

**Towards personalized care in  
pregnancy-associated breast cancer:**  
*the importance of the clinicopathologic profile*

Britt B.M. Suelmann

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# **Towards personalized care in pregnancy-associated breast cancer:** *the importance of the clinicopathologic profile*

**Op naar gepersonaliseerde behandeling bij  
zwangerschaps-geassocieerde borstkanker:**  
*het belang van het klinische en histopathologische profiel*

## **Proefschrift**

Ter verkrijging van de graad van doctor aan de Universiteit van Utrecht  
Op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling,  
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

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door

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*Het gaat niet op de eindbestemming,  
maar om de weg er naar toe ....*



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# 01





# General introduction

## Outline of this thesis



## GENERAL INTRODUCTION

### Cancer during pregnancy

Cancer diagnosed during pregnancy is increasingly reported, in part due to the advancing age of child-bearing women [1,2]. Still, a cancer diagnosis during pregnancy remains a relatively rare event, which affect about one in 1000 pregnancies and overall represents  $\leq 0.1\%$  all cancers [3]. Globally, the most common cancers diagnosed during pregnancy follow the prevalence patterns of cancers in the underlying young population. In Western countries, breast cancer is the most frequent malignancy diagnosed during (or shortly after) pregnancy, followed by thyroid and cervical cancer, melanoma and lymphoma [4-7].

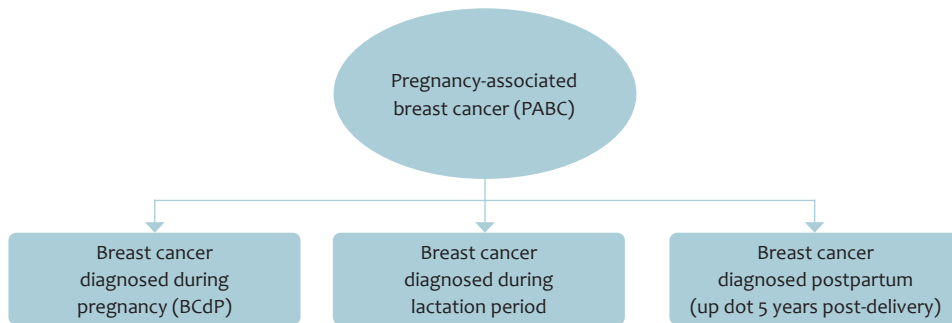
### Breast cancer during pregnancy

#### *Incidence*

The incidence of pregnancy-associated breast cancer (PABC) is reported as approximately one in every 3,000 to 10,000 pregnancies [3,4,8]. PABC is regarded as a clinically and biologically special entity that comprises only 0.2 – 0.4% of all breast cancers. However, in women younger than 45 years of age, it accounts for up to 6.9% of breast cancers [9-11], rising to a percentage of 15.6 % in women below the age of 35 [12]. The incidence of PABC has increased markedly during the last decades, and is expected to rise further, particularly in developed countries, due to the increasing age of (first) childbearing and an ongoing increase of young-onset breast cancer [13-15]. Individuals with *BRCA-1* or *BRCA-2* germline mutations are at markedly increased risk, but they do not seem to have an even further increased incidence of breast cancer during pregnancy [16,17].

#### *Definition*

Conventionally, PABC was defined as breast cancer diagnosed during pregnancy or the postpartum period. However, definitions of the duration of the ‘post-partum period’ within this definition have ranged from 0.5 to 5 years, and sometimes even longer. This variability may have led to conflicting results on the relationship between pregnancy, prior pregnancy and breast cancer outcome. In addition, there is a natural overlap between breast cancer diagnosed during pregnancy and the general risk of breast cancer in young women [18].



### Diagnosis

Physiological breast changes associated with pregnancy (including transforming glandular tissue, engorgement and nipple discharge) mask the detection of suspicious and/or persisting breast masses. In addition, the absence of routine breast cancer screening in this young population (i.e. in The Netherlands screening starts at the age of 50 years), and the reluctance to perform imaging or invasive procedures during pregnancy, commonly results in a delayed diagnosis, and usually more advanced tumor stages upon diagnosis [19-21].

### Clinicopathologic characteristics

PABC is generally characterized as a particularly aggressive type of cancer through the occurrence in a young population, the advanced tumor (T) stage at diagnosis, a high rate of lymph node involvement, higher grade tumors, a negative estrogen receptor (ER) and progesterone receptor (PR) status, and a higher rate of human epidermal growth factor receptor-2 (HER-2) amplification and overexpression [12,21,22]. However, the rarity of PABC hampers conducting large studies (with sufficient patient numbers) to assess these unique clinicopathologic features.

Whether PABC has a worse prognosis is currently the subject of debate. The existing literature gives a mixed view of whether a breast cancer diagnosis during pregnancy confers a worse prognosis than age-matched breast cancer patients not diagnosed during pregnancy [21,23-29]. Nevertheless, comparison of the available data is complex because of the small numbers of patients examined in each of the individual studies, the dissimilar reference populations, and specifically the differences in inclusion criteria.

### Treatment

Breast cancer treatment during pregnancy is a challenging situation, since the health of both the mother and the unborn child must be considered in any treatment plan. For pregnant women with PABC, therapeutic strategy should adhere as closely as possible to the standard protocols for non-pregnant patients (with consideration of unborn child safety), since delaying or postponing chemotherapy might increase the risk of cancer relapse and, as such, a worse prognosis [12]. However, due to teratogenic effects on the fetus in the first twelve weeks of pregnancy, chemotherapy is generally not administered before the second gestational trimester [7,30]. Although data for long-term outcomes after prenatal exposure to chemotherapy (i.e. after the first trimester) are scarce, they do not show any congenital, neurological, immunological or psychological abnormalities (including learning and educational behavior) in the exposed children [31,32]. Nevertheless, most complications were reported in children who were delivered prematurely, irrespective of exposure to chemotherapy. As for the maternal effect of chemotherapy, despite possible pharmacokinetic changes during pregnancy, maternal survival rates in two studies did not differ between patients who received chemotherapy during pregnancy vs. after delivery [30,33]. In addition, termination of pregnancy is not likely to improve maternal prognosis [31].

In general, surgery can be performed safely during all trimesters of pregnancy and most anesthetic agents seem to be safe for the fetus as well. The decision on whether to perform surgery during pregnancy should follow the same guidelines as for non-pregnant breast cancer patients. In contrast, radiotherapy, endocrine therapies, and targeted therapies are not recommended at any time during pregnancy. For example, monoclonal antibodies targeting HER-2, result in renal failure in the fetus as HER-2 is strongly expressed in fetal renal epithelium [31,33].

### Interactions between pregnancy and breast cancer: etiology

Pregnancy has a dual influence on breast cancer risk. The long-term protective effects (after full-term delivery and breastfeeding) has been for long acknowledged [34-36]. Yet, a temporary increase in breast cancer incidence during pregnancy and 5-10 years postpartum (or even longer in case of women aged > 35 years at first live birth) has also been observed [37-40]. It is known that the hormonal environment during pregnancy, characterized by elevated levels of circulating estrogen, progesterone and insulin-like growth factor-1 (IGF1) induces breast cell proliferation, differentiation, secretion and programmed cell death; leading to important remodeling of the glandular tissue architecture [40,41]. Although the definitive biological mechanisms underlying the role of pregnancy in breast cancer etiology still remain complex and incompletely understood, several etiological hypotheses have been proposed.

First, tumorigenesis of breast cancer during pregnancy may be driven by hormonal influences on the host stroma rather than on the mammary epithelium. As a contradiction, it was found that increasing estrogen levels promote the initiation and progression of estrogen receptor (ER) negative cancers. Thus, the tumor cells themselves do not express ER, yet they do exhibit a highly stromalized histologic phenotype, which may indicate that tumorigenesis is driven by hormonal influences on the host stroma, instead of mammary epithelial cells [42].

Second, next to elevated hormone levels during pregnancy, there is an altered immune response (the balance of pro- to anti-inflammatory signals is tipped towards suppression of overt inflammation), consisting of escape mechanisms (used by the fetal trophoblast cells, yet also by cancer cells) which may lead to tumor cell proliferation and survival [43]. Third, interestingly, the end of the lactation or pregnancy (if lactation does not occur) leads to breast involution, which differentiates the mammary gland to its pre-pregnant quiescent state, initiating a tissue-remodeling program (characterized by infiltration of macrophages, collagen deposition, massive epithelial cell death and remodeling of the stroma), which has been shown to be pro-oncogenic [44,45]. Both pregnancy and lactation generate permanent histological and molecular modifications on the breast. After full term pregnancy, the breast is characterized by a unique genomic signature that differs from nulliparous breast tissues [46].

The lack of a comprehensive understanding of the interaction between the hormonal environment during and after pregnancy, and breast carcinogenesis, indicates the need for more insights in the development of PABC, leading to improvement of outcome of patients and development of new treatment strategies. This starts with a better defined clinicopathologic footprint and definition of PABC and a deeper understanding of the molecular makeup, which may be the fundament for personalized care in this unique group of young patients.

## OUTLINE OF THIS THESIS

Research of this thesis was conducted to create a comprehensive overview of the clinicopathologic characteristics of PABC, as compared to non-pregnant young breast cancer patients. Furthermore, within PABC patients, specific subgroups were identified and analyzed for differences in outcome. The findings may contribute to an improved and more detailed definition of PABC. Lastly, novel insights into the biology of PABC tumors suggests a promising genomic biomarker. Altogether, such a PABC profile may serve as a fundament for personalized cancer treatment in PABC.

The first part of this thesis focusses on the histopathologic features of PABC. In **Chapter 2** we assess the histopathological profile of PABC in a large Dutch population-based cohort, compared to age-matched non-pregnant breast cancer patients. In addition, since more insight into the different breast cancer subgroups during pregnancy and during the time after delivery (i.e. postpartum) is warranted, we specifically focused on differences in histopathologic characteristics between the three gestational trimesters and the post-delivery period (lactating vs. non-lactating patients) in **Chapter 3**. As the divergent definitions of PABC (including breast cancer diagnosed during pregnancy and postpartum) lead to conflicting clinical and histopathological data, **Chapter 4** focusses on breast cancer diagnosed solely during pregnancy, as a separate subgroup of PABC, in an extensive review on receptor status.

While the previous chapters describe the histopathologic profile of various PABC subgroups, **Chapter 5** focusses on the outcomes of these PABC subgroups in a large-population based analysis of PABC patients subdivided according to gestational trimester of diagnosis or the postpartum period (up to six months after delivery), compared to matched non-PABC patients.

Whether the discriminatory histopathologic features are also reflected in a unique molecular signature is assessed in **Chapter 6**, where we have analyzed the genomic background of triple negative PABC tumors, the most prevalent and aggressive molecular subtype, by detection of copy number alterations (CNA).

**Chapter 7** summarizes and discusses the results of these studies within the context of the available relevant literature and speculates on areas of future research on PABC subgroups.

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# 02



# Pregnancy-associated breast cancer: nationwide Dutch study confirms a discriminatory aggressive histopathologic profile

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## ABSTRACT

### Purpose

Breast cancer is the most common type of malignancy in pregnant women, occurring approximately once in every 3,000 pregnancies. Pregnancy-associated breast cancer (PABC) is commonly defined as breast cancer diagnosed during or within one year after pregnancy, and it accounts for up to 6.9% of all breast cancers in women younger than 45 years old. Whether these cancers arise before or during pregnancy, and whether they are stimulated by the high hormonal environment of pregnancy, is currently unknown. This study assesses the histopathological profile of PABC in a large Dutch population-based cohort.

### Methods

We identified 744 patients with PABC (in this cohort defined as breast cancer diagnosed during or within six months after pregnancy) diagnosed between 1988 and 2019, in the nationwide Dutch Pathology Registry (PALGA). An age-matched PALGA cohort of unselected breast cancer patients ( $\leq 45$  years), diagnosed between 2013 and 2016, was used as a control. Histopathologic features of both cohorts were compared.

### Results

The median age of PABC patients was 34.3 years old (range: 19-45 years) and most breast cancers were diagnosed during pregnancy (74.2%). As compared to age-matched controls, PABC patients had tumors of higher Bloom-Richardson grade (grade I: 1.5% vs. 12.4%, grade II: 16.9% vs. 31.3%, grade III: 80.3% vs. 39.5%,  $p < 0.0001$ ). Furthermore, estrogen (ER) and progesterone-receptor (PR) expression was less frequently reported positive (ER: 38.9% vs. 68.2% and PR: 33.9% vs. 59.0%,  $p < 0.0001$ ), while a higher percentage of PABC tumors overexpressed HER2 (20.0% vs. 10.0%,  $p < 0.0001$ ). The most observed intrinsic subtype in PABC was triple-negative breast cancer (38.3% vs. 22.0%,  $p < 0.0001$ ), whereas hormone-driven cancers were significantly less diagnosed (37.9% vs. 67.3%,  $p < 0.0001$ ).

### Conclusion

This study, based on a large population-based cohort of 744 PABC Dutch patients, underlines the more aggressive histopathologic profile compared to age-matched breast cancer patients  $\leq 45$  years. Further in-depth genetic analysis will be performed to unravel the origin of this discriminating phenotype. It definitely calls for timely detection and optimal treatment of this small but delicate subgroup of breast cancer patients

## INTRODUCTION

Breast cancer occurs in approximately one in every 3,000 pregnancies [1-4], which makes this the most common type of malignancy in pregnant women [5]. Pregnancy-associated breast cancer (PABC) is commonly defined as breast cancer diagnosed during or within one year after pregnancy [6], and it accounts for up to 6.9% of all breast cancers in women younger than 45 years of age [7-9]. In women below the age of 35, the proportion of PABC even rises up to 15.6% [10]. The incidence of PABC has increased markedly during the last decades [7], and is expected to rise further, particularly in developed countries, due to the increasing age of (first) childbearing and an ongoing increase of young-onset breast cancer [11-13].

PABC is generally recognized as a particularly aggressive type of cancer for several reasons: occurrence in a younger population, an advanced T stage at diagnosis [10, 14-17], a high rate of lymph node involvement [10, 16], higher grade tumors [16], a negative estrogen-(ER) and progesterone (PR)-receptor status [14-16], and a higher rate of HER2 overexpression [17-22]. These characteristics, usually common in breast cancer at young age, are however described to differ in percentages of occurrence in PABC. In addition, gestational physiologic alterations in the breast commonly result in a delayed diagnosis of breast cancer and thereby more advanced stages [23]. Furthermore, whether these cancers arise before or during pregnancy, and whether they are stimulated by the high hormonal environment of pregnancy, is currently unknown.

