Linköping University Medical Dissertations No. 1560

The receptor tyrosine kinase Met and the protein tyrosine
phosphatase PTPN2 in breast cancer

Cynthia Veenstra

Division of Surgery, Orthopaedics, and Oncology
Department of Clinical and Experimental Medicine
Faculty of Medicine and Health Sciences
Linköping University
Linköping, Sweden 2017

Copyright © 2017 by Cynthia Veenstra

This is not a work of fiction. No names, characters, organisations, and events portrayed in this book are the product of the author's imagination, but are facts as known in the scientific community today.

ISBN: 978-91-7685-603-1

ISSN: 0345-0082

Papers I and III are open access articles and published under the terms of the Creative Commons Attribution License (CC BY), meaning that authors retain ownership of the copyright for their article.

Printed in Linköping, Sweden by LiU-Tryck, March 2017

Alice : "This is impossible."

The Mad Hatter : "Only if you believe it is."

- Alice in Wonderland, 2010

SUPERVISOR

Olle Stål, Professor

Department of Clinical and Experimental Medicine
Linköping University, Linköping, Sweden

CO-SUPERVISOR

Gizeh Pérez-Tenorio, PhD

Department of Clinical and Experimental Medicine
Linköping University, Linköping, Sweden

FACULTY OPPONENT

Kristian Pietras, Professor

Department of Translational Cancer Research
Lund University, Lund, Sweden

EXAMINATION BOARD

Karl-Eric Magnusson, Professor Emeritus

Department of Clinical and Experimental Medicine
Linköping University, Linköping, Sweden

Fredrik Wärnberg, MD, Associate Professor

Department of Surgical Sciences, Endocrine Surgery
Uppsala University, Uppsala, Sweden

Karin Öllinger, Professor

Department of Clinical and Experimental Medicine
Linköping University, Linköping, Sweden

ABSTRACT

Breast cancer is the most common form of cancer in women worldwide and the second leading cause of cancer death. It is a heterogeneous disease and is subdivided into different subtypes, all with different treatment responses and survival outcomes. Luminal breast cancers are characterised by the expression of oestrogen receptor and generally have a good prognosis. More aggressive tumours are marked by the presence of growth stimulating receptor tyrosine kinase HER2 (HER2-like breast cancer) or the absence of oestrogen receptor, progesterone receptor, and HER2 (triple-negative breast cancer, TNBC). The latter is the most aggressive form and is difficult to treat due to lack of treatment targets.

This thesis aimed to explore possible prognostic and predictive biomarkers in different subtypes and study their role in breast cancer. To this aid, breast cancer tumours of pre- and post-menopausal patients enrolled in two cohorts were analysed for gene copy numbers and expression of proteins involved in cell proliferation. Gene copy numbers of receptor tyrosine kinases *MET* and *EGFR*, Met's ligand *HGF*, and protein tyrosine phosphatase *PTPN2* were determined by droplet digital PCR or quantitative PCR in both cohorts. Met, phosphorylated Met (pMet), HGF, and PTPN2 protein expression levels were analysed with immunohistochemical staining in the pre-menopausal cohort. Moreover, the role of the aforementioned proteins was investigated in breast cancer cell lines. Amplification of *MET*, *HGF*, and *EGFR* in breast tissues was found to be low (5-8%). These three genes, all located on chromosome 7, were found to be strongly correlated with each other and to be associated with shortened distant recurrence-free survival. High protein expression of Met, pMet, and HGF was found in 33%, 53%, and 49% of the breast tumours. *MET* and *EGFR* were found to be more often amplified in TNBC disease, correlating with worse survival. Moreover, stromal expression of HGF was associated with shorter survival in TNBC. EGF stimulation in TNBC cell line MDA-MB-468 led to inhibited cell

proliferation and migration. Partial knockdown of EGFR caused TNBC cells to proliferate and migrate more upon EGF treatment, mirroring EGFR inhibitor resistance. Knockdown of Met had in part the opposite effects, indicating that Met inhibitors might be useful in the treatment of TNBC. The increase in proliferation and migration upon EGFR depletion could be counteracted with simultaneous knockdown of EGFR and Met, indicating that dual inhibition of these proteins might be a future treatment option in TNBC.

Copy loss of *PTPN2* was reported in 15% of the cases in both pre- and post-menopausal cohorts. Low cytoplasmic PTPN2 protein expression was found in half of the cases. Loss of PTPN2 gene or protein was associated with a shorter distant recurrence-free survival in Luminal A and HER2-positive tumours, not in TNBC, suggesting a subtype-related prognostic value of PTPN2. Subtype relevance of PTPN2 was further implied by *in vitro* analyses. Whereas PTPN2 knockdown had no observed effect on TNBC cell lines, knockdown in the Luminal A cell line MCF7 inhibited Met phosphorylation and promoted phosphorylation of Akt, a key regulator of cellular proliferation and survival. The cell growth and survival regulating RAS/MAPK pathway remained unaffected. Knockdown in the HER2-positive cell line SKBR3 led to increased Met phosphorylation and decreased RAS/MAPK-related Erk phosphorylation as well as EGF-mediated transcription factor STAT3 phosphorylation. These results indicate that the role of PTPN2 in breast cancer is subtype-related and needs to be further investigated for future treatment options.

POPULAR SCIENTIFIC SUMMARY

The most common and second deadliest cancer amongst women worldwide is breast cancer, which is a collective name for a variety of cancers in the breast. It can be subdivided into several subtypes that are all related to different survival outcomes. Changes in the DNA (mutations) can cause excessive production of a protein (overexpression) or its loss. This can play a role in the aggressiveness of the tumour, determining how fast the tumour grows or how well it responds to treatment. The most frequent and least aggressive form of breast cancer is called Luminal A and is defined by the presence of a protein called the oestrogen receptor. Luminal A tumours generally have a good prognosis. The more aggressive tumours are characterised by overexpression of HER2, a protein involved in tumour growth. The most aggressive type of breast cancer is called triple-negative breast cancer (TNBC), a difficult to treat tumour defined by the absence of the oestrogen receptor, HER2, and another protein called progesterone receptor.

This thesis aimed to explore new proteins that can show the progression of the disease (prognostic marker) or predict how well a tumour will respond to certain treatments (predictive marker). The tumour tissues of two groups (cohorts) of patients were analysed with this aim. The first cohort consisted of breast cancer patients who have not gone through menopause (pre-menopausal), the second group have gone through menopause (post-menopausal). Normally, there are two copies of a gene, but in cancer, this can be different. A copy can be lost, which is called deletion. Gene amplification is the presence of four or more copies. Genes hold the information to build a protein. To coordinate cellular processes, proteins communicate with each other through a so-called signalling pathway. The first protein to receive a signal is called a receptor. This signal is passed on to other proteins, often through activating (phosphorylating) the next protein. The deactivation (de-phosphorylating) of proteins is done by enzymes called phosphatases.

The copy numbers of the receptors *MET* and *EGFR*, the activating signal (ligand) for Met called *HGF*, and the phosphatase *PTPN2* were determined in both cohorts. The presence of these proteins was analysed in tumour tissues of patients in the pre-menopausal cohort. Amplification of *MET*, *HGF*, and *EGFR* was not common in the tumours, only between 5-8% was found. These results were compared with the survival

of the patients, and it was found that patients with tumours with amplification of one of these genes had a shorter survival time. Overexpression of Met, activated Met, and HGF was found in 33%, 53%, and 49% of the tumours. It was found that amplification of *MET* and *EGFR* was common in the TNBC subtype and this was associated with worse survival.

The expression of Met and EGFR proteins was changed in a cell line characterised as TNBC. Taking away (knocking down) EGFR resulted in increased cell growth (proliferation) and cell movement (migration) when these cells were treated with the ligand for EGFR called EGF. This mimics EGFR inhibitor resistance in the body. Resistance is when cells do not respond to the inhibitor. Knocking down Met led to the decrease in proliferation and migration, which indicates that the inhibition of Met might be useful in the treatment of TNBC. The increase of proliferation and migration after EGFR knockdown could be cancelled out when EGFR and Met were knocked down at the same time. This indicates that the simultaneous inhibition of EGFR and Met in patients might be a future treatment option in TNBC.

Deletion of *PTPN2* was found in about 15% of the patients in both cohorts. Decreased protein expression was observed in half of the cases. Loss of *PTPN2* was associated with a shorter patient survival in Luminal A and HER2-positive tumours, but not in TNBC. This suggests that the role of *PTPN2* is dependent on the subtype. This subtype-related theory was further investigated in breast cancer cell lines representing three different subtypes. Only Luminal A and HER2-positive cell lines were affected by the knockdown of *PTPN2*, TNBC cell lines remained unaffected. The Luminal A cell line MCF7 responded to the knockdown by decreasing the activation of Met and increasing that of a protein called Akt. Activation of this protein is associated with cell growth and survival. Contrary, knocking down of *PTPN2* in the HER2-positive cell line SKBR3 led to the increase of Met activation and decreased activation of proteins important in a cell signalling pathway called RAS/MAPK. These results suggest that the role of *PTPN2* in breast cancer is subtype-dependent, which gives valuable information for the future treatment of breast cancer.

POPULAIR WETENSCHAPPELIJKE SAMENVATTING

De meest voorkomende en op één na dodelijkste vorm van kanker onder vrouwen wereldwijd is borstkanker. Dit is een verzamelnaam voor verschillende soorten kankers in de borst. Het wordt onderverdeeld in een aantal subtypes die allen andere overlevingskansen hebben. Veranderingen in het DNA (mutaties) kan tot overmatige productie van een eiwit leiden (overexpressie) of tot het verlies hiervan. Dit kan een rol spelen in de mate van agressie van een tumor en daarmee bepalen hoe snel een tumor groeit of hoe het reageert op behandeling. De meest voorkomende en minst agressieve vorm van borstkanker heet Luminaal A en wordt gekenmerkt door de aanwezigheid van een eiwit genaamd oestrogeenreceptor. In het algemeen hebben Luminaal A tumoren een goede prognose. De meer agressieve tumoren worden gekenmerkt door de overexpressie van HER2, een eiwit betrokken bij tumorgroei. De meest agressieve vorm van borstkanker wordt triple negatieve borstkanker (TNBC) genoemd. Dit is een moeilijk te behandelen tumor dat wordt gedefinieerd door de afwezigheid van de oestrogeenreceptor, HER2 en een ander eiwit genaamd de progesteronreceptor.

Dit proefschrift is gericht op het verkennen van nieuwe eiwitten die de progressie van de ziekte kunnen laten zien (prognostische marker) of kan voorspellen hoe goed een tumor zal reageren op een bepaalde behandeling (voorspellende marker). De tumorweefsels van twee groepen (cohorten) patiënten werden geanalyseerd met dit doel. De eerste cohort bestond uit borstkankerpatiënten die nog niet door de menopauze zijn gegaan (pre-menopauze), de tweede groep bestaat uit patiënten die door de menopauze zijn gegaan (post-menopauze). Normaal gesproken zijn er twee kopieën van een gen, maar in kanker kan dit anders liggen. Er kan een kopie ontbreken, dit wordt deletie genoemd. Genamplificatie is de aanwezigheid van vier of meer kopieën. Genen bevatten de informatie om een eiwit op te bouwen. Om acties van de cel te reguleren kunnen eiwitten met elkaar communiceren via zogenoemde signaalwegen. Het eerste eiwit dat een signaal ontvangt wordt een receptor genoemd. De receptor geeft het signaal door aan andere eiwitten, vaak door activering (fosforylering) van het volgende eiwit. De deactivering (de-fosforylering) van eiwitten wordt uitgevoerd door enzymen, zogenoemde fosfatasen.

Het aantal kopieën van de receptoren *MET* en *EGFR*, het activerende signaal (ligand) van Met genaamd *HGF*, en de fosfatase *PTPN2* werd bepaald in beide cohorten. De

aanwezigheid van deze eiwitten werd geanalyseerd in het tumorweefsel van de patiënten in de pre-menopauze cohort. Amplificatie van *MET*, *HGF* en *EGFR* kwam niet vaak voor in de tumoren, slechts tussen de 5-8% amplificatie werd gevonden. Deze resultaten werden vergeleken met de overleving van de patiënten waaruit bleek dat patiënten met tumoren met amplificatie van één van deze genen een kortere overlevingstijd hebben. Overexpressie van Met, geactiveerde Met en HGF werd gevonden in 33%, 53% en 49% van de tumoren. Amplificatie van *MET* en *EGFR* was meer gebruikelijk in TNBC en dit was verbonden met slechtere overlevingskansen.

De expressie van de Met en EGFR eiwitten werd veranderd in een cellijn dat gekarakteriseerd wordt als TNBC. Het uitschakelen van EGFR leidde tot een verhoogde celgroei (proliferatie) en beweging (migratie) wanneer deze cellen werden behandeld met het EGFR-ligand EGF. Dit bootst EGFR-remmer resistentie na in het lichaam. Resistente cellen reageren niet op behandeling met de remmer. Uitschakeling van Met leidde tot de vermindering van proliferatie en migratie. Dit geeft aan dat het remmen van Met nuttig kan zijn in de behandeling van TNBC. De toename van proliferatie en migratie kan worden opgeheven wanneer EGFR en Met tegelijkertijd uitgeschakeld worden. Dit toont aan dat de gelijktijdige remming van EGFR en Met in patiënten een toekomstige behandelingsoptie in TNBC kan zijn.

Deletie van *PTPN2* werd bij ongeveer 15% van de patiënten waargenomen in beide cohorten. Verminderde eiwitexpressie werd gevonden in de helft van de gevallen. Verlies van *PTPN2* was gerelateerd een kortere overleving van patiënten met Luminaal A en HER2-positieve tumoren, maar niet in TNBC-tumoren. Dit suggereert dat de rol van *PTPN2* afhankelijk is van het subtype. Deze theorie werd verder onderzocht in borstkankercellijnen die de drie subtypes vertegenwoordigen. Alleen Luminaal A en HER2-positieve cellijnen werden beïnvloed door de uitschakeling van *PTPN2*, TNBC-cellijnen bleven onaangetast. De Luminaal A-celijn MCF7 reageerde op de uitschakeling door een verlaging van de activatie van Met en een verhoging van het eiwit Akt. Activatie van dit eiwit leidt tot celgroei en overleving. Aan de andere kant, uitschakeling van *PTPN2* in HER2-positieve cellijn SKBR3 leidde tot de toename van Met activering en een verminderde activering van eiwitten in de RAS/MAPK signaalweg. Deze resultaten suggereren dat de rol van *PTPN2* in borstkanker subtype-afhankelijk is. Dit kan belangrijke informatie geven over de toekomstige behandeling van borstkanker.

POPULÄR VETENSKAPLIG SAMMANFATTNING

Den vanligaste och näst mest dödliga cancerformen bland kvinnor i världen är bröstcancer, som är ett samlingsnamn för olika cancertyper i bröstet. Det finns flera subtyper som alla är förenade med olika överlevnad. Förändringar i DNA (mutationer) kan leda till för hög produktion av ett protein (överuttryck) eller till förlust. Detta kan spela roll för graden av aggressiviteten hos en tumör och därigenom bestämma hur snabbt en tumör växer eller hur väl den svarar på behandlingen. Den vanligaste och minst aggressiva typen av bröstcancer kallas Luminal A och kännetecknas av uttryck av ett protein som kallas östrogenreceptorn. Luminal A tumörer har i allmänhet en god prognos. De mer aggressiva tumörformerna karaktäriseras av överuttryck av HER2, ett protein involverat i tumörtillväxt. Den mest aggressiva typen kallas trippelnegativ bröstcancer (TNBC), en svårbehandlad tumörform som definieras genom frånvaron av östrogenreceptorn, HER2, och ett annat protein som kallas progesteronreceptorn.

Denna avhandling syftar till att utforska nya proteiner som kan visa på utvecklingen av sjukdomen (prognostisk markör) eller förutsäga hur väl en tumör kommer att svara på vissa behandlingar (prediktiv markör). Tumörvävnad från två grupper (kohorter) patienter analyserades för detta ändamål. Den första kohorten bestod av bröstcancerpatienter som ännu inte har gått igenom menopausen (premenopausala), den andra gruppen består av patienter som har gått igenom menopausen (postmenopausala). Normalt finns det två kopior av en gen, men i cancer kan det vara olika. Om en kopia förloras kallas detta för deletion. Genamplifiering är förekomsten av fyra eller fler kopior. Gener innehåller informationen för att bygga ett protein. För att reglera cellulära processer kommunicerar proteiner med varandra genom en så kallad signalväg. Det första proteinet som tar emot en signal kallas en receptor. Denna signal förs vidare till andra proteiner, ofta genom aktivering (fosforylering) av nästa protein. Inaktiveringen (defosforylering) av proteiner sker genom enzymer, så kallade fosfataser.

Antalet kopior av *MET* och *EGFR*, den aktiverande signalen (liganden) av Met, som kallas *HGF*, och fosfataset *PTPN2* bestämdes i båda kohorterna. Närvaron av dessa proteiner analyserades i tumörvävnad från patienter i den premenopausala kohorten. Amplifiering av *MET*, *HGF* och *EGFR* var inte vanligt i tumörerna, bara mellan 5-8 % hittades. Dessa resultat jämfördes med överlevnaden av patienterna, som visade att

patienter med tumörer med amplifiering av en av dessa gener har kortare överlevnadstid. Överuttryck av Met, aktiverat Met och HGF hittades i 33 %, 53 %, och 49 % av tumörerna. Amplifiering av *MET* och *EGFR* var vanligare i TNBC och detta var förknippat med sämre överlevnad.

