Obesity, Adipocytes and Breast Cancer – Insights from Translational Studies

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– Insights from Translational Studies

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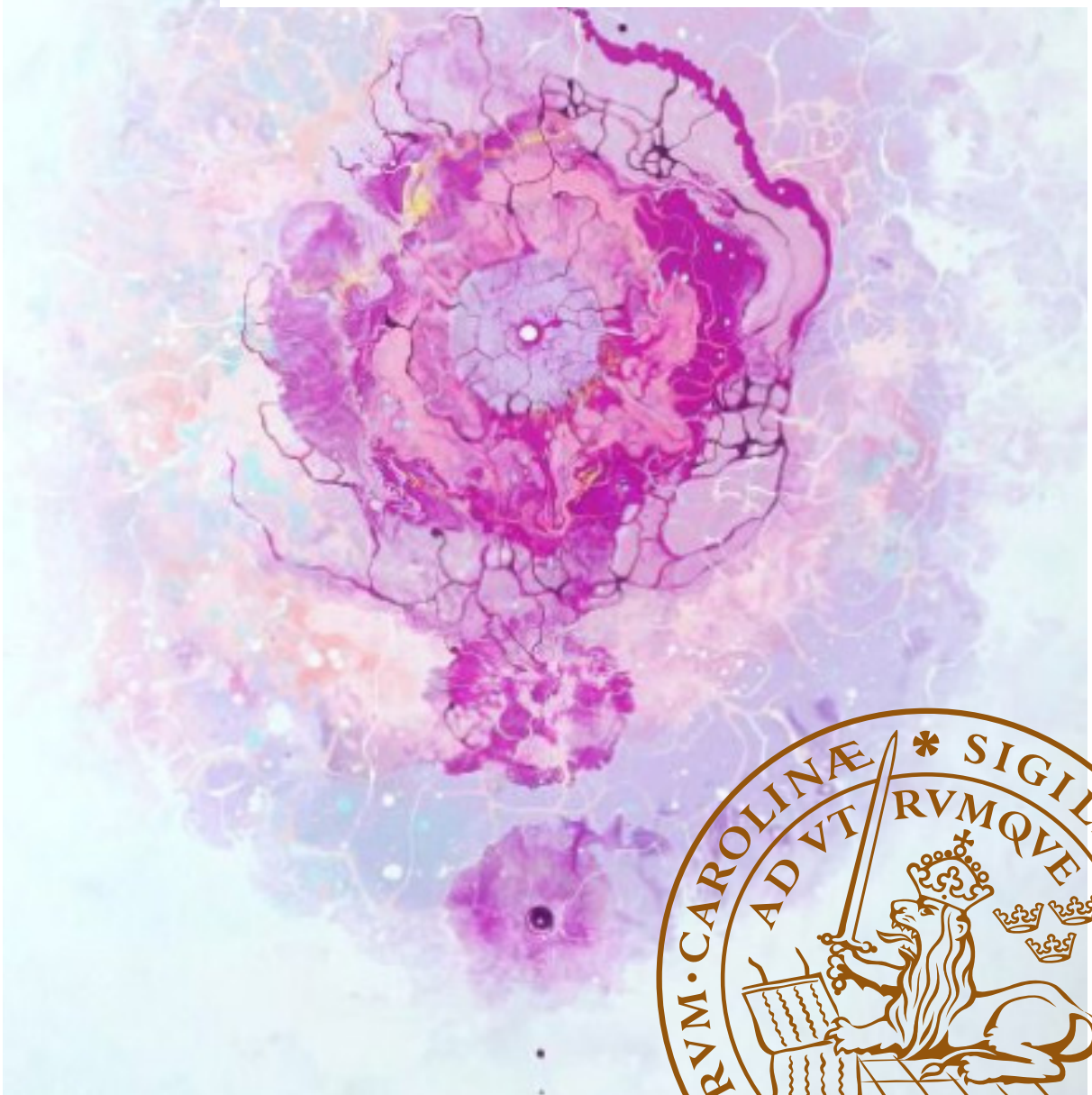

The positive thinker
sees the invisible,
feels the intangible
and achieves the impossible

— Winston Churchill

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Malin Bergqvist

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Background: Being overweight is becoming the new normal, and more than half of the adult Swedish population is overweight which poses a risk to public health. Overweight and obese women have both an increased risk and a worse prognosis for breast cancer, compared with women of normal weight. The breast cancer incidence in Sweden is increasing, and about one in nine women will be diagnosed with breast cancer during her lifetime. Adipose tissue is in close proximity to tumor cells in the breast microenvironment, and excess body fat and an altered metabolic state may lead to local and systemic molecular changes favoring tumor progression. The underlying biological mechanisms, however, are still not fully understood. The overall objective of this thesis was to bring new insights into the biological processes linking obesity to breast cancer, both through preclinical experimental studies of the interactions of adipocytes and breast cancer cells, as well as through epidemiological studies of women and their body constitution in relation to breast cancer risk and subsequent clinical outcome.

Methods: In Paper I-II, an *in vitro* model mimicking the microenvironment in normal- and obese-like breast cancer patients was established. Effects of the adipocytes secretome on breast cancer cell morphology, proliferation, and motility were investigated. The adipokine secretome was analyzed by proteome profiler array for putative biological mediators. The phosphorylation patterns of protein kinases in breast cancer cells in response to normal- and obese-like adipocyte secretome were analyzed and potential signaling pathways highlighted. The adipokine receptor CAP1 was silenced using small interfering RNA knockdown. In Paper I, the association between CAP1 mRNA expression, in a set of 1,881 breast cancer patients and prognosis were investigated. In Paper III, tumor-specific CAP1 protein expression in 718 primary breast cancers from the Malmö Diet and Cancer Study (MDCS) were analyzed with immunohistochemistry in relation to body constitution and breast cancer outcome. In Paper IV, prediagnostic NLR levels, body constitution and risk of breast cancer was explored among the 16,459 women in MDCS.

Results: In Paper I-II: Adipokines induced a more aggressive phenotype, higher proliferation, increased motility, and induced phosphorylation of proteins within key cellular processes in the breast cancer cells, effects that were more pronounced in obese-like conditions compared with normal-like. In a panel of adipokines, resistin was found upregulated in the adipocyte secretome during obese-like conditions. The receptor for resistin, CAP1 had a higher mRNA expression in estrogen receptor-negative breast cancer cells and was associated with shorter overall and relapse-free survival among breast cancer patients. Knockdown of CAP1 decreased the breast cancer cell proliferation and reduced the expression of the majority of phosphokinases. In Paper III, low tumor-specific CAP1 protein expression in patients was associated with older age at diagnosis, higher adiposity, unfavorable tumor characteristics, and poor breast cancer-specific and overall survival compared to women with tumors of high expression. In Paper IV, high prediagnostic NLR was associated with established breast cancer risk factors at study inclusion, but not with breast cancer risk overall, nor by specific tumor characteristics or by body constitution.

Conclusion/Implications: Adipocyte secretome stimulates molecular and cellular features in breast cancer cell associated with tumor progression. The adipokine receptor CAP1 displayed a divergent role for breast cancer prognosis where high CAP1 gene expression and low tumor-specific CAP1 protein level were associated with poor breast cancer prognosis. Prediagnostic NLR was not associated with overall breast cancer risk. Further studies regarding adipokines' roles in obesity-related breast cancer, the posttranslational regulation of CAP1, and studies of NLR in terms of potential short-term effects on breast cancer risk are needed.

Key words: breast cancer, risk, prognosis, obesity, adipocyte, adipokines, CAP1, NLR

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Obesity, Adipocytes and Breast Cancer

- Insights from Translational Studies

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Till alla kvinnor som kämpar

”Och vi ska slåss
Ja, vi ska slåss mot Goliat
Så tro på mig för jag vet att
du är modigast”
– Laleh, Goliat

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Adipocytes and obesity-related conditions jointly promote breast cancer cell growth and motility: associations with CAP1 for prognosis
Frontiers in Endocrinology 2018; 9:689

II. **Bergqvist M**, Elebro K, Borgquist S, Rosendahl AH
Adipocytes under obese-like conditions change cell cycle distribution and phosphorylation profiles of breast cancer cells: the adipokine receptor CAP1 matters
Submitted manuscript 2020

III. **Bergqvist M**, Elebro K, Sandsveden M, Borgquist S, Rosendahl AH
Effects of tumor-specific CAP1 expression and body constitution on clinical outcomes in patients with early breast cancer
Breast Cancer Research 2020; 22:67

IV. **Bergqvist M**, Borgquist S, Elebro K*, Rosendahl AH*
Prediagnostic neutrophil-to-lymphocyte ratio and risk of breast cancer; associations with body constitution
Manuscript *Joint senior authors

List of Abbreviations

AI	Aromatase inhibitor
Akt	Protein kinase B
AMPK α	AMP-activated protein kinase alpha
AP	Activator protein
BF%	Body fat percentage
BMI	Body mass index
BRCA	Breast cancer gene
CAAs	Cancer-associated adipocytes
cAMP	cyclic AMP
CAP1	Adenylate cyclase-associated protein 1
CARP	C-terminal actin-binding CAP and X-linked retinitis pigmentosa protein 2
CDKN1B	Cyclin-dependent kinase inhibitor 1B/p27
CLS	Crown-like structures
CREB1	cAMP-responsive element binding protein 1
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
GLUT4	Glucose transporter type 4
GOBO	Gene Expression-based outcome for Breast Cancer Online
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
IL	Interleukin
JAK	Janus-activated kinase
MDCS	Malmö Diet and Cancer Study
NF	Nuclear factor
NLR	Neutrophil-to-lymphocyte ratio
OC	Oral contraceptives
p70S6K	Ribosomal protein S6 kinase
PAM50	Prediction analysis of microarray 50
PDGF Rβ	Platelet-derived growth factor receptor β
PR	Progesterone receptor

PYK2	Protein tyrosine kinase 2
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction
SERMs	Selective ER modifier
siRNA	small interfering RNA
SRB	Sulforhodamine B
STAT3	Signal transducer and activator of transcription protein
TCGA	The Cancer Genome Atlas
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TMA	Tissue-microarray
TNF	Tumor necrosis factor
TNM	Tumor-node-metastasis
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAT	White adipose tissue
WHR	Waist-to-hip ratio
WNK-1	Lysine deficient protein kinase 1

Avhandlingen på en minut

Fetma och bröstcancer är båda sjukdomar som i en alarmerande takt drabbar allt fler kvinnor i västvärlden. Fetma ökar också risken för en kvinna att få bröstcancer, och dessutom med en sämre sjukdomsprognos.

I denna avhandling, för att bättre förstå sambandet mellan fetma och bröstcancer, genomfördes experiment där fettceller och bröstcancerceller undersöktes i olika miljöer som simulerade normala respektive fetma–lika tillstånd. I forskningsdatabaser över friska kvinnor och kvinnor med bröstcancer undersöktes kvinnornas kroppsmått och jämfördes med olika aspekter av bröstcancersjukdomen.

Vi visade att bröstcancerceller påverkas av fettceller, både vad gäller ökad tillväxt och ökad rörelseförmåga; två faktorer som ger en mer aggressiv bröstcancer. CAP1, receptor till en fettcellsutsöndrad faktor, kopplades till sämre bröstcancerprognos för de kvinnor vars tumörer antingen hade högt uttryck av CAP1 genen, eller lågt uttryck av CAP1 proteinet.

Fetma anses ge en diskret, så kallad låggradig inflammation i kroppen, och därför undersöktes också NLR, en markör för inflammation, i blodprov från friska kvinnor. NLR kunde inte kopplas till risk för bröstcancer.

Sammantaget bidrar denna avhandling till bättre kunskap och förståelse för hur fettceller och fetma påverkar bröstcancerceller och bröstcancersjukdomen.

Populärvetenskaplig sammanfattning

”*Vetenskap och vardag hänger samman och varken kan eller bör hållas isär.”*

– Rosalind Franklin (fritt översatt)

Bröstcancer är en sjukdom som var nionde kvinna i Sverige kommer att diagnostiseras med under sin livstid. Antal fall ökar och år 2018 drabbades närmare 8000 kvinnor av bröstcancer i Sverige, och över 100000 kvinnor levde med sjukdomen. Samtidigt har överlevnaden vid bröstcancer förbättrats, och mer än 80% av bröstcancerpatienterna är fortfarande vid liv 10 år efter sin diagnos.

Cancer uppstår när normala celler förändras så att de okontrollerat delar sig och bildar en knöl, en tumör. För att en bröstcancer ska bli aggressiv och sprida sig i kroppen, måste cellerna genomgå flera förändringar. Cellens utseende ändras, från en rundare till ett mer avlångt utseende. Dessutom behöver kopplingarna som håller ihop cellerna försvinna, så att cellerna blir som enskilda öar istället för att som normala celler hållas ihop som landskap av celler. Sådana förändringar gör cellerna mer rörliga och innebär att bröstcancercellerna kan förflytta sig, och så småningom bilda dottersvulster på andra ställen i kroppen. Om dottersvulsterna endast finns i lymfkörtlar går cancersjukdomen fortfarande att bota, medan dottersvulster på andra ställen i kroppen innebär s.k. spridd, och idag obotbar, sjukdom. Även om nyare behandlingsalternativ kan förlänga överlevnaden är prognosen för spridd bröstcancer är dålig, endast hälften av dessa patienter lever efter 3 år.

