cancer cells will remain in the lung, where high levels of CXCL12 and MIF provide the cancer cells with survival and growth factors directly or indirectly through formation of new blood vessels.\textsuperscript{3} The metastatic niche formation is known to be enhanced by the primary tumor, whose secreted factors create an environment that favors homing and growth of metastasis at a specific distant site.\textsuperscript{8, 9} The results show that accidental irradiation of normal lung tissue enhances the formation of this metastatic niche and the persistent growth of metastasis. Local CXCL12 and MIF production causes attraction and activation of stem cells (bone marrow-derived), endothelial progenitor cells, immune cells and local stromal cells, which creates a suitable “soil” in the irradiated lung.\textsuperscript{10-16} Stimulated attraction of circulating cancer cells (“seeds”) is seen,
accompanied by a stimulation of transendothelial migration, extravasation and growth of the present cancer cells.\textsuperscript{17-19} Interestingly high levels of CXCR4 are observed in breast cancer stem cells that initiate metastasis formation.\textsuperscript{20}

\section*{8.1 References}


Chapter 9

Role of CXCR4 pathway as therapeutic target or prognostic/diagnostic marker in TNBC
9.1 Therapeutic target

One of the research goals was to investigate if inhibition of the CXCR4 pathway could undo the pro-metastatic effects of radiation. In the investigation, we made use of an allosteric inhibitor of the CXCR4 receptor, AMD3100. When blocking the CXCR4 receptor, both in vitro and in vivo, a decrease in in vitro metastatic-promoting characteristics\(^1\) and a decrease of metastatic load in vivo is seen (Fig. 4A). In a preliminary experiment we treated mice with AMD3100, starting 5 days after orthotopical injection of 4T1_luc cells (6 days after irradiation). Mice were randomized to have the same mean tumor size in all groups. One group with 10% irradiation of the right lung, were injected twice a day into the peritoneum, with AMD3100 dissolved in PBS\(^{D-}\) (1,25 mg/kg mice).\(^2\) Control groups (10% irradiation right lung and sham) were injected with PBS\(^{D-}\) (vehicle). This was repeated 5 times a week until day 28, when the mice were sacrificed. Bioluminescent quantification of the lungs showed that treatment with AMD3100 decreased metastasis formation by 59% compared to untreated irradiated lungs (PRL vs. PRL +AMD3100: 1,42 x 10\(^6\) p/s vs. 5,76 x 10\(^5\) p/s, mean data were not significant). When compared to non-irradiated lungs, the metastatic signal was similar (sham vs. PRL + AMD3100: 5,9 x 10\(^5\) p/s vs. 5,76 x 10\(^5\) p/s). Primary tumor growth was similar in all 3 groups, showing that AMD3100 had no effect on primary tumor growth (Fig. 4B). Kozin et al.\(^4\) revealed that the combination of AMD3100 with radiotherapy induces a significant delay in tumor growth and curability of breast cancer cells. However this was only seen when AMD3100 treatment started immediately after radiation exposure. When treatment was started 5 days after radiotherapy, no significant effect on breast cancer cells was seen.\(^4\) This study shows not only the importance of the identification of an optimal treatment schedule, but also the temporal role of CXCL12/CXCR4 pathway in tumor progression. A major problem with AMD3100 are the long-term toxic effects.\(^5\) Kalatskaya et al.\(^6\) showed also an agonistic effect of
AMD3100 on the CXCR7 receptor, this probably contributes to the long-term toxic effects.

**Figure 4:** *In vivo* effect of AMD3100 treatment. 4 week old BALB/c female mice were injected orthotopically with $1 \times 10^6$ 4T1_luc cells in 0.1 mL of serum-free DMEM with 50% Matrigel. **A.** 28 days after implantation of the cells, mice were sacrificed and total lung metastasis was quantified by bioluminescent imaging. PRL + placebo and PRL + AMD3100: n=6 and sham + placebo: n=5. Lung was measured as one. **B.** Tumor formation was monitored for 28 days by caliper measurement. Tumor volumes were measured as indicated. PRL + placebo and PRL + AMD3100: n=6 and sham + placebo: n=5.