Förändringar i uttrycket av Met och EGFR skapades i en cellinje som representerar som TNBC. Borttagning av EGFR resulterade i ökad celltillväxt (proliferation) och cellrörelse (migration) när dessa celler behandlades med liganden för EGFR som kallas EGF. Detta påminner om resistens EGFR hämmare. Resistent celler svarar inte på behandling med hämmaren. Borttagning av Met ledde till en minskning i proliferation och migration, vilket tyder på att hämningen av Met kan vara användbar vid behandling av TNBC. Ökningen av proliferation och migration efter EGFR borttagning kan avbrytas genom samtidig borttagning av EGFR och Met. Detta visar att den samtidiga hämningen av EGFR och Met i patienter skulle kunna vara ett framtida behandlingsalternativ i TNBC.

Deletion av *PTPN2* observerades hos ca 15 % av tumörerna i båda kohorterna. Minskade proteinuttryck hittades i hälften av fallen. Förlust av *PTPN2* var associerad med en kortare patientöverlevnad för Luminal A och HER2-positiva tumörer, men inte för TNBC. Detta tyder på att den roll *PTPN2* spelar är beroende av subtyp. Denna teori undersöktes ytterligare i bröstcancer cellinjer som representerar de tre subtyperna. Endast Luminal A och HER2-positiva cellinjer påverkades av borttagning av *PTPN2*, TNBC cellinjen blev opåverkad. Den Luminal A lika cellinjen MCF7 reagerade på borttagningen genom att minska aktiveringen av Met och med ökning av aktiverat Akt-protein. Aktivering av detta protein leder till celltillväxt och överlevnad. Däremot ledde borttagning av *PTPN2* i den HER2-positiva cellinjen SKBR3 ledde till en ökning av Met-aktivering och minskad aktivering av proteiner som är viktiga i en signalväg som kallas RAS/MAPK. Dessa resultat tyder på att rollen för *PTPN2* vid bröstcancer är subtyps-beroende, vilket ger värdefull information som kan få betydelse för den framtida behandlingen av bröstcancer.

LIST OF PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

Paper I

CYNTHIA VEENSTRA, Gizeh Pérez-Tenorio, Anna Stelling, Elin Karlsson, Sanam Mirwani Mirwani, Bo Nordenskjöld, Tommy Fornander, and Olle Stål.

Met and its ligand HGF are associated with clinical outcome in breast cancer

Oncotarget. 2016 Jun 14;7(24):37145-37159

Paper II

CYNTHIA VEENSTRA, Anna Stelling, Krista Briedis, Bo Nordenskjöld, Tommy Fornander, Olle Stål, and Gizeh Pérez-Tenorio

The significance of Met and EGFR crosstalk in triple-negative breast cancer

Manuscript

Paper III

Elin Karlsson, CYNTHIA VEENSTRA, Shad Emin, Chhanda Dutta, Gizeh Pérez-Tenorio, Bo Nordenskjöld, Tommy Fornander, and Olle Stål

Loss of protein tyrosine phosphatase, non-receptor type 2 is associated with activation of Akt and tamoxifen resistance in breast cancer

Breast Cancer Res Treat. 2015 Aug;153(1):31-40

Paper IV

CYNTHIA VEENSTRA, Elin Karlsson, Sanam Mirwani Mirwani, Jon Gårsjö, Bo Nordenskjöld, Tommy Fornander, Gizeh Pérez-Tenorio, and Olle Stål

The effect of PTPN2 loss on cell signalling and clinical outcome in relation to breast cancer subtypes

Manuscript

PAPERS OUTSIDE THIS THESIS

Elin Karlsson, CYNTHIA VEENSTRA, Jon Gårsjö, Bo Nordenskjöld, Tommy Fornander, and Olle Stål

PTPN2 deficiency together with activation of nuclear Akt predict endocrine resistance in breast cancer

Under review for publication in The Breast

ABBREVIATIONS

4EBP1	EIF4E-binding protein 1
ANOVA	Analysis of Variance
bp	Base Pair
BSA	Bovine Serum Albumin
CIS	Carcinoma <i>In Situ</i>
CMF	Cyclophosphamide, Methotrexate, and 5-Fluorouracil
Ct	Cycle Threshold
ddPCR	Droplet Digital PCR
DAB	3,3'-Diaminobenzidine Hydrochloride
DCIS	Ductal Carcinoma <i>In Situ</i>
DRFS	Distant Recurrence-Free Survival
ECL	Enhanced Chemiluminescence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor
FBS	Foetal Bovine Serum
FFPE	Formalin-Fixed, Paraffin-Embedded
GAB1	GRB2-Associated Binder 1
GRB2	Growth Factor Receptor Bound Protein 2
GSK3	Glycogen Synthase Kinase 3
Gy	Gray (unit of ionising radiation)
HER	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
HGFR	Hepatocyte Growth Factor Receptor
HR	Hazard Ratio
HRP	Horseradish Peroxidase
IGF	Insulin-Like Growth Factor
IHC	Immunohistochemistry
IPT	Immunoglobulin-Plexin-Transcription
kDa	Kilo Dalton

ABBREVIATIONS

LCIS	Lobular Carcinoma <i>In Situ</i>
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
mTOR	Mammalian Target of Rapamycin
NHG	Nottingham Grade
NLS	Nuclear Localisation Sequence
NRG	Neuregulin
NSAID	Non-Steroidal Anti-Inflammatory Drug
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-Kinase
PR	Progesterone Receptor
PSI	Plex-Semaphorin-Integrin
PTEN	Phosphatase and Tensin Homolog
PTP	Protein Tyrosine Phosphatase
PTPN2	Protein Tyrosine Phosphatase, Non-Receptor Type 2
qPCR	Quantitative Real-Time PCR
RAS	Rat Sarcoma
RISC	RNA-Induced Silencing Complex
RNAi	RNA Interference
RTK	Receptor Tyrosine Kinase
S6K1	p70 ribosomal S6 Kinase 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
siRNA	Short Interference RNA
SPF	S-Phase Fraction
TCPTP	T-Cell Protein Tyrosine Phosphatase
TGF α	Transforming Growth Factor α
TMA	Tissue Microarray
TNBC	Triple-Negative Breast Cancer
TNM	Tumour, Node, Metastasis
Tyr	Tyrosine
Y	Tyrosine

TABLE OF CONTENTS

ABSTRACT.....	i
POPULAR SCIENTIFIC SUMMARY	iii
POPULAIR WETENSCHAPPELIJKE SAMENVATTING	v
POPULÄR VETENSKAPLIG SAMMANFATTNING	vii
LIST OF PAPERS INCLUDED IN THIS THESIS	ix
PAPERS OUTSIDE THIS THESIS	x
ABBREVIATIONS.....	xi
TABLE OF CONTENTS	xiii
INTRODUCTION	1
The female breast.....	1
Anatomy	1
Breast development.....	1
Hormonal influences.....	3
Breast cancer.....	4
Epidemiology.....	5
Risk factors	5
Tumour Classification	6
TNM staging.....	6
Nottingham grading.....	7
Breast cancer subtypes.....	8
Luminal breast cancer	8
HER2-like breast cancer	9
Triple-negative breast cancer	9
Treatment	10
Local treatment	10
Surgery	10
Radiotherapy.....	11
Systemic treatment.....	11
Chemotherapy.....	11
Endocrine therapy	12
Targeted therapy.....	12

Signalling pathways in breast cancer	13
PI3K/Akt pathway.....	13
Receptor tyrosine kinases	14
Protein tyrosine phosphatase family	18
PTPN2	19
AIMS	23
COMMENTS ON MATERIALS AND METHODS.....	25
Patient cohorts (Papers I-IV)	25
Sample preservation.....	26
Cell culture (Papers II and IV)	26
Small interfering RNA (Papers II and IV)	27
Gene copy number assessment	28
qPCR (Paper III).....	28
Droplet Digital PCR (Papers I, II, IV)	29
Western blot (Papers II and III).....	29
Immunohistochemistry (Papers I and IV)	30
MTS proliferation assay (Paper II).....	31
Transwell assay (Paper II)	32
Statistical analyses (Papers I-IV)	32
RESULTS AND DISCUSSION.....	35
Genes and proteins of interest in breast cancer cell lines	35
Genes of interest in breast cancer tumours	36
Proteins of interest in breast cancer tumours	38
Genes and proteins in relation to clinicopathological characteristics	39
Prognostic values.....	41
Predictive values.....	43
EGF-induced growth inhibition.....	45
Met and EGFR transactivation	46
The effect of PTPN2 loss is subtype-related	48
CONCLUSIONS	51
ACKNOWLEDGEMENTS	53
REFERENCES	57

"Exploration is in our nature.
We began as wanderers, and we are wanderers still."

- Carl Sagan

INTRODUCTION

Even though the breast cancer incidence has been increasing in the last decennia, fewer people die of the disease thanks to scientific advances in screening, early detection, treatment, and a better understanding of the biology of breast cancer. Still and all, breast cancer remains the second leading cause of cancer mortality in women all over the world, preceded by lung cancer, and the research needs are many.

The female breast

The female breast is subjected to radical changes during different stages of life, to wit: infancy, puberty, pregnancy, lactation, and post-menopause. The breasts start developing during infancy, with the most radical changes during puberty. However, the breasts are not completely developed until the end of the first full-term pregnancy.

Anatomy

The breasts are located over the pectoral muscles of the chest wall, where they are attached by Cooper's ligaments. The female breast largely consists of adipose (fat) tissue, connective tissue, lobes, and ducts. An average breast harbours between 15-20 lobes, all separated by adipose and connective tissue. The ratio of fat-to-connective tissue determines the breast density. Lobes are made up of smaller lobules, which in turn are composed of milk-producing alveoli. The lobes are connected by ducts, which transport the milk to the nipple. The ducts can expand near the nipple, forming sinuses to store milk (Figure 1).

Breast development

The structure in the infant's breast is undeveloped and just consists of small ducts. Between infancy and puberty, the breast does not develop and the tissue will merely keep up with the growth of the body. At this point, the male and

female breast are identical. As puberty arrives, the ducts will branch into to type 1 lobules, the least developed type of lobules. During the first few years after menarche, the first menstruation, some lobules will advance into type 2 lobules, a more developed type of lobule. At this point, the breast consists of around 75% of type 1 and 25% of type 2 lobules. If pregnancy does not occur, the breast will not develop further [1, 2].

Figure 1 | Sagittal section of the female breast. The healthy human female breast consists of adipose tissue and glandular tissue that is constructed of lobes. Ducts drain the lobes of milk and transport it to the nipple or store it in sinuses. Cooper's ligaments attach the breast to the chest wall.

The structure of the breasts changes throughout the menstrual cycle. In the start of the menstrual cycle, the follicular phase, the lobules are small and cells sparsely proliferate. The lobules will develop more during the luteal phase, the

second half of the cycle after ovulation, and more mitotic activity is seen. If no pregnancy occurs, these changes degenerate [3].

In the first 20 weeks of pregnancy, hormones like oestrogen, progesterone, and prolactin, influence further growth of the breast. The type 1 and 2 lobular structures increase in numbers and the breasts double in size. The second half of the pregnancy is characterised by the maturation of type 1 and 2 lobules into type 4. Type 4 lobules are able to secrete milk. After breastfeeding, the type 4 lobules regress to type 3 lobules [4, 5]. Epigenetic changes in this type of lobules decrease the risk of developing cancer.

During menopause, the breasts of both nulliparous (not given birth) and parous (given birth) women degenerate to type 1 lobules. However, the previous mentioned epigenetic changes stay present in the regressed lobules. This means that parous women are still less prone to breast cancer, even post-menopause [2].

Hormonal influences

The breast development is dependent on the steroid hormones oestrogen and progesterone, which both promote cell proliferation and differentiation. These hormones act on their receptors, the oestrogen receptor (ER) and the progesterone receptor (PR). There are three primary forms of oestrogen: oestrone (E1), oestradiol (E2), and oestriol (E3). Oestradiol, the most potent oestrogen, is the most prevalent form prior menopause while oestrone is the most predominant in post-menopausal women. Oestriol is most dominant during pregnancy [6-8].

The binding of hormones to their receptors stimulates the production of growth factors as hepatocyte growth factor (HGF), transforming growth factor α (TGF α), epidermal growth factor (EGF), insulin-like growth factor (IGF), amphiregulin, and neuregulin (NRG), which are all part of signalling pathways that lead to cell proliferation and cell survival. HGF is particularly important in the ductal development [9].

Breast cancer

Breast cancer is a heterogeneous disease that can arise in different parts of the breast and that can be either non-invasive or invasive (Figure 2). Tumours mostly emerge in the ducts (80%) or lobules (10%). Non-invasive breast cancer, or *carcinoma in situ* (CIS), is traditionally thought to be a precursor to invasive cancer. Most CIS are ductal CIS (DCIS) and often discovered in mammography screening. An estimated 20-50% of untreated DCIS will progress to invasive carcinoma [10].

Figure 2| The structure of normal and malignant ducts and lobules. Normal ducts and lobules are lined with a single layer of epithelial cells. Cancerous cells can be found within the ducts or lobules. If these cells are contained inside the structure, it is classified as *in situ* or non-invasive. As soon as cancer cells break through the structure, the cancer has become invasive. DCIS: Ductal carcinoma *in situ*; LCIS: Lobular carcinoma *in situ*.

Epidemiology

Breast cancer is the most common cancer in women worldwide and the second most common cancer overall. It accounted for 1.67 million new breast cancer cases globally in 2012. In the same year, breast cancer was responsible for 521,900 deaths, making it the fifth cause of death from cancer [11, 12]. It has been estimated that an astounding one in eight to ten women will get breast cancer at one point during their lifetime.

Risk factors

Many risk factors can contribute to the development of breast cancer. These factors are either uncontrollable or environmental/lifestyle-related.

The single greatest risk for developing breast cancer is being female. Although men can develop breast cancer, it is a hundred times more common amongst women. As for most cancers, the older the person, the higher the risk of breast cancer. Inheritance plays a significant part in the development of cancer; 5-10% of all breast cancer cases are hereditary. A family history of the disease, especially with first-degree relatives, increases the risk of breast cancer significantly. Well-known genes predicting breast cancer are *BRCA1* and *BRCA2*. Women who have inherited the mutated form of one of these genes have a 40-80% probability of developing breast cancer [13]. Other uncontrollable risk factors are race and ethnicity; Caucasian women are more susceptible to breast cancer than African-American women are, though the latter are more likely to succumb to the disease. Asian, Hispanic, and Native American women have the lowest risk of getting breast cancer [12, 14]. Other unmodifiable factors increasing the risk of breast cancer are dense breasts (higher percentage non-fatty tissue), early menarche (before age 12), and late menopause (after age 55) [15, 16].

Some risk factors can be controlled by adopting a different lifestyle. The risk of developing breast cancer is 1.5 times as high in women drinking 2-5 alcoholic

drinks a day, though limited wine consumption (half a bottle a week), has been correlated with decreased risk [17, 18]. Overweight or obesity in pre-menopausal women correlates with a slightly lower risk of developing breast cancer as compared with a healthy weight. Conversely, overweight or obesity in post-menopausal women is associated with a higher breast cancer risk [19, 20]. Women taking oral contraceptives have a slightly higher probability of developing breast cancer. This risk disappears when the woman stops taking contraceptives [21]. Not having children increases the risk of breast cancer as lobules of type 1 and 2 are more cancer-susceptible [20].

Early parity (pregnancy before the age of 30) is a long-term protective factor against breast cancer development. As explained previously, pregnancy results in the growth of type 4 lobules, which have been shown to be less prone to develop cancer. However, the longer the interval between menarche and the first pregnancy, the more the breast cancer risk increases. The loss of protection against breast cancer and the increase in the risk starts with an interval over 14 years [22-24]. Even though this protective factor has been known for many years, it is still not fully understood as to why early parity protects from breast cancer and late parity does not. Other protective factors are physical activity, breastfeeding, and non-steroidal anti-inflammatory drugs (NSAIDs) usage in pre-menopausal women [19, 25-27].

Tumour Classification

Tumours are clinically described by using the TNM-system and the Nottingham Histologic Score system.

TNM staging

The TNM system is applied in most solid tumours and determines the stage of the tumour. TNM stands for tumour, nodes, and metastasis. This means that the TNM system indicates the extent of the tumour by describing the size of the primary tumour, how many regional lymph nodes are affected by the cancer cells, and whether the cancer has metastasised (spread to a different part of the

body). The stage, as indicated by their Roman numerals, are prognostic indicators and help doctors to decide on the course of treatment. A stage 0 tumour contains abnormal cells and is the so-called 'pre-cancerous' carcinoma *in situ* (CIS). Stage I tumours are less than 2 cm and do not include the lymph nodes. Tumours with nodal involvement are classed as stage II or III. If the cancer has spread to distant parts of the body, the cancer is categorised as stage IV, also known as advanced breast cancer [28].

The use of TNM staging has become controversial. Many clinicians argue that the system is outdated and it no longer aids in deciding the course of (personalised) treatment, including surgery and adjuvant systemic treatment. Moreover, the TNM system does not take common biomarkers like ER into consideration [29, 30].

Nottingham grading

The Nottingham grade (NHG) system determines the grade of the tumour, which is different from stage. It characterises how abnormal and aggressive the tumour is. A pathologist who studies the tumour tissue determines the grade of the tumour. Three distinct features of the cells in the tumour are studied, and a score between 1 and 3 is given to each feature. The three features are tubule formation, nuclear pleomorphism, and mitotic count. Tubule formation is studied to determine how much of the tumour tissue has normal tubulare/ductal structures. Normal breast tissue is made up of more than 75% tubules, whilst an aggressive tumour can be made up of less than 10% tubules. Nuclear pleomorphism shows how much the nuclei in the tumour's cells have changed in shape and size. A nucleus is supposed to be uniform and small; aggressive tumours show larger nuclei with different shapes. The mitotic count demonstrates how many cells in the tumour tissue are actively dividing. In normal breast tissue, a low number of mitotic figures is seen, as the tissue grows slowly. Aggressive tumour tissue grows at a much faster rate than less aggressive tissue and thus shows more mitotic figures.

