Multiple primary malignancies in breast cancer patients

From population study to genetics

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Now God gave Solomon [exceptional] wisdom and
very great discernment and breadth of mind,
like the sand of the seashore (1 king 4:29)

Gud gav Salomo visdom och förstånd i mycket rikt mått
och så mycken kunskap att den kunde liknas
vid sanden på havets strand (1 kung 4:29)

ABSTRACT

Breast cancer (BC) is one of the most common causes of cancer-related death among women worldwide. Due to early detection of BC and more tailor-made treatments, patients live longer despite their illness. Studies have shown that BC patients are at greater risk of developing new tumors in organs other than the breast, mainly caused by BC treatment. These tumors do not originate from the breast and are not considered to be metastases, but primary tumors. However, BC patients have also been shown to be at greater risk of developing other malignancies, even before their BC. Thus far, previously diagnosed malignancies have not been investigated to a great extent. The etiology of multiple primary malignancies (MPMs) can be explained by intrinsic-, extrinsic-, and therapeutic factors. In addition, genetic factors are postulated to contribute to the development of breast cancer and MPMs. To avoid the toxicity of repeated cancer treatment, it is important to predict and prevent the development of other primary malignancies in cancer patients. These patients are in need of individually tailored cancer therapies and special follow-up programs.

The aim of this thesis was to investigate the prevalence of other previous primary malignancies (OPPMs) before a BC diagnosis and identify specific genetic changes and prognostic factors associated with high-risk patients. In the first work, we reviewed the medical records of 8,031 patients who received a BC diagnosis at Sahlgrenska University Hospital in Gothenburg between 2007 and 2018. In total, 414 patients had one or more OPPMs prior to their BC and subsequent treatment. Consequently, the incidence of OPPMs increased from approximately 3% in 2007 to 8% in 2016 ($p<0.001$). A population-based study was then conducted for 5,132 BC patients diagnosed between 2007 and 2017 using data from the Swedish Cancer Registry at the National Board of Health and Welfare. Though not statistically significant ($p>0.05$), OPPM incidence rates increased (from 8% to 10%) during this time period. In the second work, FOXA1 and Nestin protein expression was found to be associated with prognosis and aggressive tumor features for metastatic BC. In the third work, 26 tumor pairs from young women (≤50 years) with BC and OPPMs were analyzed to identify common genetic alterations. Few genetic alterations were shared by the tumor pairs. In the fourth work, next generation sequencing analysis of a blood sample from an elderly BC patient who developed five MPMs within 16 years showed the presence of possible pathogenic variants in RAD51 and RAD54. Cancer diagnoses not only affect the physical and mental health of the patient but also close relatives, frequently due to changes in financial security (sick leave and high medical costs). For patients with MPMs, these burdens will naturally multiply. Therefore, it is important that we have a better understanding of MPMs to be able to identify patients at risk of developing MPMs at an early stage.

Keywords: breast cancer, multiple primary malignancies, other previous primary malignancies

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SAMMANFATTNING PÅ SVENSKA

Vi vet idag att bröstcancer (BC) är den vanligaste orsaken till cancerrelaterad död bland kvinnor i världen. På grund av tidigare upptäckt och mer skräddarsydda behandlingar lever dessutom patienterna allt längre med sin sjukdom. Studier världen över har visat att det har blivit allt vanligare att BC patienter får nya tumörer i andra organ än bröstet efter sin genomgångna BC behandling. Dessa tumörer är då inte utgångna från bröstet utan har ett helt annat vävnadsursprung, inte att förväxla med dottertumörer (metastaser). I tidigare studier har man förklarat andra maligniteter efter genomgången BC som delvis orsakat av själva BC behandlingen. Då andelen andra tumörer hos patienter med BC före genomgången BC behandling inte är lika väl undersökt, har ansatsen i denna avhandling varit att undersöka och beskriva förekomsten av dessa. Man har ämnat identifiera om det finns några specifika riskgrupper samt om det finns genetiska förändringar som skulle kunna förklara maligniteterna. Vi vet sedan tidigare vet att det finns 72 bröstcancergener varav 17 av dem är kopplade till andra maligniteter. Tillsammans med den kliniska utvecklingen av behandlingsmetoder har även diagnostiska möjligheter ökat inom patologin. Två specifika proteiner har ingått i avhandlingen som ett analytiskt led av potentiella framtida prognostiska markörer och för att bedöma brösttumörers aggressivitet.

Genom att undersöka de patienter som under 2007–2018 erhållit BC diagnos på Sahlgrenska universitetssjukhuset i Göteborg har vi samlat ihop kliniska data bestående av alla BC inom Göteborgs upptagningsområde (n=8031, arbete 1). Av dessa 8031 BC patienter hade 414 patienter en eller fler primära maligniteter före sin BC diagnos och behandling. Förekomsten 2007 av multipla primära maligniteter var ca 3% jämfört med 2018 då förekomsten var 8% ($p<0.001$). En epidemiologisk ansats gjordes genom ett populationsbaserat registerutdrag från Socialstyrelsen. I detta material kunde man inte se samma dramatiska ökning av förekomsten av multipla primära maligniteter även om trenden påvisades. Utav dessa 414 patienter har några riskgrupper identifierats. En av dessa är de 26 unga patienterna (50 år och yngre) som drabbats av flera primära maligniteter (arbete 3). Här har vi genom genetiska analyser på tumörklossarna undersökt huruvida det föreligger några gemensamma genetiska förändringar i patientens båda tumörer. Vi ser att det är större likhet mellan brösttumörerna än mellan de olika tumörparen. Vi har dock sett att det i vårt material finns kliniskt signifikanta förändringar i tumörerna som i sig kan ge andra maligniteter och som skulle vara intressanta att undersöka vidare i en större kohort. I arbete 2, analyserades två specifika proteiner (FOXA1 och Nestin) som ett led av potentiella framtida prognostiska markörer och för att bedöma brösttumörers aggressivitet. De patienter med FOXA1 uttryck hade en godare prognos till skillnad från de med uttryck av Nestin. I arbete 4 undersöktes en patient med 5 olika primära maligniteter genetiskt. Inte heller här kunde man hitta några övertygande genetiska förändringar.

Att drabbas av en cancersjukdom är en belastning och en utmaning för såväl kropp som själ. Det påverkar även patientens närstående och inte minst ekonomin med sjukskrivning och medicinska kostnader. Att drabbas av cancer flera gånger är naturligtvis ytterligare en belastning på dessa områden och en mycket dyrköpt erfarenhet. Eftersom vår kropp endast klarar en viss mängd cellgifter och

strålbehandling är det än viktigare att förekomma sjukdomen. Vi behöver därför urskilja dessa patienter med flera olika cancrar, för att ytterligare skräddarsy cancerbehandlingar och även uppföljningar. Kan vi redan i förtid förutse vilken patient som drabbas av vilken cancer, eller som i detta fall, vilka cancrar, vore det en vinst såväl mänskligt som samhällsekonomiskt. Vi ser härmed vikten av att fortsätta bedriva forskningen kring dessa frågor för om möjligt kunna förekomma sjukdom och lidande.

LIST OF PAPERS

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

I. **Nyqvist J**, Parris TZ, Helou K, Kenne Sarenmalm E, Einbeigi Z, Karlsson P, Nasic S, Kovács A. Previously diagnosed multiple primary malignancies in patients with breast carcinoma in Western Sweden between 2007 and 2018. Breast Cancer Res Treat (2020). DOI: 10.1007/s10549-020-05822-z.

II. De Lara S*, **Nyqvist J***, Werner Rönnerman E, Helou K, Kenne Sarenmalm E, Einbeigi Z, Karlsson P, Parris TZ, Kovács A. The prognostic relevance of FOXA1 and Nestin expression in breast cancer metastases: a retrospective study of 164 cases during a 10-year period (2004-2014). BMC Cancer (2019). DOI: 10.1186/s12885-019-5373-2. *=contributed equally

III. **Nyqvist J**, Kovács A, Einbeigi Z, Karlsson P, Forssell-Aronsson E, Helou K*, Parris TZ*. Genetic alterations associated with multiple primary malignancies. *=contributed equally (manuscript)

IV. **Nyqvist J**, Persson F, Parris TZ, Helou K, Kenne Sarenmalm E, Einbeigi Z, Borg Å, Karlsson P, Kovács A. Metachronous and synchronous occurrence of 5 primary malignancies in a female patient between 1997 and 2013: A case report with germline and somatic genetic analysis. Case Rep Oncol (2017). DOI: 10.1159/000484403.

CONTENT

ABBREVIATIONS

BC	Breast Cancer
B.C.	Before Christ
bp	Base pair
CTLP	Chromothripsis-like pattern
Chr	Chromosome
dCNA	DNA Copy Number Alteration
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FOXA1	Forkhead Box A1
GM	Gastrointestinal malignancies
gnomAD	The Genome Aggregation Database
HER2	Human Epidermal growth factor Receptor
HM	Hematopoietic malignancies
HRT	Hormone Replacement Therapy
IHC	Immunohistochemistry
MM	Malignant Melanoma
MPM	Multiple Primary Malignancy
OPPM	Other Previous Primary Malignancies

PgR	Progesterone Receptor
RCC	Regional Cancer Center
SCB	Statistiska Centralbyrån
SNP	Single Nucleotide Polymorphism
SU	Sahlgrenska University Hospital, Gothenburg
TM	Thyroid malignancies
UV	Ultra Violet

1 INTRODUCTION

Cancer is one of the leading causes of death worldwide [1]. Due to longevity and more effective diagnostic methods, the number of new cancer cases (incidence) is constantly increasing. Lung cancer is the most common cancer form worldwide, closely followed by breast cancer (BC) and prostate cancer [2]. However, cancer is not a new phenomenon. Cancer has even been found in dinosaur fossils and Hippocrates (460-370 B.C.) described cancer in humans as early as 400 B.C. In ancient Greece, human pathology was divided in four different fluids: black bile, yellow bile, mucus, and blood. Too much black bile was suspected to cause cancer [3].