Bröstcancer är ett samlingsnamn för tumörer i bröstet. Brösttumörer kan ha olika kännetecken och egenskaper som på olika sätt påverkar kvinnans prognos och vilken behandling som fungerar bäst. Cirka 80% av alla brösttumörer är hormonkänsliga, vilket betyder att de svarar på det kvinnliga könshormonet östrogen. Den medicinska beteckningen för dessa tumörer är ER-positiv, för östrogenreceptor-positiv. Efter en operation av bröstet kan dessa tumörer behandlas med östrogendämpande medicin, antihormonell behandling, för att minska risken för återfall. ER-positiv bröstcancer har generellt bra prognos. Dock kan motståndskraft, resistens, mot anti-hormonell behandling utvecklas, och mycket forskning letar därför efter sätt att stoppa denna resistensutveckling, samt försöker hitta nya behandlingsmetoder. En annan variant av bröstcancer är trippel-negativa tumörer, som inte svarar på hormon. Patienter med trippel-negativ bröstcancer har

en mycket sämre prognos än de med ER-positiv bröstcancer eftersom tumören växer aggressivt, lätt sprider sig, och är svårbehandlade på grund av avsaknad på hormonreceptorer. Mycket fokus inom bröstcancerforskning är också inriktad på förebyggande arbete, prevention, av sjukdomen, bland annat genom att främja en hälsosam livsstil.

Idag är över hälften av Sveriges befolkning överviktiga och det kan på så vis ses som det ”nya normala” i vårt samhälle. Personer som lider av övervikt eller fetma har en ökad mängd kroppsfett som består av fler och större fettceller (adipocyter). Övervikt och fetma kan leda till allvarliga komplikationer som har med vår ämnesomsättning (metabolism) att göra; såsom nedsatt känslighet för insulin (insulinresistens) och diabetes typ-2 (”åldersdiabetes”). Sådana metabola förändringar kan påverka cancercellernas egenskaper och göra dem både snabbväxande och mer rörliga. Förändringarna har kopplats till försämrad bröstcancerprognos hos kvinnor med övervikt eller fetma jämfört med friska och normalviktiga kvinnor. Det finns även en ökad risk att drabbas av bröstcancer, framförallt ER-positiv, hos kvinnor efter klimakteriet som lider av övervikt och fetma.

I den experimentella delen av vårt forskningsprojekt utsattes fettceller, s.k. adipocyter, för olika normal-lika och fetma-lika tillstånd, och vi undersökte hur ämnen som utsöndras från adipocyterna, s.k. adipokiner, påverkade bröstcancercellerna. Vi såg att bröstcancerceller växte snabbare när de stimulerades av adipokinerna, och att de hade en förändrad cellstruktur och en ökad cellrörlighet. Bröstcancercellerna ändrade sin ursprungliga celltyp mot en mer aggressiv variant. Dessa förändringar ökade ytterligare i den fetma-lika miljön, vilket antyder att fetma-relaterade adipokiner förändrar tumörmiljön på ett negativt sätt. Vi identifierade ett flertal olika signalvägar som fetma kan verka genom och påverka bröstcancerceller. Signalvägar beskriver hur ämnen som adipokiner kommunicerar och ger kommandon i och mellan celler, och sådana signalvägar kan vara intressanta måltavlor t ex för nya behandlingar. I detta fall är signalvägarna intressanta då de ger underlag för hur fetma-relaterade adipokiners negativa effekter på bröstcancer kan förhindras.

En adipokin med ökad nivå i den fetma-lika miljön var resistin, ett ämne som tidigare visats vara en viktig länk mellan fetma, insulinresistens och typ 2 diabetes. Resistins receptor är adenylate cyclase-associated protein 1 (CAP1), ett ämne som bland annat är viktigt för cellernas förflyttningsmekanism. I en forskningsdatabas över 1881 bröstcancerpatienter analyserades CAP1s genuttryck i brösttumörer, och ett samband sågs mellan högt CAP1 tumörgenuttryck, aggressiva tumöregenskaper och försämrad bröstcancerprognos, jämfört med kvinnor vars tumörer hade lågt CAP1 genuttryck.

I en populationsbaserad studie, Malmö Kost Cancer studien (MKC), utforskades vidare sambandet mellan CAP1s proteinuttryck hos 718 bröstcancerpatienter och

deras prognos över lång tid. Vi såg att lågt CAP1 proteinuttryck var kopplat till övervikt och sämre tumöregenskaper vid bröstcancerdiagnosen. Dessa kvinnor hade också försämrad överlevnadsprognos, framförallt för kvinnor med ER-positiv cancer som också vara smala, jämfört med kvinnor vars tumörer hade högt CAP1 proteinuttryck. Fynden för genuttryck och proteinuttryck stod alltså i motsatsförhållande till varandra, vilket var överraskande. Dessa kontrasterande resultat kan bero på ett flertal faktorer, såsom förändringar som sker när gener översätts till protein och olika ålder hos kvinnorna som ingick i de två studierna.

Slutligen undersökte vi kopplingen mellan inflammationsmarkören neutrofil-till-lymfocyt ratio (NLR) och bröstcancerutveckling, d.v.s. bröstcancerrisk. NLR är kvotvärdet mellan två typer av vita blodkroppar som mäts vid vanligt blodprov, och har tidigare visats vara kopplat till övervikt. Vid studiestart i MKC-studien togs blodprov på 16459 kvinnor. Av alla kvinnor som var friska vid studiestart utvecklade 1116 kvinnor bröstcancer under uppföljningstiden. Vi undersökte också om övervikt hos kvinnan, i samband med NLR, påverkade kvinnans risk för bröstcancer, eftersom ett tydligt sådant samband har setts mellan övervikt och inflammation i många tidigare studier. Vi såg att högt NLR var kopplat till ett flertal kända riskfaktorer för bröstcancer. Däremot kunde vi inte bekräfta något samband mellan NLR och bröstcancerrisk. Detta kan bero på att uppföljningstiden för studien var lång och NLR endast mättes vid studiestart. Således kan NLR värdet ha ändrats ett flertal gånger under denna tid. Framtida studier där tidsaspekten mellan NLR och bröstcancerdiagnos studeras mer i detalj skulle kunna vara av värde för att bättre förstå varför högt NLR var kopplat till riskfaktorer men inte till bröstcancerrisk.

Sammanfattningsvis visade våra experiment att fettceller i en fetma-lik miljö stimulerade bröstcancercellers tillväxt och rörelseförmåga. Således kan fetma leda till en miljö i kroppen som gynnar brösttumörers tillväxt och spridning. Denna tumörgynnsamma miljö bör tas i beaktande hos de bröstcancerpatienter som lider av övervikt och fetma, och en högre beredskap bör finnas för att hantera en snabbt växande, mer aggressiv tumör hos dessa kvinnor. Vidare fann vi ett samband mellan högt genuttryck och lågt proteinuttryck av CAP1 i brösttumörer och en försämrad bröstcancerprognos. Ytterligare studier krävs för att förstå hur CAP1 gener utvecklas till protein för att kunna tolka resultaten vi såg. I den sista studien sågs inget samband mellan inflammationsmarkören NLR och en långsiktig bröstcancerrisk. Resultaten i denna avhandling kan hjälpa till med att förklara den försämrade överlevnaden som observerats hos bröstcancerpatienter med övervikt och fetma.

Introduction

"I didn't want to just know names of things. I remember really wanting to know how it all worked."

– Elizabeth Blackburn

The oldest evidence of invasive cancer among our human ancestors dates back to about 1.7 million years ago and appeared to have been a type of bone cancer (osteosarcoma) in the foot [1]. Already in 3000 BC, written Egyptian descriptions of eight cases of ulcers or tumors of the breast were found in the Edwin Smith Papyrus. Cauterization was the treatment, and breast cancer was referred to as untreatable [2]. However, the actual term carcinoma was not founded until 400 BC by the "Father of Medicine", Hippocrates [2]. In modern history, in 1971 the President of the United States of America (USA), Richard Nixon, signed the National Cancer Act and declared war on cancer, a fight that is still ongoing [3].

In 2018, more than 18 million new cancer diagnoses and about one in six deaths were attributed to cancer, accounting for 9.6 million cancer deaths worldwide. Among women worldwide, breast cancer is the most frequently occurring cancer and the leading cause of cancer deaths; however, in some highly developed countries, such as the USA, Sweden, and Canada, lung cancer has surpassed breast cancer as the leading cause of cancer-related deaths [4].

Breast cancer

More than two million women worldwide were diagnosed with breast cancer in 2018, and more than 600,000 died from breast cancer-related deaths [4]. In Sweden in 2016, approximately 108,000 women with prevalent breast cancer, 7,600 women diagnosed with breast cancer, and 1,400 deaths from breast cancer were reported. While the incidence rate has increased over the last several decades, the mortality rate has stabilized due to improvements in screening and treatment (Figure 1) [4, 5].

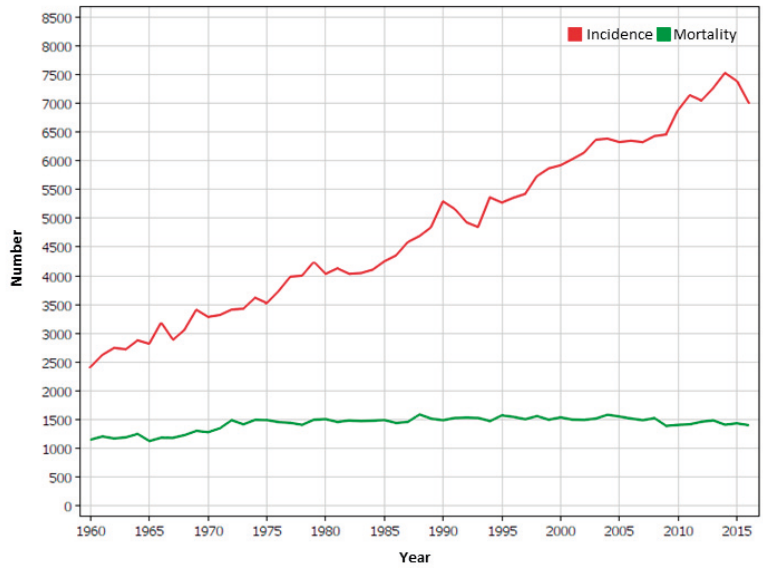

Figure 1. Breast cancer incidence and mortality among women in Sweden between the years 1960 to 2016. Printed from NORDCAN Asscociation of the Nordic Cancer Registries. Accessed November 9, 2020.

Development

Most breast cancers develop over a long period of time and originate from the epithelial cells lining the mammary milk ducts (ductal carcinomas) or milk-producing lobules (lobular carcinomas). Tumors that have not spread through the basal layer are called carcinoma *in situ*, and infiltrating tumors are known as *invasive*. Approximately 80% of all invasive breast tumors are invasive ductal carcinoma, 10%-15% invasive lobular carcinoma, and the rest mainly consist of tubular and medullary carcinomas [6].

The processes by which normal cells develop into cancer cells is called carcinogenesis. Carcinogenesis and the transformation of a normal cell into a malignant tumor cell is a complex and multistep process at the genetic, epigenetic, and cellular levels. The hallmarks of cancer consist of ten characteristics that explain this transformation (Figure 2). Hanahan and Weinberg first proposed six acquired characteristics that tumors share and which constitute carcinogenesis: (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals and evading apoptosis, (3) limitless replicative potential, (4) sustained angiogenesis, (5) tissue invasion, and (6) metastasis [7]. With the significant progress in cancer research and the enhanced conceptual understandings of tumor biology, a later update modified the initial hallmarks and introduced four additional characteristic traits: (1) genome instability and mutation, (2) tumor-promoting inflammation, (3) deregulating cellular energetics, and (4) avoiding immune destruction [8].

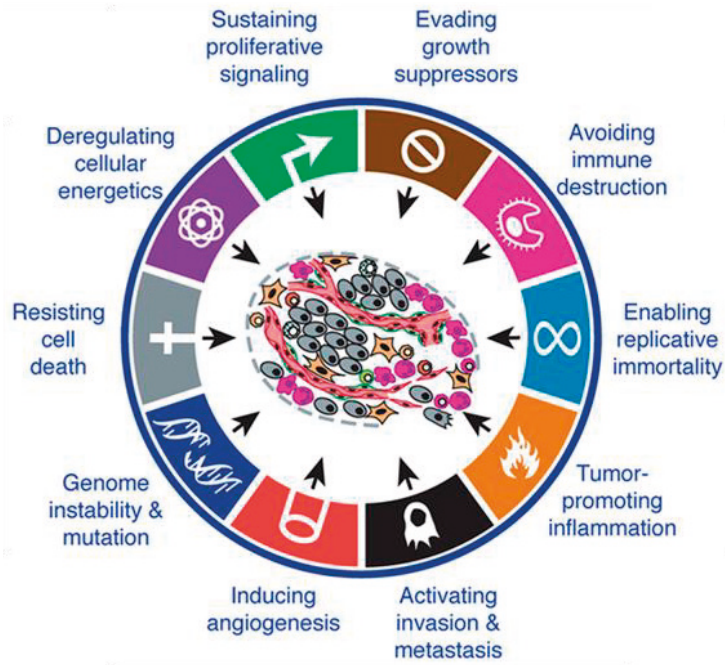

Figure 2. The hallmarks of cancer, introduced by Hanahan and Weinberg. Adapted and reprinted with permission from Hanahan & Weinberg 2011 [8].