All scores added together make up the grade of the tumour. A score of 3-5 means that the tumour is grade 1. These well-differentiated tumours grow slowly and are less likely to spread. Grade 2 tumours score a 6 or 7 and are defined as moderately differentiated with some risk of spreading. A score of 8 or 9 means the tumour is grade 3, a poorly differentiated tumour that grows fast and is likely to spread [31].

Breast cancer subtypes

While breast cancer was previously considered a single homogeneous disease, today it is known as a heterogeneous disease with different subtypes. The distinction between the molecular tumour subtypes is mainly based on three biomarkers, to wit: the ER, the PR, and the human epidermal growth factor receptor 2 (HER2). The expression levels of these receptors, as established with immunohistochemistry (IHC), give rise to three major classes, with different treatment responses and survival outcomes. A summary of the breast cancer intrinsic subtypes is shown in Table 1.

Luminal breast cancer

Luminal tumours are the most common type of breast cancer. These types are the tumours expressing hormone receptors (ER and PR). This class is subdivided into Luminal A and B. Both forms are ER and/or PR-positive whilst Luminal A tumours are HER2-negative and Luminal B tumours HER2-positive. However, if a tumour is ER/PR-positive, HER2-negative but highly proliferative, as measured by staining for proliferation marker Ki-67, this tumour is classed as Luminal B. If Ki-67 staining is not available, NHG could be used as tumour proliferation assessment. In this thesis and its articles, Luminal B is split to Luminal B1 (HER2-negative) and Luminal B2 (HER2-positive) [32-35]. Around 75% of the breast tumours are Luminal; these tumours are generally well-differentiated and less aggressive than ER-negative tumours [36-38]. Luminal A tumours are often NHG 1 or 2 and Luminal B tumours NHG 2 or 3. As Luminal tumours are ER-positive, the prognosis is better than other

subtypes, though patients with Luminal A tumours have a better outcome than patients with Luminal B.

HER2-like breast cancer

HER2-like tumours overexpress the HER2 protein and are negative for hormone receptors. HER2-like breast cancer is seen in around circa 10% of all breast cancer cases [39]. HER-2 positivity in general is seen in 15-20%. HER2-like tumours are usually associated with poor patient prognosis as this subtype has a higher relapse risk [34, 35, 40].

Triple-negative breast cancer

Triple-negative breast cancer (TNBC) is characterised by the lack of expression of the three biomarkers. Of all breast cancer cases, about 15% is triple-negative. This subtype remains the most fatal of the breast cancers, mainly because of the aggressiveness of the tumour and the unavailability of targeted therapy [41-43]. Whilst TNBC is negative for the three prognostic biomarkers it has been shown that 50-60% of all TNBC cases overexpresses the epidermal growth factor receptor (EGFR), proved to be associated with poor patient outcome [44-47].

Table 1| Definitions of clinicopathological breast cancer subtypes and their treatment.

<i>Subtype</i>	<i>Definition</i>	<i>General Outcome</i>	<i>Systemic treatment</i>
Luminal A	ER/PR-positive HER2-negative Ki-67 low	Good	Endocrine therapy
Luminal B1	ER/PR-positive HER2-negative Ki-67 high	Intermediate	Endocrine therapy Chemotherapy
Luminal B2	ER/PR-positive HER2-positive	Poor	Endocrine therapy Chemotherapy Anti-HER2 therapy
HER2-like	ER/PR-negative HER2-positive	Poor	Chemotherapy Anti-HER2 therapy
TNBC	ER/PR-negative HER2-negative	Poor	Chemotherapy

Treatment

Despite major advancements and efforts in breast cancer research, clinical treatment protocols are still highly dependent on the expression of the biomarkers ER, PR, and HER2, the stage and grade, and thus subtypes (Table 1).

The treatment plan consists of a primary treatment (often surgery), with the option to have follow-up therapy (adjuvant therapy) or a therapy prior to the primary (neo-adjuvant therapy). Neo-adjuvant therapy is usually given to shrink the tumour prior surgery. How many different therapies are needed to treat the breast cancer depends on the type of breast cancer and how advanced the disease is.

Local treatment

Local treatment refers to treatment that only affects the tumour and the surrounded tissue, without affecting the whole body.

Surgery

Breast surgery is the main treatment in most breast cancer cases. The extent of the surgery is considered per case and depends on the tumour size, the location of the tumour, and possible nodal involvement.

The surgery options are a complete mastectomy or breast-conserving surgery (lumpectomy). With a mastectomy, the entire breast is removed; this has the advantage that the woman (most often) does not need radiotherapy if the cancer was early stage. A lumpectomy is the removal of the tumour with a slight margin of healthy tissue, which has a less emotional toll on the woman. Studies revealed lumpectomy plus radiotherapy to be associated with a longer overall survival as compared with complete mastectomy [48-50].

Radiotherapy

Radiotherapy is usually given post-operatively, aimed at any remaining tumour cells, to control local recurrence and increase the breast cancer-specific survival. Conventional radiotherapy is generally given in fractions of 2 Gray (Gy) per session, with a total of 25 sessions (total dose 50 Gy). It uses high-energy beams targeted at the tumour area. The radiation causes DNA damage to the cells, so severe it cannot be repaired by the cell's repair mechanisms, and programmed cell death (apoptosis) is induced. This happens more so in tumour cells than in healthy cells [50].

Systemic treatment

Systemic treatment refers to the treatment that affects cells in the whole body. This therapy targets cells that may have spread from the breast tumour to other parts of the body.

Chemotherapy

Chemotherapy targets the cell's ability to replicate by interfering with the different phases of the cell cycle (the process leading to cell division). There are different groups of chemotherapy drugs, grouped together by their mode of action or chemical structure. Chemotherapy drugs are generally given intravenously in either an adjuvant or neo-adjuvant setting. In most cases, chemotherapy is given as a combination of two or three drugs, which is proven more efficient than the treatment with merely one drug [51].

Chemotherapy is indicated for tumours with high NHG, high Ki-67 staining, low or negative ER/PR status, positive HER2 status, and TNBC tumours [52]. Luminal tumours generally do not respond well to chemotherapy [53]. The more proliferative tumours are associated with a beneficial response towards the chemotherapy [54].

Endocrine therapy

As ER-positive tumours are dependent on oestrogen for their growth, these types of tumours can be treated relatively well by targeting the oestrogen receptor with hormonal, or endocrine, therapy [53]. This anti-oestrogen therapy decreases the oestrogen levels in the body (aromatase inhibitors) or prevents the hormone from binding to their receptors (selective oestrogen receptor response modulators (SERMs)). Aromatase inhibitors are commonly given to post-menopausal breast cancer patients to prevent oestrogen from being produced in the body fat. Tamoxifen is a well-known SERM, given to both pre- and post-menopausal women [55, 56].

Another form of hormonal treatment is ovarian ablation or suppression for pre-menopausal women. This is to prevent oestrogen production by the ovaries, practically making the woman post-menopausal. This can be done permanently by surgical removal of the ovaries (oophorectomy) or temporary shutdown with drugs as leuprolide or goserelin. These drugs are luteinising hormone-releasing hormone (LHRH) agonists [57].

Targeted therapy

As more research is done on breast cancer, more is known about which genes or proteins differ in the tumour cells as compared with healthy cells. These changes can be targeted with drugs to halt the tumour growth. Where chemotherapeutic drugs attack all fast-growing cells, targeted therapy is aimed mainly at hallmark of the tumour cells, avoiding as much as possible to damage healthy cells. A well-known example of targeted therapy is trastuzumab (Herceptin). Trastuzumab is a humanised monoclonal antibody targeting HER2 [58].

Signalling pathways in breast cancer

A signalling pathway is a collection of proteins communicating together to control cell functions, like protein synthesis or cell division. A receptor receives a signal, like a hormone or growth factor, and passes this signal through the membrane onto other proteins through post-translational modifications, activating or inactivating the next protein. A common type of this modification is protein phosphorylation, the addition of a phosphate group to an amino acid. Phosphorylation only occurs on serine (Ser or S), threonine (Thr or T), or tyrosine (Tyr or Y).

Changes in signalling pathways often occur in breast cancer, leading to a shift in the sensitive balance between cell death and cell growth.

PI3K/Akt pathway

There are multiple pathways critical in breast cancer; a specific important one in breast cancer is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. This pathway signals towards protein synthesis, cell survival, cell proliferation, and cell migration. This pathway is overly activated in 70% of the breast cancer cases, leading to uncontrolled cell growth, amongst others.

In short, one of its receptors activates the pathway, upon which PI3K is activated. This protein forms a heterodimer with the catalytic subunit p110 and the regulatory subunit p85. The gene encoding the p110 subunit (*PIK3CA*) is commonly mutated in breast cancer, leading to a constitutively active kinase [59]. The activation of PI3K leads to the phosphorylation of Akt, the key factor in the PI3K/Akt pathway. Akt, in turn, activates several proteins, regulating protein synthesis, cell proliferation and survival (Figure 3).

Another main pathway in breast cancer is the RAS/MAPK pathway, important in the regulation of cell growth, differentiation and survival [60].

Figure 3 | Two main signalling pathways in breast cancer. Various receptor tyrosine kinases can activate several signalling pathways. The two major signalling pathways in breast cancer are the PI3K/Akt and the RAS/MAPK pathway. Through a cascade of several proteins, activation of these pathways lead to cell proliferation, survival, and protein synthesis.

Receptor tyrosine kinases

Receptors that regulate through the addition of a phosphate group on a tyrosine are called receptors tyrosine kinases (RTKs). Upon ligand binding, the RTK can activate certain signalling pathways. The same RTK can activate different pathways, and the same pathway can be activated by multiples RTKs.

Met receptor

The Met oncoprotein is a transmembrane RTK; the gene is localised on chromosome 7q31. Met has been shown to be overexpressed in 20-30% of all breast cancer cases and specifically in 50-60% of the TNBC cases; this is correlated with decreased patient survival [61-64]. The receptor is activated by its only known human ligand HGF, the gene of which is in proximity to *MET* on chromosome 7q21. Therefore, the receptor is also known as hepatocyte growth factor receptor (HGFR). Despite beliefs that Met is named after the mesenchymal-to-epithelial transition, it was named Met after being discovered by treatment with methylnitronitrosoguanidine [65].

Met is formed through the proteolytic cleavage of the 170 kDa precursor protein (pro-Met). The final protein consists of an extracellular 50 kDa α -chain and a 145 kDa β -chain, linked together by a disulphide bond. The α -subunit consists solely of the semaphorin (Sema) domain. The β -subunit consists extracellularly of the Sema domain, the plex-semaphorin-integrin (PSI) domain, and four immunoglobulin-plexin-transcription (IPT) domains, connecting the PSI domain to the transmembrane. The intracellular part of the β -chain consists of the juxtamembrane, a regulator of the catalytic functions, the kinase domain with the tyrosines Y1234 and 1235, and lastly the multi-functional docking site in the carboxy-terminal tail with the tyrosines Y1349 and Y1356 (Figure 4) [66, 67].

Upon ligand binding, the receptor homo-dimerises and auto-phosphorylation of the receptor on Y1234/1235 is induced. Then, a docking site is formed by the activation of Y1349 and Y1356. The phosphorylated residues interact with growth-factor-receptor-bound protein 2 (GRB2)-associated binder 1 (GAB1) after which PI3K is recruited. The RAS/MAPK pathway can be activated by the binding of GAB1 to GRB2 and SHP2 (Figure 3) [68].

Figure 4| The domain structure of the Met RTK. The extracellular part consists of the α -subunit and the Sema, PSI and IPT part of the β -subunit. Intracellularly, the membrane contains the juxtamembrane domain, the kinase domain holding the catalytic tyrosines Y1234/1235, followed by the multi-functional docking site with the tyrosines Y1349 and 1356.

Met has been demonstrated to be co-expressed with other RTKs. Shattuck *et al.* showed that Met and HER2 are co-expressed in HER2-overexpressing breast cancer cell lines and primary breast tumours. HER2 and Met work together, which may create a more aggressive tumour [69]. *MET* amplification and activation are associated with resistance towards EGFR tyrosine kinase inhibitors [70-72]. Met has been suggested to be a bypass resistance mechanism in several cases, most notably in EGFR inhibitor resistance in lung cancer. *MET* amplification was shown *in vitro*

to be responsible for resistance towards the EGFR inhibitor gefitinib, which could be overcome with Met inhibition [72]. Met has even been shown to interfere with the working of trastuzumab. Experiments demonstrate that the PI3K/Akt pathway is still activated by HGF-promoted signalling through GAB1, despite HER2 inhibition [69, 73].

HER family

The human epidermal growth factor receptor (HER) family plays important parts in the regulation of cell proliferation and survival. This transmembrane RTK family is composed of four members: EGFR (HER1), HER2, HER3, and HER4 and are coded by the *ERBB* genes. In general, homo and hetero-dimerisation of the family members happen upon ligand binding. Activation of the HER family leads to, amongst others, the activation of the PI3K/Akt and

RAS/MAPK pathways (Figure 3). The HER family members can even bind other RTKs for onward signalling, which is especially seen with EGFR.

EGFR

The *EGFR* gene is localised on chromosome 7p12, encoding a 170 kDa protein. Its primary ligands are EGF, TGF- α , and amphiregulin. In breast cancer, EGFR is inversely associated with ER-status, meaning expression is mainly found in the more aggressive tumours. As stated before EGFR is overexpressed in 50-60% of the TNBC cases and is related to poor patient outcome; EGFR overexpression is seen to a lesser extent in other breast cancer subtypes [44-46]. One of the main causes for EGFR overexpression is the amplification of the gene, although this has only been described in breast cancer for 1-14% of the cases [74, 75]. Being overexpressed in more than half of the triple-negative tumours, EGFR theoretically makes a good treatment target. Unfortunately, none of the clinically available EGFR inhibitors have proven to work for TNBC [76-78].

HER2

The *ERBB2* gene is localised on chromosome 17q21, and its product gives an 185 kDa HER2 protein. As previously mentioned, HER2 is often overexpressed in breast cancer and is therefore used as a prognostic and predictive biomarker. The HER2 protein does not possess an ectodomain for ligand binding and is thus an orphan receptor. To be activated, HER2 must dimerise with another member of the family. HER2 is the preferred binding partner for heterodimerisation due to its stability and a potent ability for signalling [79-81].

HER3

The *ERBB3* gene is found on chromosome 12q13, coding a 145 kDa protein. Overexpression of the protein is found in around 20% of the breast cancer cases and is associated with poor prognosis. HER3's primary ligands are the NRGs. However, it needs hetero-dimerisation with another RTK for further signalling, as it is the only one in the family that is a kinase-dead receptor. When phosphorylated by another member, HER3 serves as a potent activator of signalling proteins, with an extra affinity for PI3K [82, 83].

HER4

The *ERBB4* gene is located on chromosome 2q33, and it codes for an 180 kDa protein. Like HER3, its primary ligands are the NRGs. HER4 overexpression in breast cancer is present in 12% of the cases and is associated with ER positivity, low-grade tumours and favourable prognosis, likely due to its inhibitory effects on HER2 [83-86].

Protein tyrosine phosphatase family

In contrast to protein tyrosine kinases, protein tyrosine phosphatases (PTPs) regulate the protein signalling through removal of the phosphoryl groups from tyrosine residues. Through this feature, PTPs play a major role in suppressing tumour growth. Changes in the genetic code of PTPs, like deletion, mutation, translocation, or amplification can contribute to unlimited cell growth and ultimately to the development of cancer. Epigenetic modifications of PTP genes causing loss of gene expression are a key feature for oncogenic PTPs [87].

The PTP superfamily consists of 107 members divided over four classes. This division is based on the amino sequence of the catalytic domain. Class I is the biggest group with 99 members. This class is further divided into classical tyrosine-specific PTPs and serine/threonine dual specific phosphatases. A well-known member of the latter group is PTEN, which is often lost in cancer [59]. The classical tyrosine-specific PTPs consist of those located in the cytoplasm, the non-receptor PTP, and the transmembrane receptor-like PTPs (Figure 5) [88-91].

Figure 5 | An overview of the protein tyrosine phosphatase superfamily. The members are subdivided primarily into four classes and class I is further distributed in several groups based on their function. The number of members in the different groups is shown in brackets.

PTPN2

A well-known non-receptor PTP is PTPN2, and it is found to be ubiquitously expressed, though it is primarily found in haematopoietic tissues. The phosphatase recognises a variety of substrates and has been linked to several diseases. PTPN2 was first cloned from a T-cell library and is therefore also known as T-cell protein tyrosine phosphatase (TCPTP) [92]. The human *PTPN2* gene is located on chromosome 18p11. Alternative splicing produces two main isoforms, to wit, the original 48.5 kDa (dubbed TC48) and a 45 kDa isoform (TC45) [92]. The two variants have identical N-termini but differ in C-termini.

The nuclear localisation sequence (NLS) is masked in the original isoform by the C-terminus, making it impossible for the protein to translocate to the nucleus; therefore, it resides in the endoplasmic reticulum. This hydrophobic C-terminus is lost in the 45 kDa isoform; hence this protein is more mobile than its original counterpart. Due to access to the NLS, it is mainly found in the nucleus, though it can exit the nucleus on appropriate stimuli and perform in the cytoplasm and at the plasma membrane. A third, less-known, isoform is a 41 kDa isoform (TC41). All isoforms carry exon 1-7, both TC48 and TC45 code for exon 8, TC48 then codes for the whole exon 9 (a + b) and misses exon 10. TC45 skips the b-part of exon 9 but codes for exon 10. TC41 skips exon 8 and 9b, but codes for 10. Exon 9a encodes the NLS, whilst exon 9b encodes a hydrophobic sequence that inhibits the NLS (Figure 6) [93-96].