The term "cancer" is currently used for a disease with abnormal, uncontrolled cell division and sometimes invasive properties that may occur in any cell, in any part of the body. These damaged cells ignore the normal signals for a cell to stop dividing and avoid programmed cell death (apoptosis). The type of malignant tumor is based on the site of origin. For example, abnormal uncontrolled cell division in the breast will lead to breast carcinoma. Likewise, abnormal uncontrolled cell division in the colon will lead to colon adenocarcinoma. This abnormal cellular activity is in part caused by genetic alterations in genes that control cell growth and division. These genetic changes (mutations) can be hereditary (germline changes) or caused by exposure to environmental toxins during an individual's lifetime (somatic changes). The three main genetic drivers of cancer include tumor suppressor genes (inhibit cell growth and division), proto-oncogenes (stimulate cell growth and division), and DNA repair genes (fix damaged DNA) [4].

Malignant tumors can be classified according to the cell type from which the tumor originates:

Carcinomas are formed by altered epithelial cells (cells that cover in- and outside surfaces) and are divided in specific categories. For example, adenocarcinoma (originates mainly in glands), basal cell carcinoma (from the basal layer in the skin), squamous cell carcinoma (e.g. skin, larynx, lungs) and transitional cell carcinoma (e.g. ureters, renal pelvis, the urinary bladder), etc. *Malignant melanoma* originates in the melanocytes which usually produce melanin most commonly in the skin but may also be represented in the eye and

in the surface covered with epithelial tissue. *Sarcomas* are formed by cells in soft tissues, such as muscles, fat, ligaments, tendons, joints, blood vessels, nerves, and lymph vessels. Sarcomas are divided into different types such as Kaposi sarcoma, liposarcoma, leiomyosarcoma. *Multiple myeloma* originates in plasma cells. *Lymphoma* is derived from T- or B-lymphocytes and mainly comprised of Hodgkin lymphoma or Non-Hodgkin lymphoma. *Leukemia* originates from blood-forming tissue (bone marrow) and is divided into four common types: acute myeloid, chronic myeloid, acute lymphoblastic or chronic lymphoblastic. There are also other tumor types such as *tumor in the central nervous system (*named by cells they originate from), *germ cell tumor* and *neuroendocrine tumor* (like carcinoids; produces and releases hormones) [5].

1.1 BREAST CANCER

1.1.1 ETIOLOGY & EPIDEMIOLOGY

Epidemiology

Breast cancer is one the most commonly diagnosed cancers among women. The lifetime risk of dying of breast cancer is approximately 3.4%, though breast cancer incidence varies from country to country. The highest incidence of breast cancer is in northern Europe and USA and lowest in Asia [6]. However, breast cancer incidence has been increasing since 1950 in both high-risk Western countries and lower risk countries. One explanation for this increase in incidence is longevity and mammography screening programs, such as those used in Sweden, England, Wales, and USA. However, breast cancer incidence nearly doubled in low-risk countries (Japan, Singapore, and China) where modern lifestyle changes were introduced [7, 8]. Migration affects the pattern of susceptibility for different cancers, including breast cancer. Previous studies suggest that lifestyle factors in the destination country influence the risk of developing breast cancer by adopting the lifestyle of the new country [9].

Etiology
The etiology of both breast cancer and multiple primary malignancies (MPMs) can be explained by four different factors: intrinsic, extrinsic, genetic, and therapeutic factors.

Intrinsic factors are defined as factors of embryonic and endocrine development and can be congenital or acquired. This category also includes immune status and susceptibility. Time for menarche, time for menopause, number of pregnancies and duration of nursing are all important factors that affect hormone levels throughout life. An early menarche, late menopause and/or nulliparity increases the risk of breast cancer.

Extrinsic factors are described as toxins, environmental factors, exposure to UV rays and lifestyle such as smoking, low physical activity, obesity, and alcohol consumption [10-26]. Age is also a risk factor for both breast cancer and MPMs [27].

Therapeutic factors, including hormone substitution could contribute to the risk of developing receptor-positive breast carcinoma.

Genetic factors play a role in the development of breast cancer and MPMs and will be discussed in a later chapter.

1.1.2 DIAGNOSIS

Breast cancer screening programs with mammography have been conducted in Sweden since 1977, starting as a trial in Dalarna and Östergötland (1977-1984). The trial showed that the mortality rate among women (40-74 years), could be reduced with 31% if mammography was performed every 24-33 months [28]. Nowadays, breast cancer screening is a routine procedure in Sweden that is performed every 18-24 months [29]. It is a well-known fact that individuals with high breast density constitute a 4 times higher risk of developing breast cancer. Even family history of breast cancer and heredity should be mentioned as risk factors [30]. Breast density decreases with anti-hormone therapy such as tamoxifen or raloxifene and increases in women with hormone replacement therapy (HRT).

1.1.2.1 BIOMARKERS (TRADITIONAL & FUTURE)

Different biomarkers are analyzed in the histologic sample/surgical specimen of suspected breast cancers. These biomarkers could be considered as either prognostic and/or predictive factors (Table 1). Prognostic markers provide information about clinical outcome at the time of diagnosis, regardless and independent of therapy (will the patient survive without treatment?). In contrast, predictive markers are dependent on therapy (what outcome may be expected with the planned therapy) [31, 32].

Nowadays, common markers for both prognostic and predictive clinical use are tumor size, histologic grade, lymph node status, ER, PgR, HER2 status, and Ki-67% [29]. Total extent in millimeters and focality (uni- or multifocal) are used as additional predictive histologic markers (Table 1). The most common histologic grading system in clinical use is the Elston/Nottingham score, which is composed of the assessment of a) tubulus formation (score 1-3), b) nuclear pleomorphism (score 1-3), and c) mitotic activity (score 1-3). The final total score (total score of 3-9) is then calculated by adding the scores of the individual factors, where a low score shows a highly differentiated tumor (total score 3-4-5, good prognostic outcome) and a high score shows a lowly differentiated tumor (total score 8-9, poor prognostic outcome).

Traditional markers (ER, PgR, HER2, Ki-67)

The estrogen receptor (ER) is a receptor in the nuclei of luminal epithelial cells of the breast. It is a transcription factor that controls cell proliferation by stimulating the growth of both tumor and normal cells [33]. The expression of ER and its distribution in the tumor tissue can be visualized using immunohistochemistry with monoclonal antibodies. In Sweden, the cut off point for ER-positivity is ≥10% positive tumor cells. Estrogen hormone is produced in the ovaries and peripheral fatty tissue. Tamoxifen therapy blocks the estrogen receptor and therefore reduces the amount of estrogen-related growth in ER-positive cells. Therapy such as aromatase inhibitors in post-menopausal women (or men) block conversion of testosterone and androstenedione in fatty tissue to estradiol and estrone.

Progesterone receptor (PgR) is also defined as a nuclear receptor, and is dependent on the estrogen receptor. However, the role of PgR is not yet fully

understood. Earlier studies suggest that PgR may play a role in lobular development during and after puberty [34, 35].

Human epidermal growth factor receptor (HER2), also called erbB-2 (shows homology with erythroblastosis-B-retrovirus of birds), is encoded by an oncogene known as *erbB2/HER2* that is located on chromosome 17 (17q12). The HER2 protein belongs to the ERBB family of receptor tyrosine kinases, which also includes epidermal growth factor receptor (EGFR, also known as ERBB1/HER1). HER2 expression can be detected (score 0, 1+, 2+, 3+) using immunohistochemical analysis (HercepTest). An amplification of HER-genes can be identified by *in situ* hybridization (ISH) using different tests such as FISH (fluorescent), SISH (silver), or CISH (chromogenetic).

The Ki-67 proliferation marker shows the fraction of Ki-67 positive tumor cells (the Ki-67 labeling index). Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent in resting cells (G0). Ki-67 is well accepted as a prognostic and predictive factor for breast cancer. A high Ki-67 value correlates well with poor outcome and higher sensitivity to chemotherapy [36].

Future markers (*FOXA1, NESTIN*)
Forkhead box protein A1 (FOXA1 or Hepatocyte nuclear factor 3-alpha/HNF-3A) is a DNA-binding protein encoded by the FOXA1 gene. FOXA1 is expressed in lung, colon, prostate gland, urinary bladder, liver, pancreas, and breast tissue. In ER-positive breast cancer, FOXA1 contributes to endocrine signaling (mediator of nuclear steroid receptor signaling via regulation of both androgen and estrogen receptor activity) and protein expression of ER, GATA3, and PgR, which in turn contributes to poor outcome and treatment resistance [37]. In ER-negative breast cancer, FOXA1-positivity means the opposite, i.e. improved disease-free survival and low-grade morphology [38].

Nestin (*NES*, neuroectodermal stem cell marker) is an intermediate filament type IV protein that is expressed in the axon (nerve cells) and stem cells, as well as, muscle cells. In 2007, Teranishi *et al.* showed that Nestin is an angiogenesis marker for proliferating endothelial cells in colorectal cancer tissue [39]. Triple-negative breast cancers have significantly higher *NES* mRNA

expression than the other breast carcinoma subtypes [40]. Nestin was significantly associated with angiogenesis and vascular invasion as a sign of early hematogenic spread, but not with lymphatic involvement [41].