Diagnosis

Breast cancer is most often diagnosed following a detected abnormality on a routine screening mammography or as an interval cancer discovered by a self-detected lump in the breast or lymph nodes [9]. Other symptoms that causes the woman to seek health care include nipple secretion or retraction and changes in breast shape, size, color, and/or skin texture [9, 10].

Any findings or symptoms suggestive of breast cancer should be confirmed or ruled out by triple diagnostics: (1) clinical examination, (2) pathology assessment, and (3) breast imaging. At a clinical visit, a medical history, including hereditary factors and menstruation history is obtained, and a clinical examination, including palpation of the breasts and axillary lymph nodes is performed. A core needle biopsy is taken to determine the invasiveness, histology, grade, hormone receptor, and human epidermal growth factor receptor 2 (HER2) status, and proliferative index (Ki67) of the tumor. The selected imaging diagnostics are usually mammography and ultrasound, and on occasion, magnetic resonance imaging [10]. A mammography is a two-dimensional X-ray that detects differences in breast density that could be

indicative of cancer. If cancer is confirmed or not safely ruled out following triple diagnostics, a multidisciplinary conference consisting of a panel of a surgeon, radiologist, pathologist, coordinator, contact nurse, and an oncologist, will discuss the potential diagnosis and subsequent treatment options [10].

Since 1985, mammography screening has been recommended in Sweden. In 1997, a national screening program was introduced to increase the detection of early breast cancer and to improve prognosis. It is recommended that all women in Sweden undergo mammography starting at age 40 and continue to do so up to every other year until 74 years of age [11]. The majority of breast cancers, 64%, are detected by routine mammography screening [9, 12], and a 41% reduced risk of death from breast cancer within 10 years of diagnosis for women within the screening program compared with non-participants have been found [11].

Risk factors

Breast cancer is over 100 times more common in women than men; thus, being a woman is the largest risk factor for breast cancer. The proposed underlying biological mechanism to the gender difference is the female exposure to hormones secreted by the ovaries, including estrogen and progesterone [13, 14]. Reproductive factors that cause a woman to have more menstrual cycles during her lifetime, such as early age at menarche, nulliparity, late age of first childbirth, few children, and late age at menopause increase the risk of breast cancer. Hormone stimulation from usage of hormone replacement therapy (HRT) can also increase the risk [10, 13, 14].

Aging is the second largest risk factor for breast cancer. In Sweden, the highest incidence rate for invasive breast cancer is in the age group of 60 to 69 years old with the average age at diagnosis being 66 years (Figure 3) [12]. While the cancer risk increases with age, recurrence and mortality rates are higher among younger breast cancer patients [15]. Breast cancers in young women are characterized by a higher degree of triple-negative phenotype (hormone receptor and HER2-negative), HER2-overexpression phenotype, and/or lymph node involvement [16].

Additional risk factors include height, mammographic dense breast, high socioeconomic status, alcohol consumption, physical inactivity, and overweight/obesity among postmenopausal women [10, 14]. Previous exposure to radiation also influences breast cancer risk. Conflicting evidence regarding the usage of oral contraceptives (OC), hypertension, and smoking as risk factors has also been suggested [10, 17]. Hereditary factors contribute to the risk of breast cancer, and mainly due to breast cancer gene 1 (BRCA1) and BRCA2 mutations [18].

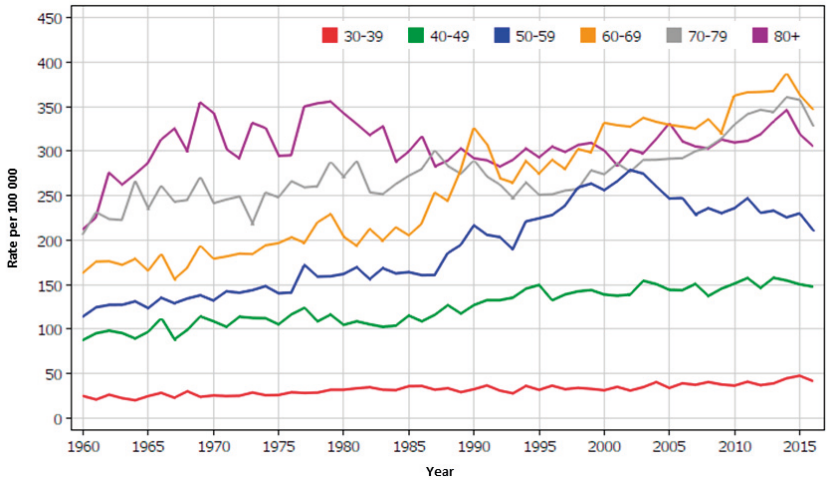

Figure 3. Breast cancer incidence in Sweden per age category between the years 1960 and 2016. Printed from NORDCAN Asscociation of the Nordic Cancer Registries. Accessed November 10, 2020.

Classifications

Breast cancer is a heterogeneous disease due to the high diversity both between and within tumors. Breast cancer can be classified according to different characteristics that have been shown to aid in predicting the outcome following diagnosis. Among these factors is the tumor-node-metastasis (TNM) staging system. T describes the extent of the primary tumor with respect to the size, location, and growth into neighboring tissue. N describes the degree of lymph node involvement, and M indicates whether the cancer has (or has not) metastasized to secondary sites. Each category is assigned a number, and a higher total number indicates a worse prognosis [19]. The most common sites for breast cancer metastasis are lymph nodes, bone, lung, brain, and liver [20]. Advanced breast cancers with confirmed distant spread are considered incurable [10].

Tumors are further categorized according to the Nottingham's histological grade. The grade displays cell abnormality and differentiation based on the presence of tubular structures within the invasive tumor, nuclear atypia, and mitotic count [21]. Low-grade tumors are well-differentiated, which means that the cells have a more normal-like phenotype with slow growth and metastasis rates and usually a good prognosis. High-grade tumors, in contrast, are poorly differentiated, display abnormal cell phenotype, and generally have a poor prognosis [21].

Estrogen receptor-α and progesterone receptor (ER and PR, respectively) are predictive of endocrine treatment sensitivity. In Sweden, tumors are considered ER- or PR-positive if more than 10% of the tumor cell nuclei are stained [10]. Approximately 80%-85% of all breast cancers are ER-positive and sensitive to

endocrine treatment. In addition, tumors cells are assessed for Ki67 expression (low, intermediate, high), which is an indicator of cell proliferation rate.

Gene expression analysis to classify tumors can be used to aid in the selection of a treatment regimen and will likely be implemented as standard clinical practice within the near future in Sweden. In current Swedish clinical practice, breast cancers are subtyped into Luminal A-like, Luminal B-like (HER2-negative), Luminal B-like (HER2-positive), HER2-positive, or triple-negative based on routine histological and immunohistochemical (IHC) evaluations [10]. The majority of breast cancer patients, 73.9%, had a Luminal (A or B) subtype in Sweden in 2019 [12]. Luminal A-like tumors are ER-positive, HER2-negative, and either Ki67 low with histological grade I to II or Ki67 intermediate with histological grade I or II and PR-positive (≥ 20%) [10]. Luminal B-like tumors are ER-positive, HER2-negative, and either Ki67 high with histological grade II to III or Ki67 intermediate with histological grade II to III and PR-positive (<20%) [10]. The least common subtypes are HER2 overexpressing tumors, either classified as luminal B (ER-positive) or non-luminal (ER- and PR-negative), and triple-negative tumors, also called basal-like tumors, which are ER-, PR-, and HER2-negative [12].

Treatment

Depending on the tumor characteristics and the decision from the multidisciplinary board, the woman may be offered a range of different treatment modalities, including surgery, radiation therapy, and various systemic therapies.

Surgery is often the primary treatment of curative intent for breast cancer and is often performed in combination with post-operative adjuvant therapy. In cases of large tumors that could hinder a successful operation or tumors of poor prognosis, neoadjuvant therapy may be used pre-operatively to downstage and shrink the tumor [10]. The breast surgery involves either a full mastectomy, in which the entire breast is removed, or a partial mastectomy, during which the tumor and margins are removed in a breast-conservative fashion [10].

Adjuvant radiation therapy is recommended for patients with higher recurrence risk in order to reduce the risk of locoregional recurrences [22]. Partial mastectomy followed by radiotherapy has increased in Sweden since the survival rates are equivalent to those of mastectomy, while the quality of life is considered higher among women with breast-conserving surgery [23, 24].

The goal of systemic therapy is to eliminate micro-metastases and thus reduce the risk of relapse. Systemic therapy includes several options: (1) chemotherapy, (2) endocrine therapy, (3) HER2-targeted therapy, and (4) bisphosphonate therapy [10]. Chemotherapy and HER2-directed therapy can be given either neoadjuvant or adjuvant therapy. Endocrine treatments are selective ER modifiers (SERMs) or aromatase inhibitors (AIs) used to treat the hormone-receptor positive cancers [25].

The most commonly prescribed SERM is tamoxifen, a competitive ER antagonist that reduces the risk of recurrence and mortality. Routinely, tamoxifen is prescribed to premenopausal patients for a period of five years, but an additional benefit has been demonstrated with ten years of use [25]. In postmenopausal patients, the first choice of adjuvant endocrine therapy are AIs; however, if these cannot be tolerated due to side effects, tamoxifen is used.

Obesity and breast cancer

Changing lifestyle patterns over the last several decades with an altered energy-dense diet together with sedentary behavior and decreased physical activity have caused a global obesity epidemic [26]. Since 1980, the number of people who are overweight and obese as generally defined by body mass index (BMI; kg/m^2) have more than doubled. Currently almost two billion adults worldwide are overweight (BMI $\geq$ 25-< 30), and 650 million of those people are considered obese (BMI $\geq$ 30) [27]. Obesity is associated with multiple comorbidities and metabolic disorders, such as metabolic syndrome, low-grade inflammation, and insulin resistance. Compared with lean people, overweight and obese individuals are further at increased risk of cardiovascular disease, arthritis, and mental health problems. It is now increasingly accepted that being overweight or obese also increases the risk of several types of cancer, including breast cancer (Figure 4) [28-32].

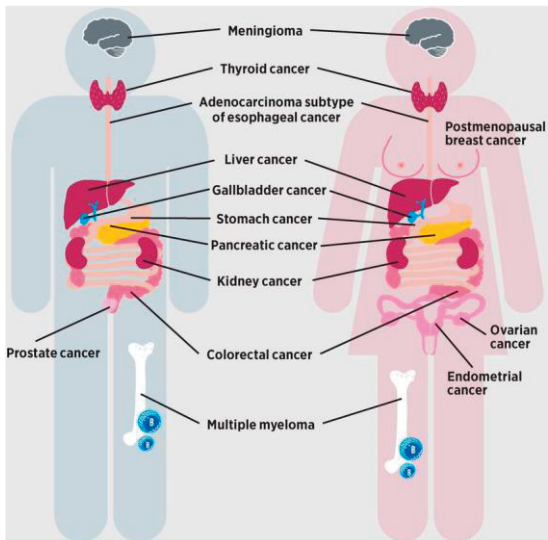

Figure 4. Obesity is associated with risk of 14 types of cancers. Reprinted with permission from Davidson *et al.* 2016 [32].

The World Health Organization (WHO) defines overweight and obesity as "abnormal fat accumulation that presents a risk to health" [27]. Currently, the most commonly used measure of overall obesity is the BMI. However, BMI has some limitations as its relationship with body fat is influenced by bone density and muscle mass; thus, this index has a low sensitivity for detecting adiposity [33]. The best reflective anthropometric measure of excess body fat or obesity (Table 1) is further complicated by different properties and effects of various fat deposits with regards to disease development and outcome. With respect to cancer risk, measurements of central adiposity, such as waist circumference and waist-to-hip ratio (WHR), have an added predictive value [34]. Another measurement of overall obesity is body fat percentage (BF%). Women with normal BMI but high BF% have been reported to have increased breast cancer risk, thus suggesting BF% to be superior compared to BMI in predicting breast cancer [35]. However, BMI is a convenient and an easily assessed anthropometric measure, while BF% require technical equipment that is not widely available.

Table 1. Established classifications for normal weight, overweight, and obesity.

	Underweight and normal	Overweight	Obese
BMI	<25	25-<30	≥30
Waist circumference	≤80	81-87	≥88
WHR	≤0.80	0.81-0.84	≥0.85
BF%	≤24	25-31	≥32

The first piece of evidence that obesity increases the risk of breast cancer in postmenopausal women was already demonstrated in 1974, and since then, multiple studies have supported that finding [36, 37]. In a previous Lancet publication, a meta-analysis compared the highest versus the lowest categories of BMI (>28 versus < 22 kg/m^2) to the relative risk of breast cancer in pre- and post-menopausal women [38]. A protective association was found in pre-menopausal women with a BMI above 28 kg/m^2. Obese post-menopausal women had a 20% increased risk of breast cancer. Results regarding pre-menopausal breast cancer and obesity have been inconclusive, which might due to lack of consideration of hormonal status in most studies or that studies have been limited by the size of study populations [39, 40]. A review demonstrated that obese pre-menopausal women had an increased risk of ER-negative, predominantly triple-negative, breast cancer, but had a decreased risk of ER-positive breast cancer compared to women with normal BMI [37]. Obesity was also shown to be associated with an increased risk of inflammatory breast cancer, a rapidly growing and aggressive form of breast cancer [41].

Obese post-menopausal women have been shown to be more likely to present with breast tumors of less favorable tumor characteristics, such as larger breast tumor size, higher histological grade, and increased lymph node involvement at diagnosis [26, 42, 43]. Furthermore, poor clinical outcome with increased recurrence, risk of

distant metastasis, and higher mortality rate are observed among both pre- and post-menopausal obese breast cancer patients compared with lean breast cancer patients [44, 45]. Obesity is, however, a modifiable risk factor, and studies have demonstrated a reduced breast cancer risk and mortality for women with a physically active lifestyle compared with a sedentary one [46].