Figure 6| The gene structure of the different isoforms of human PTPN2. Alternative splicing forms either the endoplasmic 48.5 kDa isoform, the nuclear 45 kDa isoform, or in few cases the 41 kDa isoform. TC48 is prohibited from entering the nucleus by a hydrophobic sequence in the c-terminus as encoded by exon 9b.

PTPN2 was first found to be a regulator of haematopoiesis, and soon it was found to be involved in insulin signalling, inflammatory response, and leptin regulation [97-100]. Several substrates are under the influence of PTPN2. Amongst those important in tumourigenesis are RTKs as Met, EGFR, insulin receptor, and platelet-derived growth factor receptor β , and other protein tyrosine kinases as JAK and STAT [101-106]. Interestingly, many of the PTPN2 substrates are linked to the same signalling pathways.

PTPN2 is associated with diseases as Crohn's disease, rheumatoid arthritis, and type 1 diabetes. The importance of PTPN2 in cancer is currently emerging;

PTPN2 has a role in preventing genomic instability by regulating the DNA replication checkpoint response by managing STAT3 and cyclin D1 activation levels [107]. The enzyme plays an important suppressive role in acute lymphoblastic leukaemia. Knocking down PTPN2 *in vitro* leads to a decreased sensitivity to the acute leukaemia drug imatinib, showing the importance of PTPN2 [108, 109]. Recent reports show PTPN2 to be frequently lost in breast cancer; nearly half of the ER-negative tumours has lost protein expression and even more so in TNBC cases [110]. Deficiency of PTPN2 can lead to increased phosphorylation of its substrates, which in turn leads to increased tumour growth.

AIMS

1. To study the role of Met and HGF in breast cancer prognosis and radiotherapy response (**Paper I**).
2. To study the potential crosstalk between EGFR and Met in triple-negative breast cancer (**Paper II**).
3. To study PTPN2 in regard to its role in breast cancer signalling and to patient survival and therapy response (**Papers III and IV**).

COMMENTS ON MATERIALS AND METHODS

Patient cohorts (Papers I-IV)

In all four papers in this thesis, two cohorts simultaneously started by the Stockholm Breast Cancer Study Group in 1976 have been used for analyses. Both cohorts were included in a randomised clinical trial aiming to compare post-operative radiotherapy and adjuvant chemotherapy. Patients included in the trial were all considered high-risk patients with node-positive disease and/or a tumour size exceeding 30 mm. All patients received modified radical mastectomy as the primary surgery. As the importance of hormone receptors had not been established at this point, ER-positivity was not a selection criterion. The two cohorts were separated based on the menopausal status of the patients, the pre-menopausal and the post-menopausal cohort. Patients in the post-menopausal cohort were further randomised to receive either tamoxifen or no endocrine treatment.

Mid-1970s chemotherapy was still an experimental therapy; previous clinical trials had shown improved survival with adjuvant chemotherapy in patients with node-positive disease. The Stockholm clinical trials were initiated to compare the standard post-operative radiotherapy and the experimental cytotoxic chemotherapy to obtain more evidence on the benefit of the latter in standard treatment. Patients randomised to receive radiation were given 2 Gy per fraction with a total of 46 Gy, targeted to the chest wall and internal nodes. Chemotherapy was given per the Milan trial protocol consisting of 12 courses of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) [111-113]. Retrospective studies to evaluate prognostic and predictive biomarkers were approved by the ethics committee at Karolinska Institute in Stockholm, Sweden. An overview of the randomisation of both cohorts is shown in Figure 7.

Figure 7 | An overview of the treatment arms of the randomised Stockholm Breast Cancer Trial. The trial was divided into pre- and post-menopausal patients. Both cohorts were randomised to receive either radiotherapy or chemotherapy. The post-menopausal cohort was further randomised to tamoxifen treatment or no endocrine treatment.

Sample preservation

During the Stockholm breast cancer trial, samples obtained from surgery were transported on ice to the pathologist and immersed in formalin or snap frozen in liquid nitrogen immediately after histological analysis. Fresh frozen tissues were stored in liquid nitrogen and formalin-fixed, paraffin-embedded (FFPE)-tissues were stored at room temperature. DNA extracted from the tumour samples were kept at -70°C for long-term storage and -20°C for short-term storage during experimental procedures. As recommended, sections from tumour tissues in the form of tissue microarrays (TMA) were stored at 4°C with an extra thick layer of paraffin to reduce oxidation and preserve antigens [114].

Cell culture (Papers II and IV)

Cell lines are an essential aid in cancer research and account for many research papers. The first human cell line was established in 1951, derived from a cervical carcinoma. The cell line was named HeLa after Henrietta Lacks, the patient it was isolated from [115]. The first breast cancer cell line, BT-20, was established in 1958, but it was not until the 1970s that multiple breast cancer cell lines were created [116].

One of the major advantages of using cells in research is that they are a virtually infinite source of cancer cells with the same genotype and phenotype, they are easy to handle, and the results are reproducible. Moreover, it is relatively easy to manipulate protein and gene expression in cells, and there are several functional studies available. Cell lines consist of a homogenous cell population: they exist of only one cell type, without the interference of other cell types. At the same time, this is even one of the drawbacks in cell culture. Cells can respond differently to certain manipulations when surrounded by other cell types (like stromal cells). Another disadvantage is that, when being kept in culture for too long, cell lines can shift geno- and/or phenotype. However, this can be prevented by freezing stocks of each cell line in a low passage and discarding cell lines after a certain period of time or amount of passages. A serious complication in cell culture is the contamination with microorganisms, most notoriously Mycoplasma. Mycoplasma infection can change the behaviour of the cells and their gene expression. As such, research done on Mycoplasma-infected cells should be regarded as invalid [117].

The breast cancer cell lines used in this thesis are MCF7 (**Paper IV**), MDA-MB-231 (**Paper IV**), MDA-MB-468 (**Papers II and IV**), and SKBR3 (**Paper IV**). MCF7 is a cell line isolated in 1970 from a metastatic site of a Luminal A invasive ductal carcinoma belonging to a 69-year-old Caucasian female. MDA-MB-231 was derived in 1970 from a metastasis of a 51-year-old Caucasian female with an adenocarcinoma with a triple-negative subtype. MDA-MB-468, derived from a metastatic site, is from a triple-negative adenocarcinoma isolated from a 51-year-old Black female in 1977. SKBR3 is derived from a metastasis of an HER2-like adenocarcinoma from a 43-year-old Caucasian female in 1970 [118-121].

Small interfering RNA (Papers II and IV)

The introduction of RNA interference (RNAi) has opened a new chapter in the book of cancer research. RNAi is a natural process knocking down the expression of a target gene. This process can be utilised in research to efficiently and specifically downregulate the expression of particular genes of interest. This

is often done by the use of small interfering RNA (siRNA), a class of 21-25 bp double-stranded RNA with a 3' overhang. The guide strand, or antisense strand, of the siRNA is integrated into an RNA-induced silencing complex (RISC), which then cleaves and degrades the target mRNA. The siRNA is often delivered in the cytoplasm by transfection, for which there are many reagents on the market [122, 123].

RNA silencing by siRNA is an inexpensive, simple and quick method allowing for knocking down specific genes. While the specificity of siRNA is generally high, unintended mRNA suppression can occur, known as off-target effects. Off-target effects can happen specifically when the siRNA sequence is a close homology of other than the target mRNA. Non-specific off-targets effects include disturbances in gene expression unrelated to the targeted silencing, for example cellular toxicity or immune responses. These off-target effects are increased with higher siRNA doses [124].

Gene copy number assessment

qPCR (Paper III)

One of the most used methods within bioscience is polymerase chain reaction (PCR), a method to amplify nucleic acid sequences. However, PCR is not a quantitative method. Quantitative real-time PCR (qPCR) monitors and quantifies the PCR-product in real-time. The reaction mix is similar to that of a normal PCR with the addition of a probe labelled with a reporter dye and a quencher. The quencher suppresses the fluorescence signal from the reporter. During the elongation phase of the PCR reaction, the probe gets degraded, resulting in the separation of the reporter and quencher, stopping the quencher from suppressing the fluorescence signal. This fluorescence signal is constantly measured throughout the whole PCR reaction. Increased PCR product is proportional to increased fluorescence signal. Once the fluorescence signal is detectable, after a certain number of cycles, the cycle threshold (or Ct value) is reached, which correlates with the amount of template [125].

Gene copy numbers can be measured by the standard curve method or the $\Delta\Delta C_t$ method. In the case of **paper III**, the $\Delta\Delta C_t$ method was used. This approach calculates the gene copy number by normalising the gene of interest against a reference gene and a calibrator (either DNA from a normal tissue or a cell line known to have two copies of the gene of interest).

Droplet Digital PCR (Papers I, II, IV)

Droplet Digital PCR (ddPCR) is a form of digital PCR, which was first developed in the 1990s and is a method to measure nucleic acids without using standard curves [126]. ddPCR was commercially introduced in 2011 and was demonstrated to provide absolute quantification of DNA molecules. The method divides the fluorescent probe-based PCR sample mixture into 20,000 water-in-oil droplets, randomly distributing target and background DNA amongst the droplets. The PCR sample mixture includes primers against both the target gene and a reference gene. A PCR reaction will occur in each droplet. After thermal cycling, the fluorescence signal is measured in each droplet and defined as either positive or negative. The concentration is estimated by Poisson distribution, and absolute gene copy numbers are then measured by the ddPCR software and are the ratio of the target molecule concentration to the reference molecule concentration, times the number of copies of the reference gene (two). The strength of ddPCR lies in the large number of replicates, which is made possible by sample partitioning in droplets and provides high precision and sensitivity to identify copy number variations. Moreover, opposed to qPCR, DNA copy numbers can be quantified absolutely rather than relatively [127-129].

Western blot (Papers II and III)

Western blot is a qualitative method to detect protein expression and relies on antigen-antibody reactions [130]. After cells are lysed in a specific lysis buffer, the samples are denatured to allow antibodies to access all epitopes. Boiling the samples with loading buffer containing sodium dodecyl sulfate (SDS) unfolds the protein. Apart from denaturing the protein, SDS also negatively charges the proteins. The samples are loaded onto a polyacrylamide gel, separated by their

molecular weight via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a membrane for antibody binding. Non-specific binding sites on the membrane are blocked with a blocking buffer containing non-fat milk or bovine serum albumin (BSA) prior to incubation with a primary antibody. After thorough washing, a secondary antibody conjugated to horseradish peroxidase (HRP) is added to the membrane. These secondary antibodies target antigens of a specific species and are matched to the species the primary antibody was developed in. HRP catalyses the oxidation of luminol in enhanced chemiluminescence (ECL) reagents, resulting in a light emission that can be captured on a charged coupled device (CCD) camera in the form of bands [131, 132].

A disadvantage of Western blot is that the result is the mean of all cells in a lysate and not in single cells. With Western blot, the protein cannot be localised to a cell compartment, and the method is non-quantitative. Moreover, a Western blot is time-consuming and due to the amounts of steps prone to failure. However, with Western blot low amounts of protein of interest can be detected in a sample if the method is properly optimised and executed. The method has a high specificity because of the separation by size on the gel and the specificity of the antigen-antibody interaction, provided that a specific antibody has been used.

Immunohistochemistry (Papers I and IV)

Immunohistochemistry (IHC) is a method used for detecting proteins in tissues. Like Western blot, it is based on the antigen-antibody reaction. IHC is commonly used in clinical practice to detect breast cancer biomarkers in tumour tissue. In research, IHC is used to determine the frequency of proteins of interest and their localisation in the cell and/or tissue.

Staining for a specific protein is achieved by using antibodies recognising the antigen. If the procedure is carried out correctly, the antibody will only bind to the antigen of interest. For an antibody to recognise the epitope of a specified antigen, antigen-retrieval needs to be performed. Sample fixation can cause

cross-links that mask epitopes, which can be reversed by antigen retrieval. Blocking of all epitopes on a tissue slide with a serum or protein solution prevents non-specific binding of the secondary antibody and thereby reducing false positive results and high background. After these steps, the primary antibody is allowed to attach to its target antigens. After incubation and thorough washing of the tissue the, in this case, HRP-conjugated secondary antibody is added, which binds to the primary antibody. HRP catalyses the oxidation of 3,3'-Diaminobenzidine Hydrochloride (DAB), which gives the tissue where the protein of interest is located a brown colour. Nuclei are then visualised with haematoxylin staining and the protein expression can be analysed. At least two independent investigators that have to be blinded to the clinical data grade the staining.

Apart from distinguishing expression levels and localisation of the protein of interest, other advantages of IHC are the low cost and the simplicity of the technique. A major limitation of IHC is the subjective and non-automated evaluation of the staining. Discrepancies in research articles can occur due to the lack of defined grading guidelines.

MTS proliferation assay (Paper II)

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) tetrazolium assay is a colourimetric technique for quantifying cells in proliferation. Viable cells reduce the MTS tetrazolium compound to a coloured formazan product. This can only be produced by metabolically active cells through NAD(P)H-dependent dehydrogenase enzymes. The formed product can be quantified by measuring the absorbance at 490 nm [133].

The advantage of the MTS assay is the straightforwardness of the method, the safety and the high-throughput. Moreover, cells can be measured without the removal of the culture medium. A limitation of the method is that it is less sensitive than fluorescent and luminescent methods.

Transwell assay (Paper II)

The transwell assay, or the Boyden chamber assay, is a cell migration assay to study how cells respond to a chemical signal. A transwell consists of two compartments separated by a microporous membrane with small pores, 8 μm in the case of this thesis. The cells are seeded in the upper compartment, the lower compartment is filled with medium and/or chemo-attractants, the cells are then allowed to migrate through the membrane after which those cells passed through the membrane are fixed, stained, and quantified by counting them under a microscope.

While there are multiple techniques to study cell migration, the transwell assay is considered the preferred method over for example scratch assays. The assay takes relatively little time and separates proliferation from migration. Transwell assays are easy to analyse, though the counting of cells can be tedious. It should be kept in mind that cell invasion cannot be studied with this method. However, if the membrane is coated with an extracellular matrix invasion can be analysed.

Statistical analyses (Papers I-IV)

Distant recurrence-free survival (DRFS) is the period of time between the diagnosis of the primary tumour and the distant recurrence. The same is true for loco-regional recurrence-free survival, though in relation to local recurrence. Breast cancer-specific survival relates to the number of patients in the study who have not died from breast cancer. People who have died from causes other than breast cancer are not included in this measurement. These endpoints were used in this thesis to estimate patient survival in different groups with Kaplan-Meier. The Kaplan-Meier estimator is a non-parametric test and in combination with the log-rank Mantel-Cox to compare curves for statistical significance gives information on patient survival time [134].

The Hazard ratio (HR) indicates how often a specified event happens over time in one group as compared with another group. This is a particular helpful test within cancer research to measure, for example, the benefit of a new treatment

versus an old treatment or a placebo. An HR of 1 reflects there are no differences between the groups, greater or less than means a difference in survival between the groups. HRs are often reported with a 95% confidence interval (CI), that is to say, the precision of the test is 95% sure. The Cox proportional hazards regression can be used as a regression model. A regression model analyses the effect of multiple variables on the event time and smooths out the data assuming the hazard ratio is the same throughout the study [135, 136].

The Pearson's Chi-squared test for independence is used to evaluate if differences between variables are by chance or not.

The one-way analysis of variance (ANOVA) compares groups to determine if there are statistical differences between them. The two-way ANOVA analyses the effect of independent variables and the interaction between them. However, the ANOVA test will only state if there is an interaction between some variables, but not between which ones. To find between which variables the interaction lies, a second statistical method is needed. A post-hoc Bonferroni compares all groups and will give a statistical difference accordingly.

RESULTS AND DISCUSSION

Genes and proteins of interest in breast cancer cell lines

The copy numbers of the genes researched in this thesis were established with ddPCR in DNA from seven commonly used breast cancer cell lines and one non-tumourigenic breast cell line (MCF10A). The genes of interests were *MET*, its ligand *HGF*, HER family members *EGFR* and *ERBB2*, and the tyrosine phosphatase *PTPN2*. HER2-positive cell lines BT-474 and SKBR3 showed increased copy numbers of all genes, most notably for *ERBB2*, apart from *PTPN2* in SKBR3. Luminal A cell line MCF7, non-tumourigenic cell line MCF10A, and TNBC cell line MDA-MB-231 showed no aberrations in gene copy numbers, safe from MCF7 that was found to have one copy of *ERBB2*. TNBC cell line MDA-MB-468 showed extremely high *EGFR* copy numbers, and elevated *MET* and *PTPN2* copy numbers. Luminal A cell line T-47D showed one extra gene copy for *HGF*, *EGFR*, and *ERBB2*. Luminal B1 cell line ZR-75-1 had one extra copy for *PTPN2* (Table 2).

Table 2| Gene copy numbers of genes of interest in breast cancer cell lines.