1.1.2.2 MOLECULAR ANALYSIS

Nowadays, histopathologic reports are the basis for decisions to administer additional treatment such as chemotherapy. However, several upcoming prognostic multigene assays for breast cancer are commercially available (ProSigna/PAM50, Mammaprint, OncoTypeDX, MapquantDX, Theros, Mammostrat, Endopredict). The prognostic relevance of these kinds of tests has only been validated in patients with ER-positive, HER2-negative disease, but have yet to be validated for their predictive ability [29]. The aim with these tests is to be able to tailor treatment to a greater extent. In Scandinavia, the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay is used for node-negative patients (50–80 years) with grade 2 breast carcinomas measuring 10-50 mm. In cases where the test shows intermediate or high risk of recurrence, the patient will be offered adjuvant chemotherapy [42].

Table 1. Known prognostic and predictive markers for breast cancer [43-56]

	Prognostic *patient's overall cancer outcome regardless of therapy*	**Predictive** *the effect of a therapeutic intervention*
Tumor size *(in situ or invasive)*	X[47, 53]	
Total extent *(a total size including both in situ and invasive component)*	X[53]	
Histologic grade	X[47, 52, 53]	X[47]
Lymph node status	X[44, 46, 47, 50, 53]	
Focality *(uni- or multifocal)*	-	-
ER status	X[43, 45, 50, 53]	X[43, 45, 50]
PgR status	X[43, 45, 50, 53]	X[43, 45, 50]
Ki-67% *(proliferation marker)*	X[44, 53]	X[44]
HER2 status	X[43, 45, 50, 53]	X[45, 50]
FOXA1	X[54-56]	
Nestin	X[51, 54]	
ProSigna (PAM50-based) *Breast Cancer Prognostic Gene Signature Assay*	X[44, 53]	X [49]

1.1.3 TREATMENT

1.1.3.1 BC TREATMENT

Nowadays, treatment is individually tailored to each breast cancer patient during a multidisciplinary conference comprised of pathologists, oncologists, surgeons, radiologists, and nurses. Depending on the patients' physical status and based on the biomarkers of the breast tumor in the pathology report, a range of treatments are offered such as surgery, radiation therapy, optional anti-hormonal therapy, and HER2-blockade therapy. The size of the tumor relative to the breast size and axillary lymph node status are considered when choosing surgery type (partial or total mastectomy) and neoadjuvant chemotherapy. In the majority of cases, an analysis of the sentinel node is routine procedure. In cases of macro metastasis ($\geq$ 2 mm), axillary dissection is still the main choice of treatment in Sweden [29]. Neoadjuvant therapy may consist of both anti-hormonal therapy, chemotherapy, and eventually HER2-blockade therapy. Adjuvant treatment such as radiotherapy is routine for almost all patients who have undergone a partial mastectomy. Other adjuvant therapies such as chemotherapy, anti-hormonal therapy, and HER2-blockade therapy are entirely dependent on the patient's biological age, clinical stage, biomarker status in the pathology report and nowadays even gene expression analysis [29].

1.1.3.2 CONSEQUENCES OF BC TREATMENT

According to Spratt *et al.*, chemo- and radiation therapy increase the risk of developing cancer, while also increasing patient survival [57-61]. Chemotherapy increases the risk of hematological malignancies such as leukemia, while radiotherapy increases the risk of soft tissue malignancies in the thorax [60-62]. Chemotherapy also has a potential protective effect in other cancer types such as lung cancer, head & neck cancer, ovarian cancer, and colon cancer [59]. Anti-hormonal treatment of breast cancer could increase the risk of developing gynecological malignancies (endometrium). Hormonal substitutes given for menopausal symptoms act likewise [63].

1.1.4 GENETICS & BC IN THE EYES OF A SURGEON

The vast majority of breast cancer cases are sporadic due to random mutations. However, some genetic factors need to be taken into consideration because they play a role in both breast cancer and/or MPMs. A family history of breast cancer should be regarded as an important risk factor, particularly if the cancer occurred in early adulthood. Two well-known genes associated with hereditary breast cancer include pathological germline variants in *BRCA1* and *BRCA2* (Table 2). The lifelong risk of developing breast cancer with mutations in *BRCA1* is 65% and 45% for *BRCA2*, whereas the estimated lifelong risk of developing ovarian cancer with mutations in *BRCA1* is 39% and 11% for *BRCA2* [64]. Mutations in *CHEK2* 400 delC is associated with a two 2-fold increased risk of developing BC [65, 66].

When mutations in critical genes that control division, cell growth, and DNA repair occur, the risk for cancer development increases. There are two kinds of mutations, namely somatic and germline mutations. Somatic mutations are acquired during the lifetime of an individual and are represented only in the tumor cells in the breast tissue. Although these kinds of mutations are not inherited, they represent the most common cause of breast cancer development and progression. On the other hand, germline mutations occur in all cells and are inherited. Of course, lifestyle factors will contribute to increased risk of developing breast cancer. These germline mutations could have a high-, medium-, or low penetrance. A number of germline mutations in genes with high penetrance have been described. In these cases, family clusters of breast cancer alone or together with other malignancies are known in both men and women [67]. Approximately 1-5% of breast cancers are estimated to be due to the inheritance of highly penetrant BRCA1 or BRCA2 germline mutations[68]. The number of patients with these types of mutations is higher in younger patients[69].

Table 2. Genes and germline mutations associated with specific malignancies

Function of protein	Gene	Chr	Organ	Syndrome
Tumor suppressor	*BRCA1*	17q21.31	Breast, ovary[70]	
	BRCA2	13q13.1	Breast, ovary[70], stomach[71]	
	PTEN	10q23.31	Breast[72-74]	Cowden[75]
			Endometrium[72-74], thyroid[72-74]	
	CDH1	16q22.1	Breast, Diffuse gastric cancer[76, 77]	
	TP53	17q13.1	Breast, leukemia, adrenal cortex malignancies, brain tumor[78, 79]	Li-Fraumeni[77, 78, 80, 81]
	STK11 / LKB1	19p13.3	Breast, ovary, GI-malignancies[82]	Peutz-Jeghers syndrome[83-85] (Hamartom in GI)
DNA repair	*RAD51*	15q15.1	Breast[86]	
	CHEK2 / RAD53	22q12.1	Breast, prostate, sarcoma, colon, lung, thyroid[87, 88]	E-cadherin loss; favors metastases

	ATM	11q22.3	Breast, lymphoma[89]	

Mutations in a number of these genes increase the risk of developing breast cancer, but also several other types of cancer during an individual's lifetime (Table 2). Although these mutations occur in much less than 1% of the population, cross talk between several genes has been found, regardless of the level of penetrance[88, 90]. Nor is it just as simple as distinguishing genes and their significance from one another. Stolarova *et al.* and Laitman *et al.* describe the ATM-CHEK2-p53 axis. After DNA damage, the CHEK2 protein is activated by ATM and subsequently activates BRCA1 and TP53. BRCA1 plays a lead role in DNA repair and apoptosis [88, 91-93].

Somatic mutations in *TP53* occur in almost every cancer. Cowden syndrome (PTEN) [75] , Lynch syndrome (*MSH2, MLH1, MSH6, PMS2*) [94-97], Peutz-Jeghers syndrome (STK11/LKB1) [83-85], hereditary breast and ovarian cancer (BRCA1/2) [70, 84], Li-Fraumeni syndrome (TP53) [77, 80, 81] are examples of known and established syndromes associated with multiple primary malignancies and germline mutations. In addition to well-known genetic variants, Ghoussaini *et al.* describes potential gene drivers located in close proximity to breast cancer susceptibility loci (Figure 1).

Moreover, genetic variation between different individuals occur that are called single nucleotide polymorphisms (SNPs). Each SNP represents a change in a single nucleotide (adenine, thymine, guanine, cytosine) in the DNA, for example an adenine (A) is replaced with a thymine (T). This replacement is to some extent normal and contributes to our different looks and personalities. Consequently, different SNPs can help us identify disease-associated genes if they occur in the same region as a regulatory gene. SNPs also pinpoint differences in our susceptibility to a wide range of diseases such as cystic fibrosis, sickle-cell anemia, and β-thalassemia [98-104].

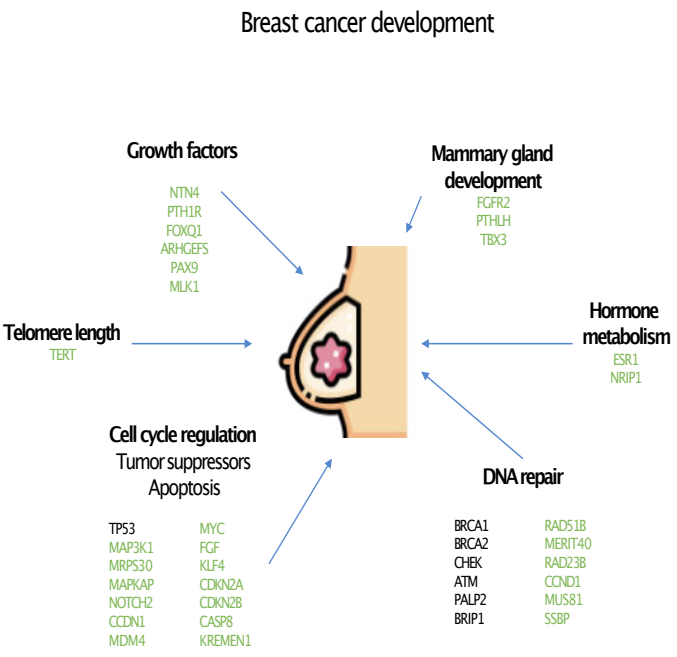

Figure 1. **Different pathways involved in the development of breast cancer.** Genes in green represent those located in close proximity to breast cancer susceptibility loci. Ghoussaini *et al.* describes that it is unclear whether such genes are genetic drivers or not (except, MYC, FGFR2, CCND1, and CASP8) [105]. Free picture of breast cancer from https://www.flaticon.com/.