Adipose tissue biology

Obesity is characterized by expanding and metabolically active fat tissue, the adipose tissue, that induces local and systemic changes (Figure 5) [47]. Adipose tissue is an important endocrine organ and can be divided into white, beige, and brown tissues [47]. The beige and brown adipose tissues are thermogenic; thus, they contribute to energy expenditure and can act protectively against obesity. Activation of the brown adipose tissue thermogenic program has been suggested as a novel therapeutic approach for treating obesity [48].

White adipose tissue (WAT), in contrast, is the dominant type and primary energy storage site and consists of adipocytes, blood vessels, immune cells, and extracellular matrix. Obesity is caused by a state of excess energy, which is converted to triglycerides and stored in lipid droplets in the adipocytes, which subsequently expand in size (hypertrophy) and number (hyperplasia) [47]. The expansion of mainly WAT during obesity causes an increase in production of adipokines, hormones, and inflammatory cytokines (Figure 5). The obesity-induced metabolic reprogramming of adipose tissue creates a microenvironment favoring pathophysiological breast developments.

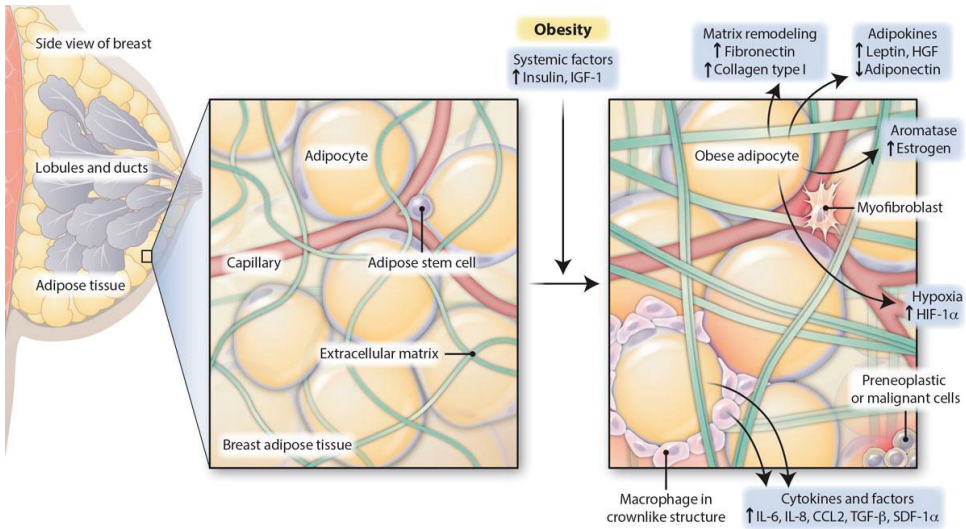

Figure 5. The human breast consists of adipose tissue and epithelial compartments with lobules and ducts and the stroma. The adipose tissue is reprogrammed during obesity, and altered levels of adipokines and cytokines are secreted. Reprinted with permission from Arendt & Kuperwasser 2015 [49].

Adipocytes

The primary component of adipose tissue is composed of adipocytes, which can be divided into three groups: (1) pre-adipocytes, (2) mature adipocytes, and (3) adipose-derived stem cells. An additional group, cancer-associated adipocytes (CAAs), has been proposed to exist in breast tumors. Both peritumoral adipocytes and adipocytes co-cultured with cancer cells *in vitro* display an altered phenotype with release of lipids and increased expression of matrix metalloproteases, such as MMP-11, and pro-inflammatory cytokines, such as interleukin (IL)-6 and -8, and tumor necrosis factor (TNF)-β [50]. The close proximity of adipocytes and their secretome to the breast tumors enables crosstalk and can induce a pro-tumorigenic microenvironment (Figure 6).

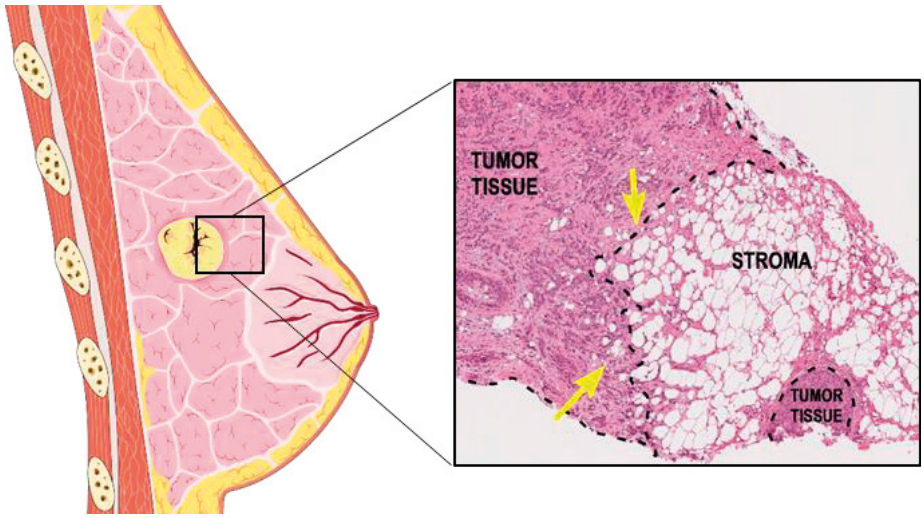

Figure 6. Adipocytes close proximity to breast cancer cells. Edited and reprinted with permission from Paré *et al.* 2020 [51] and SMART Servier medical art [52].

Endocrine function of the adipose tissue

At menopause, the ovaries cease to produce estrogen, and the main production site of estrogen is shifted to the adipose tissue [53]. Elevated estrogen production by increased aromatase activity in the excess adipose tissue is proposed to be one of the main contributing factors to the increased risk of ER-positive postmenopausal breast cancer among obese women.

Furthermore, increased secretion of adipokines and cytokines in obesity has been proposed to affect several hallmarks of cancer, such as matrix remodeling, hypoxia, immune cell recruitment, and low-grade inflammation [8, 54].

Leptin, an appetite-regulating adipokine, which can affect peripheral organs, recruits immune cells, triggers low-grade inflammation, and acts as a mitogenic and pro-angiogenic factor, is upregulated in obesity and has been suggested to be involved in obesity-mediated tumorigenesis. Leptin can affect several processes in cancer: (1) focal adhesion kinase (FAK)-mediated cell migration, (2) modulation of ERα through stimulation of the janus-activated kinase/signal transducer and activator of transcription protein/protein kinase B (JAK/STAT3/Akt) signaling pathway, (3) regulation of angiogenesis-associated vascular endothelial growth factor (VEGF) by activation of hypoxia-inducible factor (HIF)-1 and nuclear factor (NF)-κB, (4) transactivation of HER2 through activation of epidermal growth factor receptor (EGFR) and JAK2/STAT3, and (5) increased aromatase expression via activator protein (AP)-1 [55-59].

Adiponectin, an adipokine downregulated in obese individuals, has the opposite function of leptin in many regards. Adiponectin can lead to inhibition of leptin-induced proliferation, activation of apoptosis through caspase-3, a decrease macrophage infiltration and angiogenesis, and stimulation of sensitivity to insulin. Adiponectin is inversely associated with breast cancer risk, particularly in non-HRT users, and can prolong breast cancer survival [60-64].

Resistin, for resistance to insulin, is a 12 kDa hormone secreted by adipocytes, monocytes, and macrophages and has been considered a link between obesity and insulin resistance [65, 66]. Overexpression of resistin in obese adipose tissue is associated with insulin resistance [67]. Resistin can stimulate signaling pathways, such as phosphoinositide-3 kinase (PI3K)/Akt, and MAPKs associated p38, extracellular signal-regulated kinase (ERK) 1/2, STAT3, and NF-κB, all of which promote angiogenesis, metastasis, and proliferation of cancer cells [68-71]. The associations between resistin and breast cancer and resistin and obesity are conflicting. Several studies have reported a positive association, while others have found associations ranging from no association to an adverse one [72]. However, a metaanalysis found higher circulating resistin levels in breast cancer patients when compared with controls [72].

The two most recognized receptors for resistin are the toll-like receptor 4 (TLR4), which can increase macrophage infiltration, and adenylate cyclase-associated protein-1 (CAP1), which can induce NF-κB-related inflammation [73, 74]. Additional CAP1- signaling pathways stimulated by resistin include cyclic AMP (cAMP)/protein kinase A/NF-κB pathway, ERK/c-Myc, STAT3, and PI3K/Akt/protein kinase C, all of which are associated with insulin resistance, innate immunity, inflammation, proliferation, and apoptosis [75].

Adenylate cyclase-associated protein-1 (CAP1)

Apart from the recently identified role as a resistin receptor, CAP1 has actin-binding properties of importance for cell dynamics and motility, which are key processes in cancer progression. The highly conserved, ubiquitously expressed CAP1 is a 475 amino acid long, multi-domain protein. CAP1 consists of an N-terminus with oligomerization and helical-folded domains, a central part with two proline-rich motifs separated by a Wiskott-Aldrich syndrome protein (WASP)-homology 2 domain, and a C-terminal actin-binding CAP and X-linked retinitis pigmentosa protein 2 (CARP) domain and a second dimerization motif [76-78].

The capability of CAP1 to regulate actin dynamics and cytoskeletal rearrangements engage both the N- and C-terminal domains of CAP1 with distinct functions, which together stimulate rapid actin turnover that is essential for cell motility [79]. CAP1 further co-operates with cofilin, an actin depolymerizing protein, to disassemble and sever F-actin, accelerate actin nucleotide exchange, and constitute a part of the

autoinhibitory-mechanism of the actin polymerization factor, inverted formin 2 [77, 80, 81].

CAP1 deficiency has been demonstrated to cause a decrease in growth, alter morphology, and reduce migration in both yeast, epithelial, and cancer cells, thus linking CAP1 to several hallmarks of cancer [76, 77, 82, 83]. Depletion of CAP1 in cancer cell lines is further associated with reduced proliferation, migration, and cell adhesion. Furthermore, high CAP1 expression has been associated with poor prognosis in glioma, liver, lung, pancreatic, and ovarian cancers [84-88]. In breast cancer, CAP1 expression has been associated with conflicting results. While some demonstrate that high CAP1 is a poor prognostic marker, others have described the opposite effect [89-91]. A potential cell-type dependence on estrogen has been reported that includes a stimulatory effect of CAP1 silencing in ER-negative cell lines and an inhibitory effect in ER-positive cells lines [82].

Insulin resistance

Long-term harmful effect of glucose is a key factor explaining the increased health risks observed in obese individuals. Following oral intake, nutrients are broken down to glucose and enter the bloodstream to finally be used as the primary energy substrate in cell metabolism. Import of glucose into cells is mediated by the glucose transporter type 4 (GLUT4), which is activated by insulin that is produced by the β-cells of the pancreas. During overnutrition, increased insulin concentrations are required to activate GLUT4 and decrease blood glucose concentrations, and hyperinsulinemia can occur. After long-term glucose excess, such as found in obesity, cell sensitivity to insulin can decrease and blood glucose levels remain elevated (hyperglycemia). This metabolic condition is usually called insulin resistance [92]. Furthermore, a deficiency for producing insulin by β-cells results in decreased insulin levels and type 2 diabetes [93].

Insulin resistance is an important intermediary between obesity and the increased risk for type 2 diabetes, hypertension, and cardiovascular disease. The excess energy associated with overnutrition in obesity can impair the metabolic homeostasis causing insulin resistance and induce an inflammatory processes in the adipose tissue, associated with atherosclerosis and the metabolic syndrome [94-96]. Insulin resistance is further associated with an increase in risk of postmenopausal breast cancer and poor disease outcome [97].

Obesity-associated inflammation

Obesity can cause chronic and low-grade inflammation, both locally in the breast and systemically, which is a hallmark of cancer. Rapidly expanding adipose tissue in the early stages of obesity can cause insulin insensitivity, hypoxia, and

overexpression of HIF-1α with subsequent activation of the pro-inflammatory signaling NF-κB pathway [98, 99]. Furthermore, adipocytes in obese women can induce inflammation through various mechanisms, such as macrophage infiltration, activation, and polarization [100]. Crown-like structures (CLS) are composed of necrotic/damaged, usually hypertrophic adipocytes, surrounded by macrophages and are often found in the WAT of obese individuals and breast cancer patients [101, 102]. Positive correlations between CLS and pro-inflammatory macrophages with adipocyte size and BMI have been reported [101, 103]. Adipocyte-associated macrophages are usually highly pro-inflammatory, so-called M1-like macrophages, and adipose tissue with the presence of CLS have higher levels of the cytokines, IL-6 and transforming growth factor (TGF)-α [104, 105]. Furthermore, CLS are associated with NF-κB and aromatase activation in breast cancer [101].

Neutrophil-to-lymphocyte ratio

An indicator of low-grade inflammation and immune activation is the neutrophil to lymphocyte ratio (NLR), measured from peripheral blood sampling [106, 107]. High NLR is further correlated with obesity and the metabolic syndrome [108-110].