The lack of a comprehensive understanding of the interaction between pregnancy and breast carcinogenesis indicates the need for more insights in the development of PABC, which may ultimately lead to personalized PABC treatment. This starts with clear insights in the histopathologic profile of PABC, which may identify clues for further in-depth research. However, the relative rarity of the disease precludes conducting large studies with sufficient patient numbers. Therefore, this study assesses the histopathological profile of PABC in a large Dutch population-based cohort, compared to age-matched non-pregnant breast cancer patients, to increase the understanding of the histopathologic characteristics of PABC. This will serve as a starting point for further in-depth molecular research within the same patient cohort.

## METHODS

### Data source

Data were extracted from the nationwide Dutch network and registry of histo- and cytopathology (PALGA) [24], which contains excerpts of all pathology reports from Dutch

laboratories [25]. All data within the PALGA research database are pseudonymized, both in the laboratories and by a trusted third party (ZorgTTP, Houten, The Netherlands). This study was approved by the PALGA scientific and privacy committee, and all data were handled in compliance with the General Data Protection Regulation Act (GDPR).

### Study population

Excerpts were extracted from all resection specimen reports of women with a diagnosis of invasive breast cancer (IBC) and a mention of pregnancy, offspring, placenta, lactation or abortion in their pathology report between January 1, 1988 and July 1, 2019 (n=1,941) (Supplementary Figure 1). All patients with a diagnosis of IBC during pregnancy, or up to six months postpartum, were included, irrespective of pregnancy outcome, and type and timing of breast cancer treatment. Patients with sarcomas or a Phyllodes tumor, as well as patients with a history of breast cancer before their pregnancy, defined as a recurrence of invasive breast cancer in the contralateral or ipsilateral breast or chest wall at any time, were excluded (Supplementary Figure 1).

A control cohort was drawn from unselected Dutch breast cancer patients (extracted from the same PALGA database) with a synoptic IBC resection-specimen report between January 1, 2013 and December 31, 2016 (n=46,563 reports) (Supplementary Figure 1). From this cohort, we excluded patients without a primary tumor in their resection specimen (n=2,104 reports) and patients  $\geq 46$  years old, as the eldest PABC patient was 45 years of age (n=41,458 reports). In addition, males (n=13) and overlapping (i.e. PABC) patients (n=55) were excluded from this control cohort. Lastly, for patients with multiple reports, only the first report was included to prevent matching the same patient twice (Supplementary Figure 1).

For both cohorts, we extracted clinicopathologic characteristics from the pathology reports, including histologic subtype, histologic grade, estrogen receptor (ER) status, progesterone receptor status (PR) and human epidermal growth factor receptor-2 (HER2) status. For both cohorts, no information was available for the mode of presentation. However presumably most patients were presented with a palpable mass, as no major screening program exists in the Netherlands for population below the age of 50 (except for patients known to have a hereditary predisposition). In addition, based on the receptor status for ER, PR, and HER2, tumors were sub-classified as ER/PR-driven (ER and/or PR+, HER2-), triple positive (TPBC: ER+, PR+, HER2+), triple negative (TNBC: ER-, PR-, HER2-), and HER2-driven (ER-, PR-, HER2+). Additionally, for the PABC cohort, gestational age at diagnosis was extracted, which was subdivided into the trimesters (trimester one: weeks 1-12, trimester two: weeks 13-26, trimester three: weeks 27-42), and the postpartum period (up to six months after delivery). For postpartum patients, a distinction was made between lactating and non-lactating women.

### Statistical analysis

Patients from both cohorts were randomly matched 1:1 on age. Clinicopathologic characteristics were summarized and differences between both cohorts were tested by means of a  $\chi^2$  test for categorical variables. For the normally distributed continuous variable (age) a t test was performed. All tests were two-sided and p-values < 0.05 were considered statistically significant. All statistical analyses were performed using IBC SPSS Statistics version 25.0.0.2.

## RESULTS

### Patient characteristics

In total, 741 of 744 eligible patients with PABC could be age-matched to 741 non-PABC patients from the control-cohort (Supplementary Figure 1). Three patients have remained unmatched as there was no patient with similar age characteristics remaining in the control group to match against. Clinicopathologic variables of both cohorts are listed in Table 1. The median age of patients in both cohorts was 34.3 years (range: 19-45 years). Statistically significant differences ( $p < 0.0001$ ) were observed for all other histopathologic characteristics. PABC-patients had more often tumors of ductal type and higher grade (grade I: 1.5% vs. 12.4%, grade II: 16.9% vs. 31.3% and grade III: 80.3% vs. 39.5%) and these tumors were less often ER-receptor positive (38.9% vs. 68.2%) and PR-receptor positive (33.9% vs. 59.0%), as compared to the non-PABC breast cancer patients. In addition, tumors of PABC patients were more often HER2-receptor positive (20.0% vs. 10.0%).

**Table 1.** Characteristics of patients with pregnancy associated breast cancer (PABC) (n=741), age-matched 1:1 to non-PABC patients with invasive breast cancer (n=741)

	PABC patients (n=741)	Non-PABC patients (n=741)	p-value
<b>Age, median (range)</b>	34.0 (19-45)*	34.0 (19-45)	1.000
<b>Histological subtype, n (%)</b>			
Ductal	707 (95.4%)	670 (90.4%)	<b>0.000</b>
Lobular	22 (3.0%)	31 (4.3%)	
Other	12 (1.6%)	40 (5.4%)	
<b>Histological grade, n (%)</b>			
Grade I	11 (1.5%)	92 (12.4%)	<b>0.000</b>
Grade II	124 (16.9%)	232 (31.3%)	
Grade III	595 (80.3%)	293 (39.5%)	
Unknown	11 (1.5%)	124 (16.7%)	
<b>ER-receptor status, n (%)</b>			
Positive	288 (38.9%)	505 (68.2%)	<b>0.000</b>
Negative	393 (53.0%)	210 (28.3%)	
Unknown	60 (8.1%)	26 (3.5%)	
<b>PR-receptor status, n (%)</b>			
Positive	251 (33.9%)	437 (59.0%)	<b>0.000</b>
Negative	415 (56.0%)	277 (37.4%)	
Unknown	75 (10.1%)	27 (3.6%)	
<b>Her2-receptor status, n (%)</b>			
Positive	149 (20.0%)	141 (10.0%)	<b>0.000</b>
Negative	483 (65.2%)	560 (75.6%)	
Unknown	109 (14.7%)	40 (5.4%)	
<b>Gestational age</b>			
First trimester	179 (24.2%)	N.a.	
Second trimester	111 (15.0%)	N.a.	
Third trimester	260 (35.1%)	N.a.	
Postpartum: not lactating	94 (12.7%)	N.a.	
Postpartum: lactating	83 (11.2%)	N.a.	
Unknown gestational age	14 (1.9%)	N.a.	

\* Up to six months postpartum

### Intrinsic subtypes

Regarding the surrogate intrinsic subtypes, notable differences were observed for triple negative and ER/PR-driven breast cancer. Breast tumors of PABC patients were significantly more often triple negative (38.3% vs. 22.0%,  $p < 0.0001$ ) and, significantly less often hormone-driven (37.9% vs. 67.3%,  $p < 0.0001$ ).

### Gestational trimesters

The majority of PABC patients were diagnosed during pregnancy (74.2%), of which nearly half during the third trimester (47.3%) (Table 1). Of all pregnant PABC patients, 38 patients (5%) terminated their pregnancy in the first or second gestational trimester. Of the postpartum PABC-patients 83 patients were lactating (47%) and 94 patients (53%) did not breastfeed after pregnancy.



**Table 2.** Surrogate intrinsic subtypes of patients with pregnancy associated breast cancer (PABC) (n=741), age-matched 1:1 to non-PABC patients with invasive breast cancer (n=741)

	PABC patients (n=741)	Non-PABC patients (n=741)	p-value
Surrogate intrinsic subtypes, n (%)			
Triple positive			
Triple positive (ER+, PR+, Her2+)	63 (8.5%)	89 (12.0%)	0.000
Triple positive unknown (ER, PR or HER2 missing)	112 (15.1%)	44 (5.9%)	
Triple negative			
Triple negative (ER-, PR-, Her2-)	284 (38.3%)	163 (22.0%)	0.000
Triple negative unknown (ER, PR or HER2 missing)	112 (15.1%)	44 (5.9%)	
Hormone-driven			
Hormone-driven (ER and/or PR+, Her2+/-)	281 (37.9%)	499 (67.3%)	0.000
Hormone-driven unknown (ER, PR or HER2 missing)	112 (15.1%)	44 (5.9%)	
HER2-driven			
Her2-driven (ER+/-, PR+/-, Her2+)	149 (20.1%)	141 (19.0%)	0.000
HER2-driven unknown (ER, PR or HER2 missing)	112 (15.1%)	44 (5.9%)	

## DISCUSSION

This large nationwide study compared the histopathologic profile of 741 PABC patients to an age-matched cohort of 741 non-pregnant breast cancer patients. A particularly aggressive histopathologic profile was observed for PABC patients, as their tumors were significantly more often of higher histologic grade, HER2 positive and ER and PR negative. Furthermore, a higher incidence of triple negative tumors in PABC patients was observed.

Our results are in line with previous studies in (smaller) PABC cohorts [10, 15, 16], which report comparable proportions of ER- and PR-negative tumors in PABC patients between 50-60%. In addition, higher grade tumors were also previously observed in PABC patients [16]. Interestingly, the majority of our PABC patients were diagnosed during pregnancy, which is in contrast to a previous literature, claiming that two third of patients are diagnosed postpartum (up to 12 months after delivery) [17, 26]. However, this may be due to our search strategy that depend on signs of pregnancy or the post-partum-stage in pathology reports and inclusion of women with a diagnosis of invasive breast cancer during pregnancy or within six months postpartum. Perhaps a concurrent pregnancy is more likely to be mentioned in a pathology report than the postpartum stage (lactating or non-lactating).

Our findings in this large nationwide cohort of PABC patients add to the existing literature, implicating that PABC may be a different breast cancer entity than breast cancer in non-pregnant young women. Although a more aggressive histopathologic profile is observed

in young breast cancer patients in general [17, 27-29]; PABC patients show an even more aggressive histopathologic profile. These findings render interesting clues for further studies to unravel the molecular and genetic background of PABC. For example, the high proportion of ER and PR negative tumors in PABC patients seems contradictory, as estrogen and progesterone levels are generally high during pregnancy. These tumors are therefore probably driven by other growth factors, or as recently suggested by Gupta et al, tumorigenesis may be driven by the influence of hormones on the host stroma, rather than the mammary epithelium itself [30]. This is supported by findings that xenograft models of PABC require systemic estrogen for their formation, and increasing the estrogen levels promotes the initiation and progression of ER negative cancer (i.e. the tumor cells do not express estrogen-receptors themselves) [30]. Further, PABC xenografts are rich in stroma and PABC cell lines do not proliferate in vitro (i.e. without their cancer associated fibroblasts in response to estrogen). Furthermore, epigenetic changes, that by itself may affect subsequent hormone concentrations, could also play a role. In addition, a Norwegian study showed that lactating PABC patients have a worse outcome [31], which may indicate a prominent role for prolactin levels.

A limitation of this study is that data are drawn from pathology reports, which usually does not include clinical information, i.e. TNM stage, imaging, maternal treatment and neonatal and maternal outcome. This precludes outcome analyses like disease-free survival (DFS) and overall survival (OS) analyses. As a second step, these pathology data will therefore be linked to clinical and follow-up data from the Dutch Cancer Registry (NCR) to investigate whether the observed aggressive histopathologic profile translates into a worse survival for PABC patients. Another limitation of this study are the missing data, especially for HER2-receptor status in the PABC cohort. This is mostly due to the fact that routine HER2-testing was only introduced around the year 2000. However, it is unlikely that the distribution of HER2-receptor status in the population that has not received HER2-testing would differ from that was observed in patient who did receive HER2-testing. There could be concerns about the time span in the different breast cancer cohorts: the PABC group covers a longer time period (1988 – 2019) in comparison with the non-PABC group (2013 – 2016). However, a short subgroup analysis between the PABC-patients in three different time lines: 1988 – 2012, 2013 – 2016, and 2017 – 2019 observed no significant differences in receptor status or grade. Beside, histologic typing, grading and detection of the ER-and PR receptor by immunohistochemistry using monoclonal antibodies have not been changed in the last decades (32-34).

Overall, this large study nonetheless renders a unique, nationwide, overview of the histopathologic profile about all Dutch PABC patients since 1988. The observations from this study serve as a starting point for further indepth research that may ultimately lead to tailored PABC treatment. Tumor tissue of al PABC patients is currently being

collected from the concerning pathology laboratories. RNA- and DNA-sequencing will be performed. Further, the role of BRCA1 and BRCA2 germline mutations and prolactin levels will be investigated.

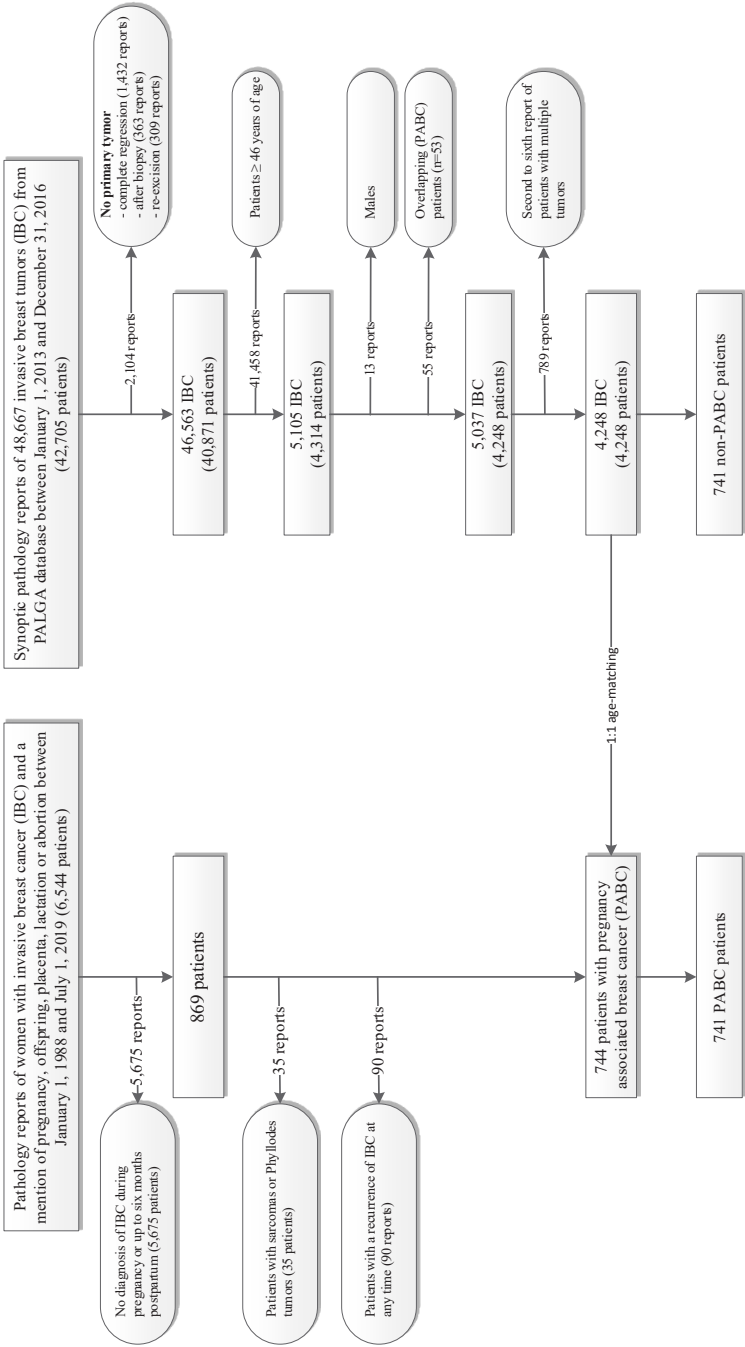
In conclusion, this large population-based cohort shows a significantly higher proportion of high-grade, and ER- and PR-negative tumors among PABC patients compared to age matched controls. This underlines a different, more aggressive histopathologic profile for PABC. Further in-depth research will be conducted to unravel the genetic background of PABC, which may render clues for personalized PABC-treatment.