1.2 MULTIPLE MALIGNANCIES

1.2.1 ETIOLOGY & EPIDEMIOLOGY IN BC & MPM

Epidemiology
As survival rates for cancer patients have improved during the past 40 years, the risk for further primary malignancies has increased with age [65, 106]. Early genetic events might influence the development of several primary malignancies at different times throughout an individual's life due to a latency period. In addition, Rubino *et al.* describes a 23–40% increased risk of a second primary malignancy in breast cancer patients (not including contralateral breast cancer) after chemotherapy (leukemia), irradiation (lung cancer, sarcoma of thorax and upper limb, esophagus cancer, thyroid gland carcinoma), and hormone therapy (gynecological malignancies) [107]. According to Donin *et al.*, approximately 17% of all yearly reported malignancies consists of multiple primary malignancies. Further, almost 1 of 12 (8.1%) cancer patients developed another primary malignancy, of which bladder malignancies were the most common first primary malignancy and lung cancer was the most common second malignancy in this study [59].

Etiology
As previously described, the etiology of both breast cancer and MPMs can be divided into four different factors: intrinsic, extrinsic, genetic, and therapeutic factors [63, 65, 108].

Intrinsic factors are connected to embryonic and endocrine development and can be congenital or acquired, including immune status and susceptibility. Toxins, environmental factors, exposure to UV rays are explained as *extrinsic* factors. Lifestyle habits such as smoking, low physical activity, and increased alcohol intake are also included here [10, 65] Age is an also a risk of both breast cancer and MPMs [27]. The number of pregnancies, duration of lactation, age at first childbirth, as well as, obesity and diet increase the risk of developing breast- and gynecological malignancies. *Therapeutic* factors could be one of

the reasons for the development of MPMs, but not breast cancer in itself. According to Spratt *et al.*, chemotherapy and radiation treatment increase the risk of developing cancer while also improving survival[57, 58]. Chemotherapy also has a potential protective effect in other subsequent cancers such as cancers of the lung, head & neck, ovary, and colon. Anti-hormonal treatment in breast cancer care could increase the risk of developing gynecological malignancies. Hormonal substitutes given for menopausal symptoms may act likewise [109].

1.2.2 GENETICS OF BC & MPM IN THE EYES OF A SURGEON

Germline mutations in the *BRCA1/2* tumor suppressor genes may explain some of the cancers as ovarian- & stomach malignancies. There is also examples of other syndromes associated with MPMs, such as Li Fraumeni syndrome type II and von Hippel Lindau syndrome[109-112]. Ghoussaini *et al.* described 72 loci in the human genome that are associated with breast cancer. Seventeen of which, for example *p53*, *KRAS*, *ERBB2*, *CDKN2A*, and *NF1*, are associated with breast cancer and MPMs [105]. The specific seventeen loci with breast cancer susceptibility located in regions associated with other malignancies of importance are located on chromosomes 1q32, 2p24, 2q31, 4q24, 5p12, 5p15, 6q25, 8q24, 9p21, 9q31, 10p12, 10q26, 11p15, 11q13, 12q24, 14q24, and 19p13. Ghoussaini highlights four of these genetic regions (5p15, 8q24, 9p21, 11q13) due to their strong association with other malignancies. The *TERT* gene, located on chromosome 5p15, is associated with glioma, lung-, pancreatic-, and basal cell cancer [113]. Six different malignancies including colon-, rectal-, and ovarian malignancies are associated with mutations in the 8q24 region (rs6983267) [113]. In the 9p21 region, tumor suppressor genes such as *CDKN2A* and *CDKN2B* are associated with seven malignancies such as glioma, lymphoblastic leukemia, basal cell carcinoma, melanoma, nasopharyngeal carcinoma, breast and pancreatic cancers. In addition, this loci is also associated with type 2 diabetes, myocardial infarction, cutaneous nevi, intracranial aneurysm, and sporadic amyotrophic lateral sclerosis [113]. In the region of 11q13, *CCND1* and several *FGFs* are found. These genes are

associated with prostate-, renal-, and breast cancers [113]. SNPs in the 12q24 region are associated with squamous esophageal carcinoma, breast cancer, liver adenoma, renal cell carcinoma, heart diseases, type 1 diabetes, blood pressure, and prostate specific antigen level and is located close to the *TBX3* (mammary gland development) and *MAPKAP* genes [105, 113].

In a study regarding MPMs, Stathopoulos *et al.* compared gene expression patterns in the peripheral blood of patients with single primary malignancies with those in patients with multiple primary malignancies [65]. A statistically significant difference was found in the expression patterns of nine genes. In addition, Stathopoulos *et al.* described three deregulated pathways of interest (pathways connected to heme biosynthesis, ubiquitin proteasome, and apoptosis signaling) in the group of multiple primary malignancies compared to single primary malignancies, all of which are associated with cancer development [65].

2 SPECIFIC AIMS

The overall aim of this thesis was to provide insight into the epidemiology and genetics of previously diagnosed primary malignancies in patients with breast cancer, thereby warranting the development of tailored follow-up programs for potential risk groups.

To investigate this, the specific aims were:

2.1 PAPER I

To assess the incidence of and characterize other previous primary malignancies (OPPMs) in patients with breast cancer at Sahlgrenska University Hospital between 2007 and 2018.

2.2 PAPER II

To evaluate the prognostic significance of FOXA1 and Nestin in metastatic breast cancer patients.

2.3 PAPER III

To identify common somatic genetic alterations in tumor pairs from patients diagnosed with breast cancer and OPPMs.

2.4 PAPER IV

To explore if any constitutional mutation or pathogenic variant could be identified in an elderly patient with five primary malignancies.

3 MATERIALS AND METHODS

3.1 PAPER I

Patient selection
During 2007-2018, 8,031 patients were diagnosed with primary breast cancer at Sahlgrenska University Hospital and included in the study. The clinical records (Melior) and pathology reports (Sympathy) for each patient were reviewed to assess whether they had any other previous malignancies. These data were validated using information provided by the Swedish Cancer Registry and the National Board of Health and Welfare. Due to the high prevalence of common skin tumors, basal cell carcinoma and squamous cell carcinoma were excluded.

As the time at risk per person may differ and patient data may be incomplete due to relocation, a population-based study was also conducted using data from the Swedish Cancer Registry (2007-2017) for four municipalities in the Gothenburg region (Gothenburg, Härryda, Mölndal, and Kungälv municipalities). Data were not yet available for 2018 when the study was performed. The identified breast cancer patients (2007-2017) were traced for any OPPM from the start of the Swedish Cancer Register 1958 until their breast cancer diagnosis. This resulted in 49 years as the longest time period before the onset of breast cancer to ensure the same length of time at risk for each patient. Male patients, metastases, benign tumors and the most common skin tumors (i.e. basal cell carcinoma and squamous cell carcinoma) were excluded from the study due to integrity issues. Very unusual types of malignancies were categorized as “other type”.

The ICD7 (International Statistical Classification of Diseases and Related Health Problems, WHO classification of diseases from 1952; ICD7 from 1958) and the histopathology diagnosis codes (SNOMED) were used to identify patients and their malignancies in the Swedish Cancer Registry. A multi-step procedure was performed to a) evaluate the prevalence of OPPMs in breast cancer patients diagnosed from 2007 to 2017, b) investigate which

malignancies each patient was diagnosed with before their breast cancer diagnosis (2007-2017), and c) evaluate the order of the OPPM diagnoses. Due to integrity issues, patients were divided into age categories at 10-year intervals (<49, 50-59, 60-69, 70-79, 80+).

Statistical analysis

Statistical analyses were performed concerning frequencies and percentages for categorical variables and as mean and range for continuous variables. Chi-square test was used to compare groups of categorical variables. A possible change over time with respect to frequencies/percentages of patients with another primary malignancy was tested by "linear-by-linear" Chi-Square test and by logistic regression with MPMs (yes or no) as outcome and year as explanatory variable. P-value <0.05 was considered to be statistically significant. The IBM SPSS v.25 statistical package was used for statistical analyses.

3.2 PAPER II

Patient selection

In total, 162 patients were diagnosed with breast cancer metastasis between 2004 and 2014 at the Department of Clinical Pathology at Sahlgrenska University Hospital (Gothenburg, Sweden). Consequently, two of the 162 patients were found to have metastases in more than one anatomical location, and hence 164 breast cancer metastases from different anatomical sites were examined. Only 9/164 metastases were regional axillary lymph node metastases.

Immunohistochemical analysis

The 164 breast cancer metastases were examined for mammaglobin, ER/PR, CK7, CK20, and HercepTest (at the time of diagnosis) and retrospectively analyzed by immunohistochemistry (IHC) for GATA3, FOXA1, and Nestin expression. Immunostaining on full-face formalin-fixed paraffin-embedded (FFPE) specimens was evaluated by a breast pathologist, blinded to patient clinical outcome. Nuclear staining of breast luminal epithelial cells was

considered to be positive for GATA3 (cutoff ≥1%), FOXA1 (cutoff ≥1%), and Nestin (cutoff ≥1%) protein expression.