Lymphocytes and neutrophils are types of white blood cells. Lymphocytes consist of three major types: (1) natural killer cells, (2) T cells, and (3) B cells. Natural killer cells are part of the innate immune system, while B cells and T cells are a part of the adaptive immune system and recognize specific antigens [111]. Neutrophils are part of the innate immune system and are the first line of defense at sites of infection and acute inflammation [112]. Neutrophil receptors recognize pathogens through pathogen-associated molecular patterns and create neutrophil extracellular traps, deliver antimicrobial molecules, and generate reactive oxygen intermediates [113]. In addition, neutrophils secret cytokines and chemokines, such as TNF-α and IL-1, which recruit the adaptive immune system to sites of inflammation [112, 114].

Numerous studies have demonstrated that in a tumor milieu of inflammation and hypoxia, continuous recruitment of neutrophils occurs, which can modulate the neutrophils to promote tumor progression. In contrast, lymphocytes are generally considered protective and anti-tumorigenic; however, high levels of tumor-infiltrating lymphocytes have been correlated with poor prognosis [111, 115]. Elevated neutrophil count, mainly in relation to lymphocyte count and NLR, both in peripheral blood and from intratumoral assessments, have been associated with poor breast cancer characteristics and outcome [116-120], particularly in ER-negative [121] and triple-negative breast cancer [122]. Furthermore, higher levels of NLR have been reported in women diagnosed with breast cancer compared when with healthy women [123, 124].

Aims

"*Basically, I have been compelled by curiosity*."

– Mary Leakey

The overall aim of this doctoral thesis was to elucidate the associations between obesity and breast cancer from a translational perspective.

The specific aims of each paper are listed below:

Paper I

- In a preclinical setting, examine the effects of adipocytes and obesity-related metabolic conditions on breast cancer cell proliferation and migration. Also, to identify adipokines that may affect the biological response, and in a clinical setting evaluate the corresponding receptor expression in relation to breast cancer outcome.

Paper II

- In a preclinical setting, further study molecular and cellular effects in breast cancer cells in response to normal or obese-like adipocyte secretome stimulation, and potential functional involvement of the adipokine receptor CAP1, to gain a better understanding of how adipokines affect breast cancer cells.

Paper III

- In a clinical setting, investigate the association between body constitution and tumor-specific CAP1 protein expression regarding breast cancer prognosis.

Paper IV

- In a prospective clinical setting, explore the association between prediagnostic NLR and risk of invasive breast cancer overall, or according to specific tumor characteristics, and whether or not different body constitutions have a modifying effect.

Material and Methods

"*Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.*"

– Rosalind Franklin

Methodology overview

The papers in this thesis are mainly based on laboratory-oriented studies with functional *in vitro* experiments and clinically-oriented epidemiology investigations using a large prospective observational study, the Malmö Diet and Cancer Study (MDCS). The methods range from cell-based molecular and cell biology techniques to molecular epidemiology and applied statistics (Table 2). Below, a brief mention of the techniques used in the thesis is included. The specifics of the different methods, however, are found in Papers I-IV and will not be discussed further here.

In the laboratory-oriented *in vitro* projects (Papers I and II), adipocyte differentiation was verified by Oil Red-O staining and breast cancer cell proliferation by a sulforhodamine B (SRB) assay. Morphological alterations were assessed by light microscopy and immunofluorescent staining, visualized, and evaluated by ZEN and Image J software. Cell migration by scratch assay (also called wound healing assay) was quantified by TScratch software [125]. For gene expression silencing, small interfering (si) RNAs by reverse transfection was used, and mRNA expression was evaluated by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Pan-adipokine levels and kinase phosphorylation were assessed by proteome profiler arrays. Protein levels were assessed by cell microarray, immunocytochemistry, and Western immunoblotting. Cell cycle distribution was evaluated by flow cytometry.

In the clinically-oriented studies (Papers III and IV), tumor-specific CAP1 protein expression was assessed by immunohistochemistry on tissue-microarrays (TMAs) and NLR was established from leukocyte counts in baseline blood samples (Table 2). Statistical analyses were performed using GraphPad Prism, Excel, and SPSS software sets.

Table 2. Methodology overview*: In vitro* models, epidemiological study populations, and the techniques used for each.

Paper	Cell models/Study populations		Techniques/Methods
I	*In vitro*	3T3-L1, T47D, MCF-7, MDA-MB-231	Oil Red-O staining, SRB, Immunofluorescent staining, Scratch Assay, Western immunoblotting, obesity-associated models
	Study populations	GOBO, TCGA	Gene expression assessment
II	*In vitro*	3T3-L1, T47D, MDA-MB-231	SRB, Western immunoblotting, Flow Cytometry, obesity-associated models, siRNA knockdown
III	*In vitro*	T47D, MDA-MB-231	Immunofluorescent staining, qRT-PCR, Western immunoblotting, cell microarray, siRNA knockdown, immunocytochemistry
	Study population	The Malmö Diet and Cancer Study (MDCS)	Immunohistochemistry, epidemiology
IV	Study population	MDCS	Epidemiology

*In vitro s*tudies

Cell models

Established cell lines derived from animals and human donors are widely used for *in vitro* studies to model the cellular environment of *in vivo* tissues [126]. The four cell lines used in this thesis were purchased from and validated by ATCC-LGC Standards. The specific breast cancer cell lines were chosen since they have different phenotypes, hormone receptor status, and gene mutations and thereby display the heterogeneity of breast cancer. For Paper I, three breast cancer cell lines (MCF-7, T47D, and MDA-MB-231) and one pre-adipocyte fibroblast cell line (3T3-L1) were used. The same cell lines, except MCF-7, were also used in Papers II and III. The different breast cell line characteristics are outlined in Table 3.

Table 3. Human breast cell line characteristics

Cell line	Subtype	Hormone receptors	Gene mutations	Phenotype	Cell-cell adhesion	Donor
T47D	Luminal A-like	ER^+, PR^+, $HER2^-$	*PIK2CA*, *TP53*	Epithelial-like, non/low invasive	Highly cohesive	54 years, woman, ductal carcinoma
MCF-7	Luminal A-like	ER^+, PR^+, $HER2^-$	*PIK3CA*, *CDKN2A*	Epithelial-like, (non/)low invasive	Highly cohesive	69 years, woman, adenocarcinoma
MDA-MB-231	Triple-negative	ER^-, PR^-, $HER2^-$	*BRAF*, *TP53*, *CDKN2A*, *KRAS*, *NF2*	Mesenchymal-like, highly invasive	Loosely cohesive	51 years, woman, adenocarcinoma

The 3T3-L1 pre-adipocyte cell line used in Papers I and II is the most frequently used cell line for modeling adipogenesis and adipocyte biology [127]. A standardized protocol for successful differentiation from precursor fibroblasts to mature adipocytes was used and is further described in Papers I and II, respectively.

Adipocyte secretome in normal or obese-like conditions

The specific collection of proteins secreted by adipocytes is called the adipocyte secretome. To obtain the adipocyte secretome for further study and simulate various obesity-related metabolic conditions, differentiated adipocytes were cultured for 24 h under metabolic conditions that mimic normal and obese-like physiology. Serum-free medium was supplemented with bovine serum albumin (0.2 mg/mL), sodium bicarbonate (1.2 mg/mL), transferrin (0.01mg/mL), antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) with the addition of the conditions described in Table 4 to reflect insulin and glucose variations associated with obesity and the development of insulin resistance. In parallel to the adipocyte-conditioned medium, an equivalent adipocyte-free control medium to be used as a negative control was collected.

Table 4. The metabolic conditions used for adipocyte-conditioned medium

	Normal	**Pre-type 2 diabetes** (Hyperinsulinemia)	**Overt type 2 diabetes** (Insulin resistance)	**Late type 2 diabetes** (Impaired insulin secretion)
Insulin	Low (0.1 ng/mL)	High (1.0µg/mL)	High (1.0 µg/mL)	Low (0.1 ng/mL)
Glucose	Low (5 mmol/L)		High (25 mmol/L)	
Used in papers	I & II	I	I & II	I

Experimental model

An experimental model was established to study the effects of adipocytes and obesity-related metabolic conditions on breast cancer cells and was used throughout the studies in Papers I and II (Figure 7). Breast cancer cells were exposed to the adipocyte secretome or control medium for 24 to 72 h. The potential changes in breast cancer cells induced by the adipocyte secretome and the different metabolic conditions, such as effects on cell proliferation, morphology, migration, and signaling pathways were assessed by different techniques. In Paper II, the breast cancer cells in the model were either CAP1 expressing or silenced.

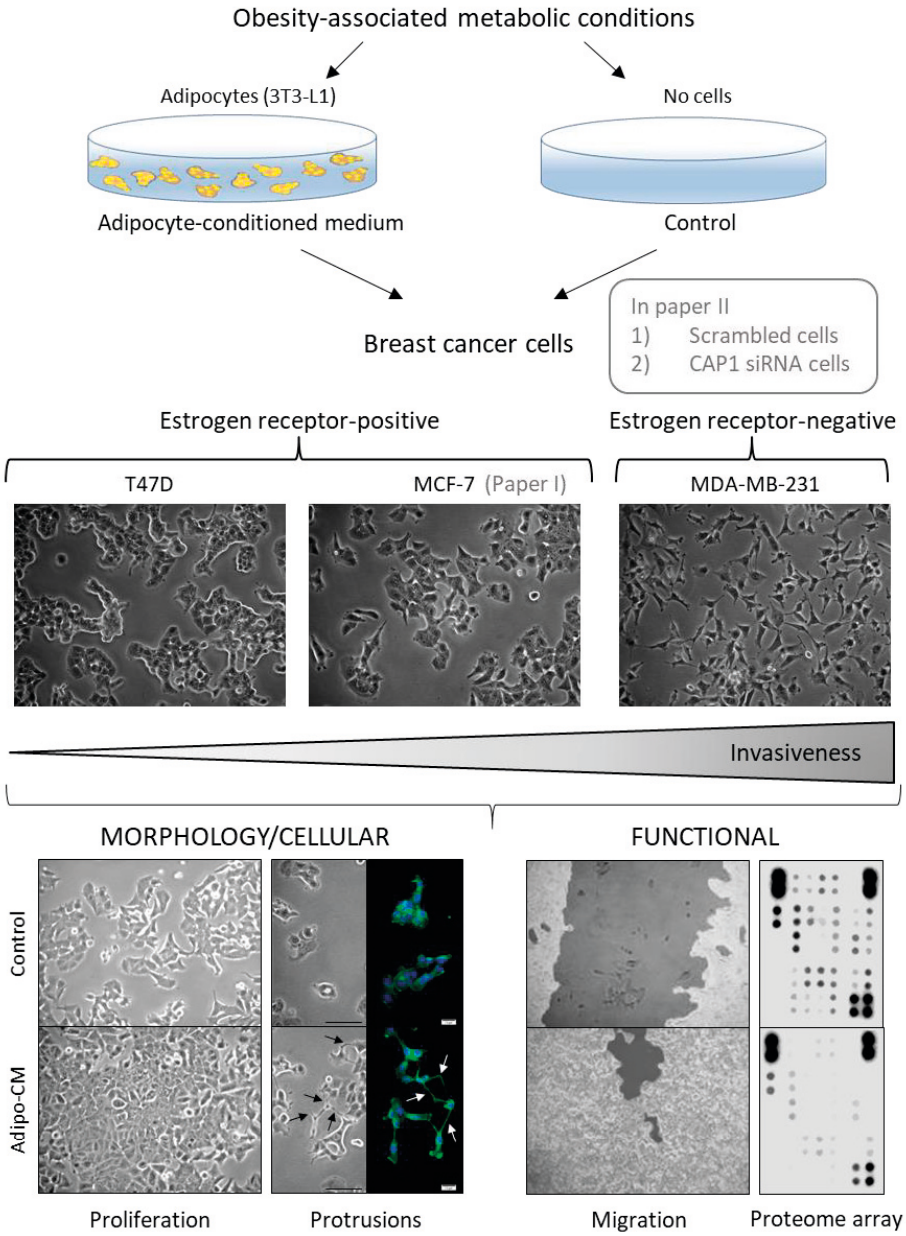

Figure 7. Experimental *in vitro* model used in Papers I and II.

Antibody validation

A CAP1 antibody validation was performed in accordance with recommendations from the *ad hoc* International Working Group for Antibody Validation to ensure target specificity and application functionality before staining the breast tumor tissue in the MDCS [128]. Antibody performance was evaluated using a genetic approach combined with an independent antibody strategy. In order to reduce the target protein level and assess antibody specificity, *CAP1* gene expression was silenced using siRNA by an optimized reverse transfection technique, and two independent anti-CAP1 antibodies that bind to different epitopes of the target protein were subsequently tested.

Anti-CAP1 antibodies

- Atlas (HPA030124); rabbit polyclonal, concentration for immunocytochemistry 1:75 and Western immunoblotting 1:750
- Abcam (ab133655); rabbit monoclonal, concentration for immunocytochemistry and Western immunoblotting 1:10,000

CAP1 mRNA expression was determined by qRT-PCR, and CAP1 protein levels were analyzed with Western immunoblotting and immunocytochemistry of a constructed cell microarray (Figure 8). The Abcam antibody was selected due to its higher specificity and performance when compared with the Atlas antibody.