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SUPPLEMENTARY



**Supplementary figure 1.** Flowchart of included patients with pregnancy-associated breast cancer (PABC) and breast cancer patients without PABC from the PALGA database.



# 03





# Pregnancy-associated breast cancer: the influence of gestational age and lactation

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## ABSTRACT

### Aims

Whether PABC tumors arise before or during pregnancy and whether histopathology is affected by gestational age is currently unclear. The present study assesses the influence of gestational age and lactation on the histopathologic profile of PABC.

### Methods

We identified 744 patients with PABC (defined as breast cancer during pregnancy or 6-months following delivery). Histopathologic features were compared between pregnant and postpartum patients.

### Results

Median age at diagnosis was 34.2 years and majority of cancers were diagnosed during pregnancy (71.3%). Within pregnant patients, tumors were significantly more often ER-negative in second and third trimesters (57.4%), as compared to first trimesters (41.9%) ( $p=0.036$ ). Similarly, a PR-negative status was reported significantly less often within first trimesters (38.0%) compared to second and third trimesters (57.1%) ( $p=0.032$ ). For HER2 status no significant differences were observed between gestational trimesters or lactating versus non-lactating patients. In postpartum patients, grade III tumors were found in over 80%, with high percentages of ER-negative tumors reaching 63% in those lactating versus 49% in non-lactating patients.

### Conclusion

This study demonstrates the varying histopathologic profile of PABC by gestational age and lactation status. Second and third trimester cancers display most typically the common ER/PR-negative phenotype, which is commonly reported in literature. The increased ER-negative status and percentage grade III tumors in lactating versus non-lactating patients also suggest presence of additional factors further diversify histology. This indicates the need for clear definitions of PABC and the role of potential subgroups, which may provide a stepping stone for further in-depth research into PABC-carcinogenesis.

## INTRODUCTION

Breast cancer is the most common type of invasive cancer among pregnant women (Smith et al. 2015) and has been reported to affect 1 in 3,000 to 10,000 pregnancies (Antonelli et al. 1996, Loibl et al. 2006). The average age of women diagnosed with pregnancy-associated breast cancer (PABC) is 32 to 38 years (Bae et al. 2018) and among women younger than 45 years, the rate of PABC varies from 2.6 to 6.9% of breast cancer cases. This proportion rises to 15.6% (of all breast cancer cases) in women younger than 35 years (Lethaby et al. 1996, Andersson et al. 2009, Moreira et al. 2010, Beadle et al. 2011).

PABC, commonly defined as breast cancer diagnosed during or within six to twelve months after pregnancy, is generally recognized as a particularly aggressive type of cancer due to the occurrence in a younger population, an advanced T stage at diagnosis, a higher rate of lymph node involvement, a higher histologic grade, a negative ER and PR status (Bonnier et al. 1997, Rodriguez et al. 2008, Murphy et al. 2012, Beadle et al. 2011, Ruiz et al. 2017), and a higher rate of human epidermal growth factor receptor-2 (HER2) overexpression (Keleher et al. 2002, Rovera et al. 2010, Asgeirsson et al. 2011, Basaran et al. 2014, Ruiz et al. 2017, Cordeiro & Gemignani 2017). In addition, gestational physiologic alterations in the breast and the reluctance to perform imaging or invasive procedures during pregnancy, commonly results in a delayed diagnosis of breast cancer and usually more advanced stages (Lethaby et al. 1996, Woo et al. 2003, Rodriguez et al. 2008, Basaran et al. 2014, Bae et al. 2018).

The interactions between pregnancy and breast cancer are complex. The hormonal environment during pregnancy, characterized by elevated levels of circulating estrogen, progesterone and insulin like growth factor-1 (IGF1) induces breast cell proliferation, differentiation, secretion and programmed cell death; leading to important remodeling of the glandular tissue architecture (Schedin 2006, Lyons et al. 2009). The definitive mechanisms driving PABC remain unclear and are incompletely understood, but several hypotheses have been launched. First, it has been suggested by Gupta et al. that PABC tumorigenesis may be driven by hormonal influences on the host stroma rather than on the mammary epithelium (Gupta et al. 2003). However, increasing estrogen levels promotes the initiation and progression of ER-negative cancers. Thus, the tumor cells themselves do not express estrogen-receptors, yet they do exhibit a highly stromalized histologic phenotype (Gupta et al. 2003). Second, next to elevated hormone levels during pregnancy, there is also an altered immune response, consisting of escape mechanisms (used by the fetal trophoblast cells, yet also by cancer cells) which may lead to tumor cell proliferation and survival (Shakhar et al. 2007). Third, interestingly, lactation or pregnancy (if lactation does not occur) leads to breast involution, which

differentiates the mammary gland to its pre-pregnant quiescent state, initiating a tissue-remodeling program (characterized by massive epithelial cell death, macrophage infiltration, collagen deposition and stromal remodeling), which has been shown to be pro-oncogenic (Strange et al. 1992, Lund et al. 1996, Watson & Kreuzaler 2011). Both pregnancy and lactation generate permanent histological and molecular modifications on the breast. After full term pregnancy, the human breast is characterized by a unique and specific genomic signature that differs from nulliparous breast tissues (Russo et al. 2008). The lack of a comprehensive understanding of the interactions between the hormonal environment during pregnancy and breast carcinogenesis indicates the need for more insights in the development of PABC.

## 3

The existing literature renders a mixed view of clinicopathologic profiles and survival outcomes of breast cancer diagnosed during pregnancy and or the postpartum period (which can extend 5 to 10 years after delivery) (Ibrahim et al. 2000, Murphy et al. 2012, Amant et al. 2013, Iqbal et al. 2017, Boudy et al. 2018, Ploquin et al. 2018, Choi et al. 2019, Shao et al. 2020).

The available results are conflicting and inconsistent, most likely as they focused on different breast cancer populations: a) the entire PABC population consisting of both pregnant and postpartum patients, b) the postpartum patients with a varying follow-up period, up to ten years after delivery, c) breast cancer diagnosed during pregnancy (BCdP) solely (without differentiation to gestational trimester at breast cancer diagnosis). Nevertheless, comparison of this available data is complex because of the small numbers of patients examined in each of the prior individual studies and due to the dissimilar reference populations. Therefore, a distinction between both entities of BCdP and breast cancer diagnosed during the postpartum period (PPBC) is necessary as Amant et al. suggested, to improve the understanding of the biology of breast cancer during pregnancy, lactation, involution and thereafter (Amant et al, 2021). For example, the longer after the delivery date breast cancer is diagnosed, the less clear the association with pregnancy become. In addition, these cases may be largely underreported, since the likelihood of linking breast cancer to the previous pregnancy (months or years before) decreases.

Earlier, we assessed the histopathologic profile of BCdP and PPBC (up to six months after delivery) in a large Dutch population-based cohort (Suelmann et al. 2021). This analysis rendered an exclusive overview of the histopathologic characteristics of BCdP and PPBC compared to age-matched non-pregnant breast cancer patients ( $\leq 45$  years); which underlined a different, more aggressive histopathologic profile for BCdP and short-term postpartum patients with significantly higher proportion of high-grade, and ER- and PR-negative tumors.

In the present paper, we specifically focus on the different histopathologic characteristics between the three gestational trimesters and the lactation status in the first six months post-delivery to maximally enrich for hormonal influences of pregnancy. This study demonstrates a unique histopathologic profile of PABC subgroups in a previously established large Dutch population-based cohort, to better define the histopathologic footprint and definition of PABC. This may ultimately serve as a fundament for further molecular analysis and finally to improved care in this distinctive group of young patients.

## METHODS

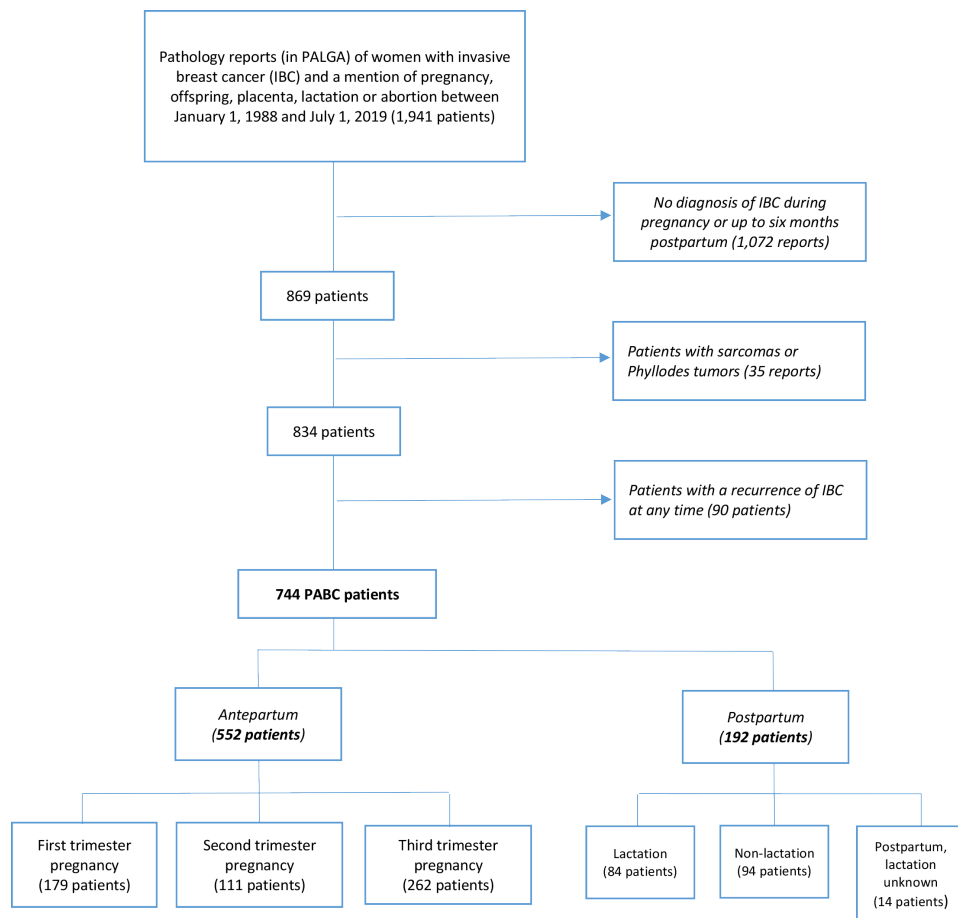
### Data source

Data were extracted from excerpts of all pathology reports in the Netherlands, which were derived from the nationwide Dutch Network and Registry of Histo- and cytopathology (PALGA) (Casparie et al. 2007, Foundation PALGA 2017). All data within the PALGA research database are pseudonymized, both in the laboratories and by a trusted third party (ZorgTTP, Houten, The Netherlands). This study was approved by the PALGA scientific and privacy committee, and all data were retrieved and handled in compliance with the General Data Protection Regulation Act (GDPR).

### Study population

We extracted all resection specimen reports of women with a diagnosis of invasive breast cancer (IBC) and a mention of pregnancy, offspring, placenta, lactation or abortion in their pathology report between January 1, 1988 and July 1, 2019 (n=1,941) (Figure 1). All patients with a diagnosis of IBC during pregnancy, or up to six months after delivery, irrespective of pregnancy outcome, and type and timing of breast cancer treatment were included. The six-month period was chosen to stay as closely as possible to the pregnancy/lactation period, and thereby to maximally enrich the study population for truly PABC. Patients with a history of invasive breast cancer or carcinoma *in situ* before their pregnancy (defined as recurrence of invasive breast cancer or carcinoma *in situ* in the contralateral or ipsilateral breast or chest wall at any time), as well as patients with sarcomas or a phyllodes tumor were excluded (Figure 1). We extracted clinicopathologic characteristics from the pathology reports, including age at diagnosis, histologic grade -and subtype, estrogen -and progesterone receptor (ER and PR) status, and HER2 receptor status. Lastly, gestational age at diagnosis was extracted from the pathology reports by distinction between duration of amenorrhea, time of abortion, miscarriage, date of delivery, date of submission of a placenta or umbilical cord. Thereafter, gestational age was subdivided into the gestational trimesters; first trimester: weeks 1-12, second trimester: weeks 13-26, third trimester: weeks 27-42, and the postpartum period (up to six months after delivery).

For the postpartum period, differentiation was made between lactating and non-lactating women by use of information from the pathology reports (date of delivery, date of submission of a placenta or umbilical cord, information of lactation or breastfeeding) (Figure 1).



**Figure 1.** Flowchart of included patients with pregnancy-associated breast cancer (PABC)

### Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (version 25.0.0.2). Clinicopathologic characteristics of all PABC patients were summarized and features were compared between the three gestational trimesters, and for post-partum PABC patients between lactating and non-lactating women. Separate analyses were performed for the first versus the second and third trimester.

Differences were tested by means of a  $\chi^2$  test for categorical variables. For the normally distributed continuous variable (age) a t-test was performed. All tests were two-sided and p-values <0.05 were considered statistically significant.