Statistical analysis

A 0.05 *P*-value cutoff was used in R/Bioconductor (version 3.3.2) and all *P*-values were two-sided. Using Fisher's exact test two-tailed test, the relationship between clinicopathological features and FOXA1 and Nestin protein expression patterns was evaluated. Overall survival (OS) and distant metastasis-free survival (DMFS) were calculated by univariate Cox proportional hazard model for FOXA1 and Nestin. Multivariate analysis was conducted using the Cox proportional hazard model for OS and DMFS with FOXA1 and Nestin expression after adjusting for clinicopathological features (age at diagnosis, metastatic site, Mammoglobin status, GATA3 status, histological grade, axillary lymph node status, ER/PgR status, HER2/*neu* status, and triple-negative status). The definition of survival rates was specified as a) time from diagnosis of the primary breast malignancy to death from any cause for OS and b) time from diagnosis of the primary breast cancer to distant metastasis for DMFS. Kaplan–Meier curves were used to analyze survival rates and tested with log-rank test.

3.3 PAPER III

Patient selection

In total, 414 of 8,031 patients (described in **Paper I**) with primary breast cancer were diagnosed with OPPMs at Sahlgrenska University Hospital (Gothenburg, Sweden)[114]. Of the 414 breast cancer patients with OPPMs, 26 patients were ≤ 50 years and were regarded as young patients. Clinical data for the patients were collected from the Swedish Cancer Registry, the National Board of Health and Welfare, and Sahlgrenska University Hospital (Departments of Clinical Pathology and Oncology). A breast pathologist confirmed the different tumors as primary malignances (not metastases) using formalin-fixed paraffin-embedded (FFPE) sections stained with hematoxylin and eosin. Only one patient had three tumors (patient 25) the rest of the patients had two primary malignancies each, including breast cancer.

OncoScan CNV Plus Assay

Genomic DNA was extracted from two to three 10 µm FFPE sections for the 53 tumor samples using the AllPrep DNA/RNA. Of the 53 samples, 47 were analyzed by Affymetrix OncoScan® Arrays according to standard protocols at the Array and Analysis Facility (Uppsala University, Uppsala, Sweden) regarding genome-wide copy number alterations and mutations. Due to low DNA concentration or lack of DNA amplification, five samples were excluded. Only pairwise samples (A and B samples) were included in the analysis. Sample 25C was therefore excluded. The OncoScan somatic mutation panel consisted of 64 mutations in nine genes (*BRAF*, *EGFR*, *IDH1* and *2*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, and *TP53*). DNA copy number and mutation analysis, similarity and clonality analysis, and genetic instability analysis were performed to identify common genetic alterations between the tumor pairs or within the tumor groups.

DNA copy number and mutation analysis

Mutations identified in the OncoScan somatic mutation panel (*e.g.* missense mutations) and allelic imbalance data (*e.g.* $\log_2$ratio, allele difference, BAF, and LOH) were extracted from ChAS. Additional analysis to compare genomic profiles for the two tumors in the same patient or between different cancer diagnosis was performed using Nexus Copy Number (BioDiscovery v8.1) with normalized OSCHP files and a 25% differential threshold between groups (P<0.05). Descriptive statistics (mean ± standard error of the mean (SEM) and range) for the number of genetic alterations in each tumor were calculated using Microsoft Excel (v16.16.27). Box plots were constructed using the ggplot2 (v3.3.1) and ggpubr (v0.3.0) R packages with the Wilcoxon test.

Similarity and clonality analysis

To evaluate whether the genomic profiles for tumors from the same patient were similar, hierarchical clustering, calculation of the Similarity Index (SI), and clonality testing was performed. Tumors from the same patient were considered to be similar if they clustered together in the terminal branch of the dendrogram. SI was calculated by determining unique, shared, and opposite CNA or LOH changes between tumor pairs using CNA $\log_2$ratio thresholds. Clonality were tested to determine whether tumors from the same patient were clonal or independent entities.

Genetic instability analysis
To identify genetically unstable tumors, three analyses were performed with segmented CNA data, i.e. Genetic instability index (GII), Complex arm-wise aberration index (CAAI), and Chromothripsis-like pattern (CTLP) detection. CAAI detects complex focal rearrangements in the genome containing narrow regions of high copy number gain.

3.4 PAPER IV

Patient selection
When analyzing the cohort of patients with breast cancer and OPPMs (**Paper I**) at Sahlgrenska University Hospital between 2007 and 2018, a female patient with five different malignancies was identified. To ensure that all five malignancies constituted five primary tumors and not metastases, immunohistochemical staining with cytokeratin 7, cytokeratin 20, mammaglobin, and GATA3 was performed.

Genetic mutation analysis
Genetic mutation analysis was performed using DNA extracted from EDTA blood samples. The DNA sample was fragmented by ultrasonication, and custom SureSelectXT library kits (Agilent) were used to capture fragments from target genes in gene panels including *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CDH1*, *PALB2*, *RAD51C*, *RAD51D*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*, *APC*, *MUTYH*, *STK11*, *BMPR1A*, *SMAD4*, *PTEN*, *POLE*, *POLD1*, *GREM1*, and *GALNT12* high-risk cancer genes. The patient sample was analyzed with respect to possible mutations in the coding regions and splice sites. Variants detected with next-generation sequencing were confirmed with Sanger sequencing.

4 RESULTS AND DISCUSSION

4.1 PAPER I

During the past 40 years, the overall survivor rates for patients with malignant tumors have improved due to individually tailored therapies and screening programs. During a 12-year period (2007-2018), the prevalence of new diagnosed breast cancer patients with OPPMs (n=414) at Sahlgrenska University Hospital increased from 17 to 59 patients yearly (2.6-8.2%; $P<0.001$; Table 3). Our study revealed a significant increase in the number of OPPMs in breast cancer patients, which correlated well with other international studies[115-117]. In this study, we also attempted to validate these findings using population-based data found in the Swedish Cancer Registry at the National Board of Health and Welfare. These data showed an increasing trend of OPPMs from 2007 to 2017, though not statistically significant. This could be due to selection bias and differences in the person time at risk.

Table 3. The distribution of patients with newly diagnosed breast cancer and OPPMs comparing pathology reports and medical records from Sahlgrenska University Hospital (2007-2018) with data from Swedish Cancer Registry (2007-2017).

	Sahlgrenska University Hospital **2007-2018**		**Swedish Cancer Registry** **2007-2017**	
	Patients with newly diagnosed breast cancer **(n = 8,031)**	**Breast cancer patients with OPPMs** **(n = 414)**	**Patients with newly diagnosed breast cancer** **(n = 5,132)**	**Breast cancer patients with OPPMs** **(n = 473)**
2007	**545**	**18** (3.3%)	**394**	**32** (8.1%)

2008	**657**	**17** (2.6%)	**468**	**37** (7.9%)
2009	**521**	**21** (4.0%)	**347**	**19** (5.5%)
2010	**688**	**32** (4.7%)	**471**	**53** (11.3%)
2011	**678**	**29** (4.3%)	**485**	**41** (8.5%)
2012	**634**	**29** (4.6%)	**439**	**45** (10.3%)
2013	**731**	**27** (3.7%)	**477**	**44** (9.2%)
2014	**800**	**54** (6.8%)	**520**	**55** (10.6%)
2015	**745**	**49** (6.6%)	**506**	**44** (8.7%)
2016	**718**	**59** (8.2%)	**506**	**49** (9.7%)
2017	**725**	**40** (5.5%)	**519**	**54** (10.4%)
2018	**589**	**39** (6.6%)	**-**	**-**
		P <0.001[1]		P <0.075[2]

([1] p-value for trend over time, tested by Poisson regression for counts and by linear regression with rates/fractions as outcome, p-value=0.075 in both cases)

The number of primary breast cancer cases identified at Sahlgrenska University Hospital during the study period agreed with data from the Regional Cancer Center (RCC). The number of breast cancer patients may have differed between the two cohorts (Sahlgrenska University Hospital and the Swedish Cancer Registry) due to different coverage areas. To obtain data from the Swedish Cancer Registry, we needed to specify which municipalities we wanted data from. We chose four municipalities, namely Gothenburg, Kungälv, Härryda, and Mölndal, which all are included in the catchment area for Sahlgrenska University Hospital. However, there have been changes to the catchment area in recent years, which could have led to different statistical outcomes.

The most commonly occurring OPPM included malignant melanoma, gastrointestinal malignancies, and gynecological cancers. The increased prevalence of malignant melanoma could be due to changes in lifestyle habits regarding tanning and vacationing combined with the very light skin complexions of most Swedish people. The increase in gynecological malignancies could in part be explained by modern lifestyle, where many

women have children later in life. Apart from this, we are also aware of the known *BRCA1* and *BRCA2* mutations that are associated with elevated risk of developing both breast- and ovarian cancer. Studies have revealed that number of *BRCA1* and *BRCA2* are prevalent in western Sweden[118, 119]. The increase in gastrointestinal malignancies can be due to modern eating habits and obesity [120]. The population is getting older, both due to better treatments and, in certain, population groups healthier lifestyles, which also increases the risk of developing malignancies.

Spratt *et al* proposes a theory regarding different mutations and doubling rate/time. [57, 58]. If we suppose that a mutation has appeared in the genome, that particular mutation could lead to several different primary malignancies but in different organs and time points. The malignancies with different origin have various proliferation rates resulting in different clinical manifestation. Namely, when one malignant tumor (tumor #1) has already been detected, there can still be a subclinical tumor (tumor #2) at another anatomical site. When tumor #1 is treated by chemotherapy, at the same time subclinical tumor #2 may disappear without being diagnosed.