	<i>MET</i>	<i>HGF</i>	<i>EGFR</i>	<i>ERBB2</i>	<i>PTPN2</i>
BT-474	2.94	2.92	4	38.7	4.29
MCF7	2.01	2.36	1.54	1.06	1.57
MCF10A	2.19	2.22	1.89	2.06	2.11
MDA-MB-231	2.1	2.09	1.98	2.15	1.99
MDA-MB-468	3.2	1.86	27.2	2.07	5.72
SKBR3	7.3	4.42	4.73	27.3	1.96
T-47D	1.32	2.5	2.65	2.83	1.32
ZR-75-1	2.06	2.06	1.95	2.08	2.53

Protein expression levels of Met, EGFR, and PTPN2 in the aforementioned cell lines were analysed by Western blot. Discordant with *MET* copy numbers, Met expression was shown to be high in both TNBC MDA cell lines and merely a slight expression was seen in SKBR3. Were increased *EGFR* gene copy numbers shown in BT-474, MDA-MB-468, SKBR3, and T-47D, protein expression was not detected in BT-474. Consistent with the high number of gene copy numbers, MDA-MB-468 showed excessive expression of EGFR. PTPN2 showed bands at different kDAs, for the three main isoforms (TC48, TC45, and TC41). Protein expression of one or more isoforms was found in all cell lines at varying expression levels (Figure 8). Copy numbers and protein expression do not necessarily have to be in line with each other; protein expression is subjected to control at transcriptional, translational, and post-translational level [137].

Figure 8| Met, EGFR, and PTPN2 protein expression in breast cancer cell lines.

Genes of interest in breast cancer tumours

Gene copy numbers of *MET*, *HGF*, *PTPN2*, and *EGFR* were analysed with ddPCR, or qPCR in the case of **Paper III** (Table 3). Amplification was defined as more than three copies while gain was defined at more than two copies. Amplification rates were low in both cohorts of all investigated genes and did not exceed 8%. Copy gain rate was higher, with the highest found for *MET*. In both cohorts, *MET* and *HGF* were significantly correlated with each other ($p<0.01$). *MET* amplification has not been reported to be common in breast cancer, in the present study only 8% was found (**Paper I**) [138, 139]. However, copy gain has been reported for *MET* in 27% HER2-positive metastatic breast

cancer cases, as in the pre- and post-menopausal patients here, unrelated to subtype [140]. *HGF* copy gain was previously reported in 65% of the HER2-positive cases, considerably higher than the 21-27% found in all patients in the two cohorts, here [140].

Table 3 | Copy number variations of the genes of interest in the pre- and post-menopausal breast cancer patient cohorts.

Gene	Cohort	Amplification (>3 copies)	Gain (>2 copies)	Deletion	Paper
<i>MET</i>	Pre-menopausal	8% (17/205)	33% (66/205)	n/a	I
	Post-menopausal	8% (15/184)	27% (50/184)	n/a	I
<i>HGF</i>	Pre-menopausal	6% (11/205)	21% (41/205)	n/a	I
	Post-menopausal	7% (12/184)	27% (50/184)	n/a	I
<i>EGFR</i>	Post-menopausal	5% (9/184)	18% (33/184)	n/a	II
<i>PTPN2</i>	Pre-menopausal	n/a	29% (61/214)	15% (33/214)	IV
	Post-menopausal	n/a	13% (28/215)	16% (34/215)	III

EGFR amplification has been reported in other breast cancer studies at an equally low rate (0.8-14%) [74, 141, 142]. *MET* and *HGF* gene amplification strongly correlated with *EGFR* amplification. Interestingly, these genes are all three located on chromosome 7. Another gene located on this chromosome is *EPHB4* (located on chromosome 7q22), the gene coding for the erythropoietin-producing hepatoma 4 (EPHB4) protein. We have previously analysed this gene in the post-menopausal cohort with ddPCR and found 3% (5/184) amplification. *EPHB4* and *EGFR* amplification correlated strongly with each other. Chromosome 7q amplification has previously been reported in breast cancer [143]. The results in the post-menopausal cohort give more insight as to which genes are amplified on 7q (*MET*, *HGF*, and *EPHB4*), and shows that not only genes on the q-arm might be affected but even those on the p-arm (*EGFR*). These findings indicate that genes on chromosome 7 can be co-amplified. To test this further, a combined variable was created representing chromosome 7

amplification. It was shown that chromosome 7 amplification was present in 13% of the tumours and was associated with a shorter distant recurrence-free survival ($p=0.007$, unpublished results). All these RTKs are involved in the activation of signalling pathways like the PI3K/Akt and RAS/MAPK pathway. These findings imply that gene amplification on chromosome 7 leads to higher expression levels of receptor tyrosine kinases driving tumour proliferation.

As *PTPN2* is a phosphatase and a proposed tumour suppressor, gene loss might influence the tumour growth. Therefore, gene deletion was reported for *PTPN2*. The deletion rate for this gene was essentially the same in both cohorts (~15%). Moreover, *PTPN2* gene copy number has been analysed in our lab by qPCR in a low-risk post-menopausal breast cancer cohort, and an equal rate of 18% (26/146) was found [144]. In addition, relative *PTPN2* mRNA expression levels were determined in 86 tumours with qPCR in **Paper III**. These levels were found to correlate with *PTPN2* gene copy number in the same paper ($p=0.038$). The correlation between copy loss and low mRNA levels has previously been demonstrated in breast cancer in a study identifying loss of the 18p chromosomal region [145].

Proteins of interest in breast cancer tumours

Protein expression levels of Met, phosphorylated Met Y1349 (pMet), HGF, and *PTPN2* were analysed with IHC in **Papers I** and **IV** in the pre-menopausal cohort (Table 4). Met was localised to the plasma membrane and the cytoplasm. High membranous total Met expression was found in 20% of the cases, cytoplasmic in 33%. High pMet expression was recorded in 25% and 53% in the membrane and cytoplasm, respectively. HGF expression was found in stromal cells and the tumour cytoplasm in respectively 51% and 49%. Met is a protein that has been studied in multiples breast cancer cohorts. While in the early years after its discovery Met was thought to be downregulated or absent in breast cancer, later studies showed a clear presence of the receptor in malignant breast tissue [146-148]. High Met protein expression levels fluctuate in studies, varying from 22-72% [64, 149-156]. HGF is not largely studied in breast cancer, protein

expression in the literature range from 46-66%, when not considering localisation [150, 155-157].

Low cytoplasmic PTPN2 expression, considered 'protein loss', was found in 50% of the cases, in concordance with unpublished findings in our lab where 53% (354/664) low cytoplasmic staining was found [144]. PTPN2 loss has been previously reported in 67% of the TNBC cases [110].

Table 4| Proteins expression levels of proteins of interest in the pre-menopausal breast cancer patient cohorts.

Protein	Localisation	High expression	Definition High expression	Paper
Met	Membrane	20% (45/223)	Moderate/Strong	I
	Cytoplasm	33% (73/223)	Moderate/Strong	I
pMet Y1349	Membrane	25% (55/218)	Positive	I
	Cytoplasm	53% (116/218)	Positive	I
HGF	Stroma	51% (110/214)	Positive	I
	Cytoplasm	49% (105/215)	Strong	I
PTPN2	Cytoplasm	50% (110/219)*	Negative/Weak*	IV

*Low expression

Genes and proteins in relation to clinicopathological characteristics

Met and EGFR are reported to be involved in aggressive disease [138, 153, 156, 158-160]. An aggressive disease is indicated if the tumour is ER-negative, HER2-positive or triple-negative, and highly proliferative (as measured by NHG or S-phase fraction). *MET* amplification was found to be correlated with high cell proliferation in both pre- and post-menopausal cohorts, the same was true for *HGF* and *EGFR* gain in the latter cohort. *EGFR* gain was correlated with a negative ER status. Increased *MET* amplification in both cohorts was inversely correlated with ER status and furthermore with Luminal A disease. Low membranous Met expression was more often found in the Luminal A subtype. Additionally, tumours with *HGF* amplification and gain in the post-menopausal cohort tended to be negative for ER. This indicates that Met and EGFR are related to the more aggressive subtypes. Indeed, in concordance with other

Figure 9| *MET* and *EGFR* amplification are associated with a shorter DRFS. Amplification of *MET* in the pre-menopausal cohort (A). *MET* (B) and *EGFR* (C) amplification in the post-menopausal cohort.

studies, *MET* and *EGFR* amplification was more frequently found in TNBC in the post-menopausal cohort [44, 161, 162]. *MET* gain was found to be correlated with the HER2-like subtype in the pre-menopausal cohort, in agreement with a 2013 study [163]. Moreover, a correlation was found between pMet and HER2 status. Contrarily, no correlation was found between *MET* gain and HER2-like disease in the post-menopausal cohort, which was likewise shown in a meta-analysis of multiple studies [162]. Interestingly, cytoplasmic pMet tended to be more abundant in the less-aggressive Luminal A subtype. Contradicting previous findings indicating Met to be involved in aggressive disease, suggesting the activated state and likely the location of Met is important in tumour progression.

In neither of the cohorts was PTPN2 deletion correlated with any clinicopathological parameters. High PTPN2 protein expression was correlated with ER-positive status, suggesting a tumour suppressive role of PTPN2. Cytoplasmic HGF, membranous and cytoplasmic pMet were more often present in PTPN2-positive tumours. Met has been previously proposed to be a substrate to PTPN2, which was not indicated by these

results [104]. Amongst Luminal A tumours, *PTPN2* deletion correlated with high pAkt expression in the post-menopausal cohort. Low mRNA levels in the same cohort were found to be related to high pAkt staining and *PIK3CA* wild-type status. Contrastingly, high *PTPN2* expression was found to be associated with high pAkt in the pre-menopausal cohort.

Prognostic values

Met and EGFR overexpression have been shown to be poor prognostic factors for breast cancer [62-64, 150, 151, 157, 160, 164-167]. Here, Met nor HGF protein expression were found to be correlated with prognosis. However, high *MET* gene copy number showed a trend towards shorter DRFS in both cohorts (HR = 1.76; 95% CI: 0.94-3.30, $p=0.08$ and HR = 1.9; 95% CI: 0.99-3.72, $p=0.05$ in the pre- and post-menopausal cohort, respectively). Tumours with *EGFR* amplification were associated with a higher relapse rate as compared with those without amplification (HR = 3.71; 95% CI: 1.40-9.85, $p=0.008$) (Figure 9). Furthermore, amplification of both genes showed worse DRFS in TNBC patients: in the pre-menopausal cohort for *MET* (HR = 6.5; 95% CI: 1.98-21.1, $p=0.002$) and the post-menopausal cohort for *EGFR* (HR = 3.63; 95% CI: 0.99-13.38, $p=0.052$) (Figure 10).

Figure 10| The prognostic value of *MET* and *EGFR* amplification in TNBC. *MET* amplification in the pre-menopausal cohort (A), *MET* (B) and *EGFR* (C) amplification in the post-menopausal cohort.

This is in line with previous studies showing both genes to be involved in triple-negative disease [70, 71]. Moreover, stromal, but not tumoural, HGF expression was correlated with a shorter DRFS in TNBC tumours (HR = 2.93; 95% CI: 1.06-8.09, $p=0.04$ and HR = 1.0; 95% CI: 0.4-2.4, $p=0.9$, respectively). Met and EGFR have been revealed to be co-amplified in TNBC, possibly related to shorter relapse-free survival [61, 168]. Contrary, patients having tumours with *EGFR* amplification and less than four copies for *MET* were associated with a higher relapse rate (HR = 5.7; 95% CI: 2.01-16.21, $p=0.001$), which was not clear for co-amplified tumours (HR = 1.42; 95% CI: 0.37-5.55, $p=0.611$) (Figure 11). The same was true in relation to *HGF* (1-3 *HGF* copies: HR = 5.18; 95% CI: 1.84-14.61, $p=0.002$, *HGF* amplification: HR = 3.02; 95% CI: 0.6-15.36, $p=0.181$). As *MET* and *HGF* amplification were shown to be highly correlated with each other, it is likely this is not mutually exclusive.

Figure 11 | Patients with tumours without *MET* amplification and *EGFR* amplification were shown to have a worse distant recurrence-free survival than patients without *EGFR* (A), whilst *MET* amplification did not affect survival in relation to *EGFR* amplification (B).

Patients with tumours with *PTPN2* protein loss (pre-menopausal cohort) and *PTPN2* deletion (post-menopausal cohort) were related to a shorter DRFS (Figure 12) and moreover, a shorter breast cancer survival in the post-menopausal cohort (HR = 2.23; 95% CI: 1.34-3.72, $p=0.002$). In both cohorts, this was especially true for Luminal A tumours and in the pre-menopausal

cohort even for HER2-positive tumours (HR = 0.28; 95% CI: 0.11-0.74, $p=0.010$), but not for TNBC (HR = 0.83; 95% CI: 0.35-1.97; $p=0.67$). These results suggest that the prognostic value of PTPN2 is subtype-related.

Figure 12 | *PTPN2* loss in relation to DRFS. Protein loss (A) and gene copy loss (B) in the pre- and post- menopausal cohort respectively were related to a shorter DRFS.

Predictive values

In vitro studies suggest Met and its ligand to play a role in radioresistance [169-171]. Whereas in the post-menopausal cohort no correlations were found in relation to treatment resistance, pre-menopausal patients with increased *MET* or *HGF* tumours had more benefit from radiotherapy than from chemotherapy (CMF), compared with those with no copy gain (*MET* copy gain: HR = 0.15; 0.03-0.7, $p=0.001$, no *MET* gain: HR = 0.6; 0.3-1.4, $p=0.23$, interaction $p=0.09$. *HGF* copy gain: HR = 0.16; 0.04-0.74, $p=0.019$, no *HGF* gain: HR = 0.6; 0.3-

1.39, $p=0.2$, interaction $p=0.1$). The same was true for high cytoplasmic pMet (high: HR = 0.18; 0.05-0.63, $p=0.007$, low: HR = 0.8; 0.36-1.74, $p=0.55$, interaction $p=0.05$) or HGF expression (high: HR = 0.23; 0.08-0.7, $p=0.007$, low: HR = 0.90; 0.41-1.99, $p=0.8$, interaction $p=0.04$) (Figure 13). This might indicate that the activated state of Met is of importance for the beneficial radiotherapy response.

Figure 13| *MET* gain (A and B) and high cytoplasmic pMet expression (C and D) were associated with more benefit from radiotherapy than chemotherapy and compared with no gain or low pMet expression.

In the post-menopausal cohort, 1-2 *EGFR* copies were significantly associated with a favourable response towards radiotherapy compared with chemotherapy. However, this appeared to be true for *EGFR* amplification as well, indicating that *EGFR* copy number is of no influence on radiotherapy resistance.

Patients with normally *PTPN2*-expressing tumours showed a better benefit from radiotherapy than chemotherapy in terms of loco-regional recurrence-free survival as compared with those with low *PTPN2* expression in **Paper IV** (low *PTPN2* expression: HR = 0.721; 95% CI: 0.330-1.572, $p=0.410$ and high protein expression: HR = 0.301; 95% CI: 0.101-0.896, $p=0.031$, test for interaction: $p=0.2$). ER-positive breast cancer and deletion of *PTPN2* was related to poor benefit from tamoxifen as compared with normal gene copy number in **Paper III** (HR = 1.90; 95% CI: 0.73-4.96 $p=0.18$, HR = 0.48; 95% CI: 0.28-0.84, $p=0.009$, test for interaction: $p=0.011$). This indicates *PTPN2* deletion to be a marker for tamoxifen resistance.

EGF-induced growth inhibition

As previously mentioned, EGFR has been shown to play a role in TNBC. Theoretically, EGFR inhibition would impair tumour growth. However, clinical trials with EGFR kinase inhibitors and monoclonal antibodies showed no benefit in survival compared with the control arm, with the exception of one trial [172-176]. The difference between the successful trial and the others is that the drug (panitumumab) was given in a neo-adjuvant setting to patients with primary TNBC [176]. In the other trials, the EGFR inhibitors were given to patients with metastatic disease. Lately, clues have arisen of an EGFR paradox in breast cancer. *EGFR*-amplified tumours have been shown to be correlated with aggressive disease and reduced survival rate, demonstrated *in vitro* by showing that increased proliferation of EGFR-positive cell lines was accomplished with EGF stimulation. These cells responded well to EGFR inhibition. Interestingly, once these cells were transformed to metastases *in vivo*, EGFR inhibitor resistance had emerged and cell proliferation was inhibited upon EGF treatment [177]. Thus, the EGFR paradox suggests there is a switch in EGFR performance from primary tumours to metastases. Here, we have taken to investigate how EGF treatment affects proliferation and migration in triple-negative cell line MDA-MB-468. This is an EGFR-overexpressing cell line derived from pleural effusion and not from the primary tumour. Even though EGF stimulation could induce phosphorylation on Y1068 and Y845

(Figure 14A), proliferation was stagnated (Figure 14B). This EGF-induced growth inhibition was also reported in the epidermoid carcinoma A431 cell line. Both A431 and MDA-MB-468 express exceptionally high levels of EGFR and it has been suggested that elevated levels of EGFR are necessary for EGF-induced growth inhibition [178, 179]. Although a plausible mechanism, inhibition of growth upon EGF treatment has also been demonstrated in (breast cancer) cell lines not expressing such excessively high EGFR levels [180, 181]. Another possibility is the induction of apoptosis by EGF. This phenomenon has been previously described in MDA-MB-468 cells [182, 183].

Figure 14| EGF stimulation in MDA-MB-468. Cells were serum-starved for 24 hours. **A)** Cells were treated with 5 or 30 ng/mL EGF for 15 or 60 min. Lysis was followed by immunoblotting against pMet Y1349, Met, pEGFR Y1068 and Y845, and EGFR. **B)** MTS proliferation assay of cells left either untreated or treated with 30 ng/mL EGF during indicated number of hours. The bars represent the mean of five replicates, with error bars corresponding to SEM. ****: $p < 0.0001$.