## RESULTS

A total of 744 patients with PABC were identified. The median age of the entire PABC population was 34.2 years (range: 19-45 years). The study population was divided into pregnant patients (n = 552) and postpartum patients (n = 192). The majority of pregnant PABC patients were diagnosed during the third trimester (n=262, 47.5%), followed by the first trimester (n=179, 32.4%) and the second trimester (n=111, 20.1%). Of all pregnant PABC patients, 38 (5.0%) terminated their pregnancy in the first or second gestational trimester. The remaining 192 PABC patients were diagnosed within six months after delivery; 84 patients (43.8%) were lactating during their diagnosis of IBC, and 94 postpartum patients (49.0%) were not. In fourteen patients (7.2%) there was no description of gestational age, delivery date and lactation status at the time of diagnosis. This group was excluded from the analysis given the potential distortion of research outcomes and limited number of patients involved.

### Histopathologic characteristics

Histopathological features of all PABC patients divided by pregnant and postpartum patients are listed in Table 1. Among the entire PABC population, the most common histological subtype was invasive ductal carcinoma, accounting for 95.4% (n = 710). We observed a higher proportion of grade III tumors within the second (87.4%) and third trimester (79.4%), as compared to the first trimester (73.7%) (p = 0.014). In postpartum PABC patients a high percentage of grade III tumors was observed which was independent of lactation status (grade III tumors 84.5% vs 81.9%) (p = 0.66)) (Table 1).

**Table 1.** Histopathologic variables by gestational age and lactation

	Pregnant patients			p-value	Postpartum patients		p-value
	1st trimester (T1) n = 179	2nd trimester (T2) n = 111	3rd trimester (T3) n = 262		Non-lactating (PP-NL) n = 94	Lactating (PP-L) n = 84	
<b>Age (years)</b>							
Median, range	34.5 (24-45)	34.1 (23-44)	33.9 (23-45)	0.413	34.4 (19-45)	35.0 (27-45)	0.278
<b>Histopathologic subtype</b>							
Ductal	171 (95.5%)	106 (95.5%)	247 (94.3%)	0.292	91 (96.8%)	81 (96.4%)	0.709
Lobular	6 (3.4%)	3 (2.7%)	10 (3.8%)		1 (1.1%)	2 (2.4%)	
Other	2 (1.2%)	2 (1.8%)	5 (1.9%)		2 (2.1%)	1 (1.2%)	
<b>B&amp;R grade</b>							
Grade I	6 (3.4%)	0 (0.0%)	2 (0.8%)	0.014	2 (2.1%)	1 (1.2%)	0.660
Grade II	38 (21.2%)	13 (11.7%)	48 (18.5%)		14 (14.9%)	9 (10.7%)	
Grade III	132 (73.7%)	97 (87.4%)	208 (79.4%)		77 (81.9%)	71 (84.5%)	
Unknown*	3 (1.7%)	1 (0.9%)	4 (1.5%)		1 (1.1%)	3 (3.6%)	
<b>ER-receptor</b>							
Positive	85 (47.5%)	38 (34.2%)	95 (36.3%)	0.013	39 (41.5%)	26 (31.0%)	0.150
Negative	75 (41.9%)	63 (56.8%)	151 (57.6%)		46 (48.9%)	53 (63.1%)	
Unknown*	19 (10.6%)	10 (9.0%)	16 (6.1%)		9 (9.6%)	5 (5.9%)	
<b>PR-receptor</b>							
Positive	86 (48.0%)	37 (33.3%)	91 (34.7%)	0.217	28 (29.8%)	24 (28.6%)	0.270
Negative	68 (38.0%)	64 (57.7%)	149 (56.9%)		54 (57.4%)	55 (65.5%)	
Unknown*	25 (14.0%)	10 (9.0%)	22 (8.4%)		12 (12.8%)	5 (5.9%)	
<b>HER2-receptor</b>							
Positive	31 (17.3%)	21 (18.9%)	64 (24.4%)	0.377	21 (22.3%)	9 (10.8%)	0.104
Negative	118 (65.9%)	75 (67.6%)	163 (62.2%)		58 (61.7%)	62 (73.7%)	
Unknown*	30 (16.8%)	15 (13.5%)	35 (13.4%)		15 (16.0%)	13 (15.5%)	

\* not considered in chi-square analysis

Within the pregnancy group, the percentage of ER negative cancers significantly increased at an advanced gestational age: an ER negative status was observed in 41.9% within the first trimester, in 56.8% within the second trimester and in 57.6% within third trimester ( $p = 0.013$ ). A similar pattern was observed for PR; the proportion of a PR-negative status within the first trimester was 38.0%, within the second trimester 57.7%, and within the third trimester 56.9%, though these differences were not statistically significant ( $p = 0.217$ ) (Table 1). When specifically focusing on early pregnancy (first trimester) versus advanced pregnancy (second and third trimester), a significantly higher percentage of tumors were ER-negative in the second and third trimester (57.4%), as compared to the first trimester (41.9%) ( $p = 0.036$ ). Similarly, a PR-negative status was reported significantly less often within the first trimester (38.0%) as compared to the second and third trimester (57.1%) ( $p = 0.032$ ) (Table 2). Although non-significant, in postpartum lactating patients, the frequency of ER-and PR-negative tumors increased even further to 63.1% (ER) and 65.5% (PR), in contrast to the postpartum non-lactating patients ((ER:48.9%),  $p = 0.15$ , (PR:57.4%),  $p = 0.27$ ). For HER2, no significant differences were observed between the trimesters (first trimester: 17.3%, second trimester 18.9%, third trimester 24.4% ( $p = 0.377$ )). However, notably, the overexpression of HER2 in



lactating patients decreased (not significantly) to 10.8%, in contrast to non-lactating patients; 22.3% ( $p = 0.104$ ) (Table 1).

**Table 2.** Histopathologic variables for the 1<sup>st</sup> versus the 2<sup>nd</sup> and 3<sup>rd</sup> gestational trimesters

	Pregnant patients		p-value
	1 <sup>st</sup> trimester n = 179	2 <sup>nd</sup> and 3 <sup>rd</sup> trimester n = 373	
<b>ER</b>			
Positive	85 (47.5%)	133 (35.7%)	0.036
Negative	75 (41.9%)	214 (57.4%)	
Unknown	19 (10.6%)	26 (6.9%)	
<b>PR</b>			
Positive	86 (48.0%)	128 (34.3%)	0.032
Negative	68 (38.0%)	213 (57.1%)	
Unknown	25 (14.0%)	32 (8.6%)	
<b>HER2</b>			
Positive	31 (17.3%)	85 (22.8%)	0.800
Negative	118 (65.9%)	238 (63.8%)	
Unknown	30 (16.8%)	90 (24.4%)	
<b>Triple negative</b> (ER-, PR-, HER2-)	55 (30.7%)	154 (41.3%)	0.0086
<b>Triple positive</b> (ER+, PR+, HER2+)	14 (7.8%)	39 (10.5%)	
<b>ER/PR driven</b> (ER and/or PR+, HER2-)	68 (38.0%)	91 (24.4%)	

## DISCUSSION

Over the last decades breast cancer diagnosed in women aged 25 – 39 years significantly increased (Lima et al. 2020), which inevitably results in a raise of breast cancer diagnoses in or shortly after pregnancy. In this large Dutch population-based study, we retrospectively analyzed 744 pregnant and short-term postpartum patients to identify specific histopathologic profiles of pregnancy-associated breast cancers by gestational age at diagnosis and postpartum lactational status.

According to the current literature, the hormonal, immunological and inflammatory mechanisms that play a role in the development and progression of PABC (before or during pregnancy) (Ruiz et al. 2017), remain unclear and incompletely understood. The relative rarity of the disease precludes conducting large studies with sufficient patient numbers. Several studies, with (relatively) small patient cohorts (< 500 patients) demonstrated histopathologic and clinical features of the entire PABC population (consisting of both pregnant and postpartum patients) (Elledge et al. 1993, Antonelli et al. 1996, Bonnier et al. 1997, Beadle et al. 2009, Murphy et al. 2012, Langer et al. 2014,

Wang et al. 2019). Nevertheless, they often focus on the oncological and obstetric management of PABC, as well as neonatal and maternal outcomes (Elledge et al. 1993, Loibl et al. 2012, Troisi et al. 2018, De Haan et al. 2018, Gomez-Hidalgo et al. 2019). However, these studies did not analyze the influence of gestational age and lactation status on the histopathologic features, which may potentially render clues for a better understanding of PABC tumorigenesis. In addition, PABC patients in these studies (60-70% of the PABC population) were predominantly diagnosed postpartum (up to 12-24 months after delivery) (Ives et al. 2005, Ruiz et al. 2017), in contrast to our study population in which more than 70% of patients were pregnant at diagnosis.

## 3

Recently, we compared this large nationwide PABC cohort with over 700 age-matched non-pregnant breast cancer patients. The results implicated that breast cancer during pregnancy (BCdP) and short-term postpartum (within 6 months after delivery) could be considered as a different breast cancer entity with tumors which were of higher histologic grade and significantly more often ER- and PR-receptor negative (Suelmann et al. 2021). Furthermore, a higher incidence of TNBC subtypes in these patients was observed in line with other smaller PABC studies (Bonnier et al. 1997, Middleton et al. 2003, Beadle et al. 2009, Murphy et al. 2012, Langer et al. 2014, Wang et al. 2019, Bae et al. 2018), compared to the general breast cancer population in which  $\pm 15\%$  TNBC is described (Lachapelle et al. 2011)

In follow-up of this analysis, we now demonstrate a significantly higher percentage of ER- and PR negative status at advanced gestational trimesters, whilst a similar pattern is observed for lactating versus non-lactating breast cancer patients. These findings add to the existing literature by implicating that PABC should not be considered a homogenous group of breast cancer patients; second and third trimester cancers display most typically the common ER/PR negative phenotype. Hypothetically, it would be possible that breast cancers in the first gestational trimester may have initiated before pregnancy and postpartum breast cancers may be influenced by epigenetic changes following delivery, postpartum cancers in lactating patients possibly being more dependent on prolactin than estrogen/progesterone. The frequent ER/PR negative phenotype may well underlie the previously reported poorer prognosis of postpartum lactating patients; implicating a pro-oncogenic role for prolactin that should be further elucidated.

Distinction according to gestational age and lactation status therefore renders interesting clues for further analysis to unravel the molecular and genetic background of these PABC subgroups which potentially could lead to different therapeutic (adjuvant) approaches during pregnancy and early stage postpartum. For example, the higher proportion of ER- and PR negative tumors in advanced gestational trimesters seems contradictory, as ER- and PR-levels generally rise during pregnancy. These tumors are

therefore probably driven by other growth factors, or, as previously suggested, by the influence of hormones on the host stroma, rather than the mammary epithelium itself. This is supported by findings that xenograft models of PABC require systemic estrogen for their formation, whilst increasing the estrogen levels promotes the initiation and progression of ER-negative cancer (i.e., the tumor cells do not express estrogen-receptors themselves) (Gupta et al. 2003). Furthermore, Hsiao et al. (Hsiao et al. 2010) detected complex patterns of specific tumoral gene expression in PABC patients, in which epithelia exhibit a gain in copy number of DNA coding genes for morphogenesis, angiogenesis and metastases and a loss of DNA coding genes for tumor suppressors and cell adhesion, leading to increased invasiveness and aggressiveness.

This supports the distinct biological nature of PABC and as Allouch et al. suggested underlines the need for further molecular subtyping and identification of complete gene and miRNA profiles (to identify novel targets and biomarkers) (Allouch et al. 2020). However, this starts with a better defining of the histopathologic footprint as defined in this paper, so further differentiation in gene expression between gestational trimesters and early stage lactation can be studied.

In view of the short interval between pregnancy and remodeling of the mammary gland during lactation, it seems likely that the increased estrogen levels (during pregnancy) will act synergistically with the pro-inflammatory environment to promote oncogenesis. This process is possibly enhanced by the influence of prolactin (Tworoger et al. 2006, Clevenger et al. 2003), since prolactin and its receptors have also been implicated as promoters of tumor cell growth and progression, especially in an estrogen high environment.

Altogether, pregnancy can lead to the development of a unique form of breast cancer (PABC) with particularly poor prognostic characteristics (e.g. advanced tumor (T) stage and nodal involvement at diagnosis) (Stensheim et al. 2019, Rodriguez et al. 2008, Johansson et al. 2013, Bae et al. 2018, Madaras et al 2014). The existing literature renders a mixed view on whether this aggressive breast cancer entity results in a worse prognosis than the prognosis of patients with breast cancer diagnosed outside of pregnancy. And if so, whether pregnancy itself negatively influences prognosis remains the subject of debate. In addition, a Norwegian study observed a poorer outcome in lactating patients, which may implicate a role for prolactin levels (Stensheim et al. 2009). However, El Shamy demonstrated opposite results with a protective effect of longer duration of breastfeeding, resulting in full terminal differentiation of all oncogene-overexpressing cells in women who lactate for longer periods (El Shamy et al. 2016). Functional studies with e.g. organoids could include the specific effect of lactation and possibly speculate about the favorable or unfavorable influences of lactation on prognosis.

The inconsistent data, together with the observed discriminatory histopathologic findings between the PABC subgroups within our nationwide cohort, underline the importance of redefining PABC with more than two entities (e.g. BCdP and PPBC), before further molecular and survival analyses should be performed.

A limitation of our study is that data on outcome and treatment were lacking at the time of this study, precluding disease-free survival (DFS) and overall survival (OS) analyses with correction for treatment confounders. A next step will therefore be linking this large pathologic database to clinical and follow-up data from the Dutch Cancer Registry (NCR), to investigate whether the unique histopathologic profile of PABC patients by gestational trimester and postpartum (lactation versus non-lactation) translates into different clinical entities and overall survival.

Another limitation of this study are the missing data, especially for HER2-receptor status. This is mostly due to the fact that HER2-testing was only introduced around the year 2000. However, it is unlikely that the distribution of HER2-receptor status in the population that has not received HER2-testing would differ from the distribution that was observed in patients who did receive HER2-testing. Finally, we currently have insufficient data on *BRCA* germline status of our patients, which could have been important because such patients have an increased risk of additional cancers and second breast cancers. Tumor tissues of our PABC patients are currently being collected from pathology laboratories to fill in missing receptor data and allow RNA-and DNA-sequencing.

We chose to limit the postpartum period of our PABC patients to cancers diagnosed within six months after delivery to stay as closely as possible to the hormonal milieu of the pregnancy/lactation period and to exclude confounding factors as concomitant pregnancy and advanced post-lactational breast involution. As the breast cancer interacts with the hormonal environment during pregnancy which changes during the trimesters, these different trimesters may reflect an impact on the breast cancer itself. Therefore, the histopathological characteristics may be influenced by the trimester of diagnosis. Furthermore, breast tumors diagnosed during the first trimester might have, for example, developed before the pregnancy, and thus possibly differ from breast cancers which arise during the second and third trimester and developed in the exceptional hormonal milieu of pregnancy.