During this study, the question arose whether the histopathology of BC+OPPM differed with those in patients with only BC. After receiving data from National Board of Health and Welfare for 2018, this analysis was possible (Table 5). Both cohorts were relatively similar, with the exception of fewer HER2-positive and PgR-positive BCs, larger tumor size, and higher number of BCs with axillary lymph node metastases in the SU dataset. This would result in differences in staging: tumor stage pT1 in the national data among patients with only BC compared to a higher tumor stage, pT2, among patients with BC and OPPM. Tumor size is one of the most important prognostic factors for BC (besides axillary lymph node metastases, tumor differentiation grade and histopathological subtype: e.g. ductal, lobular morphological subtypes, etc.). Although tumor size is an independent prognostic factor by itself, it is not sufficient because breast cancer prognostication demands on all the four above-mentioned factors.

Table 4. Comparison of histopathological data for BC patients with OPPMs and patient with only BC. Differences marked in bold.

	Newly diagnosed primary invasive breast carcinomas in Sweden in 2018 according to National Board of Health and Welfare (17.02.2020)			**Newly diagnosed primary invasive breast carcinomas with OPPMs at Sahlgrenska University Hospital, Gothenburg, Sweden between 2007-2018 according to Sympathy and RCC**	
n	**7735**			**414**	
Histological grade	Grade 1	21.1%		Grade 1	19.0%
	Grade 2	52.9%		Grade 2	56.3%
	Grade 3	26.0%		Grade 3	24.7%
ER-status	Positive	86.0%		Positive	84.7%
PgR-status	Positive	71.4%		Positive	**60.6%**
HER2-status	Positive	13.8%		Positive	**7.3%**
Ki-67	Low <10%	35.4%		Low	38.9%
	Intermediate 10-19%	16.4%		Intermediate	14.5%
	High >20%	48.2%		High	46.6%
Tumor size	mm	19.0 mm (T1≤2cm)		mm	**28.1 mm (T2>2cm)**

Axillary lymph node	Metastasis (yes)	25.8%		Metastasis (yes)	**29.9%**

These data suggest that unknown or poorly described gene mutations and syndromes may drive the development of multiple synchronous and metachronous primary malignancies. In future studies, we plan to explore this hypothesis in different subgroups of the current study cohort, i.e. patients with at least three different primary malignancies and patients under 50 years of age with breast cancer who developed two different primary malignancies. Another possible explanation for the increase in MPMs is the aging population with defective DNA repair mechanisms combined with Western lifestyle-related factors. Life expectancy in Sweden increased with almost 2 years during 2007-2018 (for women 83.0-84.3 years of age; Figure 2).

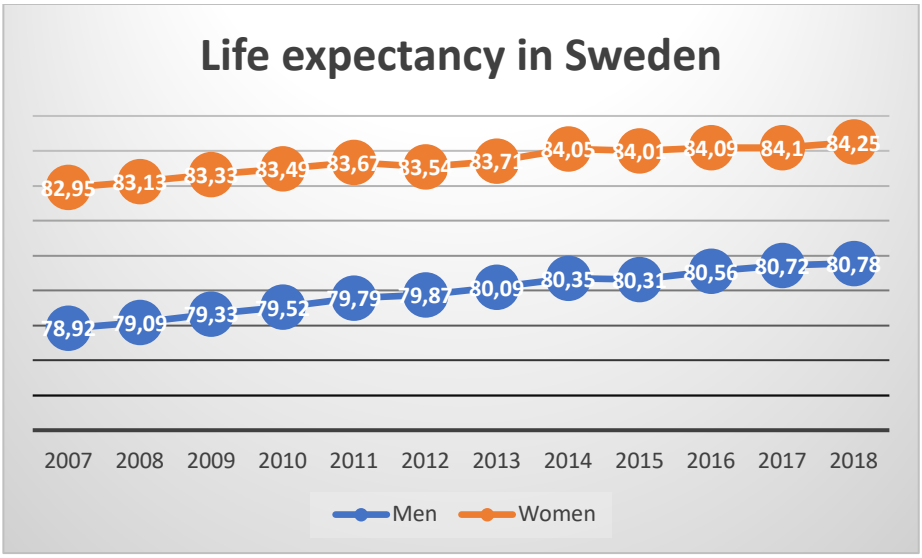

Figure 2. Life expectancy in Sweden (2007-2018) according to data from SCB.

Summarized with the above-mentioned data, more studies need to be done to ensure these results even with a nationwide cohort.

4.2 PAPER II

Before the era of molecular pathology, the only diagnostic tool for stratification of patients with breast cancer was IHC. There was an attempt to identify additional biomarkers besides ER, PR, Ki-67 and HercepTest that could be used to individually tailored breast cancer treatment. In this study, FOXA1 and NESTIN protein expression were evaluated in 162 female BC patients with 164 BC metastases. The average age at the time of breast cancer metastasis was 62 years. The youngest breast cancer patient with BC metastasis was a 31 year old and the oldest patient was 90 years of age, both of which had axillary lymph node metastasis. Of the 164 BC metastases, 11 were detected in the regional axillary lymph nodes (7%). Nine of the 162 patients had only regional axillary metastases. The other 155 metastases were distantly located: 27% in the abdomen, 23% in the bones, 18% in the brain, 12% in the thorax/lungs, 7% in the skin, 5% in the pelvis, and 2% in the lymph nodes in the neck region.

When the pathologist analyzes a metastasis, it is necessary to verify the primary tumor site. Nowadays all breast cancer metastases are stained not only with standard biomarkers for BC (ER, PR, Ki-67 and HercepTest), but even with GATA3- and Cytokeratin 7 antibodies to confirm that the primary tumor originated from mammary tissue. An overall assessment of FOXA1 and Nestin protein expression was performed using IHC on FFPE slides for the breast cancer metastases, without focusing solely on hot spots. For each BC metastasis, the pattern of nuclear FOXA1 and cytoplasmic Nestin staining was evaluated together with the staining extent (% of positively stained tumor cell nuclei for FOXA1 and tumor cell cytoplasm for Nestin; Figure 3).

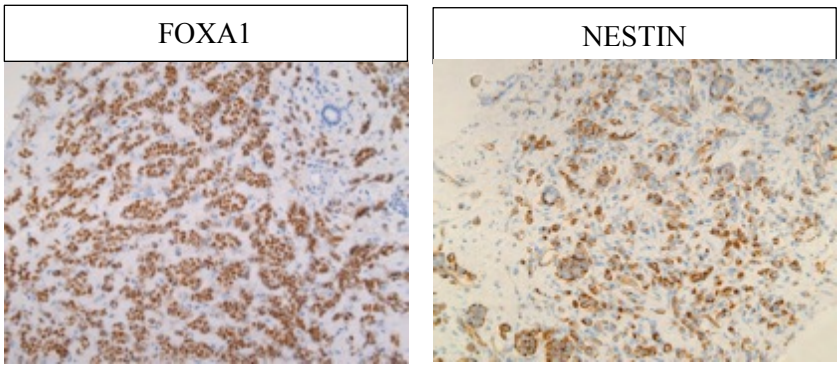

Figure 3. Breast cancer metastasis in the liver: positive FOXA1 and Nestin immunostaining (nuclear staining with the FOXA1 antibody and cytoplasmic staining with the Nestin antibody).

Positivity thresholds (cut-off point) were set at ≥1% for FOXA1 and Nestin. However, < 20% was rarely noticed among positive cases, with only 4 metastases showing less than 20% positivity with FOXA1 immunostaining (2.4%) and only ten cases showing less than 20% positivity with Nestin staining (6.1%). Of 164 BC metastases, 6 cases were FOXA1 and Nestin immunopositive (3 cases in the pleura, 1 metastasis in the brain, 2 metastases in the liver). All six double positive metastases belonged to the Luminal B subtype. Moreover, 2/6 metastases were even HER2 amplified. In the 15 double negative (FOXA1- and Nestin-negative) metastases, 2 belonged to the triple-negative molecular subtype (1 in the brain and 1 in the skin). Only one bone metastasis belonged to the Luminal B/HER2-positive subtype. Twelve cases of double negativity belonged to Luminal B/HER2-negative subtype (five metastases in the bone, three metastases in the ovarium, one metastasis in the liver, one metastasis in the esophagus, and one metastasis in the axilla).

Those breast cancer patients ERα-positive and FOXA1-positive tumors frequently had a better clinical outcome. On the contrary, expression of Nestin was found to be a marker of poorer clinical outcome, mainly in patients with triple-negative status. In a review article made by Zhang *et al.* the link between Nestin and its role as an independent prognostic factor for worse BC- specific survival and overall survival were confirmed [51]. Taken together, FOXA1 and Nestin expression in breast cancer metastases were correlated with specific

breast cancer subtypes (luminal phenotype and triple-negative subtype) and may therefore be regarded possible future prognostic biomarkers.

4.3 PAPER III

In **paper III**, genome-wide profiling was used to identify potential biomarkers associated with common somatic genetic alterations in primary tumors (BC and OPPM) from the same patient. Pairwise primary tumors (not to be confused with metastases) from 26 young breast cancer patients (≤ 50 years) with OPPMs were analyzed to evaluate whether somatic genetic alterations were accountable for the occurrence of the MPMs and could therefore be possibly used to guide future cancer treatment choices for patients with MPMs. To the best of our knowledge, this is one of the first studies to assess an association between somatic genetic alterations and MPMs. Martin *et al.* performed both germline and somatic genetic analyses in a patient with four different primary tumors without identifying any common patterns [121], which is in agreement with the present study. Although all tumors (n=47) were found to be genetically unstable, BC had the highest number of dCNAs. These findings are in agreement with a study by Zhou *et al.*, which demonstrated that breast carcinomas had the highest number of driver somatic dCNAs [122].