Met and EGFR transactivation

The suggested crosstalk between Met and EGFR was examined in MDA-MB-468 by studying cell proliferation and migration. First, cell proliferation was analysed by transfecting cells with siRNA against Met and/or EGFR. Due to major overexpression of EGFR, full knockdown was not achieved in MDA-MB-468. Knockdown was best visualised by pEGFR Y1068 (Figure 15). The partial knockdown of EGFR increased

Figure 15| Transfection with scrambled, EGFR, and Met siRNA and EGF treatment. Cells were transfected for 48 hours in complete medium, serum-starved for 24 hours and treated with 30 ng/mL EGF for 15 minutes.

Figure 16 | The proliferative effect of Met and EGFR knockdown on EGF stimulation. MDA-MB-468 cells were transfected with siRNA against a scrambled control, Met, EGFR, or both 48 hours prior further experimentation. Transfected cells were seeded in 96-well plates, allowed to grow in either complete medium or serum-low medium supplemented with 30 ng/mL EGF and harvested at indicated time points. The proliferation was measured by MTS proliferation assay. **A** and **B** show the proliferation rate at indicated time points without the addition of EGF. **C** and **D** show proliferation rate of cells grown in medium supplemented with 30 ng/mL EGF. Each bar represents the mean $\pm$ SEM of five replicates. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

proliferation after 72 hours (Figure 16A and B). This was even more prominent when the cells were treated with EGF (Figure 16C and D). Dual knockdown of Met and EGFR could counteract this proliferation (Figure 16C and D).

To study the effect of Met and/or EGFR knockdown on migration, transfected cells were allowed to migrate through the porous membrane of a Transwell migration assay for 24 hours. In general, migration upon EGF treatment was shown to be lower compared with non EGF-stimulated cells (Figure 17). This reduced migration rate upon EGF treatment, could be reverted by EGFR knockdown, which in turn could be counteracted by dual knockdown (Figure 17B). As previously shown in another TNBC study, Met deficiency could reduce cell migration, with or without EGF stimulation [61]. Dual knockdown of EGFR and Met did not change the level of migrating cells as compared with mono-knockdown of Met, indicating that Met knockdown inhibits cell migration

regardless of EGFR status. Simultaneously, the presence of Met seems important for migration in the absence of EGFR (Figure 17).

Figure 17 | The effect of Met and EGFR knockdown on cell migration. Transfected cells were allowed to migrate through the porous membrane of a Transwell migration assay for 24 hours. The lower Transwell compartment was either filled with complete medium (A) or serum-low medium supplemented with 30 ng/mL EGF (B). Ten random fields from two replicates were counted. Representative images from one random field per condition at 400x total magnification are shown to illustrate cell migration (C). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

The effect of PTPN2 loss is subtype-related

The breast cancer cell lines MCF7 (Luminal A), MDA-MB-231 (TNBC), MDA-MB-468 (TNBC), and SKBR3 (HER2-positive) were subjected to transfection with PTPN2 siRNA to study which proteins, commonly altered in breast cancer, are affected by PTPN2 loss. For this purpose, cells were transfected for 48 hours in complete medium after which they were serum-starved for 24 hours. Cellular signalling was stimulated with EGF and/or HGF.

Depletion of PTPN2 in Luminal A cell line MCF7 decreased the basal phosphorylation level of pMet Y1349 and increased both basal and HGF-induced pAkt S473 expression. This further implies the relation between PTPN2, Akt and the Luminal A subtype. The decreased Met phosphorylation

Figure 18| Effect of PTPN2 knockdown and EGF/HGF stimulation on expression of proteins related to PTPN2. Cells were transfected with 10 nM PTPN2 siRNA for 48 hours and serum-starved 24 hours upon 30-minute 30 ng/mL EGF and/or 50 ng/mL HGF treatment. **A)** MCF7 cell lysate, **B)** MDA-MB-468 cell lysate, **C)** SKBR3 cell lysate. The panels show representative images of Western blots; the experiments were repeated three times. GAPDH was used as loading control.

upon PTPN2 deficiency suggests that increased Akt phosphorylation in PTPN2-depleted cells was not Met-dependent (Figure 18A).

Copy number variation and protein expression were shown not to be prognostic in TNBC. To further establish that PTPN2 does not play a role in TNBC, two TNBC cell lines were transfected with siRNA against PTPN2. In disagreement with Shields *et al.*, none of the tested proteins showed alterations in the phosphorylation levels upon PTPN2 depletion in MDA-MB-468 (Figure 18C) or MDA-MB-231 (results not shown) [110].

Contrarily, Met phosphorylation levels were increased after PTPN2 knockdown in HER2-positive cell line SKBR3, without affecting pAkt levels nor that of

pErk. pSTAT3 slightly decreased upon transfection. The increase of pMet upon PTPN2 loss suggests this RTK to be a PTPN2 substrate, as proposed in a study on HeLa cells [104] (Figure 18B). The different responses to PTPN2 loss in these two cell lines imply that PTPN2 plays separate roles in Luminal A and HER2-positive disease.

CONCLUSIONS

In conclusion, *MET* and *EGFR* amplification are associated with shorter survival in TNBC. EGFR knockdown in TNBC cell line MDA-MB-468 leads to increased proliferation and migration upon EGF treatment. Dual EGFR and Met depletion reduce this increase in proliferation and migration, indicating that double inhibition of EGFR and Met in TNBC might be a future treatment option. Knockdown of Met relates to decreased proliferation and migration, suggesting Met to be a treatment target in TNBC. However, patients with tumours with high cytoplasmic pMet have more benefit from radiotherapy than chemotherapy regarding loco-regional recurrence-free survival, while this is not seen in patients with non-phosphorylated Met expression. Therefore, more research is needed on the benefit of Met inhibitors in TNBC.

PTPN2 is a tumour suppressor, and gene or protein loss leads to worse recurrence-free survival. The role of PTPN2 in breast cancer is subtype-related. While PTPN2 appears to be less relevant in TNBC, Luminal A cells respond to PTPN2 loss by downregulating pMet and upregulating pAkt. HER2-positive cells, on the other hand, will respond with a pMet increase. Moreover, *PTPN2* deletion in oestrogen receptor-positive breast cancer is associated with poor benefit from tamoxifen. Patients with normally PTPN2-expressing tumours have more benefit from radiotherapy than chemotherapy, as compared with those with low expression of PTPN2. These results suggest that more research is needed for possible future treatment options.

ACKNOWLEDGEMENTS

Olle Stål, my main supervisor, thank you for taking me in eight and a half years ago. One student project became three and merged into one big PhD-adventure. Throughout the years, you have proven to be a true Gandalf, you believed in me, supported me, pushed me, and let me go when I needed to figure things out on my own. Thank you for sharing your vast knowledge and wisdom. I have learned so incredibly much these past years, in so many ways and you have made that all possible!

Gizeh Pérez-Tenorio, my co-supervisor, you are my light of Eärendil, you are my light in dark places when all other lights go out. You show me hope where I see none. You have no idea how much your presence and always good spirit has helped me throughout the years. Thank you for all the great (pep)talks and discussions, especially those about science and books.

Liam Ward, thank you for your great friendship, for those surprise visits in the years you were back in England, for all the festival fun in the Netherlands and the Czech Republic, the gigs, countless trips, dinners, and all the *functional* evenings (days). Most of all, thank you for coming back to Sweden!

Emina Vorkapić, my private punch bag, thank you for all the sweaty boxing sessions and keeping me motivated. I had a fantastic time on our holidays; you are a perfect travel companion! Thank you for your friendship and for always being there for me. I'm not quite sure you know how amazing you are.

Erik Hilborn, you crazy Britney Spears fan, it has been great sharing an office with you, thank you for the needed hugs, all the discussions about everything and nothing, the after works, the boxing. I am glad the rock-you come out on our brilliant three-bands-one-evening rock night!

My Group, present and past: **Agneta** Janson, the workout addict, I always look forward hearing your stories, the medical student labs are so much more bearable with you. **Birgit** Olsson, ever kind and ever caring, you showed me so many practical things, and your patience with my (then) terrible Swedish helped me a lot. **Birgitta** Holmlund, your kindness and empathy are unlimited. **Elin** Karlsson, always so helpful and interested in others. You have infected me with your interest for PTPN2! **Helena** Fohlin, I cannot help but take notes every time you talk about statistics. **Linda** Bojmar, the New Yorkified Swede, your energy, ambition, and motivation are outright insane. **Josefine** Bostner, you are always interested in my results and what they mean (and you're on standby for consoling hugs when the

results were *#%&\$!). **Piiha-Lotta** Jerevall Jannok, the very first person I had contact with when I was still in the Netherlands. Thank you for all your help, who knows where I would have ended up without you! **Sander** Ellegård, it has been great to have someone to share this HER2 interest with. It is always so clearing to discuss theories with you. **Tove** Sivik, you'd always have good input and feedback to presentations and project plans. **My fantastic students:** **Lisa** Ehrlich, **Sanam** Mirwani, **Krista** Briedis, and **Jon** Gårsjö who have helped to obtain data for this thesis. Everyone, present and past, in **Xiao-Feng Sun's Group** and **Charlotta Dabrosin's** group. **Annelie** Abrahamsson, thank you for showing me how great body pump is. You are my role model!

Thank you to all the patients who made these studies possible, to all co-authors, and to all other (previous) **co-workers at KEF** for all fikas, lunches, workouts, after works.

Thank you to everyone at the University, PhD students, and others, whom I have not mentioned here but have been there one, a few, or all steps along the way.

Charlotta Dabrosin, discipline coordinator, for giving me the opportunity to do my PhD studies here at Linköping University.

Fredrik Nyqvist, Crazy Freddie, you are a great friend; I know you are there for me whenever (with a glass of wine or four). **Ivana** Tichá, my beautiful crazy Czech lady, my dear friend. Whatever life throws at you, you kick back. Your strength is amazing. I admire you. Děkuji Ti za všechno, čím pro mě jsi. **Jakob** Domert, one of my first friends in Sweden, building snowflamingoes, weird holiday celebrations, sipping wine in the park, you forcing me to watch film classics, it was all great! Jobke, you grew on me. **Natalie** Beaconsfield-Herbert, oh, the adventures we had! It all started by talking about music, and we never stopped talking. I love our talks and discussions. You bloody well know you started my British grammar/language obsession! **Susanna** Lönnqvist, Susie, my very first training buddy, I would have skipped so many workouts if it wasn't for you! Kiitos for all the great times, at work and outside (time for good-food-Wednesday soon?). **Fylleryd-gänget** and consorts, with in particular: **Anna** Dahlqvist, **Erik** Ianke, **Jelena** Gacic, **Johan** Noreng, **Jon** Bjärkefur, **Klas** Tybrandt, **Maria** von Knorring, **Ola** Tybrandt, **Olle** Westman, **Oskar** Törnqvist, and **Susanna** Lagerlöf: I can honestly say, without a doubt, if it weren't for you, I would not be here. I would have gone back to the Netherlands after a year, as planned. Thank you for all the crazy parties (it's not a party unless you've danced on the table!), the corridor dinners, introducing me to new food, film nights, conversations, teaching me Swedish, and what not!

Antonio Lentini, **Chris** Sackmann, **Emelie** Blixt, **Jasen** Sackmann, **Riccardo** Barchiessi; **Robert** Lindau, **Stefan** Ljunggren, **Valerie** Sackmann, thank you all for your friendship. Thank you for the amazing and memorable Canada trip! Ghostbusters will never be the same; I'm blue, da ba dee!

Friends and family in the Netherlands, in particular:

Vrienden en familie in Nederland, met in het bijzonder:

Renée Drent, mijn allerliefste vriendinnetje, al zoveel jaren ben je er altijd voor me, al wonen we nog zover uit elkaar. Jij bent mijn Noorden! **Arnoud** Mollema, wat hebben we samen veel avonturen beleefd! Bedankt voor alle reisjes, bezoeken, festivals, concerten en wat niet! **Anita** Oppedijk-Hoeksema, bedankt voor je vriendschap, voor al je interesse, onze lunches en voor dat ik altijd Ruurdje mag 'lenen' als ik zin heb om te rijden! **Bertha** Cazemier, dankzij jou (en Carlo) heb ik het plezier in het rijden weer teruggekregen. Ik kijk altijd uit naar onze 'inhaal-avonden' als ik weer even in Nederland ben!

Papa, Mama, en Fiënna: Zonder jullie zou ik hier niet staan. Zonder discussie en vol vertrouwen dat het goed zou komen lieten jullie me in 2008 naar Zweden vertrekken, voor een jaar. Ook toen dat verblijf werd verlengd, en daarna nog een keer, waren jullie niets dan steun en liefde. Ik houd oneindig veel van jullie!

"Was er iets waar ik om wenste, voordat de put droog kwam te staan, dan was het 'lang zullen ze leven', familie waar ik veel van hou, en voor wie ik sterven zou."

"Maybe it's sad that these are now memories.
and maybe it's not sad."

— Charlie in Stephen Chbosky's
— The Perks of Being a Wallflower

REFERENCES

1. Russo J, Hu YF, Yang X, Russo IH: **Developmental, cellular, and molecular basis of human breast cancer.** *J Natl Cancer Inst Monogr* 2000(27):17-37.
2. Russo J, Rivera R, Russo IH: **Influence of age and parity on the development of the human breast.** *Breast Cancer Res Treat* 1992, **23**(3):211-218.
3. Potten CS, Watson RJ, Williams GT, Tickle S, Roberts SA, Harris M, Howell A: **The effect of age and menstrual cycle upon proliferative activity of the normal human breast.** *Br J Cancer* 1988, **58**(2):163-170.
4. Geddes DT: **Inside the lactating breast: the latest anatomy research.** *J Midwifery Womens Health* 2007, **52**(6):556-563.
5. Russo J, Russo IH: **Development of the human breast.** *Maturitas* 2004, **49**(1):2-15.
6. Hall JM, Couse JF, Korach KS: **The multifaceted mechanisms of estradiol and estrogen receptor signaling.** *J Biol Chem* 2001, **276**(40):36869-36872.
7. Speirs V, Walker RA: **New perspectives into the biological and clinical relevance of oestrogen receptors in the human breast.** *J Pathol* 2007, **211**(5):499-506.
8. Anderson E, Clarke RB, Howell A: **Estrogen responsiveness and control of normal human breast proliferation.** *J Mammary Gland Biol Neoplasia* 1998, **3**(1):23-35.
9. Soriano JV, Pepper MS, Orci L, Montesano R: **Roles of hepatocyte growth factor/scatter factor and transforming growth factor-beta1 in mammary gland ductal morphogenesis.** *J Mammary Gland Biol Neoplasia* 1998, **3**(2):133-150.
10. Yeong J, Thike AA, Tan PH, Iqbal J: **Identifying progression predictors of breast ductal carcinoma in situ.** *J Clin Pathol* 2017, **70**(2):102-108.
11. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: **Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.** *Int J Cancer* 2015, **136**(5):E359-386.
12. Torre LA, Siegel RL, Ward EM, Jemal A: **Global Cancer Incidence and Mortality Rates and Trends--An Update.** *Cancer Epidemiol Biomarkers Prev* 2016, **25**(1):16-27.
13. Fackenthal JD, Olopade OI: **Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations.** *Nat Rev Cancer* 2007, **7**(12):937-948.
14. Smigal C, Jemal A, Ward E, Cokkinides V, Smith R, Howe HL, Thun M: **Trends in breast cancer by race and ethnicity: update 2006.** *CA Cancer J Clin* 2006, **56**(3):168-183.
15. Pettersson A, Graff RE, Ursin G, Santos Silva ID, McCormack V, Baglietto L, Vachon C, Bakker MF, Giles GG, Chia KS *et al*: **Mammographic density phenotypes and risk of breast cancer: a meta-analysis.** *J Natl Cancer Inst* 2014, **106**(5).
16. Collaborative Group on Hormonal Factors in Breast C: **Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies.** *Lancet Oncol* 2012, **13**(11):1141-1151.
17. Allen NE, Beral V, Casabonne D, Kan SW, Reeves GK, Brown A, Green J, Million Women Study C: **Moderate alcohol intake and cancer incidence in women.** *J Natl Cancer Inst* 2009, **101**(5):296-305.
18. Breimer LH, Theobald H: **RE: Moderate alcohol intake and cancer incidence in women.** *J Natl Cancer Inst* 2009, **101**(15):1093.