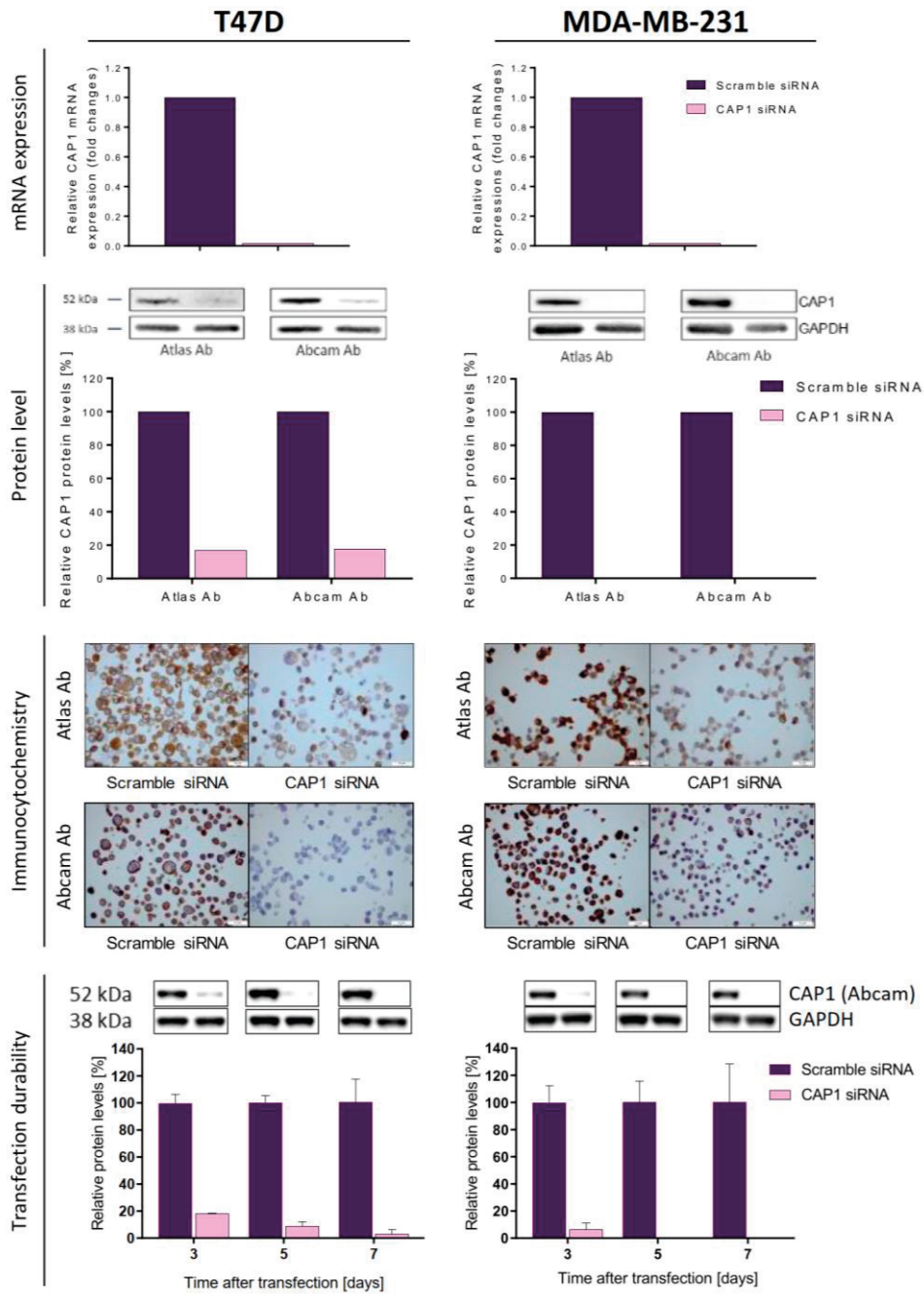

Figure 8. Two independent adenylate cyclase-associated protein-1 (CAP1) antibodies were validated for their target specificity and functional application validity in CAP1 expressing or CAP1 silenced T47D and MDA-MB-231 cells at the protein level via Western immunoblotting and immunocytochemistry of a constructed formalin-fixed paraffin-embedded cell microarray.

Epidemiological studies

Study populations

In Paper I, two publicly available data sets were used: (1) the Gene Expression-based Outcome for Breast Cancer Online (GOBO) and (2) The Cancer Genome Atlas (TCGA) [129, 130]. GOBO contains gene expression data from eleven breast cancer cohorts. A total of 1881 primary breast tumors and 51 breast cancer cell lines were used (47 in Paper I). *CAP1* mRNA expression was examined with regards to breast cancer subtypes (cell line data) and according to the Prediction Analysis of Microarray 50 (PAM50) subtypes (primary breast tumors) [131]. PAM50 consists of 50 classifier genes and five reference genes that are used to categorize tumor samples into intrinsic breast cancer subtypes [132]. Within the subset of 1,105 invasive breast carcinomas in the TCGA project, *RETN* (resistin gene) and *CAP1* mRNA and protein expression were examined, and co-expressed genes and biological network analyses were performed.

The MDCS, used in Papers III and IV, is one of the largest population-based prospective cohorts in Sweden [133]. The overall objective of the MDCS is to explore the association between dietary habits and cancer risk. Between 1991 and 1996, all women (born between 1923 and 1950) living in Malmö, the third-largest city in Sweden, were invited to participate in the study. In total, 17,035 (42.6%) women completed baseline examinations and questionnaires and were included in the study. The baseline examination included drawn blood samples, anthropometric measurements recorded by a trained nurse, and an extensive questionnaire that covered medical and reproductive history, demographics, detailed dietary measurements, and lifestyle factors. Information on cancer incidences, tumor characteristics and treatments, and causes-of-death were retrieved from patient charts and record-linkage to the Swedish Cancer Registry, Regional Tumor Registry for Southern Sweden, and the Swedish Cause of Death Registry. Exclusion criteria were limited to causes affecting the ability to complete the questionnaire. An overview of the study populations included in Papers III and IV is illustrated in Figure 9. Tumor-specific CAP1 expression was examined in Paper III and pre-diagnostic NLR levels in peripheral blood in Paper IV. More extensive information can be found in the specific papers.

The studies included in the thesis were approved by the Regional Ethical Committee at Lund University, Sweden.

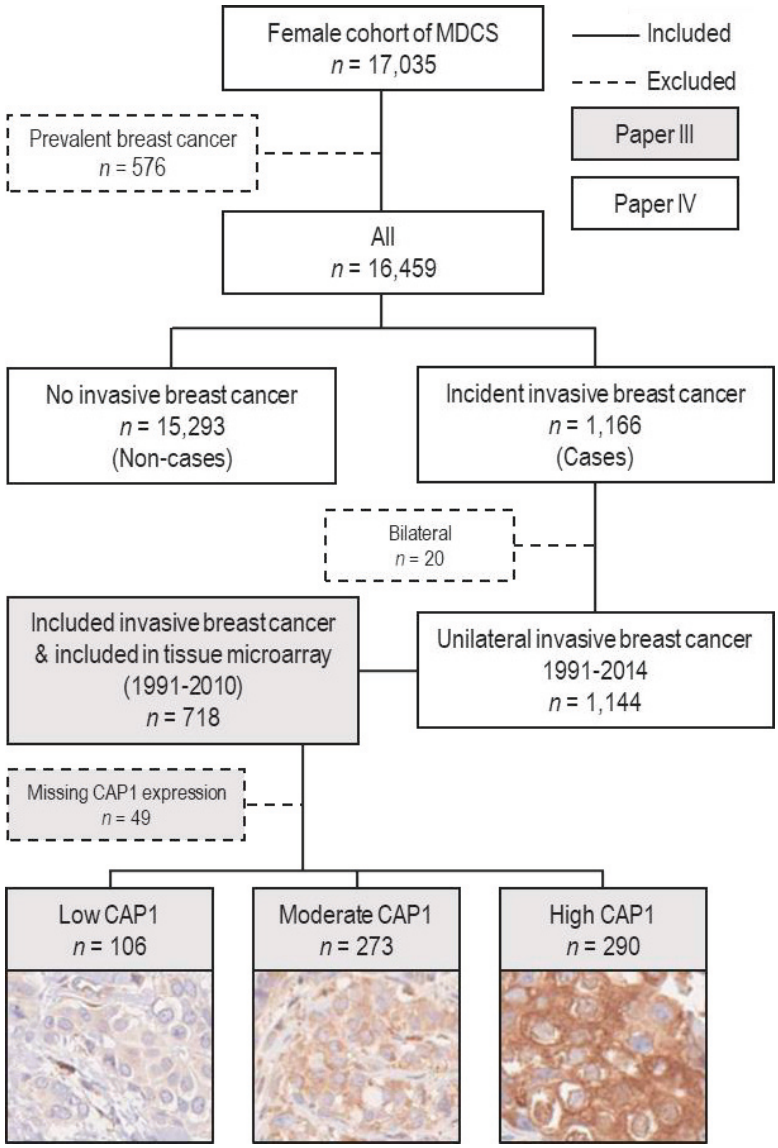

Figure 9. An overview of the study populations in Paper III (grey background) and Paper IV (white background) of the included women from the Malmö Diet and Cancer Study.

Tissue microarray and immunohistochemistry

The TMA technique is a high-throughput method that was developed to analyze large numbers of tissue samples concurrently [134]. This technique was introduced in 1998 by Kononen *et al.* and allowed for faster throughput of molecular analyses of multiple specimens compared with the conventional labor-intense technology for

tumor analysis using whole-tissue sections. In addition, less tumor material is required. Today TMAs are rapid, cost-effective, and compatible with IHC, immunofluorescence *in situ* hybridization, and RNA *in situ* hybridization [135]. TMAs were used in Paper III, and the TMA technique is illustrated in Figure 10. In brief, tumor specimens embedded in donor paraffin block are evaluated by a histopathologist and core tissue biopsies from selected areas are taken and mounted into a recipient paraffin block that can hold over a hundred tissue specimens. The recipient block is cut into 3- to 4-µm-thin sections and mounted onto glass slides.

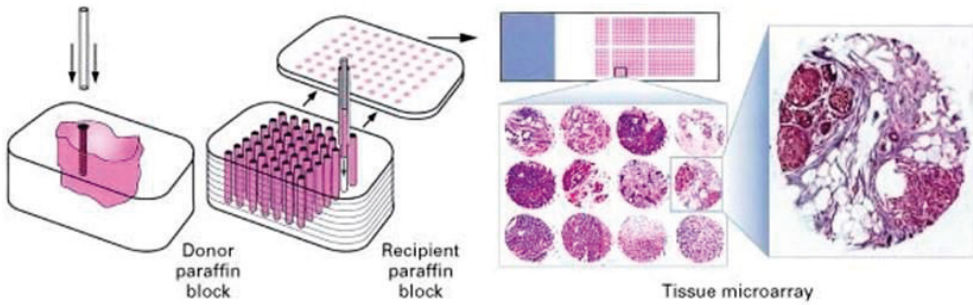

Figure 10. Illustrations of the construction of a tissue microarray. Reproduced with permission from Hedenfalk *et al.* [136], Copyright Massachusetts Medical Society.

IHC is a common method used in medical research and clinical diagnostics to detect proteins in tissues, either whole-tissue sections or TMAs, using an antibody specific for the target protein of interest. First, antigen retrieval is performed to unmask antigens that may have been concealed by protein cross-linking due to the formalin fixation and which could restrict antibody-antigen binding. A primary antibody is then applied, and the bound protein of interest is visualized by a secondary antibody, usually as a brown-colored substrate [137].

In paper II, tumor-specific CAP1 protein expression was evaluated in duplicate tissue cores from breast cancer tumors in the MDCS using light microscopy. Based on the intensity of the staining, CAP1 was graded into negative, weak, moderate, strong, or intense. If the score was borderline, the higher score was applied. The staining fraction was not considered since more than 75% of the cells were positively stained for CAP1. The evaluation was performed twice and the discrepancy between the readings was 4%.

Statistical analyses

In Papers I, II, and IV, two-way analysis of variance (ANOVA) with either Sidak's or Bonferroni's post hoc test was used to estimate differences between multiple groups means. The two-way ANOVA analysis only identifies whether a difference across means exists, but does not distinguish which difference. To identify potential differences and to reduce potential type I errors, post-hoc tests can be performed, such as the Sidak and Bonferroni.

Parametric tests, such as the t-test used in Paper I, are used to compare the distribution between groups when the data are normally distributed. When the data have a skewed distribution, non-parametric tests, such as the linear-by-linear association chi-square test (used in Paper II and IV) for categorical data and the Mann-Whitney U test (used in Paper IV) for continuous data, are used instead. The Jonckheere-Terpstra test (used in Paper II) was used to examine whether an ordered difference in medians for non-parametric data existed.

Univariable survival analyses of breast cancer outcome were performed by LogRank trend test and Kaplan-Meier estimates in Papers I and III. The LogRank test is used to compare the linear trend between two or more survival curves. The Kaplan-Meier survival analysis estimates survival over time based on the survival probability. Certain assumptions must be fulfilled to use this method: (1) the censoring must be unrelated to the outcome, (2) the expected survival should be constant over time, and (3) the time of events should be known. Uni- and multivariable Cox regression analyses with calculated hazard ratios with 95% confidence intervals adjusted for potentials factors were used in Paper III. In Paper IV, logistic regression analysis providing odds ratio was used to estimate breast cancer risk since the assumption for proportional hazards required by Cox regression was not met.

Results and Discussion

"*Science makes people reach selflessly for truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist*."

– Lise Meitner

This section discusses the results from both preclinical and clinical settings with the aim of translationally elucidating the potential effects of obesity on the development, progression, and clinical outcomes of breast cancer.