This limitation is also related to the selection bias inherent to the available data, as probably not all patients will notify the treating physician of the previous pregnancy, let alone that a pathologist will be notified of this clinical characteristic. This has led to relatively smaller numbers of postpartum patients, in comparison with antepartum PABC patients. However, the highest proportion of postpartum cancers occurs within

two months after delivery and declines slowly thereafter (Lee et al. 2012). Several studies demonstrate this transient increase in the risk of PABC in the months after pregnancy, followed by a long-term protective effect. Therefore, the postpartum women who develop invasive breast cancer months or years after delivery could possibly have different tumor biology. Additionally, the numbers of pregnancies and duration of breastfeeding will be of influence on PABC (Han et al. 2020).

Overall, this large study nonetheless renders a unique, nationwide overview of the histopathologic profile by gestational age and postpartum status of Dutch PABC patients since 1988. The observations from this study form a starting point for further in-depth research that may ultimately lead to personalized PABC treatment by gestational age. Additionally, this subgroup analysis indicates that further research on PABC should focus specifically on the second and third gestational trimesters.

## CONCLUSION

This study, based on a large population-based cohort of 744 PABC patients, is the first to demonstrate the varying histopathologic profile of PABC by gestational age and lactation status for post-partum PABC-patients. These data show biologically distinct entities, with frequent negative ER- and PR status at advanced gestational trimesters and during lactation. These novel findings add to the existing literature, and imply that PABC should not be considered a homogenous group of breast cancer, which should therefore be redefined according to trimester of diagnosis (for pregnant patients) and lactational status for early stage postpartum patients. Further in-depth molecular research is needed to unravel the genetic and molecular background and the potential differences according to gestational trimester, and lactation status allowing the discovery of new avenues for PABC treatment.

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04



# Receptor status of breast cancer diagnosed during pregnancy: a literature review

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## ABSTRACT

The definition of PABC is inconsistently given as either breast cancer diagnosed exclusively during pregnancy, or combined with breast cancer diagnosed within six months to five years after delivery, and sometimes even longer. The longer away from the delivery date breast cancer is diagnosed, the less clear this association with pregnancy may become. Therefore, breast cancer diagnosed during pregnancy (BCdP) may not necessarily be the same disease entity as PABC. This review aims to provide an overview of BCdP receptor status, as this has not been assessed before. BCdP tumors were predominantly ER negative (56.6%), PR negative (57.2%) or both ER and PR negative (47.9%). Moreover, HER2-overexpression was seen in 33.2% of BCdP patients and 27.6% had triple negative disease. This predominantly ER and PR negative profile with more often HER2 overexpression is aggressive and distinct from non-pregnant similar-aged patients, warranting future comparative research.

## INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy during (or shortly after) pregnancy<sup>1</sup> with a reported incidence of 2.38 diagnoses per 100.000 deliveries.<sup>2</sup> Unfortunately, this incidence is increasing; possibly due to the overall increase of breast cancer incidence and the delayed onset of childbearing.<sup>3,4</sup> Pregnancy-associated breast cancer (PABC) is commonly defined as breast cancer diagnosed during pregnancy or within one to two year(s) after delivery.<sup>5,6</sup> However, the variety of this definition amongst different research papers<sup>7</sup> may cause confusion. The longer away from the delivery date breast cancer is diagnosed, the less clear the association with pregnancy may become. Therefore, breast cancer diagnosed during pregnancy (BCdP) may not necessarily be the same disease entity as PABC.

In general, studies describing the receptor status of PABC indicate a more frequent negative hormone receptor (estrogen- (ER) and progesterone receptor (PR)) status and a higher rate of human epidermal growth factor receptor-2 (HER2) overexpression, when compared to non-pregnancy-associated breast cancer cases in the same age group.<sup>6,8–11</sup> However, it has been hypothesized that this increased hormone receptor negativity might be caused primarily by women diagnosed with invasive breast cancer during pregnancy.<sup>12</sup> Therefore, this review aimed to describe the hormonal status of BCdP cases solely, as this approach limits the influence of postpartum variation.

To our knowledge, an extensive review on the receptor status of BCdP has not been carried out in the last decade. Moreover, most PABC reviews include studies describing postpartum patients until several years after delivery, possibly resulting in a different histopathologic profile. Thus, this review strives to add specific information on the receptor status of BCdP to the existing literature, to shed new light on the influence of pregnancy on the development of breast neoplasms, and to set a reference for comparison with postpartum PABC.

## METHODS

### Literature Search

The target-of-search for this review consisted of articles describing receptor status of patients diagnosed with BCdP. The search strategy for this review comprised the search terms ‘breast cancer’, a variety of terms to include pregnant cases only (e.g., ‘gestational age’, ‘pregnancy duration’ etc.) and several terms to describe histopathology (e.g., ‘estrogen’, ‘progesterone’, ‘HER2’, ‘hormonal’, ‘receptor’, ‘histopathology’ and ‘clinicopathological’). The full search strategy can be found in Appendix A.

Medline (PubMed), Embase and the Cochrane Library were searched on the 8<sup>th</sup> of July 2020. In accordance to standard practice in reviews, both title and abstract (if provided) of all articles were screened, after which the full text of potentially eligible studies was read. These studies were then assessed using our inclusion criteria. The program Rayyan was used for the registration of all stages of screening.<sup>13</sup> The reference lists of all included articles were searched to find additional records. The abstracts of these records were subsequently assessed and, if potentially eligible, the full text was read.

### Inclusion criteria

The criteria for inclusion in our study were: (1) only patients with a diagnosis of BCdP, (2) description of ER, PR, and/or HER2, (3) minimum of five BCdP patients and (4) only diagnoses of primary invasive breast cancer. There were no language or publication year restrictions for the articles.

### Quality assessment

All included studies were qualitatively analyzed with the Joanna Briggs Institute (JBI) Checklist for Case Series.<sup>14</sup> This checklist was chosen because of the nature of the data of interest. Some of the included studies are, in fact, case-control or cohort studies. However, from the concerning articles, only data on the receptor- and HER2 status of BCdP patients was used, as this is the main interest of this review. Therefore, these studies were also considered as case series, enabling their assessment using the Checklist for Case Series.

### Data collection

The data of interest in all studies comprised expression of ER, PR and/or HER2 for patients who were diagnosed with BCdP. Additionally, the method of hormone receptor assessment (e.g., immunohistochemistry (IHC), Dextran Coated Charcoal etc.) was retrieved from the text if mentioned. All cases after 2002 were considered to be assessed by immunohistochemistry when this was not otherwise specified in the article, as immunohistochemistry had by then become the standard method worldwide. HER-2 amplification was mainly determined by immunohistochemistry, or the method was unspecified as the data were derived from central registries. When there was no mention of patients with recurrent disease, it was assumed all diagnoses concerned primary breast cancer.

The results of all selected studies were extracted and double checked. The accuracy of the presented data was assessed as extensively as possible (e.g., calculations were carried out to check whether the total number of patients was correct etc.). When a percentage was provided, this percentage was recalculated into the number of patients based on the total number of patients (minus ‘missing values’, if applicable). Afterwards,

the calculated numbers were summed up, to check whether this sum matched the total number of patients.

## RESULTS

The strategy of our search resulted in 1,081 articles after duplicate removal. Title and abstract screening provided 988 exclusions, resulting in 93 articles for full text screening using our inclusion criteria. Of these studies, 15 were considered relevant for the scope of this review and were therefore included. The reference lists of all included articles were subsequently searched for additional records. This resulted in 13 additional inclusions. The quality assessment performed led to the exclusion of Wallack et al. (1983), as inclusion and reporting were unclear.<sup>15</sup> In total, 27 studies were therefore included. Two studies, both with a multi-center international approach, scored lower than other included studies on the quality assessment, Cardonick et al.<sup>16</sup> (possible differences in methodology and inclusion amongst participating centers) and Loibl et al.<sup>17</sup> (possible differences in inclusion amongst participating centers). However, they were not excluded, as they extensively described large cohorts. Moreover, Ramírez-Torres et al.<sup>18</sup> described one postpartum patient diagnosed during lactation. As this carcinoma has a low contribution (1 of 16 cases) to the results, this study was nevertheless included in this review. The flowchart of the record selection process is shown in Figure 1. Reported mean/median age of the patients in these studies varied from 30.7 to 38 years.

After record selection, the receptor status of BCdP patients in the 27 included studies was extracted. These results are presented in Table 1. Elledge et al.<sup>20</sup> was the only article describing hormone receptor results both determined by biochemistry (Ligand Binding Assay) and immunohistochemistry (IHC). Therefore, only the IHC-data of this study are shown in Table 1.

The results of Table 1 are subsequently summarized in Table 2, providing the sum of all individual numbers per variable of interest. Differences exist on the number of patients described for every parameter, as different combinations of studies from Table 1 contribute to the summarized data of each variable in Table 2.

Table 1. Receptor status (determined by any method) of patients diagnosed with Breast Cancer during Pregnancy (BCdP) per article included in this review.<sup>1</sup>

First author of study	Patients (n)	Age (range) <sup>3</sup>	ER+	ER-	PR+	PR-	HER2+	HER2-	Triple negative	ER- PR-	ER/PR +
<b>Azim<sup>21</sup></b>	65	Median: 36 (28-47)	43	22	42	23	11	54	14	20	45
<b>Bodner-Adler<sup>22</sup></b>	5	Mean: 38 <sup>4</sup> (33-40)	2	3	2	3	1	4	2	2	3
<b>Callihan<sup>23</sup></b>	21	NA	10	11			10	11	6	11	10
<b>Cardonick<sup>46</sup></b>	115 108 102	Mean: 34.8 (23-47)	49	66	41	67	32	70			
<b>Dominic<sup>24</sup></b>	67	Mean: 34 (24-43)	21	46	15	52	12	55			
<b>Elledge<sup>20</sup></b>	12	Mean: 31.7 (26-37)	6	6	10	2	7	5	1	2	10
<b>Ezzat<sup>25</sup></b>	7	NA	1	6	1	6					
<b>Feng<sup>26</sup></b>	63	Mean: 34.5 (20-44)					27	36		34	29
<b>Framarino-dei-Malatesta<sup>27</sup></b>	22	Mean: 37.2	10	12							
<b>Giacalone<sup>28</sup></b>	15	Mean: 33.5	6	9	6	9					
<b>Gomez-Hidalgo<sup>9</sup></b>	13	Mean: 36.64	10	3	10	3	8	5	1		
<b>Greene<sup>30</sup></b>	8	Mean: 33	0	8							
<b>Ibrahim<sup>31</sup></b>	18 16	Median: 34	6	12	11	5					
<b>Ishida<sup>32</sup></b>	23 21	Mean: 32.6	7	16	6	15					
<b>Johansson<sup>33</sup></b>	82 29	NA	31	51	34	48	8	21	10	14	15
<b>Lee<sup>34</sup></b>	37	Median: 34 (25-40)	23	14	22	15	12	25			
<b>Loibl<sup>17</sup></b>	352 381 411	Median: 33 (22-51)					126	226	118	214	197
<b>Mathelin<sup>35</sup></b>	18	Mean: 33.8	8	10	5	13					
<b>Meden<sup>36</sup></b>	6	Mean: 30.7 (27-34)	1	5	1	5	4	2	1	4	2
<b>Meisel<sup>37</sup></b>	74	Median: 34 (25-40)					25	49	14	28	46



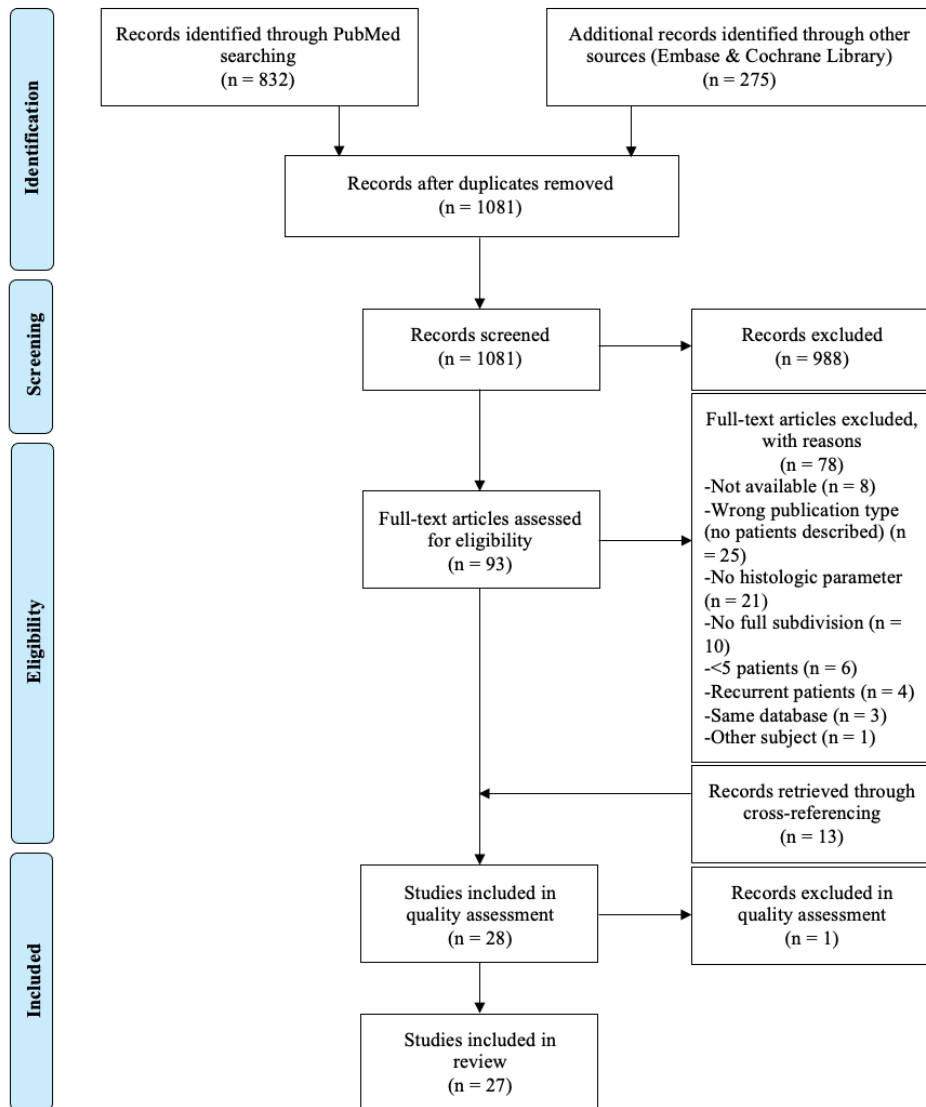
Table 1. Continued

First author of study	Patients (n)	Age (range) <sup>2</sup>	ER+	ER-	PR+	PR-	HER2+	HER2-	Triple negative	ER- PR-	ER/PR +
<b>Nugent<sup>18</sup></b>	14	Mean: 32 (26-37)	4	10							
<b>O'Sullivan<sup>39</sup></b>	57 52	Mean: 34-5	29	28			13	39			
<b>Ramírez-Torres<sup>18</sup></b>	16	Median: 35 (28-39)	7	9	4	12	5	11			
<b>Reed<sup>40</sup></b>	18	Mean: 34	4	14	6	12	8	10			
<b>Rouzier<sup>41</sup></b>	8 11	Mean: 36 (28-41)					2	6		6	5
<b>Tobon<sup>12</sup></b>	7	Mean: 32-3 (29-37)	0	7	5	2				2	5
<b>Yang<sup>43</sup></b>	15	Median: 33 (25-40)	9	6	5	10	6	9			

1 (ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor-2, NA = age-data not available).