We showed that downstream inhibition of ERK activation under control conditions results in compensatory activation of Akt and STAT3, which can result in tumor-promoting effects. Therefore, we prefer a treatment that blocks either the CXCR4 receptor or selectively blocks CXCL12 and MIF. In the development of new therapeutic strategies, the 5Rs of radiobiology will be very important where each R should be considered as a double-edged sword: taking care of the repair, repopulation, redistribution, reoxygenation and radiosensitivity of cancer cells.

Clinical studies showed that CXCR4 inhibition decreases breast cancer cell progression. Peng et al. made use of a small peptide antagonist of CXCR4 (LY2510924) that blocks binding of CXCL12 and CXCL12-induced GTP binding. The use of the antagonist reduced lung metastasis by blocking the migration/homing
process of breast cancer cells with tolerable side effects.\textsuperscript{8, 9} Treatment with CXCL12 peptide analogues (CTCE-9908) significantly reduces metastasis as well as primary tumor growth in a mouse model of breast cancer.\textsuperscript{10} Inhibition of CXCR4 or MIF and CXCL12 peptide analogues would only have a partial effect.

A better way to prevent radiation-induced metastasis is by direct neutralization of CXCL12 and MIF since both cytokines not only activate CXCR4, but also other receptors. CXCL12 is known to activate the CXCR7 receptor. Miao \textit{et al.}\textsuperscript{11} found that CXCR7 activation promotes breast cancer growth \textit{in vivo} and its expression level influences lung metastasis. MIF can activate CD74, which results in breast cancer cell growth.\textsuperscript{12} It also stimulates CXCR2 (IL-8RB) and because of its 3D similarity to IL-8, it has a chemokine-like function, but no effects are described in breast cancer cells.\textsuperscript{13} A peptide analogue would only bind to a binding site on a specific receptor. The analogue should contain amino acid sequences that fit to binding sites of all possible activated receptors, which at this moment is not available. Neutralizing CXCL12 and MIF would make the lung environment less receptive, which would lead to reduced chemotaxis and homing of BCC in the lung. It would not only affect cancer cells, but trafficking of bone marrow derived progenitor cells and local stromal cells would also be inhibited, resulting in not only a less metastatic receptive environment but also in reduced fibrosis.\textsuperscript{14} Roccaro \textit{et al.}\textsuperscript{15} showed that CXCL12 neutralization leads to a bone marrow microenvironment that is less receptive for multiple myeloma, which resulted in a reduction of melanoma cell homing and growth.\textsuperscript{15}

As shown in our \textit{in vivo} model, blocking the CXCL12-CXCR4 pathway alone had no effects on the already established primary tumor. The use of anti-CXCL12 agents would not be beneficial in patients with localized disease. It rather would be used for delaying or, in optimal conditions, preventing metastasis formation. The ultimate
goal is to find a treatment that inhibits the negative effects of radiotherapy and complement the effect of the chemotherapy.

In the literature we find that CXCR4 activation is also responsible for tumor resistance to chemo- and radiotherapy. This is achieved both directly by promoting cancer cell survival, invasion and initiation of circulating stem cells and indirectly by the recruitment of bone marrow derived cells (promoting tumor recurrence and metastasis) and by promoting angiogenesis (in)dependent of VEGF.\textsuperscript{16, 17} Inhibition of the CXCR4 pathway not only prevents side-effects of radiotherapy but also sensitizes the cancer cells to chemotherapy.

We conclude that further preclinical models and clinical studies should be developed to closely recapitulate the clinical features and the response to MIF and CXCL12 inhibitors combined with radiotherapy as treatment for TNBC patients.

**9.2 CXCL12 or MIF as biomarker**

Detection of MIF and CXCL12 concentrations in patients could be useful to determine the potential effect of radiotherapy in patients.

Measurement of CXCL12 and MIF levels in patients.