19. Barnard ME, Boeke CE, Tamimi RM: **Established breast cancer risk factors and risk of intrinsic tumor subtypes.** *Biochim Biophys Acta* 2015, **1856**(1):73-85.
20. Lambertini M, Santoro L, Del Mastro L, Nguyen B, Livraghi L, Ugolini D, Peccatori FA, Azim HA, Jr.: **Reproductive behaviors and risk of developing breast cancer according to tumor subtype: A systematic review and meta-analysis of epidemiological studies.** *Cancer Treat Rev* 2016, **49**:65-76.
21. Gierisch JM, Coeytaux RR, Urrutia RP, Havrilesky LJ, Moorman PG, Lowery WJ, Dinan M, McBroom AJ, Hasselblad V, Sanders GD *et al*: **Oral contraceptive use and risk of breast, cervical, colorectal, and endometrial cancers: a systematic review.** *Cancer Epidemiol Biomarkers Prev* 2013, **22**(11):1931-1943.
22. MacMahon B, Cole P, Lin TM, Lowe CR, Mirra AP, Ravnihar B, Salber EJ, Valaoras VG, Yuasa S: **Age at first birth and breast cancer risk.** *Bull World Health Organ* 1970, **43**(2):209-221.
23. Rosner B, Colditz GA, Willett WC: **Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study.** *Am J Epidemiol* 1994, **139**(8):819-835.
24. Lambe M, Hsieh CC, Chan HW, Ekblom A, Trichopoulos D, Adami HO: **Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden.** *Breast Cancer Res Treat* 1996, **38**(3):305-311.
25. Wu Y, Zhang D, Kang S: **Physical activity and risk of breast cancer: a meta-analysis of prospective studies.** *Breast Cancer Res Treat* 2013, **137**(3):869-882.
26. Luo T, Yan HM, He P, Luo Y, Yang YF, Zheng H: **Aspirin use and breast cancer risk: a meta-analysis.** *Breast Cancer Res Treat* 2012, **131**(2):581-587.
27. Zhang X, Smith-Warner SA, Collins LC, Rosner B, Willett WC, Hankinson SE: **Use of aspirin, other nonsteroidal anti-inflammatory drugs, and acetaminophen and postmenopausal breast cancer incidence.** *J Clin Oncol* 2012, **30**(28):3468-3477.
28. Sobin LH, Gospodarowicz MK, Wittekind C: **TNM classification of malignant tumours**, 7th ed. edn. Oxford: Wiley-Blackwell; 2010.
29. Barr LC, Baum M: **Time to abandon TNM staging of breast cancer?** *Lancet* 1992, **339**(8798):915-917.
30. Park YH, Lee SJ, Cho EY, Choi YL, Lee JE, Nam SJ, Yang JH, Shin JH, Ko EY, Han BK *et al*: **Clinical relevance of TNM staging system according to breast cancer subtypes.** *Ann Oncol* 2011, **22**(7):1554-1560.
31. Elston CW, Ellis IO: **Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.** *Histopathology* 1991, **19**(5):403-410.
32. Vallejos CS, Gomez HL, Cruz WR, Pinto JA, Dyer RR, Velarde R, Suazo JF, Neciosup SP, Leon M, de la Cruz MA *et al*: **Breast cancer classification according to immunohistochemistry markers: subtypes and association with clinicopathologic variables in a peruvian hospital database.** *Clin Breast Cancer* 2010, **10**(4):294-300.
33. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS *et al*: **Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer.** *J Natl Cancer Inst* 2009, **101**(10):736-750.
34. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al*: **Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.** *Proc Natl Acad Sci USA* 2001, **98**(19):10869-10874.
35. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S *et al*: **Repeated observation of breast tumor subtypes in independent gene expression data sets.** *Proc Natl Acad Sci USA* 2003, **100**(14):8418-8423.

36. Anderson WF, Chatterjee N, Ershler WB, Brawley OW: **Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database.** *Breast Cancer Res Treat* 2002, **76**(1):27-36.
37. Dunnwald LK, Rossing MA, Li CI: **Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients.** *Breast Cancer Res* 2007, **9**(1):R6.
38. Putti TC, El-Rehim DM, Rakha EA, Paish CE, Lee AH, Pinder SE, Ellis IO: **Estrogen receptor-negative breast carcinomas: a review of morphology and immunophenotypical analysis.** *Mod Pathol* 2005, **18**(1):26-35.
39. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A *et al*: **Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.** *Science* 1989, **244**(4905):707-712.
40. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET: **Breast cancer classification and prognosis based on gene expression profiles from a population-based study.** *Proc Natl Acad Sci U S A* 2003, **100**(18):10393-10398.
41. Malorni L, Shetty PB, De Angelis C, Hilsenbeck S, Rimawi MF, Elledge R, Osborne CK, De Placido S, Arpino G: **Clinical and biologic features of triple-negative breast cancers in a large cohort of patients with long-term follow-up.** *Breast Cancer Res Treat* 2012, **136**(3):795-804.
42. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: **Triple-negative breast cancer: clinical features and patterns of recurrence.** *Clin Cancer Res* 2007, **13**(15 Pt 1):4429-4434.
43. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V: **Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry.** *Cancer* 2007, **109**(9):1721-1728.
44. Nakajima H, Ishikawa Y, Furuya M, Sano T, Ohno Y, Horiguchi J, Oyama T: **Protein expression, gene amplification, and mutational analysis of EGFR in triple-negative breast cancer.** *Breast Cancer* 2014, **21**(1):66-74.
45. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L *et al*: **Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.** *Clin Cancer Res* 2004, **10**(16):5367-5374.
46. Burness ML, Grushko TA, Olopade OI: **Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker?** *Cancer J* 2010, **16**(1):23-32.
47. Prenzel N, Zwick E, Leserer M, Ullrich A: **Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification.** *Breast Cancer Res* 2000, **2**(3):184-190.
48. van Maaren MC, de Munck L, de Bock GH, Jobsen JJ, van Dalen T, Linn SC, Poortmans P, Strobbe LJ, Siesling S: **10 year survival after breast-conserving surgery plus radiotherapy compared with mastectomy in early breast cancer in the Netherlands: a population-based study.** *Lancet Oncol* 2016, **17**(8):1158-1170.
49. Kurian AW, Lichtensztajn DY, Keegan TH, Nelson DO, Clarke CA, Gomez SL: **Use of and mortality after bilateral mastectomy compared with other surgical treatments for breast cancer in California, 1998-2011.** *JAMA* 2014, **312**(9):902-914.
50. Early Breast Cancer Trialists' Collaborative G, Darby S, McGale P, Correa C, Taylor C, Arriagada R, Clarke M, Cutter D, Davies C, Ewertz M *et al*: **Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials.** *Lancet* 2011, **378**(9804):1707-1716.

51. Carrick S, Parker S, Thornton CE, Ghersi D, Simes J, Wilcken N: **Single agent versus combination chemotherapy for metastatic breast cancer.** *Cochrane Database Syst Rev* 2009(2):CD003372.
52. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, Panel m: **Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011.** *Ann Oncol* 2011, **22**(8):1736-1747.
53. Brenton JD, Carey LA, Ahmed AA, Caldas C: **Molecular classification and molecular forecasting of breast cancer: ready for clinical application?** *J Clin Oncol* 2005, **23**(29):7350-7360.
54. Iwamoto T, Bianchini G, Booser D, Qi Y, Coutant C, Shiang CY, Santarpia L, Matsuoka J, Hortobagyi GN, Symmans WF *et al*: **Gene pathways associated with prognosis and chemotherapy sensitivity in molecular subtypes of breast cancer.** *J Natl Cancer Inst* 2011, **103**(3):264-272.
55. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M *et al*: **Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen.** *J Clin Oncol* 2010, **28**(3):509-518.
56. Burstein HJ, Griggs JJ, Prestrud AA, Temin S: **American society of clinical oncology clinical practice guideline update on adjuvant endocrine therapy for women with hormone receptor-positive breast cancer.** *J Oncol Pract* 2010, **6**(5):243-246.
57. Francis PA, Regan MM, Fleming GF, Lang I, Ciruelos E, Bellet M, Bonnefoi HR, Climent MA, Da Prada GA, Burstein HJ *et al*: **Adjuvant ovarian suppression in premenopausal breast cancer.** *N Engl J Med* 2015, **372**(5):436-446.
58. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M *et al*: **Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.** *N Engl J Med* 2001, **344**(11):783-792.
59. Miller TW, Rexer BN, Garrett JT, Arteaga CL: **Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer.** *Breast Cancer Res* 2011, **13**(6):224.
60. Molina JR, Adjei AA: **The Ras/Raf/MAPK pathway.** *J Thorac Oncol* 2006, **1**(1):7-9.
61. Kim YJ, Choi JS, Seo J, Song JY, Lee SE, Kwon MJ, Kwon MJ, Kundu J, Jung K, Oh E *et al*: **MET is a potential target for use in combination therapy with EGFR inhibition in triple-negative/basal-like breast cancer.** *Int J Cancer* 2014, **134**(10):2424-2436.
62. Zagouri F, Bago-Horvath Z, Rossler F, Brandstetter A, Bartsch R, Papadimitriou CA, Dimitrakakis C, Tsigginou A, Papaspyrou I, Giannos A *et al*: **High MET expression is an adverse prognostic factor in patients with triple-negative breast cancer.** *Br J Cancer* 2013, **108**(5):1100-1105.
63. Ghoussoub RA, Dillon DA, D'Aquila T, Rimm EB, Fearon ER, Rimm DL: **Expression of c-met is a strong independent prognostic factor in breast carcinoma.** *Cancer* 1998, **82**(8):1513-1520.
64. Camp RL, Rimm EB, Rimm DL: **Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma.** *Cancer* 1999, **86**(11):2259-2265.
65. Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, Vande Woude GF: **Molecular cloning of a new transforming gene from a chemically transformed human cell line.** *Nature* 1984, **311**(5981):29-33.
66. Trusolino L, Bertotti A, Comoglio PM: **MET signalling: principles and functions in development, organ regeneration and cancer.** *Nat Rev Mol Cell Biol* 2010, **11**(12):834-848.

67. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF: **Met, metastasis, motility and more.** *Nat Rev Mol Cell Biol* 2003, **4**(12):915-925.
68. Cecchi F, Rabe DC, Bottaro DP: **Targeting the HGF/Met signalling pathway in cancer.** *Eur J Cancer* 2010, **46**(7):1260-1270.
69. Shattuck DL, Miller JK, Carraway KL, 3rd, Sweeney C: **Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells.** *Cancer Res* 2008, **68**(5):1471-1477.
70. Gusenbauer S, Vlaicu P, Ullrich A: **HGF induces novel EGFR functions involved in resistance formation to tyrosine kinase inhibitors.** *Oncogene* 2013, **32**(33):3846-3856.
71. Mueller KL, Madden JM, Zoratti GL, Kuperwasser C, List K, Boerner JL: **Fibroblast-secreted hepatocyte growth factor mediates epidermal growth factor receptor tyrosine kinase inhibitor resistance in triple-negative breast cancers through paracrine activation of Met.** *Breast Cancer Res* 2012, **14**(4):R104.
72. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J *et al*: **MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling.** *Science* 2007, **316**(5827):1039-1043.
73. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, Toschi L, Rogers A, Mok T, Sequist L *et al*: **Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC.** *Cancer Cell* 2010, **17**(1):77-88.
74. Ro J, North SM, Gallick GE, Hortobagyi GN, Gutterman JU, Blick M: **Amplified and overexpressed epidermal growth factor receptor gene in uncultured primary human breast carcinoma.** *Cancer Res* 1988, **48**(1):161-164.
75. Spyrtas F, Delarue JC, Andrieu C, Lidereau R, Champeme MH, Hacene K, Brunet M: **Epidermal growth factor receptors and prognosis in primary breast cancer.** *Breast Cancer Res Treat* 1990, **17**(2):83-89.
76. Mueller KL, Hunter LA, Ethier SP, Boerner JL: **Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells.** *Cancer Res* 2008, **68**(9):3314-3322.
77. Carey LA, Rugo HS, Marcom PK, Mayer EL, Esteva FJ, Ma CX, Liu MC, Storniolo AM, Rimawi MF, Forero-Torres A *et al*: **TBCRC 001: randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer.** *J Clin Oncol* 2012, **30**(21):2615-2623.
78. Baselga J, Albanell J, Ruiz A, Lluch A, Gascon P, Guillem V, Gonzalez S, Sauleda S, Marimon I, Tabernero JM *et al*: **Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer.** *J Clin Oncol* 2005, **23**(23):5323-5333.
79. Nielsen DL, Andersson M, Kamby C: **HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors.** *Cancer Treat Rev* 2009, **35**(2):121-136.
80. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA: **The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen.** *Nature* 1984, **312**(5994):513-516.
81. Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Symmans WF, Pusztai L, Hortobagyi GN: **Targeted therapy in breast cancer: the HER-2/neu gene and protein.** *Mol Cell Proteomics* 2004, **3**(4):379-398.
82. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA: **Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors.** *Proc Natl Acad Sci U S A* 1989, **86**(23):9193-9197.
83. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM: **Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer.** *J Pathol* 2003, **200**(3):290-297.

84. Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M: **Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family.** *Proc Natl Acad Sci U S A* 1993, **90**(5):1746-1750.
85. Ghayad SE, Vendrell JA, Ben Larbi S, Dumontet C, Bieche I, Cohen PA: **Endocrine resistance associated with activated ErbB system in breast cancer cells is reversed by inhibiting MAPK or PI3K/Akt signaling pathways.** *Int J Cancer* 2010, **126**(2):545-562.
86. Barnes NL, Khavari S, Bolland GP, Cramer A, Knox WF, Bundred NJ: **Absence of HER4 expression predicts recurrence of ductal carcinoma in situ of the breast.** *Clin Cancer Res* 2005, **11**(6):2163-2168.
87. Jacob ST, Motiwala T: **Epigenetic regulation of protein tyrosine phosphatases: potential molecular targets for cancer therapy.** *Cancer Gene Ther* 2005, **12**(8):665-672.
88. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T: **Protein tyrosine phosphatases in the human genome.** *Cell* 2004, **117**(6):699-711.
89. Tonks NK: **Protein tyrosine phosphatases: from genes, to function, to disease.** *Nat Rev Mol Cell Biol* 2006, **7**(11):833-846.
90. Julien SG, Dube N, Hardy S, Tremblay ML: **Inside the human cancer tyrosine phosphatome.** *Nat Rev Cancer* 2011, **11**(1):35-49.
91. He RJ, Yu ZH, Zhang RY, Zhang ZY: **Protein tyrosine phosphatases as potential therapeutic targets.** *Acta Pharmacol Sin* 2014, **35**(10):1227-1246.
92. Cool DE, Tonks NK, Charbonneau H, Walsh KA, Fischer EH, Krebs EG: **cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family.** *Proc Natl Acad Sci U S A* 1989, **86**(14):5257-5261.
93. Ibarra-Sanchez MJ, Simoncic PD, Nestel FR, Duplay P, Lapp WS, Tremblay ML: **The T-cell protein tyrosine phosphatase.** *Semin Immunol* 2000, **12**(4):379-386.
94. Tiganis T, Kemp BE, Tonks NK: **The protein-tyrosine phosphatase TCPTP regulates epidermal growth factor receptor-mediated and phosphatidylinositol 3-kinase-dependent signaling.** *J Biol Chem* 1999, **274**(39):27768-27775.
95. Lorenzen JA, Dadabay CY, Fischer EH: **COOH-terminal sequence motifs target the T cell protein tyrosine phosphatase to the ER and nucleus.** *J Cell Biol* 1995, **131**(3):631-643.
96. Andersen JN, Jansen PG, Echwald SM, Mortensen OH, Fukada T, Del Vecchio R, Tonks NK, Moller NP: **A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage.** *FASEB J* 2004, **18**(1):8-30.
97. You-Ten KE, Muisse ES, Itie A, Michaliszyn E, Wagner J, Jothy S, Lapp WS, Tremblay ML: **Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice.** *J Exp Med* 1997, **186**(5):683-693.
98. Galic S, Hauser C, Kahn BB, Haj FG, Neel BG, Tonks NK, Tiganis T: **Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP.** *Mol Cell Biol* 2005, **25**(2):819-829.
99. Loh K, Fukushima A, Zhang X, Galic S, Briggs D, Enriori PJ, Simonds S, Wiede F, Reichenbach A, Hauser C *et al*: **Elevated hypothalamic TCPTP in obesity contributes to cellular leptin resistance.** *Cell Metab* 2011, **14**(5):684-699.
100. Heinonen KM, Nestel FP, Newell EW, Charette G, Seemayer TA, Tremblay ML, Lapp WS: **T-cell protein tyrosine phosphatase deletion results in progressive systemic inflammatory disease.** *Blood* 2004, **103**(9):3457-3464.
101. Tiganis T, Bennett AM, Ravichandran KS, Tonks NK: **Epidermal growth factor receptor and the adaptor protein p52Shc are specific substrates of T-cell protein tyrosine phosphatase.** *Mol Cell Biol* 1998, **18**(3):1622-1634.

102. Galic S, Klingler-Hoffmann M, Fodero-Tavoletti MT, Puryer MA, Meng TC, Tonks NK, Tiganis T: **Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP.** *Mol Cell Biol* 2003, **23**(6):2096-2108.
103. Persson C, Savenhed C, Bourdeau A, Tremblay ML, Markova B, Bohmer FD, Haj FG, Neel BG, Elson A, Heldin CH *et al*: **Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase.** *Mol Cell Biol* 2004, **24**(5):2190-2201.
104. Sangwan V, Paliouras GN, Abella JV, Dube N, Monast A, Tremblay ML, Park M: **Regulation of the Met receptor-tyrosine kinase by the protein-tyrosine phosphatase 1B and T-cell phosphatase.** *J Biol Chem* 2008, **283**(49):34374-34383.
105. ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, David M, Shuai K: **Identification of a nuclear Stat1 protein tyrosine phosphatase.** *Mol Cell Biol* 2002, **22**(16):5662-5668.
106. Simoncic PD, Lee-Loy A, Barber DL, Tremblay ML, McGlade CJ: **The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3.** *Curr Biol* 2002, **12**(6):446-453.
107. Shields BJ, Hauser C, Bukeczynska PE, Court NW, Tiganis T: **DNA replication stalling attenuates tyrosine kinase signaling to suppress S phase progression.** *Cancer Cell* 2008, **14**(2):166-179.
108. Kleppe M, Lahortiga I, El Chaar T, De Keersmaecker K, Mentens N, Graux C, Van Roosbroeck K, Ferrando AA, Langerak AW, Meijerink JP *et al*: **Deletion of the protein tyrosine phosphatase gene PTPN2 in T-cell acute lymphoblastic leukemia.** *Nat Genet* 2010, **42**(6):530-535.
109. Mattila E, Marttila H, Sahlberg N, Kohonen P, Tahtinen S, Halonen P, Perala M, Ivaska J: **Inhibition of receptor tyrosine kinase signalling by small molecule agonist of T-cell protein tyrosine phosphatase.** *BMC Cancer* 2010, **10**:7.
110. Shields BJ, Wiede F, Gurzov EN, Wee K, Hauser C, Zhu HJ, Molloy TJ, O'Toole SA, Daly RJ, Sutherland RL *et al*: **TCPTP regulates SFK and STAT3 signaling and is lost in triple-negative breast cancers.** *Mol Cell Biol* 2013, **33**(3):557-570.
111. Bonadonna G, Brusamolino E, Valagussa P, Rossi A, Brugnattelli L, Brambilla C, De Lena M, Tancini G, Bajetta E, Musumeci R *et al*: **Combination Chemotherapy as an Adjuvant Treatment in Operable Breast Cancer.** *N Engl J Med* 1976, **294**(8):405-410.
112. Rutqvist LE, Cedermark B, Glas U, Johansson H, Rotstein S, Skoog L, Somell A, Theve T, Askergrén J, Friberg S *et al*: **Radiotherapy, chemotherapy, and tamoxifen as adjuncts to surgery in early breast cancer: a summary of three randomized trials.** *Int J Radiat Oncol Biol Phys* 1989, **16**(3):629-639.
113. Rutqvist LE, Johansson H: **Long-term follow-up of the Stockholm randomized trials of postoperative radiation therapy versus adjuvant chemotherapy among 'high risk' pre- and postmenopausal breast cancer patients.** *Acta Oncol* 2006, **45**(5):517-527.
114. Economou M, Schoni L, Hammer C, Galvan JA, Mueller DE, Zlobec I: **Proper paraffin slide storage is crucial for translational research projects involving immunohistochemistry stains.** *Clin Transl Med* 2014, **3**(1):4.
115. Gey GO: **Some aspects of the constitution and behavior of normal and malignant cells maintained in continuous culture.** *Harvey Lect* 1954, **50**:154-229.
116. Lasfargues EY, Ozzello L: **Cultivation of human breast carcinomas.** *J Natl Cancer Inst* 1958, **21**(6):1131-1147.
117. Hay RJ, Macy ML, Chen TR: **Mycoplasma infection of cultured cells.** *Nature* 1989, **339**(6224):487-488.
118. Cailleau R, Young R, Olive M, Reeves WJ, Jr.: **Breast tumor cell lines from pleural effusions.** *J Natl Cancer Inst* 1974, **53**(3):661-674.