2 The patients of whom age data was presented in the articles, did not always fully overlap with the BCdP-patient cohort due to possible missing values in the receptor status data.

3 Mean age was described as 37 years in the article text. However, when calculated from the presented study data, the mean age was 38 years.



**Figure 1.** Flowchart<sup>19</sup> of the record-selection process of papers describing receptor status of breast cancer diagnosed during pregnancy.

**Table 2.** Summarized results of published studies on receptor status in patients with breast cancer diagnosed during pregnancy determined by any method.

Estrogen Receptor			
Patients (n)	Studies (n)	ER+ (%)	ER- (%)
661	23	287 (43.4%)	374 (56.6%)
Progesterone Receptor			
Patients (n)	Studies (n)	PR+ (%)	PR- (%)
528	18	226 (42.8%)	302 (57.2%)
HER2			
Patients (n)	Studies (n)	HER2+ (%)	HER2- (%)
955	18	317 (33.2%)	638 (66.8%)
Triple negative			
Patients (n)	Studies (n)	Triple - (%)	Other
606	9	167 (27.6%)	439 (72.4%)
Estrogen and Progesterone Receptor negative			
Patients (n)	Studies (n)	ER- PR- (%)	Other (ER/PR +)
704	11	337 (47.9%)	367 (52.1%)

Receptor status of BCdP patients in eight studies in total were (presumably or definitely) not determined using IHC, but with alternative methods (e.g., Radioligand Binding Assay, Dextran Coated Charcoal or other).<sup>25,28,30,31,32,36,38,42</sup> All of these studies presented ER data, of which six also described PR data and two reported intrinsic subtype (hormone receptor negative) data. Table 3 shows the breakdown of receptor status in studies using IHC or biochemical methods. The percentage of ER negative cases was significantly higher for the biochemical methods, while for PR and ER/PR combined negativity the 95% confidence intervals overlapped.

Of the biochemical studies, only Meden et al.<sup>36</sup> described information on HER2-overexpression, which was determined using IHC. Therefore, the data for HER2 and triple negative subtype did not change and are thus not presented in Table 3.

**Table 3.** Summarized results of the receptor status of patients with breast cancer during pregnancy, determined by immunohistochemistry (IHC) or biochemistry.

Patients	Studies	Immunohistochemistry		Patients	Studies	Biochemistry	
		ER+	ER-			ER+	ER-
563	15	262 (46.5%) (95% CI 42.4% - 50.6%)	301 (53.5%) (95% CI 49.4% - 57.6%)	98	8	25 (25.5%) (95% CI 16.9% - 34.1%)	73 (74.5%) (95% CI 65.9% - 83.1%)
		PR+	PR-			PR+	PR-
456	12	196 (43.0%) (95% CI 38.5% - 47.5%)	260 (57.0%) (95% CI 52.5% - 61.5%)	72	6	30 (41.7%) (95% CI 30.3% - 53.1%)	42 (58.3%) (95% CI 46.9% - 69.7%)
		ER- PR-	Other (ER/PR +)			ER- PR-	Other (ER/PR +)
691	9	331 (47.9%) (95% CI 44.2% - 51.6%)	360 (52.1%) (95% CI 48.4% - 55.8%)	13	2	6 (46.2%) (95% CI 19.1% - 73.3%)	7 (53.8%) (95% CI 26.7% - 80.9%)

## DISCUSSION

This review comprises, to our knowledge, the most extensive description of the existing literature on BCdP receptor status. Here, we focused on pregnant breast cancer patients only, as this is the clearest and best defined group of pregnancy-associated breast cancers. For example in postpartum patients, other confounding factors may play a larger role (e.g., additional pregnancies, the potential role of lactation in carcinogenesis, environmental factors as advanced age, et cetera). Therefore, BCdP patients form the most homogenous and well defined group of breast cancers related to pregnancy.

As can be seen in Tables 2 and 3, a majority of BCdP tumors is ER negative and PR negative, and approximately one third of all patients show HER2-overexpression. Negativity for both ER and PR is also very common, as well as triple negativity. The detection method for HER2-overexpression was mainly IHC for all articles, whilst the detection methods for ER and PR varied. Older studies used biochemical methods, which showed an even higher percentage of ER negative cases. However, as these biochemical studies only contributed 13-15% of cases, the older studies have likely only influenced the overall results minimally. A few articles did not specify the methods used, for which we assumed IHC was applied, based on their publication year.

The patients used in the analysis of HER2 overexpression, triple negative subtype and combined ER/PR negativity are for a relatively large proportion derived from a single study<sup>17</sup> (respectively 36.9%, 62.9% and 59.5% of the total/IHC-only group). This study did not present a breakdown into separate ER and PR proportions, and therefore only influenced HER2, triple negativity and combined ER/PR negativity results. Leaving out these data did not change the results much (HER2 negativity 66.8% vs. 68.3%, ER/PR negativity 47.9% vs. 41.8%, triple negative subtype 27.6% vs. 21.8%), as the calculated 95% CI always showed overlap (data not presented). This denies a major result-modifying effect of this large patient cohort.

In the included articles, all data on receptor information was extracted and calculated. However, when, for example, data on triple negativity or combined ER/PR negativity was presented, it was not always possible to subsequently derive the separate ER and PR proportions when data was not broken down into the individual proportions. This is the case in four studies.<sup>17,26,37,41</sup>

The mean/median age of the BCdP patients in the reviewed studies varied from 30.7 to 38 years. PABC/BCdP patients might have an elevated risk for an unfavorable receptor status due to both their age and the association with pregnancy. In general, a younger age ( $\leq 40$  years) at diagnosis, irrespective of pregnancy status, is associated with more frequent hormone receptor negativity and HER2 positivity.<sup>44-46</sup> Therefore, age is already

a risk factor for adverse tumor characteristics in the BCdP patient group. However, also when compared to breast cancer patients of similar age without pregnancy-association, PABC/BCdP cases still demonstrated increased ER/PR negativity and a higher rate of HER2 overexpression.<sup>6,9–11,47</sup> This suggests that the pregnancy itself has an additional effect on the hormone and HER2 receptor status, increasing the likelihood of ER/PR negativity and HER2 overexpression.

Subsequently, a comparison between BCdP and postpartum breast cancer was attempted. However, as there was, to the best of our knowledge, no large description of the receptor status of breast cancer diagnosed postpartum solely, the results of this review were juxtaposed to two large PABC cohorts including both pregnant and postpartum cases. Both cohorts (Rodriguez et al.<sup>48</sup> and Wang et al.<sup>8</sup>) consisted largely of patients diagnosed until one year postpartum (respectively 76.5% and 78.9% of all included patients). This first PABC cohort of over 500 patients described by Rodriguez et al., reported 57.0% ER negative tumors and 59.1% PR negative tumors, percentages comparable to the results of this review. The second PABC cohort, consisting of 140 patients described by Wang et al., reported 45.7% of tumors to be negative for ER and 45.7% for PR, and 30% HER2 overexpression cases. These proportions are lower than the 56.6%, 57.2% ER respectively PR negativity and 33.2% HER2 positivity described in the present review. These varying proportions may be caused by differences in case selection (during pregnancy or postpartum, and/or the allowed postpartum interval), geographical differences, or varying distributions of gestational trimesters of diagnosis between the patient cohorts. Nevertheless, these results once again illustrate the complexity of the relationship between pregnancy and breast cancer and the necessity for further research on specific PABC-subgroups. For example, there is currently little information on the influence of a breast cancer diagnosis during the various pregnancy trimesters, which requires further investigation. In the studies included in this review, a coupling between gestational trimester of diagnosis and receptor status was only possible for 36 patients in total from five papers.<sup>20,22,30,36,42</sup> Possible differences between the gestational trimesters may be of interest in the future, as a recent publication by Johansson et al.<sup>49</sup> indicated a higher hazard ratio (HR) for mortality in breast cancer diagnosed during the second and third gestational trimester, when compared to first gestational trimester patients. Therefore, differences in receptor status between the trimesters may further elucidate the cause of this interesting finding.

Strengths of this study are the high number of included articles and the relatively 'simple' data, providing a lower chance of data-extraction errors. Moreover, possible deviations due to different receptor assays (IHC vs. others) were assessed. Furthermore, this review focused on one specific PABC subgroup, being BCdP, which led to the inclusion of only those studies that separately or solely described patients diagnosed

with breast cancer while being pregnant. This review therefore enables comparisons of BCdP patients to other PABC-subgroups based on, for example, lactational status and time after pregnancy (e.g., postpartum patients 1-5 years after delivery).

A limitation of this study may be variation in patient selection which occurred in some of the included studies. For example, Ezzat et al.<sup>25</sup> excluded patients with distant metastases, while Feng et al.<sup>26</sup> excluded patients with locally widespread and/or stage IV disease. These exclusions may have led to less aggressive hormonal profiles in our results. In contrast, Giacalone et al.<sup>28</sup> and Dominici et al.<sup>24</sup> only included patients who received chemotherapy, while Rouzier et al.<sup>41</sup> only described patients of whom treatment had yet been initiated during pregnancy. These criteria may have therefore led to the description of more aggressive hormonal profiles in our results.

As a general remark, many articles did not specifically describe if a consecutive and/or complete inclusion had occurred. This may be a point of attention for case series on this subject that will be published in the future.

In conclusion, this review defined the receptor status landscape of breast cancers diagnosed during pregnancy, being predominantly ER and PR negative and often HER2 positive with higher percentages than published before in large studies that also included postpartum cases. BCdP may therefore be a separate entity with a more aggressive profile when compared to PABC and non-pregnant breast cancer patients of similar age. This different histopathologic profile sets the stage for further in depth research comparing breast cancers diagnosed during the different gestational trimesters and those diagnosed postpartum (lactating and non-lactating). To shed light on possible differences in tumor (micro)environment and tumor characteristics between the various subgroups of breast cancer related to pregnancy, further molecular and genetic research is required. Specific molecular features may eventually serve as a steppingstone for further improvement of the existing therapies for BCdP.

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05



# Prognosis of Pregnancy-associated Breast Cancer: Inferior Outcome in Patients Diagnosed during Second and Third Gestational Trimesters and Lactation

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## ABSTRACT

### Purpose

Pregnancy-associated breast cancer (PABC), although most commonly defined as breast cancer diagnosed during pregnancy or  $\leq 1$  year following delivery, knows a variety of definitions, likely related to the diversity of reported clinicopathological features and prognosis. More insight into the different breast cancer subgroups during pregnancy, time after delivery and the postpartum period is therefore warranted.

### Methods

Patients with breast cancer diagnosed during pregnancy or  $\leq 6$  months postdelivery were included, and subdivided according to gestational trimester, and postpartum patients according to lactational status. Subgroups were compared to matched non-PABC patients, to investigate the influence of pregnancy and lactation on clinical course and outcome.

### Results

Overall, 662 PABC patients were included (median age 34 years, median follow-up 6.5 years). PABC patients showed an advanced stage at diagnosis and an inferior 5-years-OS (75.4% vs. 83.2%,  $p=0.000$ ) compared to 1,392 matched non-PABC patients. In subgroup analysis, first trimester PABC patients showed a significantly lower tumor size and stage as compared to other trimesters. Patients diagnosed during the first trimester and postpartum non-lactating patients had a relatively good OS (81.3% and 77.9%, respectively) versus patients diagnosed during the second and third trimesters and during lactation (OS 60.0%, 64.9% and 65.6%, respectively,  $p=0.003$ ).

### Conclusion

In this large (uniquely specified) PABC cohort, an inferior outcome was found for patients diagnosed within the second and third gestational trimesters and during lactation. These findings indicate that PABC is clinically a heterogeneous group of breast cancer patients that should be redefined based on trimester of diagnosis and lactational status.

## INTRODUCTION

Breast cancer (BC) is the most common cancer, the leading cause of cancer death in women worldwide, and the most common cancer in pregnant women [1-4]. Pregnancy has a dual influence on breast cancer risk; it has long-term protective effects (after full-term delivery and breastfeeding) [5-7], however, there is a temporary increase in breast cancer incidence during pregnancy and 5-10 years postpartum (or even longer in case of age >35 years at first live birth) [8-11].

Whether pregnancy-associated breast cancer (PABC), commonly defined as breast cancer diagnosed during or within one year following delivery, has a worse prognosis, is currently the subject of debate. Evolving evidence indicates that women with PABC have a poorer prognosis as compared to women with non-pregnancy-related breast cancers [12-18]. These studies and our previous study [19] demonstrated that pregnancy-associated breast tumors exhibit adverse prognostic characteristics, consisting of an advanced tumor (T) stage at diagnosis, nodal involvement (possibly attributed to the delayed diagnosis of tumors during pregnancy and lactation), high histologic grade, negative estrogen (ER) and progesterone (PR) status and frequent human epidermal growth factor receptor-2 (HER-2) overexpression. The worse prognosis persists after adjustment for age at breast cancer diagnosis, year of diagnosis and tumor stage [12,15,16]. In addition, a recent meta-analysis of Shao et al. demonstrated, compared to nulliparous women, a 60% higher mortality in women with PABC diagnosed at 12 months after delivery, without significant differences at 70 months after the final delivery [20]. In contrast, other studies did not show significant differences in both disease-free-survival (DFS) and overall survival (OS) in women with PABC in comparison with breast cancer diagnosed outside pregnancy or the postpartum period [21-27].