Shu \textit{et al}.\textsuperscript{14} measured CXCL12 levels in BALF and serum of mice at different time point after whole thorax irradiation. A significant increase was observed in BALF CXCL12 level 1 day after irradiation, while a significant increase in CXCL12 serum level was observed only 3 days after irradiation.\textsuperscript{14} Significantly elevated MIF level were seen in serum samples of breast cancer patients with metastatic disease compared to healthy and localized breast cancer patients.\textsuperscript{18} Higher pretherapeutic levels of MIF were seen in non-responsive patients.\textsuperscript{18} In this study, no difference was made between non-TNBC and TNBC.\textsuperscript{18} Although BALF seems to be the most accurate specimen to study elevated levels of CXCL12 and MIF, we prefer using
blood samples because the collection procedure is less invasive for the patients. Due to the cytokine stability plasma is preferred. Platelet fragments can bind to CXCL12 in serum conditions, which reduce the available amount for quantification. Human plasma CXCL12 concentration can be stable for 2 hours, when blood is placed on ice immediately after sampling. Also measurements of MIF levels are preferred in plasma samples and immediate processing of samples is recommended to avoid haemolysis.

**What are the basal levels of both cytokines and what are the factors besides radiation that can elevate these levels?**

In cell culture and in laboratory mouse studies, this basal level is relatively constant because of the limiting environmental and disease-related factors. In humans this is not the case. Environmental factors and other diseases can cause elevated serum levels of CXCL12 and MIF. Elevation of CXCL12 and MIF serum levels are seen during acute lung injury, infectious diseases and sepsis, transplantation rejection, autoimmune diseases (lupus, rheumatoid arthritis, ...), metabolic diseases (type II diabetes), inflammatory lung disorder, asthma and arteriosclerosis.

The problem is that besides cancer-environmental cells, some breast cancer cells themselves produce MIF, which contributes to the overexpression of MIF detected in breast cancer tissues. There is evidence that MIF secretion by breast cancer cells is upregulated by exogenous MIF, and MIF levels in serum are increased in breast cancer patients independently of tumor characteristics. Hence, elevated levels could be seen before treatment and comparison of serum levels can only be done in a patient-specific manner.

**What is the time-point after irradiation to return to the basal level?**

Shu *et al.* measured significant increased levels of CXCL12 in serum up to 7 days after IR. In BALF a significant increase was measured up to 28 days after IR.14
Further clinical investigation needs to be performed to determine at which time points a peak and the return to the basal level is seen.

**9.3 CXCR4 as prognostic marker**

Yu et al. reported that high level of CXCR4 in triple-negative breast cancer specimens are associated with a poor clinical outcome. High CXCR4 expression in primary tumor is related to more extensive metastasis to the lymph nodes (LN) and focal staining of CXCR4 is also associated with more metastasis to LN compared to diffuse staining of CXCR4 in primary tumor. Although CXCR4 expression is absent or very low in normal breast epithelium, CXCR4 upregulation is already present in DCIS and DCIS with invasive foci. An increase in CXCR4 level is seen as cancer progresses from atypical hyperplasia to invasive cancer.

Our research showed that radiotherapy as monotherapy could be disadvantageous for CXCR4 expressing TNBC cells. Irradiation of lung tissue induces increased secretion of CXCL12 and MIF which will interact with the CXCR4 receptor on the breast cancers cells. This will initiate signaling cascades resulting in metastasis-promoting events. So, combined-therapy of radiotherapy with CXCL12- and MIF-neutralizing compounds should be preferred.

**9.4 CXCR4 as diagnostic marker**

Liang et al. developed a biotinylated probe for CXCR4, that can be used as radiotracer to detect CXCR4 in living animals with PET scan. [Cu] AMD3100 was successfully used by Nimmagadda et al. to detect lung metastasis in an in vivo breast cancer model. This can be useful to detect metastasis by molecular imaging in patients.

The challenge is making a difference between CXCR4-bearing metastasis and CXCR4-bearing normal tissue. Another challenge is the effect of radiolabeling on the
pharmacokinetics of the tracer and its affinity for CXCR4 receptor. Since molecular imaging becomes more important in targeted therapy trials, studies on CXCR4 tracers could be very interesting.
9.5 References


Chapter 10

Future perspectives
As described in this thesis, our in vitro and in vivo experiments demonstrated that irradiation of healthy lung tissue promotes metastasis formation in TNBC through activation of the CXCR4 pathway on the breast cancer cells. To optimize radiotherapy treatment of breast cancer patient and to study its unwanted effect on healthy tissue, a more clinically relevant in vivo model is warranted.