According to the Oncoscan mutation panel, *TP53* mutations were frequently identified in BC, MM, and HM. Interestingly, only 8 tumor pairs (patient numbers 3, 11, 13, 18, 20, 22, 23, and 26) were characterized as "similar" (i.e. common genetic alterations in the tumor pairs) according to the clustering analyses with dCNA data (Figure 4). However, BC, MM, and TM frequently clustered together. Genetic alterations on chromosomes 1, 11, and 17 were frequently detected in BC specimens, suggesting that these genetic changes were breast cancer-specific. Takehisa *et al.* and Reinholz *et al.* postulated that LOH on chromosomes 1, 11, and 17 were indicators of genetic instability and may serve as prognostic factors of poor outcome in breast cancer patients [123, 124]. Therefore, these analyses demonstrated that the tumor biology of samples representing a specific cancer type were more genetically similar than tumor pairs from the sample patient. These data further confirm that the tumors included in the study were not metastases, but primary malignancies.

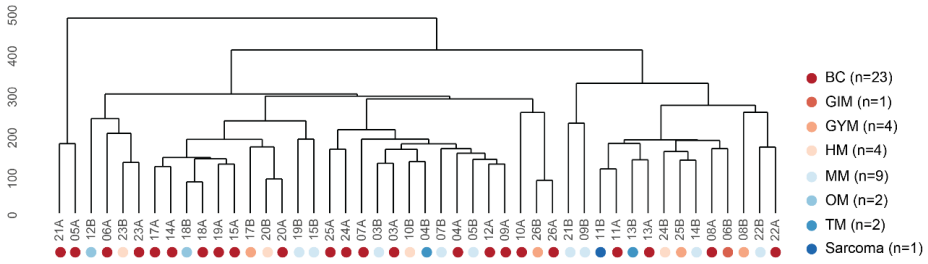

Figure 4. Hierarchal clustering analysis using dCNA data from Oncoscan. Each patient is labeled with a number, where tumor pairs are labeled as 'A' for breast cancer (BC) samples and 'B' for all other cancer types; GIM, gastrointestinal malignancies; GYM, gynecological malignancies; HM, hematological malignancies; MM, malignant melanoma; OM, oral cavity malignancies; TM, thyroid malignancies.

Although previous studies have been done with regard to MPMs in BC patients, most studies focused on MPMs arising after breast cancer treatment [116, 117, 125], suggesting that the treatment itself contributed to development of the MPMs [117, 125]. In contrast, the main focus of the present study was to reveal whether MPMs from the same patient contain common genetic alterations, indicating similar susceptible regional DNA origins

This study is unique in that we chose to select breast cancer patients with OPPMs diagnosed before breast cancer treatment. Here, the fact that BCs showed the highest number of dCNAs compared to OPPMs could be explained by time-related factors, as the patient was older when the BC was diagnosed and more genetic alterations could be accumulated. It could also be the result of cytotoxic harm due to previous treatment [27, 57, 58, 63]. Multiple primary malignancies in an aging population is to be expected due to the accumulation of genetic alterations during an individual's lifetime [63, 126-128]. However, we found young MPM patients (≤50 years) to be interesting, as they had already developed more than one primary malignancy at such a young age.

Immunohistochemical examination of the BCs included in the cohort showed a non-favorable histopathological diagnosis with a high proportion of lymph node-positive patients. Cancello *et al.* and Azim *et al.* linked poor prognosis for breast cancer patients with diagnosis at a young age, large tumor size at diagnosis, mitotic rate, higher tumor grade, node-positive status, high HER2 expression, and lower ER and PgR expression [129, 130]. Cancello *et al.* also concluded that young women with breast cancer generally have a more unfavorable outcome compared to elderly patients [129]. Taken together, these findings warrant further investigation into ways to predict which cancer patients are at risk of developing additional primary malignancies, especially young patients.

In the present study, malignant melanoma was the most frequent OPPM (11/26, 42%) occurring before the BC diagnosis. Wilson *et al.* described that dCNAs (both losses and gains) as well as tumor-specific somatic mutations (e.g. in *BRAF, NRAS, KIT*) play an integral role in melanoma pathogenesis [131]. Further, loss on chromosomes 4, 6q, 9, 10, 11, 13, 16, and 18 and gains on 1, 6p, 7, 17q, and 20 have been reported [131-136]. In BRAF-mutated malignant melanoma, gains on chromosomes 7 and 10 were observed, while loss on chromosome 11 were associated with *NRAS* mutations [131, 136, 137]. We showed that 6/9 patients (66%) with both BC and malignant melanoma had dCNAs on chromosomes 8 and 16, equally distributed between losses and gains (Figure 5-7). Besides BC (23,501±50,547.5 (±SEM), range 1,551-102,646) and GYM (21,638.7±30,142, range 3,698-63,982), malignant melanoma (26,644±48,842.5, range 3,039-100,724) were also one of the OPPMs showing the highest number of LOHs. In malignant melanoma, the highest number of LOH were identified on chromosomes 4 (3,123±4,677, range 0-9,354), 9 (3,522.5±2,006.5, range 0-4,013), and 11 (3,411±4,972, range 0-9,944).

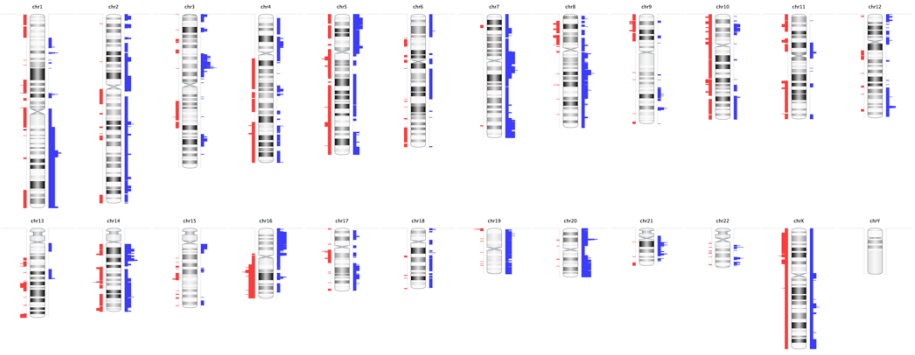

Figure 5. Karyotype containing genomic regions of gains (blue) and losses (red) in 8 patients with BC and MM.

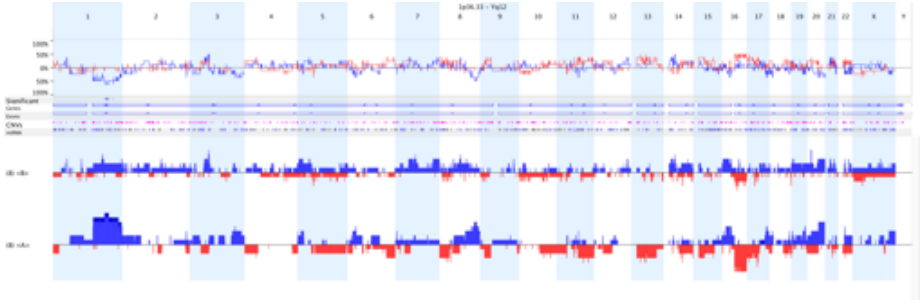

Figure 6. Frequency of dCNAs for the 8 patients with BC and MM. Red shows losses, blue shows gains.

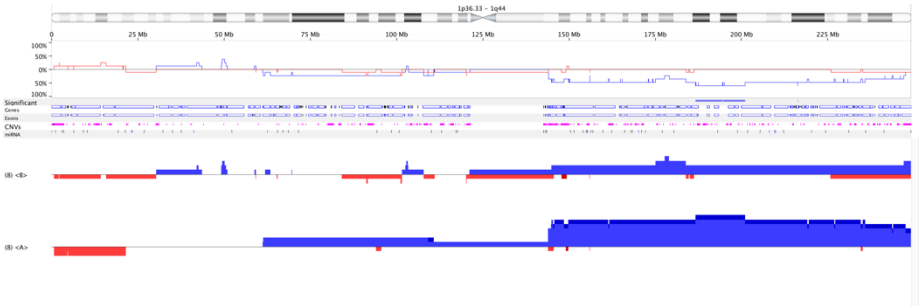

Figure 7. Frequency of dCNAs on chromosome 1 for 8 patients with BC and MM. Red shows losses, blue shows gains.

According to the Oncoscan mutation panel, 14 of the 47 tumors showed mutations in the *p53* gene, the majority (n=8) of which were BCs (Table 6). Oliver *et al.* described a strong association and linear relationship between tumor size, node positivity, and the frequency of *TP53* mutations [138]. *TP53* plays a key role in cell survival, genomic integrity, and cell proliferation.[79, 138, 139] Its role as a proliferation brake is well-known following DNA damage. Defective *TP53* promotes genomic instability and uncontrolled proliferation [138]. In the present study, 7/14 tumors with *TP53* mutations were classified as genetically unstable according to the GII and CAAI analyses and the mean tumor size for *TP53*-positive BCs was 39.2 mm, which is regarded as large. Though, only 3 patients with lymph node-positivity showed *TP53* mutations. In agreement with previous studies, these results show that *TP53* mutations are generally associated with an advanced and aggressive tumor phenotype [138, 140]. Other genetic alterations in *BRAF*, *IDH2*, *KRAS*, *NRAS*, *PIK3CA*, and *TP53* were also identified. According to the dbSNP database (https://www.ncbi.nlm.nih.gov/snp and https://cancer.sanger.ac.uk/cosmic), these specific alterations plays a clinically significant role in the development of other malignancies which is highly interesting. Encinas *et al.* described in a study concerning somatic mutations in young patients with breast and serous ovarian cancer that certain mutations are linked to age. The incidence of *TP53* somatic mutations tends to increase in the elderly [127, 128, 138]. The clinical relevance of the mutations and their reported localization were also reviewed in the dbSNP database. Nine of the SNPs that we identified were reported as possibly pathogenic for several different malignancies. For an example, missense mutations in *BRAF, KRAS* (two variants), and *NRAS* were reported

to be pathogenic in several of other malignancies including cancer in the gastrointestinal organs, hematopoietic system, brain and thyroid glandule. Similarly, *PIK3CA* and *TP53* (4 variants) mutations were reported to be pathogenic in a number of malignancies including breast- and gynecological malignancies, implying that mutations in these genes may play a key role in the development of different type of cancers.