Nevertheless, a comparison of the available data is complex because of the small numbers of patients examined in each of the individual studies, the dissimilar reference populations, and specifically the differences in inclusion criteria (e.g., the definition of PABC often includes postpartum periods up to two years). This variability may have led to the diverse reports on the relationship between pregnancy, postpartum lactation and breast cancer prognosis. Therefore, proper analysis requires a better definition of PABC with a focus on subgroups consisting of breast cancer diagnosed during the different gestational trimesters, the early and the advanced postpartum period and the influence of lactation. More insight should be obtained into the varying clinicopathologic features and prognosis of these unique breast cancer entities, as this may guide new personalized treatment strategies. Indeed, in a previous paper, we showed that the histopathological profile of PABC patients varies between the trimesters and postpartum period [28].

To the best of our knowledge, this is the first (large population based) analysis providing comprehensive insight into the association between gestational age at breast cancer diagnosis, the influence of lactation ( $\leq 6$  months postpartum) and OS, compared to matched non-PABC invasive breast cancers.

## METHODS

### Data source and study population

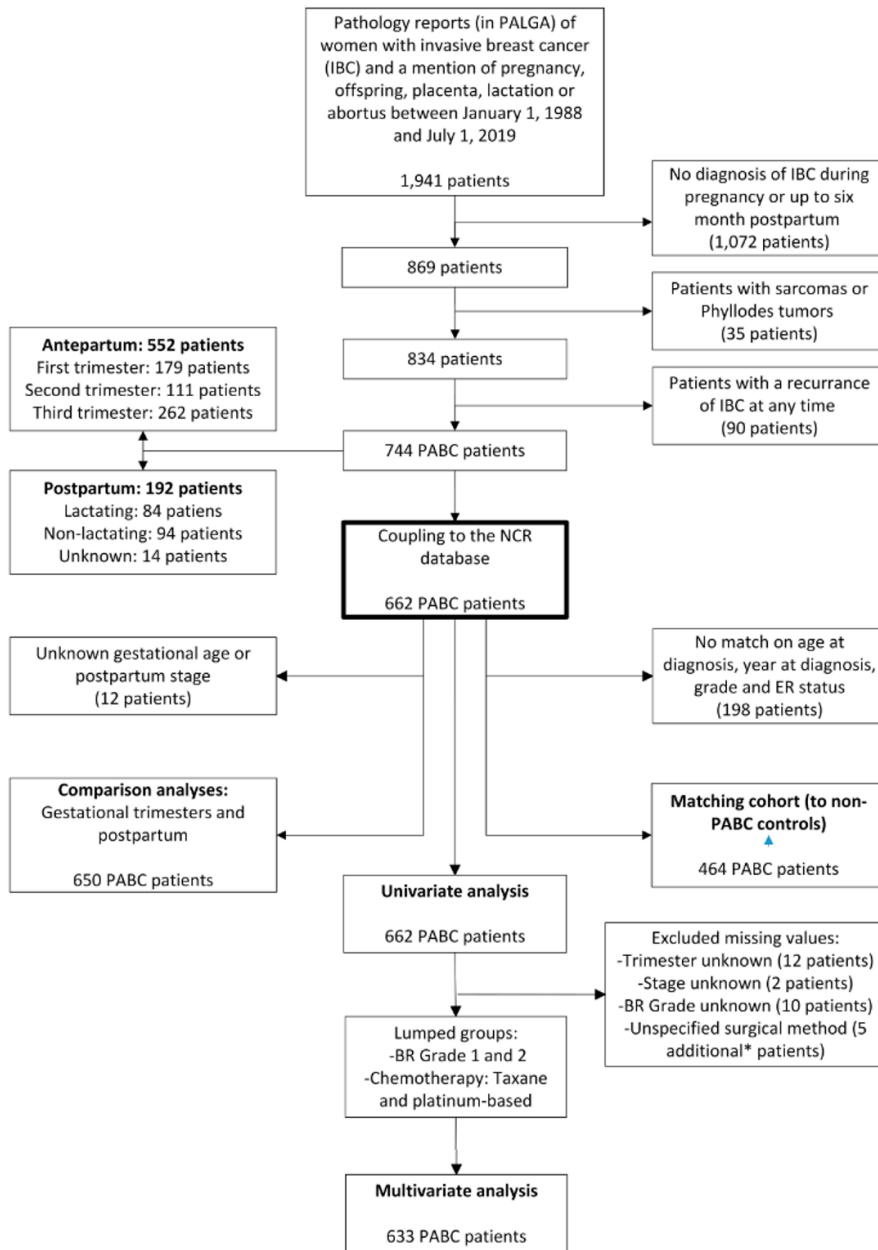
Using the Dutch nationwide network and registry of histo- and cytopathology (PALGA) [29,30], we reviewed excerpts of pathology reports in The Netherlands of all breast cancer (BC) cases of women  $\leq 45$  years of age, diagnosed between January 1<sup>st</sup>, 1988 and July 1<sup>st</sup>, 2019.

Of these reports, all patients with a diagnosis of invasive BC and a mention of pregnancy, amenorrhea duration, offspring, placenta, lactation or abortion in their pathology report ( $n=1,941$ ), were included in the PABC cohort when a diagnosis of BC was made during pregnancy or within six months after delivery (irrespective of pregnancy outcome and BC treatment) ( $n=744$ ) (Figure 1). Patients with a history of invasive BC or carcinoma *in situ* before their pregnancy, as well as patients with sarcomas or a phyllodes tumor were excluded. The remaining BC patients ( $\leq 45$  years of age) were selected as control non-PABC patients. Clinicopathological data from pathology reports of both groups, including age at diagnosis, tumor subtype, Bloom Richardson (BR) grade, ER-, PR- and HER-2 expression, were obtained. In addition, gestational age at diagnosis was extracted from the pathology reports by distinction between duration of amenorrhea, time of abortion, miscarriage, date of delivery, date of submission of a placenta or umbilical cord. Thereafter, gestational age was subdivided into the gestational trimesters; first trimester (weeks 1-12), second trimester (weeks 13-26), third trimester (weeks 27-42), and the postpartum period. Here, we opted for a more restricted definition: we limited the postpartum period to BC diagnoses up to six months after delivery, to stay as closely as possible to the hormonal milieu of the pregnancy/lactation period and to exclude confounding factors as concomitant pregnancy and advanced post-lactational breast involution. For the postpartum period we separated women into lactating and non-lactating.

In the second step, we linked our database of 744 PABC patients to the Netherlands Cancer Registry (NCR) (controlled by the Netherlands Comprehensive Cancer Organization (IKNL)) for additional clinical information (e.g., TNM stage at diagnosis, BC treatment and outcome) of each BC patient in our cohort. Eighty-two patients were excluded, as their BC-diagnose originated mostly from before 2000, at which time the



clinical data was not exclusively stored in the NCR database. This resulted in 662 PABC patients with full relevant clinical information (Figure 1).



**Figure 1.** Schematic presentation of case selection and inclusion.

### Study approval and privacy

All data were pseudonymized by a trusted third party (ZorgTTP, Houten, The Netherlands). This study was approved by scientific and privacy committees of both PALGA and IKNL. All data were thereby retrieved and handled in compliance with the General Data Protection Regulation Act (GDPR).

### Control non-PABC cohort

A control (non-PABC) cohort of young patients diagnosed with BC without being pregnant or postpartum over more than one year was assembled (derived from the NCR database). In order to prevent influences of differences in age at diagnosis, treatment modalities over the last decades and histopathologic profiles, 662 PABC patients were matched for age at diagnosis, year at diagnosis, grade and ER status to the non-PABC group. One-to-three matching was possible for 464 PABC patients (mainly due to an unknown ER status before 2005 and absence of extensive clinical data in the NCR), providing a control group of 1,392 non-PABC patients (Figure 1).

### Statistical analysis

Pearson Chi-square ( $\chi^2$ ) tests were used to compare the clinicopathologic characteristics between the different PABC subgroups (gestational trimesters and postpartum lactating and non-lactating status) and between the PABC- and non-PABC cohorts. OS (defined as time from initial diagnosis to the time of death or date of last follow-up) analysis was performed using the Kaplan-Meier/logrank method.

Multivariate survival analysis was performed to correct for subgroup differences in confounding variables that independently influence survival. Therefore, potential confounding variables were selected, including gestational trimester or postpartum status at diagnosis, age-, year- and stage at diagnosis, BR grade, ER-, PR- and HER-2 status, surgery and chemotherapy. Univariate analysis of all variables showed age at diagnosis and ER status to be non-significant (here considered as a p-value <0.20) contributors to survival estimation and were therefore excluded from the multivariate model (Table 4). The final multivariate Cox regression analysis in this PABC cohort included gestational trimester or postpartum status at diagnosis, year of diagnosis, PR status, stage at diagnosis and surgery.

In our multivariate analysis, 29 patients were excluded due to missing values, leaving 633 patients (Figure 1). To keep subgroups as large as possible, BR grade 1 and 2 were lumped, even as platinum based chemotherapy was lumped with taxane chemotherapy (as most platinum regimens also included taxanes). The multivariate analyses was performed using Cox regression (Backward Stepwise Likelihood Ratio; enter and remove limits 0.05/0.10), calculating Hazard Ratios (HRs) per variable. P-values  $\leq 0.05$  were considered statistically significant.

## RESULTS

### Characteristics of the PABC cohort

We identified 744 women with PABC from the PALGA database. Eighty-two women could not be linked to the clinical NCR database, leaving 662 for analysis (Figure 1). Clinicopathologic characteristics of the PABC cohort are shown in Table 1. Breast cancer was diagnosed in 73.6% during pregnancy whereas in 24.6% during the postpartum period. Most pregnant patients were diagnosed during the third gestational trimester (34.4%), followed by the first (24.5%) and second (14.7%) trimesters. Median age at diagnosis was 34 years (ranging 19-45 years) and most patients (77.2%) were diagnosed after the year 2000. Invasive ductal/no special type carcinoma was most commonly observed (83.3%) and 80.5% had high grade tumors (BR grade 3). Immunohistochemistry showed that the majority of tumors were negative for ER, PR and HER-2 (53.2%, 56.3% and 65.7%, respectively).

**Table 1.** Clinicopathologic characteristics of a nationwide Dutch cohort of pregnancy associated breast cancer (PABC) patients

	PABC patients (%)	
Total number of patients	662	
Pregnancy trimester at diagnosis		
Trimester 1	162	24.5%
Trimester 2	97	14.7%
Trimester 3	228	34.4%
Postpartum non-lactating	88	13.3%
Postpartum lactating	75	11.3%
Unknown	12	1.8%
Age at diagnosis		
18-25 years	8	1.2%
26-30 years	119	18.0%
31-35 years	295	44.6%
36-40 years	193	29.2%
>40 years	47	7.1%
Mean age in years (±SD)	34.17	(±4.198)
Median age in years (IQR)	34	(31 – 37 years)
Year of diagnosis		
≤1995	69	10.4%
1996-2000	82	12.4%
2001-2005	121	18.3%
2006-2010	136	20.5%
2011-2015	145	21.9%

**Table 1.** Continued

Total number of patients	PABC patients (%)	
	662	
>2015	109	16.5%
Clinical TNM-stage at diagnosis		
Primary tumor (T) stage		
In situ	3	0.5%
To	2	0.3%
T1	224	33.8%
T2	279	42.1%
T3	87	13.1%
T4	39	5.9%
Unknown	28	4.2%
Regional lymph node (N) stage		
No	408	61.6%
N1	185	27.9%
N2	8	1.2%
N3	25	3.8%
Unknown	36	5.4%
Distant metastases (M) stage		
Mo	555	83.8%
M1	52	7.9%
Unknown	55	8.3%
Stage at diagnosis		
1	129	19.5%
2	347	52.4%
3	130	19.6%
4	54	8.2%
Unknown	2	0.3%
Bloom Richardson grade		
1	10	1.5%
2	109	16.5%
3	533	80.5%
Unknown	10	1.5%
Estrogen receptor status		
Positive (+)	261	39.4%
Negative (-)	352	53.2%
Unknown	49	7.4%
Progesterone receptor status		
Positive (+)	227	34.3%
Negative (-)	373	56.3%
Unknown	62	9.4%
Human epidermal growth factor receptor 2		
Positive (+)	133	20.1%
Negative (-)	435	65.7%

**Table 1.** Continued

		PABC patients (%)	
Total number of patients		662	
Unknown		94	14.2%
Treatment			
Surgery			
Yes		628	94.9%
	Mastectomy with ALND#	211	33.6%
	Mastectomy without ALND	146	23.2%
	Lumpectomy with ALND	94	15.0%
	Lumpectomy without ALND	169	26.9%
	Surgical method not specified	8	1.3%
No		34	5.1%
Chemotherapy			
Yes		562	84.9%
	Anthracycline	127	22.6%
	Taxane	43	7.7%
	Anthracycline and taxane	131	23.3%
	Platinum	9	1.6%
	Platinum and taxane	4	0.7%
	Paclitaxel, trastuzumab and carboplatin	2	0.4%
	Unspecified	246	43.8%
No		100	15.1%
Median duration of follow up in days (IQR)		2378.5	(1028.5 - 4956.5 days)
5 Year Overall Survival (%) (n=534)		378	70.8%
Survival Total (%)		452	68.3%

# ALND=axillary lymph node dissection

Over a quarter of patients (27.8%) presented with an advanced tumor stage (stage  $\geq 3$ ). The majority of patients received a combination of different treatment modalities consisting of surgery and chemotherapy; unfortunately information on radiotherapy and endocrine therapy was not registered. Due to their teratogenic effects during pregnancy, radiotherapy and endocrine therapy can only start after delivery; registering this treatment modality would confound results within the entire PABC group (as the postpartum part is nearly 25%). Mastectomies were most often performed (56.8%), whilst approximately half of our PABC cohort (48.6%) underwent an additional axillary lymph node dissection (ALND). Among PABC patients, 84.9% underwent chemotherapy, of which 23.3% received a combination of anthracyclines and taxanes. The group with “unspecified” chemotherapy, 43.8% of all patients, mainly consisted of patients from the early years of this cohort. Median follow-up was 6.5 years (interquartile range 2.8–13.6 years). The 5-years OS of PABC patients as a group was 70.8%.

### Trimesters and lactational status

Of the 662 PABC patients, 650 patients were eligible for comparison, as trimester was unknown for 12 patients (Figure 1, Table 2). Age at diagnosis was comparable for all trimester subgroups and postpartum patients, and the years of diagnosis were similar for all different subgroups. However, tumor (T) stage was significantly higher ( $T \geq 2$ ) in the second and third trimesters and postpartum (non-lactating and lactation) group as compared with the first trimester (respectively, 70.1%, 69.3%, 68.2%, 62.7%, versus 40.1%,  $p=0.000$ ). Postpartum non-lactating patients were more often diagnosed with at least one positive lymph node (N+) (40.9%), whilst first trimester patients were more frequently N=0 (65.4%) ( $p=0.088$ ). Distant metastases (M) stage did not significantly differ between subgroups ( $p=0.075$ ). However, there was a significant shift in the distribution toward a more advanced overall tumor stage from the second trimester onwards with 19.7% of first trimester patients with stage 3/4 disease, compared to 29.9%, 32.9%, 28.4% and 28.0% for the second and third trimesters and postpartum non-lactating and lactating patients, respectively ( $p=0.040$ ).