119. Soule HD, Vazquez J, Long A, Albert S, Brennan M: **A human cell line from a pleural effusion derived from a breast carcinoma.** *J Natl Cancer Inst* 1973, **51**(5):1409-1416.
120. Trempe GL: **Human breast cancer in culture.** *Recent Results Cancer Res* 1976(57):33-41.
121. Cailleau R, Olive M, Cruciger QV: **Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization.** *In Vitro* 1978, **14**(11):911-915.
122. Brummelkamp TR, Bernards R, Agami R: **Stable suppression of tumorigenicity by virus-mediated RNA interference.** *Cancer Cell* 2002, **2**(3):243-247.
123. Meister G, Tuschl T: **Mechanisms of gene silencing by double-stranded RNA.** *Nature* 2004, **431**(7006):343-349.
124. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR: **Activation of the interferon system by short-interfering RNAs.** *Nat Cell Biol* 2003, **5**(9):834-839.
125. Heid CA, Stevens J, Livak KJ, Williams PM: **Real time quantitative PCR.** *Genome Res* 1996, **6**(10):986-994.
126. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA: **Quantitation of targets for PCR by use of limiting dilution.** *BioTechniques* 1992, **13**(3):444-449.
127. Beer NR, Hindson BJ, Wheeler EK, Hall SB, Rose KA, Kennedy IM, Colston BW: **On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets.** *Anal Chem* 2007, **79**(22):8471-8475.
128. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC *et al*: **High-throughput droplet digital PCR system for absolute quantitation of DNA copy number.** *Anal Chem* 2011, **83**(22):8604-8610.
129. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M: **Absolute quantification by droplet digital PCR versus analog real-time PCR.** *Nat Methods* 2013, **10**(10):1003-1005.
130. Towbin H, Staehelin T, Gordon J: **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** *Proc Natl Acad Sci U S A* 1979, **76**(9):4350-4354.
131. Burnette WN: **"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.** *Anal Biochem* 1981, **112**(2):195-203.
132. Liu ZQ, Mahmood T, Yang PC: **Western blot: technique, theory and trouble shooting.** *N Am J Med Sci* 2014, **6**(3):160.
133. Berridge MV, Herst PM, Tan AS: **Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction.** *Biotechnol Annu Rev* 2005, **11**:127-152.
134. Bewick V, Cheek L, Ball J: **Statistics review 12: survival analysis.** *Crit Care* 2004, **8**(5):389-394.
135. Hernan MA: **The hazards of hazard ratios.** *Epidemiology* 2010, **21**(1):13-15.
136. Blagoev KB, Wilkerson J, Fojo T: **Hazard ratios in cancer clinical trials--a primer.** *Nature reviews Clinical oncology* 2012, **9**(3):178-183.
137. Vogel C, Marcotte EM: **Insights into the regulation of protein abundance from proteomic and transcriptomic analyses.** *Nat Rev Genet* 2012, **13**(4):227-232.
138. Gonzalez-Angulo AM, Chen H, Karuturi MS, Chavez-MacGregor M, Tsavachidis S, Meric-Bernstam F, Do KA, Hortobagyi GN, Thompson PA, Mills GB *et al*: **Frequency of mesenchymal-epithelial transition factor gene (MET) and the catalytic subunit of phosphoinositide-3-kinase (PIK3CA) copy number elevation and correlation with outcome in patients with early stage breast cancer.** *Cancer* 2013, **119**(1):7-15.

139. Carracedo A, Egervari K, Salido M, Rojo F, Corominas JM, Arumi M, Corzo C, Tusquets I, Espinet B, Rovira A *et al*: **FISH and immunohistochemical status of the hepatocyte growth factor receptor (c-Met) in 184 invasive breast tumors.** *Breast Cancer Res* 2009, **11**(2):402.
140. Minuti G, Cappuzzo F, Duchnowska R, Jassem J, Fabi A, O'Brien T, Mendoza AD, Landi L, Biernat W, Czartoryska-Arlukowicz B *et al*: **Increased MET and HGF gene copy numbers are associated with trastuzumab failure in HER2-positive metastatic breast cancer.** *Br J Cancer* 2012, **107**(5):793-799.
141. Bhargava R, Gerald WL, Li AR, Pan Q, Lal P, Ladanyi M, Chen B: **EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations.** *Mod Pathol* 2005, **18**(8):1027-1033.
142. Al-Kuraya K, Schraml P, Torhorst J, Tapia C, Zaharieva B, Novotny H, Spichtin H, Maurer R, Mirlacher M, Kochli O *et al*: **Prognostic relevance of gene amplifications and coamplifications in breast cancer.** *Cancer Res* 2004, **64**(23):8534-8540.
143. Pinto AE, Roque L, Rodrigues R, Andre S, Soares J: **Frequent 7q gains in flow cytometric multiploid/hypertetraploid breast carcinomas: a study of chromosome imbalances by comparative genomic hybridisation.** *J Clin Pathol* 2006, **59**(4):367-372.
144. Karlsson E, Veenstra C, Gårdsjö J, Nordenskjöld B, Stål O: **PTPN2 deficiency together with activation of nuclear Akt predict endocrine resistance in breast cancer.** In: *Unpublished results*. 2017.
145. Addou-Klouché L, Adelaide J, Finetti P, Cervera N, Ferrari A, Bekhouche I, Sircoulomb F, Sotiriou C, Viens P, Moulessehou S *et al*: **Loss, mutation and deregulation of L3MBTL4 in breast cancers.** *Mol Cancer* 2010, **9**:213.
146. Tsarfaty I, Alvord WG, Resau JH, Altstock RT, Lidereau R, Bieche I, Bertrand F, Horev J, Klabansky RL, Keydar I *et al*: **Alteration of Met protooncogene product expression and prognosis in breast carcinomas.** *Anal Quant Cytol Histol* 1999, **21**(5):397-408.
147. Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM: **Expression of the Met/HGF receptor in normal and neoplastic human tissues.** *Oncogene* 1991, **6**(11):1997-2003.
148. Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, Seiwert T, Christensen JG, Jagadeeswaran R, Krausz T *et al*: **Expression and mutational analysis of MET in human solid cancers.** *Genes Chromosomes Cancer* 2008, **47**(12):1025-1037.
149. Nakopoulou L, Gakiopoulou H, Keramopoulos A, Giannopoulou I, Athanassiadou P, Mavrommatis J, Davaris PS: **c-met tyrosine kinase receptor expression is associated with abnormal beta-catenin expression and favourable prognostic factors in invasive breast carcinoma.** *Histopathology* 2000, **36**(4):313-325.
150. Kang JY, Dolled-Filhart M, Ocal IT, Singh B, Lin CY, Dickson RB, Rimm DL, Camp RL: **Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matrilysin, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer.** *Cancer Res* 2003, **63**(5):1101-1105.
151. Chen HH, Su WC, Lin PW, Guo HR, Lee WY: **Hypoxia-inducible factor-1 α correlates with MET and metastasis in node-negative breast cancer.** *Breast Cancer Res Treat* 2007, **103**(2):167-175.
152. Raghav KP, Wang W, Liu S, Chavez-MacGregor M, Meng X, Hortobagyi GN, Mills GB, Meric-Bernstam F, Blumenschein GR, Jr., Gonzalez-Angulo AM: **cMET and phospho-cMET protein levels in breast cancers and survival outcomes.** *Clin Cancer Res* 2012, **18**(8):2269-2277.

153. Inanc M, Ozkan M, Karaca H, Berk V, Bozkurt O, Duran AO, Ozaslan E, Akgun H, Tekelioglu F, Elmali F: **Cytokeratin 5/6, c-Met expressions, and PTEN loss prognostic indicators in triple-negative breast cancer.** *Med Oncol* 2014, **31**(1):801.
154. Koh YW, Lee HJ, Ahn JH, Lee JW, Gong G: **MET expression is associated with disease-specific survival in breast cancer patients in the neoadjuvant setting.** *Pathol Res Pract* 2014, **210**(8):494-500.
155. Edakuni G, Sasatomi E, Satoh T, Tokunaga O, Miyazaki K: **Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma.** *Pathol Int* 2001, **51**(3):172-178.
156. Lindemann K, Resau J, Nahrig J, Kort E, Leesser B, Annecke K, Welk A, Schafer J, Vande Woude GF, Lengyel E *et al*: **Differential expression of c-Met, its ligand HGF/SF and HER2/neu in DCIS and adjacent normal breast tissue.** *Histopathology* 2007, **51**(1):54-62.
157. Lengyel E, Prechtel D, Resau JH, Gauger K, Welk A, Lindemann K, Salanti G, Richter T, Knudsen B, Vande Woude GF *et al*: **C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu.** *Int J Cancer* 2005, **113**(4):678-682.
158. Yang H, Zhang C, Cui S: **Expression of hepatocyte growth factor in breast cancer and its effect on prognosis and sensitivity to chemotherapy.** *Mol Med Rep* 2015, **11**(2):1037-1042.
159. Toi M, Osaki A, Yamada H, Toge T: **Epidermal growth factor receptor expression as a prognostic indicator in breast cancer.** *Eur J Cancer* 1991, **27**(8):977-980.
160. Sainsbury JR, Farndon JR, Needham GK, Malcolm AJ, Harris AL: **Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer.** *Lancet* 1987, **1**(8547):1398-1402.
161. Sobande F, Dusek L, Matejkova A, Rozkos T, Laco J, Ryska A: **EGFR in triple negative breast carcinoma: significance of protein expression and high gene copy number.** *Cesk Patol* 2015, **51**(2):80-86.
162. Yan S, Jiao X, Zou H, Li K: **Prognostic significance of c-Met in breast cancer: a meta-analysis of 6010 cases.** *Diagn Pathol* 2015, **10**:62.
163. Paulson AK, Linklater ES, Berghuis BD, App CA, Oostendorp LD, Paulson JE, Pettinga JE, Melnik MK, Vande Woude GF, Graveel CR: **MET and ERBB2 are coexpressed in ERBB2+ breast cancers and contribute to innate resistance.** *Mol Cancer Res* 2013.
164. Tolgay Ocal I, Dolled-Filhart M, D'Aquila TG, Camp RL, Rimm DL: **Tissue microarray-based studies of patients with lymph node negative breast carcinoma show that met expression is associated with worse outcome but is not correlated with epidermal growth factor family receptors.** *Cancer* 2003, **97**(8):1841-1848.
165. Ho-Yen CM, Green AR, Rakha EA, Brentnall AR, Ellis IO, Kermorgant S, Jones JL: **C-Met in invasive breast cancer: is there a relationship with the basal-like subtype?** *Cancer* 2014, **120**(2):163-171.
166. Lee HJ, Seo AN, Kim EJ, Jang MH, Kim YJ, Kim JH, Kim SW, Ryu HS, Park IA, Im SA *et al*: **Prognostic and predictive values of EGFR overexpression and EGFR copy number alteration in HER2-positive breast cancer.** *Br J Cancer* 2015, **112**(1):103-111.
167. Rimawi MF, Shetty PB, Weiss HL, Schiff R, Osborne CK, Chamness GC, Elledge RM: **Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes.** *Cancer* 2010, **116**(5):1234-1242.
168. Chae YK, Gagliato Dde M, Pai SG, Carneiro B, Mohindra N, Giles FJ, Ramakrishnan-Geethakumari P, Sohn J, Liu S, Chen H *et al*: **The Association between EGFR and cMET Expression and Phosphorylation and Its Prognostic Implication in Patients with Breast Cancer.** *PloS One* 2016, **11**(4):e0152585.

169. Medova M, Aebersold DM, Zimmer Y: **MET inhibition in tumor cells by PHA665752 impairs homologous recombination repair of DNA double strand breaks.** *Int J Cancer* 2012, **130**(3):728-734.
170. De Bacco F, Luraghi P, Medico E, Reato G, Girolami F, Perera T, Gabriele P, Comoglio PM, Boccaccio C: **Induction of MET by ionizing radiation and its role in radioresistance and invasive growth of cancer.** *J Natl Cancer Inst* 2011, **103**(8):645-661.
171. Qian LW, Mizumoto K, Inadome N, Nagai E, Sato N, Matsumoto K, Nakamura T, Tanaka M: **Radiation stimulates HGF receptor/c-Met expression that leads to amplifying cellular response to HGF stimulation via upregulated receptor tyrosine phosphorylation and MAP kinase activity in pancreatic cancer cells.** *Int J Cancer* 2003, **104**(5):542-549.
172. Dickler MN, Rugo HS, Eberle CA, Brogi E, Caravelli JF, Panageas KS, Boyd J, Yeh B, Lake DE, Dang CT *et al*: **A phase II trial of erlotinib in combination with bevacizumab in patients with metastatic breast cancer.** *Clin Cancer Res* 2008, **14**(23):7878-7883.
173. Dickler MN, Cobleigh MA, Miller KD, Klein PM, Winer EP: **Efficacy and safety of erlotinib in patients with locally advanced or metastatic breast cancer.** *Breast Cancer Res Treat* 2009, **115**(1):115-121.
174. von Minckwitz G, Jonat W, Fasching P, du Bois A, Kleeberg U, Luck HJ, Kettner E, Hilfrich J, Eiermann W, Torode J *et al*: **A multicentre phase II study on gefitinib in taxane- and anthracycline-pretreated metastatic breast cancer.** *Breast Cancer Res Treat* 2005, **89**(2):165-172.
175. Engebraaten O, Edvardsen H, Lokkevik E, Naume B, Kristensen V, Ottestad L, Natarajan V: **Gefitinib in Combination with Weekly Docetaxel in Patients with Metastatic Breast Cancer Caused Unexpected Toxicity: Results from a Randomized Phase II Clinical Trial.** *ISRN Oncol* 2012, **2012**:176789.
176. Nabholz JM, Abrial C, Mouret-Reynier MA, Dauplat MM, Weber B, Gligorov J, Forest AM, Tredan O, Vanlemmens L, Petit T *et al*: **Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact.** *Ann Oncol* 2014, **25**(8):1570-1577.
177. Wendt MK, Williams WK, Pascuzzi PE, Balanis NG, Schiemann BJ, Carlin CR, Schiemann WP: **The antitumorigenic function of EGFR in metastatic breast cancer is regulated by expression of Mig6.** *Neoplasia* 2015, **17**(1):124-133.
178. Kawamoto T, Mendelsohn J, Le A, Sato GH, Lazar CS, Gill GN: **Relation of epidermal growth factor receptor concentration to growth of human epidermoid carcinoma A431 cells.** *J Biol Chem* 1984, **259**(12):7761-7766.
179. Filmus J, Pollak MN, Cailleau R, Buick RN: **MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF.** *Biochem Biophys Res Commun* 1985, **128**(2):898-905.
180. Imai Y, Leung CK, Friesen HG, Shiu RP: **Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture.** *Cancer Res* 1982, **42**(11):4394-4398.
181. Choi J, Moon SY, Hong JP, Song JY, Oh KT, Lee SW: **Epidermal growth factor induces cell death in the absence of overexpressed epidermal growth factor receptor and ErbB2 in various human cancer cell lines.** *Cancer Invest* 2010, **28**(5):505-514.
182. Armstrong DK, Kaufmann SH, Ottaviano YL, Furuya Y, Buckley JA, Isaacs JT, Davidson NE: **Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells.** *Cancer Res* 1994, **54**(20):5280-5283.

183. Prasad KA, Church JG: **EGF-dependent growth inhibition in MDA-468 human breast cancer cells is characterized by late G1 arrest and altered gene expression.** *Exp Cell Res* 1991, **195**(1):20-26.

"I have hated words and I have loved them,
and I hope I have made them right."

- Liesel in Mark Zusak's -The Book Thief

Papers

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:

[http://urn.kb.se/resolve? urn:nbn:se:liu:diva-135047](http://urn.kb.se/resolve?urn:nbn:se:liu:diva-135047)