Gene	BC	MM	TyM	GyM	HM	OM	SM	GIM
	n=24	*n=9*	*n=2*	*n=4*	*n=4*	*n=2*	*n=1*	*n=1*
TP53	TP53:p.G245S/C:c.733G>A/T (n=4 s) TP53:p.R175H:c.524G>A (n=2) TP53:p.Y163C:c.488A>G (n=1) TP53:p.Y220C:c.659A>G (n=1)	TP53:p.G245S/C:c.733G>A/T (n=1) TP53:p.Y220C:c.659A>G (n=1)		TP53:p.R175H:c.524G>A (n=1)	TP53:p.G245S/C:c.733G>A/T (n=1) TP53:p.Y220C:c.659A>G (n=1)	TP53:p.G245S/C:c.733G>A/T (n=1)		
KRAS								KRAS:p.G13D:c.38G>A (n=1)
NRAS			NRAS:p.Q61R:c.182A>G (n=1)					
IDH2	IDH2:p.R140Q:c.419G>A (n=1)							
BRAF	BRAF:p.V600E:c.1799T>A (n=1)	BRAF:p.V600E:c.1799T>A (n=1)						
PIK3CA	PIK3CA:p.H1047R:c.3140A>G (n=1)							

Table 5. Number of mutations in cancer-related genes, according to the Oncoscan somatic mutation panel BC, breast cancer; MM, malignant melanoma; TM, thyroid malignancies; GYM, gynecological malignancies; HM, hematopoietic malignancies; OM, oral cavity malignancies; SM, sarcoma; GIM, gastrointestinal malignancies.

Chromosomes 10 and 17 revealed chromothripsis-like patterns (CTLP) in several BCs spanning several cancer-related genes, e.g. *NCOA4* (chromosome 10), and *BRIP1*, *BRCA1*, *CD79B*, *CDK12*, *COL1A1*, *CLTC*, *DDX5*, *ERRB2*, *MS12*, and *PRKAR1A* (chromosome 17). CTLP events are developed during a single catastrophic event where tumors accumulate genetic rearrangements within a short period of time[141]. Therefore, genetic instability in a tumor may be associated with an increased risk of developing other primary malignancies. However, there may be ways to predict which patients may be at risk of developing specific malignancies. Spratt *et al.* describes the time labeling index that suggests that one and the same mutation could contribute to different metachronous malignancies due to differences in proliferation between tumors [57, 58]. Previous studies also highlight the importance of tailoring follow-up

programs for patients with breast cancer and OPPMs [114, 142, 143]. Lee *et al.* stated the importance of establishing guidelines for improving prognosis and quality of life [143]. Raymond *et al.* proposed that breast cancer survivors should be advised of their increased risk of developing certain cancers in their lifetime [125]. This could contribute to how a patient's lifestyle choices regarding smoking, exercise, weight, exposure to UV-radiation, etc. [18, 125]. More studies need to be done to be able to correlate mutations with patient clinical outcome [144]. Bleyer *et al.* also emphasized the importance of tailoring treatment strategies in different age groups, as cancer biology may differ depending on the age group [145].

4.4 PAPER IV

In this case report, we performed genetic analysis on a blood sample from a woman diagnosed with five primary synchronous and metachronous malignancies from 1997 to 2013 (Figure 8). An analysis of her family tree revealed two uncles and five siblings that had previously been diagnosed with cancer (Figure 9). Subsequent analysis of the patient's blood revealed no mutations using gene panels from SWEA (65 genes) or SWEN (20 genes). Retrospectively, a possible pathogenic variant (mutation class 3) could be detected in two specific genes, namely *RAD54L* and *RAD51* which are still not known as disease genes. The clinical value of these mutations and a possible link to cancer formation has not yet been clarified.

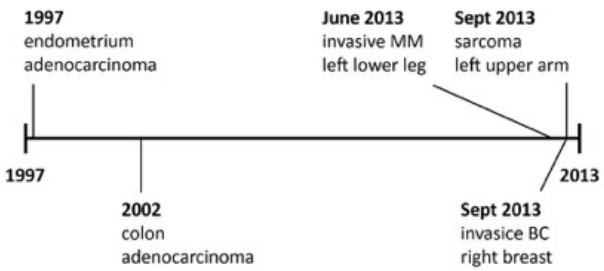

Figure 8. Timeline showing the consecutive malignancies from 1997 to 2013.

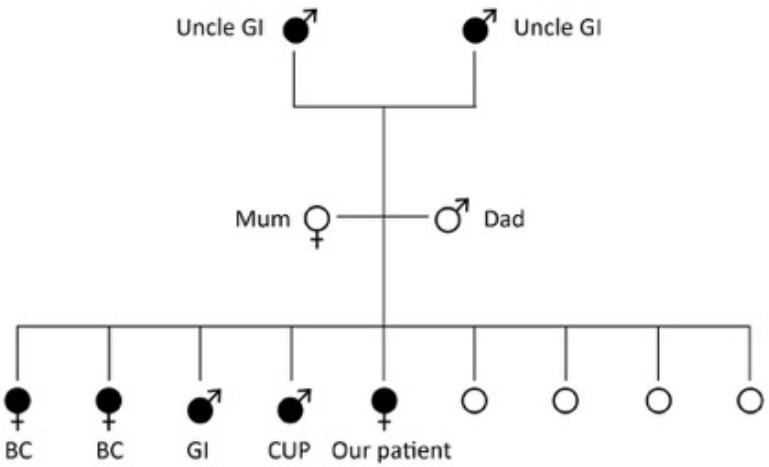

Figure 9. Family tree with several malignancies diagnosed in the patient's two uncles and five siblings. BC=breast cancer. GI=gastrointestinal malignancy. CUP=unknown primary site of malignancy. Unfilled circle=no malignancy. Filled circle=cancer diagnosis.

Human *RAD54* (hRAD54) is located on chromosome 1p32 and encodes a key protein that plays a role in homologous recombination and DNA double strand repair through its helicase activity which facilitates formation of base pairs[146-148]. However, this DNA repair is not correct, probably because of inaccurate splicing which was detected in our patient as RAD54L c.1610+1G>A. Germline (RAD54) missense variants are associated with an "inborn disease" and even somatic mutations have been identified in tumor tissue.[148] In the normal Swedish population, this type of variant shows a relatively high frequency (0.027%) compared with BC patients in the SWEA study (three such variants in 4,000 BC patients (0.075%)). Interestingly, *RAD54* mutations have been described in lymphomas, breast and colon carcinomas[149]. The *RAD51* gene functions similarly to *TP53* in that it plays a role in cell cycle checkpoint in case of DNA damage or incomplete replication[150]. Defects in *RAD51* are associated with tumor formation. Moreover, *RAD51* interacts with *BRCA1* and

BRCA2.[150] In the present study, a *RAD51* c.168_172del mutation was identified, representing a 5 bp protein truncation and possibly a loss of function. Although this mutation was found in seven of the 4,000 SWEA families, it constitutes a relatively common alteration in the European population (0.045% according to gnomAD).

None of the patient's five primary tumors resulted in distant metastases and can therefore be regarded as relatively less aggressive tumors (1. endometrium adenocarcinoma without lymph node metastasis; 2. colon adenocarcinoma stage B according to Dukes classification without lymph node metastasis; 3. invasive malignant melanoma Clark level II, Breslow 0.3 mm without regional lymph node metastases; 4. pleomorphic spindle cell sarcoma 40 mm large, grade 3 according to Trojani without regional lymph node metastases; 5. invasive mucinous breast carcinoma T1cN0Mx). No recurrence from any of the five primary tumors has been detected thus far. These findings warrant further analysis of this patient's tumor samples using whole-genome sequencing. If further genetic analysis of all five primary malignancies could be performed, it would be of interest to learn whether the identified mutations were similar or different.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In **Paper I**, we initially showed a significant increase in the number of OPPMs in breast cancer patients diagnosed at SU. Data from National Board of Health and Welfare could not confirm a statistically significant increase in OPPMs during the same time period, though both datasets showed the same trend. The most common OPPMs were found to be gynecological malignancies, malignant melanomas, and gastrointestinal malignancies. More studies have to been done, preferably with a national approach and without selection bias. In **Paper III**, no common somatic genetic alterations were identified in the pairwise tumors (BC and OPPMs). More studies are needed to reveal these changes, preferably using genome-wide sequencing on a larger cohort containing tumor and normal tissue. In **Paper IV**, genetic analysis of the five MPMs from an elderly patient revealed genetic variants in two genes, but no genetic changes of clinical significance. Therefore, whole-genome sequencing is recommended. In addition, FOXA1 and Nestin protein expression (**Paper II**) were shown to be associated with clinical outcome and different molecular subtypes of breast carcinoma, suggesting they could be used as possible prognostic biomarkers. We propose the inclusion of FOXA1 and Nestin in current immunohistochemical panels for breast carcinoma to improve prognostication and therapy choice.

An additional study is currently ongoing:

- Quality of life and sense of coherence in patients with earlier breast cancer and previous multiple malignancies. Jenny Nyqvist; Toshima Z. Parris; Khalil Helou; Zakaria Einbeigi; Elisabeth Kenne Sarenmalm; Eva-Marie Sjöberg; Per Karlsson; Anikó Kovács. Planned manuscript

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