Proposal in vivo set-up (Fig. 5)

Orthotopic BCC in 2nd mammary fat pad

↓

Surgical removal of primary tumor

↓

Fractionated RT treatment tumor bed

Figure 5: Future perspectives - in vivo set-up

Our in vivo model revealed, by bioluminescent follow-up, that 4T1_luc cells give rise to lung metastasis within 28 days. Instead of injection into the 4th mammary fat pad, a more patient relevant position can be used as injection site. When using the 2nd mammary fat pad, the breast cancer is formed located in a more similar place as in human breast cancer patients. This results in a more clinically relevant collateral damage pattern after radiotherapy. The problem with this set-up, is that thoracic bioluminescent signals can either represent breast cancer local recurrence or lung metastasis. More sophisticated imaging tools, such as small animal MRI can solve this problem.

In the future we would like to remove the primary tumor before starting radiotherapy. The tumor needs to be removed before formation of visible (lung)
metastasis. Hence, first we would need to examine the exact time point when the primary tumor results in metastasis formation in the lung.

After surgery, the primary tumor bed will be irradiated according to the patient situation. Looking at radiotherapy schedules of TNBC patients at UZ Ghent, 2 irradiation schedules are possible: normo-fractionated (25 x 2 Gy or 30 x 2Gy) or hypo-fractionated radiation schedule (15 x 2.67 Gy + 4 x 2.5 Gy). This gives us the possibility to study if there is any difference in metastasis formation dependent on irradiation schedule. SARRP optimization allows yielding beams from different arcs, thus enabling multiple field irradiations. But still a problem exists with positioning of the mouse. For each individual mouse a restrainer is needed to keep it in the same position during every radiation session.

If there is a difference in metastasis formation, we would like to set-up experiments with radiotherapy in combination with CXCR4 inhibitors or CXCL12-neutralizing or MIF-neutralizing agents or combinations of all of the above. The combination with the best outcome, with the least metastasis and the least side-effects will be used for further investigation. Furthermore, blood samples will be taken to follow-up changes in serum levels of CXCL12 and MIF. This gives us the opportunity to set-up a relevant treatment-schedule.

Post-mortem analysis will consist of H&E staining of lung tissue to visualize metastasis formation. It would be ideal to use a specific luciferase antibody for immunohistochemistry staining, so only luciferase-containing cells would be visualized.

**Proposal for patient samples (Fig. 6)**

Additional investigations of patient samples could be interesting to see if there is any change in CXCL12 and/or MIF plasma levels during treatment procedure and if these changes in plasma levels correlate to increased metastasis formation. We want
to study the basal levels of CXCL12 and MIF in TNBC patients. The cytokine with the minimal inter-patient variance would be used for further research. Follow-up of the cytokine level will be done in patients during treatment. Secondly, because chemo- or targeted therapeutics could have an effect on the cytokine level, this should be monitored as well. In patient studies a relative change in cytokine level is more important than absolute change and in our case the relative change before and after radiotherapy. We can study if there is any effect of radiation treatment schedule or patient position during radiotherapy (prone versus supine position).

The patient needs to be followed-up for at least 5 years, because the majority of metastasis in TNBC manifests 3-5y after radiotherapy. If there is a correlation between elevated serum levels of CXCL12 or MIF and lung metastasis formation after radiotherapy, patients can be selected and clinical trials can be set-up for combination therapy of radiotherapy and cytokine-inhibiting agents.

As illustrated in this discussion, the lack of research on the effect of radiotherapy on healthy tissue in patients proves that further research is needed. Do hormone receptor and/or HER2 receptor positive breast cancer cells respond the same way as

Figure 6: Proposal patient study – time schedule. The figure represents a treatment schedule of an average TNBC patient in time. Arrows indicating treatment start and end date. Asterisks indicate time points of blood sample collection and determination of the cytokine levels.
triple-negative breast cancer cells to radiotherapy, and if not which cytokines or mechanism play an important role after radiotherapy? As for triple-negative breast, the effect of other cytokines than CXCL12 and MIF need to be further investigated. Not only in breast cancer, but also in colon, prostate or other types of cancer this research could be useful. Are the same cytokine upregulation seen as in triple-negative breast cancer or are other cytokine more elevated after irradiation? Are the same mechanisms activated?
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