It is well established that obesity is adversely associated with the risk of postmenopausal breast cancer and poor breast cancer prognosis, but the biological mechanisms of the complex interactions are still poorly understood. As illustrated in figure 5, a pro-tumorigenic microenvironment can result from the systemic metabolic alterations in obese individuals, including aberrant insulin, insulin-like growth factor, and glucose homeostasis, together with the close proximity and local effects of adipocytes and their secretome can lead to a pro-tumorigenic microenvironment.

Effects of adipocyte secretome in normal and obese-like conditions

In an experimental setting, the adipocyte secretome from different metabolic conditions was assessed to gain a better understanding of how adipokines directly affect the proliferation and migratory capabilities of breast cancer cells.

Cellular features and functional changes

Paper I examined the combined effects of adipocytes and obesity-related metabolic conditions on the proliferation of breast cancer cells. Breast cancer cells exposed to the adipocyte secretome displayed enhanced proliferation compared with cells exposed to control medium. The increase in proliferation was greater for cells in the

adipocyte secretome from obese-like metabolic conditions mimicking the later stages of type 2 diabetes (insulin resistance and insulin deficiency).

Compared with ER-positive cells (T47D and MCF-7), triple-negative cells (MDA-MB-231) were more responsive to the growth-promoting effects of the adipocyte secretome, suggesting additional mediators beyond estrogen. The magnitude of induced proliferation was significantly higher in the presence of the adipocyte secretome, but a weaker proliferative effect was observed for the obese-like control medium, indicating growth stimulation by insulin and glucose. This is in line with findings from other studies, with additional joint influence by the adipocytes and the obese-like conditions [138, 139]. Insulin acts through both the insulin-like growth factor 1 receptor and the insulin receptor and has been shown to stimulate the proliferation-associated PI3K/Akt and Mitogen-activated protein kinase (MAPK) pathways [140]. Hyperglycemia may promote cell growth through the stimulation of epidermal growth factor receptor in breast cancer cells [139]. These pathways could possibly have been stimulated to an even greater extent by the obese-like adipocyte secretome that was examined.

In addition to promoting proliferation, the obese-like adipocyte secretome further induced morphological changes in the ER-positive breast cancer cell line T47D. Upon stimulation with the adipocyte secretome under obese-like conditions, the typical epithelial-like cells displayed elongated morphology with stellate protrusions, indicating the acquisition of a more motile cell phenotype. No morphological changes were observed for the ER-positive MCF-7 cell line or the mesenchymal-like triple-negative cell line MDA-MB-231. Despite this, significantly increased migration in response to the adipocyte secretome was observed for all three cell lines, which was further enhanced under the obese-like metabolic conditions.

Paper II further explored the functional effects on the molecular features by investigating the adipocyte secretome-induced changes in patterns of intracellular tyrosine kinase phosphorylation in breast cancer cells. T47D cells were exposed to the adipocyte secretome in obese-like conditions, and compared with normal conditions, upregulation occurred for several kinases involved in the regulation of cell proliferation and the control of focal adhesions and cell motility (Figure 11) [141]. The affected kinases included Akt, platelet-derived growth factor receptor β (PDGF Rβ), tumor protein 53 (TP53), cAMP-responsive element binding protein 1 (CREB1), FAK/PTK2, AMP-activated protein kinase alpha 1 (AMPKα2), and alpha 2 (AMPKα2). The induced kinase phosphorylation profiles were found to be enriched within the biological processes associated with cell population proliferation and receptor tyrosine kinase signaling.

Given the increased proliferation, elevated kinase phosphorylations, and enriched biological processes, Paper II examined the cell cycle distribution for cells in the obese-like adipocyte secretome and compared them with cells in normal and

adipocyte-free medium. A shift from G1-phase to S- and G2/M-phase was observed, which is in line with the increased proliferation found earlier. There was increased expression of lysine-deficient protein kinase 1 (WNK-1) and cyclin-dependent kinase inhibitor 1B (CDKN1B)/p27, which are regulators of the phase transition from G1 to S. This may have contributed the shift observed in the cell cycle distribution and the increased proliferation of cells stimulated by the obese-like adipocyte secretome [142, 143].

A panel of adipokines was investigated to gain deeper understanding of factors that could mediate the molecular and cellular changes induced by the adipocyte secretome in breast cancer cells. Adipocytes had increased secretion of proteins associated with inflammation, proliferation, and matrix remodeling comprising resistin, C-reactive protein, EN-RAGE, endocan, metalloproteinase inhibitor 1 (TIMP1), and TIMP3. Resistin, which is related to insulin resistance, was upregulated by the obese-like adipocyte secretome in comparison to normal conditions.

Resistin is an interesting adipokine that has been positively associated with the risk of breast cancer, and elevated levels have been positively associated with type 2 diabetes. For women with breast cancer, resistin has also been associated with poor tumor characteristics in postmenopausal women and with poor prognosis [67, 72, 144-146]. However, the associations have been conflicting, and until a few years ago, studies regarding the biological role of resistin have been hindered by the lack of a known functional receptor [71, 147, 148]. The association observed with insulin resistance in the present studies was also interesting in that the obese-like adipocyte secretome enhanced the expression of multiple phosphoproteins associated with insulin resistance in Paper II, such as Akt, CREB, AMPKα1, AMPKα2, and STAT3 (Figure 11).

In conclusion, the adipocyte secretome stimulated molecular and cellular features that promoted the proliferation and motility of breast cancer cells, which are two central hallmarks of cancer. These changes were enhanced in obese-like conditions compared with normal-like conditions. Furthermore, these studies identified several adipokines and potential signaling pathways that are plausible as mediators for these adipocyte-induced changes in obesity-associated breast cancer.

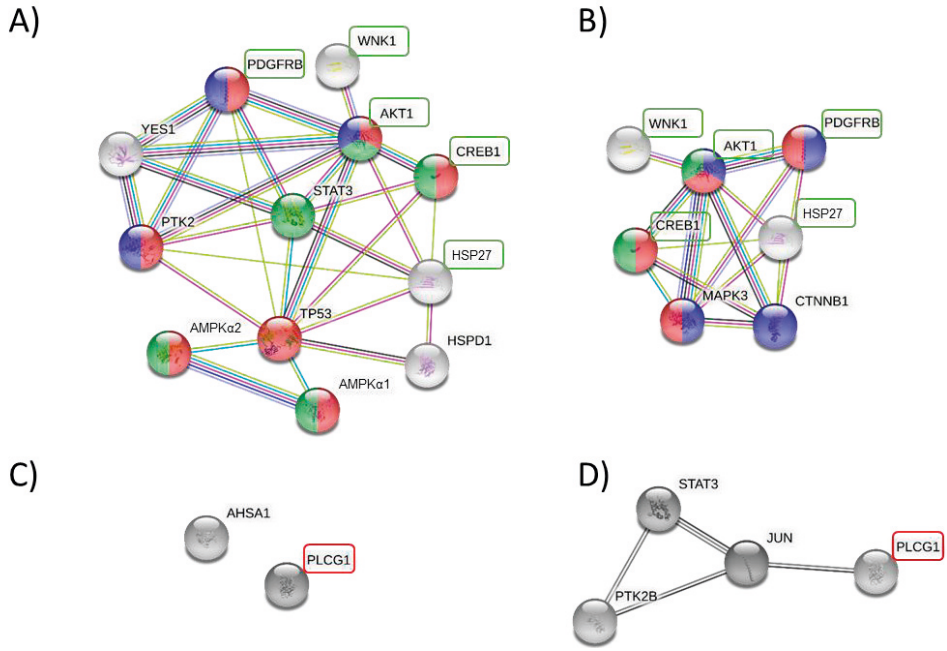

Figure 11. String analysis of phosphorylated proteins that were up- or downregulated by more than 1.3-fold by the obese-like adipocyte secretome compared with normal conditions in CAP1-expressing and silent T47D cells (Paper II) [149]. Proteins upregulated in A) CAP1-expressing cells B) CAP1-silent cells. Proteins downregulated in C) CAP1-expressing cells and D) CAP1-silent cells. Green squares indicate upregulated proteins, and red squares indicate downregulated proteins in both CAP1-silent and expressing cells. A cluster of target genes involved in the PI3K-Akt signaling pathway (red), focal adhesion (blue), and insulin resistance (green) were also identified.

Adipokine receptor CAP1

The preclinical findings were then elaborated upon in terms of the adipokine resistin receptor and actin-regulatory protein CAP1, followed by assessments of the CAP1 gene and tumor-specific protein expression. Furthermore, the perspective was broadened to assess the clinical implications of CAP1 in terms of associations with clinical outcomes and potential effect modifications by body constitution.

CAP1 in breast cancer cell lines

The *de novo* receptor for the pro-inflammatory mechanism of resistin is CAP1, which is an actin-binding protein that is involved in cytoskeletal rearrangements and important for cell division and motility [74, 79].

In Paper I, *CAP1* mRNA expression was explored in a large set of breast-cancer cell lines, and the expression was found to be cell-subtype specific, with triple-negative

(basal-like) cell lines having higher *CAP1* expression than luminal-like cell lines. The same observations were made for the cell lines in our experimental model (Figure 7). A positive correlation was observed between the three cell lines CAP1 mRNA expression and protein levels. The triple-negative MDA-MB-231 cells displayed the highest CAP1 expression, followed by the luminal-like MCF-7 and T47D cells. The established role of CAP1 in actin cytoskeleton rearrangements and in cell motility described above could explain the higher CAP1 levels identified in more mesenchymal-like cell lines.

Next, the potential role of CAP1 in the molecular and cellular changes induced by the adipocyte secretome was investigated (Paper II). CAP1 expressing ER-positive and triple-negative breast cancer cells displayed higher proliferation, while CAP1-silenced breast cancer cells showed decreased proliferation and a weaker response to the adipocyte-secretome compared to CAP1-expressing cells. The reduced proliferative response to the adipocyte secretome stimulation in CAP1 silenced cells was further reflected at the cell cycle level. The cell cycle distribution shifted from the G1-phase to the S- and G2/M-phases for CAP1-expressing cells exposed to the obese-like adipocyte secretome compared to normal cells. However, the effect was relatively reduced for CAP1-silenced T47D cells, while apoptotic cells in the sub-G1-phase were slightly increased.

The reduced adipocyte secretome-induced proliferation seen for CAP1-silenced MDA-MB-231 cells was accompanied by a lower proportion of cells in the G1-phase and a relative accumulation of cells in the S- and G2/M-phases. These results may imply an improper cell cycle progression. Furthermore, a reduced phosphorylation of proteins involved in the proliferation-associated PI3K/Akt signaling pathway was observed for CAP1-silenced cells in comparison to CAP1-expressing cells, which supports the finding of decreased proliferation (Figure 11). For future studies, further analyses should be conducted on proteins that are important for cell cycle progression, such as cyclin D1, an important regulator of the transition G1- to the S-phase, which would help to improve the understanding of the role of CAP1 in cell proliferation [150].

Unique phosphorylation patterns were observed for CAP1-expressing and silenced cells. Overall, CAP1-silenced cells had reduced expression of the phosphorylated proteins induced by the obese-like adipocyte secretome in comparison with CAP1-expressing cells. Protein tyrosine kinase 2 (PYK2) was downregulated by ≥1.25-fold in CAP1-silenced cells. PYK2, a member of the FAK family, can induce proliferation and invasion and is highly expressed in lymph node-positive breast cancer [151]. Several downregulated proteins, such as ribosomal protein S6 kinase (p70S6K) and Akt (S473), are known to be associated with the PI3K/Akt/mTOR pathway, which is a proliferation-associated signaling pathway that is commonly activated in breast cancer [152]. The reduced levels of the proliferation-associated kinases FAK, c-Jun, and Src in CAP1-silenced cells compared with CAP1-expressing cells could further explain the reduced proliferation [141].

In conclusion, the role of the adipokine resistin receptor CAP1 in the adipocyte secretome-induced cellular changes was investigated. CAP1 had higher expression in aggressive breast cancer cell lines and was important for cell proliferation. Several potential cellular and molecular mechanisms induced by the adipocyte secretome and that were modified by CAP1 were identified, and taken together, the results provide biological insights into how obesity in relation to CAP1 could affect breast cancer.

CAP1 in breast tumors

Next, CAP1 was assessed in human breast tumors in terms of mRNA expression and tumor-specific protein expression. *CAP1* gene expression was explored across 1,881 breast tumors, and a higher expression was found for ER-negative tumors than ER-positive tumors (Paper I). This is in line with the results seen in breast cancer cell lines. A weak to moderate correlation was found between *CAP1* mRNA expression and CAP1 protein levels in the breast tumors, in contrast to the strong correlation found for the breast cell lines. *CAP1* gene expression was also positively associated with higher tumor grade and processes involved in cell growth and motility. Furthermore, compared with low *CAP1* gene expression, high *CAP1* expression was associated with decreased overall survival and relapse-free survival in the overall analysis in ER-positive and lymph node-positive patients as assessed with LogRank test and multivariable Cox regression analyses.

Following these observations, further assessments of CAP1's potential clinical relevance were performed with regard to body constitution and breast cancer outcome. After thorough antibody validation, tumor material from 718 breast cancer patients included in the MDCS was stained for CAP1 expression (Paper III). Tumor-specific CAP1 staining intensity was assessed and graded into five categories ranging from negative to intense. There were seven patients with breast tumors negative for CAP1. Such a low number was expected since CAP1 is a highly conserved and ubiquitously expressed protein in breast tissue.

The staining categories were then compiled into three groups. Patients with tumors of low CAP1 protein expression had higher age at study inclusion, wider waist- and hip circumference, and higher BMI and BF% compared with patients who had tumors of moderate or high CAP1 expression. These findings contrasted with the hypothesis that CAP1 protein expression would be positively associated with obesity, given the findings from the previous studies in which elevated resistin levels and insulin resistance had been associated with obesity [67].

At diagnosis, breast cancer patients with low CAP1 expressing tumors were generally older, and their tumors more commonly displayed unfavorable tumor characteristics, such as histological grade III, high Ki67, and higher degrees of ER-negative tumors, including triple-negative tumors, than women with tumors of high

CAP1 expression. Furthermore, low CAP1 expression was associated with poor breast cancer-specific and overall survival, particularly among patients with ER-positive tumors. A similar trend was observed for patients with ER-negative tumors, but few events limited these estimations. However, in the multivariable analysis, CAP1 expression did not remain an independent prognostic marker. The results of the present study contrasted with those of two smaller previous studies that investigated CAP1 expression and breast cancer prognosis. However, those studies were limited in study population size and also analyzed CAP1 IHC staining differently [88, 89]. Since the role of CAP1 in breast cancer remains conflicting, further studies are required to fully understand the biological role of CAP1 in breast cancer.

In patients with low adiposity status, low CAP1 expression was associated with poor survival, irrespective of which anthropometric measure was used to assess adiposity. No associations between CAP1 expression and clinical outcome were found among women of higher adiposity status. A limitation of the study is that the anthropometric measures were taken at baseline, and no information was obtained regarding adiposity status at diagnosis, which could have changed from baseline to the time of diagnosis.

The MDCS is mainly composed of postmenopausal women (92%), so subgroup analyses based on menopausal status were limited. The median age of the cohort used in paper I was 10 years younger which could in part explain the conflicting result between Papers I and II. In Paper I, the highest CAP1 mRNA expression was observed in ER-negative tumors, which are most prevalent among premenopausal women [158]. The triple-negative patient subgroup generally has poor prognosis. However, in the MDCS and its very limited subgroup of triple-negative breast cancer patients, the subset of triple-negative patients with high CAP1 tumor expression had more favorable breast cancer prognoses than patients with low-CAP1 triple-negative cancers.

In conclusion, high *CAP1* gene expression was associated with poor tumor characteristics and impaired overall and relapse-free survival. Low tumor-specific CAP1 protein expression was associated with higher adiposity and poor breast cancer outcome, and it was surprisingly most pronounced in lean patients. Further studies regarding the post-translational modification of CAP1 are required to better understand these contrasting mRNA and protein results.

Neutrophil-to-lymphocyte ratio (NLR)

Finally, in a prospective, population-based setting, prediagnostic NLR from the peripheral blood of healthy women was evaluated for its potential as a predictive marker of primary breast cancer risk and whether or not that risk varies with body

constitution. NLR is a marker of low-grade inflammation (one of the hallmarks of cancer) and is associated with obesity. As such, it could potentially be associated with the risk of breast cancer. Nevertheless, in this study prediagnostic NLR was not confirmed to be a predictive marker of breast cancer risk.

In terms of distributional differences, women with the highest NLR quartile were younger and more commonly demonstrated established risk factors for breast cancer, such as nulliparity, later age at first childbirth, and use of combination HRT than women with low NLR. Furthermore, women with high NLR were more frequently premenopausal, which is in line with other studies [124, 153]. In terms of risk, however, only a slight, non-significant, increase was identified in breast cancer incidence per 100,000 person-years with increased NLR quartiles.

In terms of associations with body constitution, there was a distributional correlation between high NLR and leaner body constitution, which is an inverse association to what has been hypothesized. However, this was not apparent in the subsequent risk assessments. Thus, no modifying effect by body constitution on breast cancer risk could be confirmed. Nor was NLR associated with breast cancer risk according to different tumor characteristics. Women in the second quartile of NLR did display a significantly increased risk of ER-negative breast cancer than women in the first quartile, and this finding remained in the multivariable analysis. However, as one of many analyses, the finding may have been a chance finding, and it did not clearly comply with the proposed biological hypothesis.

Inflammation is critical for cancer progression, and an altered microenvironment can induce several malignant processes, including proliferation, tissue remodeling, angiogenesis, metastasis, and immune evasion/suppression [154]. Multiple studies have explored NLR as a biomarker for systemic low-grade inflammation, and demonstrated that NLR is significantly associated with and comparable to C-reactive protein, an established marker of inflammation [155-157]. One explanation for our neutral results could be that NLR is only predictive of breast cancer diagnosis in a short time frame from blood sampling. The median time from inclusion and blood sample collection in MDCS to the time of breast cancer diagnosis was 10.8 years, and the NLR could have changed multiple times during the follow-up period.

Although the association between NLR and obesity is disputed, and studies have demonstrated conflicting results, the finding of women with high NLR being leaner was unexpected [109, 110, 158]. Several interpretations are possible. Menopausal status and estradiol levels may affect the observed associations. As one study speculated, NLR levels may change in relation to estradiol in premenopausal women compared with postmenopausal women [124]. Furthermore, estradiol can inhibit neutrophil apoptosis and decrease lymphocyte production, thereby leading to a higher ratio [159, 160]. There may also be a normalizing effect in the ratio of neutrophils to lymphocytes by the elevated levels of lymphocyte and neutrophil in

women with high BMI compared to women with low BMI, which could explain the observed result.

In conclusion, although distributional associations were identified for women with high NLR and established risk factors for breast cancer, the findings in this study did not support prediagnostic NLR as a strong predictive marker for breast cancer risk overall or by body constitution.

Conclusions

"*The unforgotten moments of my life are those rare ones which come after years of plodding work, when the veil over nature's secret seems suddenly to lift, and when what was dark and chaotic appears in a clear and beautiful light and pattern*."

– Gerty Cori

Paper I

- Adipocyte secretome stimulated proliferation and motility of both ER-positive and triple-negative breast cancer cell, effects that were more prominent in obese-like metabolic conditions. A panel of adipokines, amongst them resistin, were enhanced under the obese-like conditions and may contribute to the identified biological responses.
- CAP1, a novel receptor of resistin, was expressed at relatively higher levels in ER-negative, compared with ER-positive, breast cancer cells and tumors.
- High *CAP1* gene expression was associated with poor tumor characteristics, and shorter overall- and relapse-free survival among breast cancer patients, when compared with low *CAP1* expression.

Paper II

- Breast cancer cells exposed to adipocyte secretome in obese-like compared to normal conditions, displayed an enrichment of protein phosphorylation patterns involved in important biological processes related to cell population proliferation.
- The proliferative response of breast cancer cells to the obese-like adipocyte secretome was associated with a cell cycle shift from G1-phase to S- and G2/M-phase.
- CAP1 silencing resulted in a weaker response to the obese-like adipocyte secretome stimulation with regards to phosphokinase induction and breast cancer cell proliferation compared with CAP1 expressing cells, thus suggesting a potential role of CAP1 in the molecular mechanisms of obesity-related breast cancer.

Paper III

- Among 669 breast cancer patients in the MDCS, the patients with tumors of low CAP1 protein expression had higher body fatness with wider waist, higher BMI and BF%, and displayed more unfavorable tumor characteristics, compared to patients with tumors of high CAP1 expression.
- Patients with low CAP1 tumors expression had poor breast cancer specific- and overall survival, most prominent among patients with ER-positive tumors or low body fatness, compared to patients with high CAP1 tumors.

Paper IV

- Women in the MDCS with high prediagnostic NLR at study inclusion were leaner and more likely to display established breast cancer risk factors, compared with women with low NLR
- No overall association between NLR and for risk of breast cancer was observed, nor in relation to specific tumor characteristics or body constitution.

Future Perspectives

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

– Marie Curie

Breast cancer is the most common malignancy in women and steadily increasing worldwide. At the same time, the prevalence of obesity is rising. Overweight, obesity, and obesity-related inflammation increase both breast cancer risk and mortality. Understanding key pathological features of the biological process underlying these associations therefore have important implications for the prevention of breast cancer and improving breast cancer outcome.

Studying the paracrine impact of adipocytes under different metabolic conditions mimicking normal-like and obese-like physiology on breast cancer cells provides insight into the mechanistic crosstalk between adipose tissue and breast cancer. While our experimental model simulates obesity-induced alterations in adipocytes and breast cancer cells, it is limited and simplified in that it does not capture the multiple different cell types and factors that interacts in the breast microenvironment and can affect tumorigenesis. The advantage of our model though, is the possibility to identify altered cellular and molecular features to investigate in more complex models and further validate for its clinical value in patient cohorts with potential to find new treatment targets.

Resistin was identified as an interesting adipokine upregulated in obese-like conditions. Its receptor CAP1 was investigated and targeted in experimental models and explored with regards to prognosis among breast cancer patients. CAP1, a highly conserved, ubiquitously expressed protein involved in cytoskeletal rearrangements, was shown by us and others to be associated with multiple cellular processes associated with tumorigenesis. Despite this, there is limited information on the regulation of CAP1 and potential implications for breast cancer. The presented divergent results for CAP1 gene and protein expression regarding breast cancer outcome may involve unknown post-translational modifications. This is supported by the weak to modest positive correlation between mRNA and protein expression in tumor samples, but more research regarding the regulation of CAP1 is needed. In addition, the observation that lean breast cancer patients, which

generally have a better prognosis than obese patients, had a poorer prognosis if their tumor had low expression of CAP1 warrants further investigation on the role of CAP1 in the complex relationship between excess adipose tissue and breast cancer.

The inflammatory indicator NLR has been positively associated with obesity and also found to be elevated at the time of breast cancer diagnosis. Despite the neutral result of prediagnostic NLR and risk of breast cancer found herein, the easy assessment and evaluation of NLR could warrant further studies in which changes in NLR over time and potential effects on short-term risk of breast cancer are investigated.

This doctoral thesis provide novel understanding to biological processes linking obesity, adipocytes, and breast cancer. To meet the challenges with an increasing obese population at risk of breast cancer with poor long-term prognosis, further translational research initiatives are required to provide mechanistic explanations and enable identification of high-risk individuals.

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"*You Have Two Hands, One for Helping Yourself, the Other for Helping Others*."

– Audrey Hepburn

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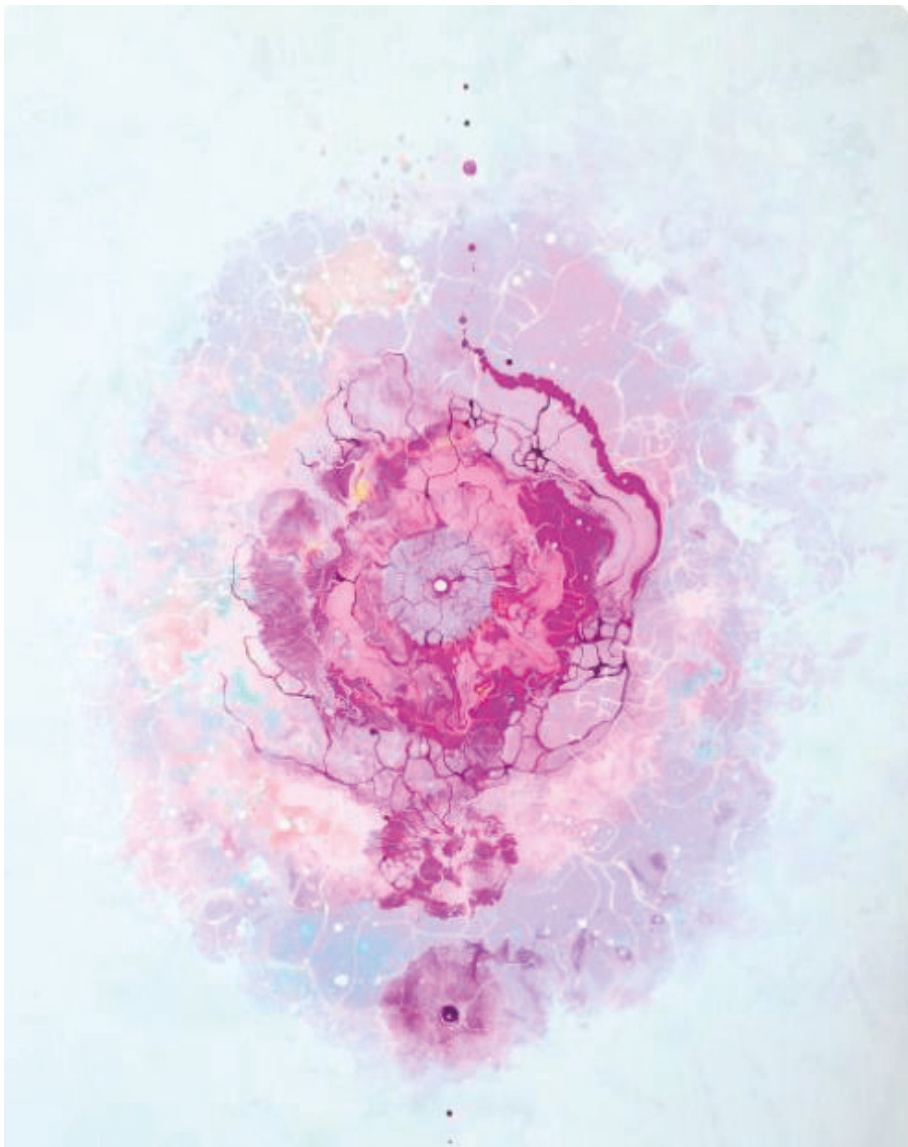
The Cover



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