**Table 2.** Clinicopathologic characteristics and survival per gestational trimester or lactation status (postpartum) at BC diagnosis of a cohort of pregnancy associated breast cancer patients

	First trimester	Second trimester	Third trimester	Postpartum (non-lactating)	Postpartum (lactating)	p-value
Total (%), n=650	162 (24.9%)	97 (14.9%)	228 (35.1%)	88 (13.5%)	75 (11.5%)	NA
Age at diagnosis						0.690*
18-25 years	1 (0.6%)	2 (2.1%)	3 (1.3%)	2 (2.3%)	0 (0%)	
26-30 years	26 (16.0%)	18 (18.6%)	44 (19.3%)	15 (17.0%)	12 (16.0%)	
31-35 years	68 (42.0%)	43 (44.3%)	109 (47.8%)	34 (38.6%)	36 (48.0%)	
36-40 years	56 (34.6%)	29 (29.9%)	58 (25.4%)	30 (34.1%)	18 (24.0%)	
>40 years	11 (6.8%)	5 (5.2%)	14 (6.1%)	7 (8.0%)	9 (12.0%)	
Mean age in years ( $\pm$ SD)	34.47 ( $\pm$ 3.968)	34.02 ( $\pm$ 4.118)	33.79 ( $\pm$ 4.125)	34.24 ( $\pm$ 4.710)	34.92 ( $\pm$ 4.126)	
Median age in years (IQR)	35 (32-37)	34 (31-37)	33 (31-37)	34 (31-38)	35 (32-37)	
Year of diagnosis						0.561*
$\leq 1995$	23 (14.2%)	7 (7.2%)	20 (8.8%)	10 (11.4%)	8 (10.7)	
1996-2000	21 (13.0%)	8 (8.2%)	31 (13.6%)	9 (10.2%)	11 (14.7%)	
2001-2005	31 (19.1%)	15 (15.5%)	40 (17.5%)	17 (19.3%)	12 (16.0%)	
2006-2010	38 (23.5%)	19 (19.6%)	48 (21.1%)	18 (20.5%)	14 (18.7%)	
2011-2015	31 (19.1%)	23 (23.7%)	55 (24.1%)	17 (19.3%)	19 (25.3%)	
>2015	18 (11.1%)	25 (25.8%)	34 (14.9%)	17 (19.3%)	11 (14.7%)	
Clinical TNM-stage at diagnosis						0.000*
Primary tumor (T) stage						
In Situ (IS)	0 (0%)	1 (1.0%)	2 (0.9%)	0 (0%)	0 (0%)	
T0	2 (1.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
T1	84 (51.9%)	26 (26.8%)	59 (25.9%)	23 (26.1%)	27 (36%)	
T2	46 (28.4%)	44 (45.4%)	104 (45.6%)	45 (51.1%)	34 (45.3%)	
T3	14 (8.6%)	16 (16.5%)	35 (15.4%)	10 (11.4%)	11 (14.7%)	
T4	5 (3.1%)	8 (8.2%)	19 (8.3%)	5 (5.7%)	2 (2.7%)	
Unknown	11 (6.8%)	2 (2.1%)	9 (3.9%)	5 (5.7%)	1 (1.3%)	

Table 2. Continued

	First trimester	Second trimester	Third trimester	Postpartum (non-lactating)	Postpartum (lactating)	p-value
Total (%), n=650	162 (24.9%)	97 (14.9%)	228 (35.1%)	88 (13.5%)	75 (11.5%)	NA
Regional lymph node (N) stage						0.088*
No	106 (65.4%)	59 (60.8%)	143 (62.7%)	45 (51.1%)	45 (60%)	
N1	43 (26.5%)	33 (34.0%)	58 (25.4%)	27 (30.7%)	23 (30.7%)	
N2	1 (0.6%)	0 (0%)	2 (0.9%)	4 (4.5%)	0 (0%)	
N3	3 (1.9%)	1 (1.0%)	12 (5.3%)	5 (5.7%)	4 (5.3%)	
Unknown	9 (5.6%)	4 (4.1%)	13 (5.7%)	7 (8.0%)	3 (4%)	
Distant metastases (M) stage						0.075*
Mo	143 (88.3%)	80 (82.5%)	192 (84.2%)	73 (83.0%)	59 (78.7%)	
M1	7 (4.3%)	5 (5.2%)	25 (11.0%)	6 (6.8%)	8 (10.7%)	
Unknown	12 (7.4%)	12 (12.4%)	11 (4.8%)	9 (10.2%)	8 (10.7%)	
Stage						0.040*
1	45 (27.8%)	12 (12.4%)	37 (16.2%)	16 (18.2%)	18 (24.0%)	
2	85 (52.5%)	56 (57.7%)	116 (50.9%)	45 (51.1%)	36 (48.0%)	
3	24 (14.8%)	24 (24.7%)	49 (21.5%)	19 (21.6%)	13 (17.3%)	
4	8 (4.9%)	5 (5.2%)	26 (11.4%)	6 (6.8%)	8 (10.7%)	
Unknown	0 (0%)	0 (0%)	0 (0%)	2 (2.3%)	0 (0%)	
Bloom Richardson grade						0.189*
1	5 (3.1%)	0 (0%)	2 (0.9%)	2 (2.3%)	1 (1.3%)	
2	35 (21.6%)	12 (12.4%)	38 (16.7%)	13 (14.8%)	9 (12%)	
3	119 (73.5%)	85 (87.6%)	185 (81.1%)	72 (81.8%)	62 (82.7%)	
Unknown	3 (1.9%)	0 (0%)	3 (1.3%)	1 (1.1%)	3 (4%)	
Estrogen receptor status						0.046*
Positive (+)	81 (50%)	35 (36.1%)	82 (36.0%)	35 (39.8%)	23 (30.7%)	
Negative (-)	67 (41.4%)	54 (55.7%)	132 (57.9%)	45 (51.1%)	48 (64%)	
Unknown	14 (8.6%)	8 (8.2%)	14 (6.1%)	8 (9.1%)	4 (5.3%)	
Progesterone receptor status						0.304*
Positive (+)	64 (39.5%)	34 (35.1%)	78 (34.2%)	26 (29.5%)	21 (28%)	
Negative (-)	78 (48.1%)	55 (56.7%)	130 (57.0%)	53 (60.2%)	50 (66.7%)	
Unknown	20 (12.3%)	8 (8.2%)	20 (8.8%)	9 (10.2%)	4 (5.3%)	
Human epidermal growth factor receptor 2						0.411*
Positive (+)	30 (18.5%)	19 (19.6%)	54 (23.7%)	20 (22.7%)	7 (9.3%)	
Negative (-)	107 (66.0%)	65 (67.0%)	143 (62.7%)	56 (63.6%)	57 (76%)	
Unknown	25 (15.4%)	13 (13.4%)	31 (13.6%)	12 (13.6%)	11 (14.7%)	
Treatment						
Surgery						
Yes	158 (97.5%)	96 (99.0%)	211 (92.5%)	84 (95.5%)	68 (90.7%)	0.002*
Mastectomy with ALND#	50 (31.6%)	41 (42.7%)	67 (31.8%)	33 (39.3%)	17 (25%)	
Mastectomy without ALND	34 (21.5%)	27 (28.1%)	53 (25.2%)	16 (19.0%)	14 (20.6%)	
Lumpectomy with ALND	37 (23.4%)	7 (7.3%)	29 (13.7%)	10 (11.9%)	8 (11.8%)	
Lumpectomy without ALND	36 (22.8%)	21 (21.9%)	59 (28.0%)	22 (26.2%)	28 (41.2%)	
Surgical method not specified	1 (0.6%)	0 (0%)	3 (1.4%)	3 (3.6%)	1 (1.5%)	
No	4 (2.5%)	1 (1.0%)	17 (7.5%)	4 (4.5%)	7 (9.3%)	

**Table 2.** Continued

	First trimester	Second trimester	Third trimester	Postpartum (non-lactating)	Postpartum (lactating)	p-value
Total (%), n=650	162 (24.9%)	97 (14.9%)	228 (35.1%)	88 (13.5%)	75 (11.5%)	NA
Chemotherapy						
Yes	131 (80.9%)	87 (89.7%)	197 (86.4%)	75 (85.2%)	62 (82.7%)	0.020*
Anthracycline	31 (23.7%)	33 (37.9%)	40 (20.3%)	10 (13.3%)	12 (19.4%)	
Taxane	12 (9.2%)	4 (4.6%)	14 (7.1%)	12 (16%)	1 (1.6%)	
Anthracycline and taxane	26 (19.8%)	20 (23.0%)	47 (23.9%)	17 (22.7%)	18 (29.0%)	
Platinum	4 (3.1%)	0 (0%)	5 (2.5%)	0 (0%)	0 (0%)	
Platinum and taxane	1 (0.8%)	0 (0%)	3 (1.5%)	0 (0%)	0 (0%)	
Paclitaxel, trastuzumab and carboplatin	0 (0%)	0 (0%)	1 (0.5%)	1 (1.3%)	0 (0%)	
Unspecified	57 (43.5%)	30 (34.5%)	87 (44.2%)	35 (46.7%)	31 (50%)	
No	31 (19.1%)	10 (10.3%)	31 (13.6%)	13 (14.8%)	13 (17.3%)	
5 year overall survival (%)	113 (81.3%) (n=139)	42 (60%) (n=70)	120 (64.9%) (n=185)	53 (77.9%) (n=68)	40 (65.6%) (n=61)	0.003**
Survival total (%)	118 (72.8%)	64 (66.0%)	144 (63.2%)	67 (76.1%)	50 (66.7%)	0.027**

NA = Not Applicable.

\*: Determined via  $\chi^2$ -test

\*\*: Determined via Log Rank (Mantel-Cox) procedure

# ALND=axillary lymph node dissection

When comparing treatment modalities per subgroup, patients in the third trimester and lactating patients underwent less frequently surgery ( $p=0.025$ ), while second trimester patients were more often treated by mastectomy (70.8%) ( $p=0.002$ ). Overall, administration of chemotherapy did not significantly differ between pregnant and postpartum subgroups ( $p=0.342$ ), although there was a significant difference in the application of the specific chemotherapeutic agents and regimens that were applied ( $p=0.020$ ). Taxane usage was most common in the postpartum non-lactating patients (16.0%), whilst anthracycline monotherapy was primarily applied in second trimester (37.9%). Besides anthracycline monotherapy, a combination of anthracyclines with taxane also widely used in all trimesters.

### Comparison of PABC and non-PABC patients

Matched PABC-patients ( $n=464$ ) presented significant differences for primary tumor size, stage at diagnosis, surgery and chemotherapy, in comparison to non-PABC patients ( $n=1,392$ ) (Table 3). PABC patients were more likely to have higher T status (T2: 42.0% vs. 38.1%, T3: 11.4 vs. 5.2% and T4: 3.0% vs. 2.4%,  $p<0.005$ ), and a higher overall tumor stage at diagnosis (stage 2: 55.8 vs. 52.6%, stage 3: 17.2 vs. 14.6%, stage 4: 5.6 vs. 3.2%,  $p<0.005$ ). PABC patients tended to be treated more often by mastectomy than the non-PABC group (56.6% vs. 49.7%).



**Table 3.** Distribution of clinical variables for the PABC and non-PABC cohorts\*

	PABC patients (%)		Non-PABC patients (%)		p-value
Total number of patients	464		1392		NA
Pregnancy trimester at diagnosis					
Trimester 1	119	25.6%			
Trimester 2	69	14.9%			
Trimester 3	149	32.1%			
Postpartum non-lactating	63	13.6%			
Postpartum lactating	56	12.1%			
Unknown	8	1.7%			
Age at diagnosis					0.879*
18-25 years	5	1.1%	16	1.1%	
26-30 years	88	19.0%	239	17.2%	
31-35 years	202	43.5%	608	43.7%	
36-40 years	136	29.3%	415	29.8%	
>40 years	33	7.1%	114	8.2%	
Mean age in years (±SD)	34.16	(±4.177)	34.31	(±4.184)	
Median age in years (IQR)	34	(31 – 37 years)	34	(31 – 37 years)	
Year of diagnosis					1.000*
≤1995	27	5.8%	80	5.7%	
1996-2000	63	13.6%	188	13.5%	
2001-2005	106	22.8%	314	22.6%	
2006-2010	108	23.3%	326	23.4%	
2011-2015	108	23.3%	324	23.3%	
>2015	52	11.2%	160	11.5%	
Clinical TNM-stage at diagnosis					
Primary tumor (T) stage					0.000*
In situ	3	0.6%	11	0.8%	
T0	1	0.2%	6	0.4%	
T1	180	38.8%	657	47.2%	
T2	195	42.0%	530	38.1%	
T3	53	11.4%	72	5.2%	
T4	14	3.0%	34	2.4%	
Unknown	18	3.9%	82	5.9%	
Regional lymph node (N) stage					0.970*
N0	316	68.1%	944	67.8%	
N1	113	24.4%	334	24.0%	
N2	3	0.6%	7	0.5%	
N3	8	1.7%	24	1.7%	
Unknown	24	5.2%	83	6.0%	
Distant metastases (M) stage					0.155*
M0	400	86.2%	1219	87.6%	
M1	24	5.2%	45	3.2%	
Unknown	40	8.6%	128	9.2%	
Stage at diagnosis					0.001*
1	99	21.3%	409	29.4%	
2	259	55.8%	732	52.6%	
3	80	17.2%	203	14.6%	
4	26	5.6%	45	3.2%	
Unknown	0	0%	3	0.2%	

**Table 3.** Continued

	PABC patients (%)		Non-PABC patients (%)		p-value
Total number of patients	464		1392		NA
Treatment					
Surgery					0.993*
Yes	452	97.4%	1355	97.3%	0.000*
Mastectomy with ALND#	151	33.4%	382	28.2%	
Mastectomy without ALND	105	23.2%	292	21.5%	
Lumpectomy with ALND	65	14.4%	189	14.0%	
Lumpectomy without ALND	128	28.3%	478	35.3%	
Surgical method not specified	3	0.7%	14	1.0%	
No	12	2.6%	37	2.7%	
Chemotherapy					0.514*
Yes	400	86.2%	1181	84.8%	0.003*
Anthracycline	74	18.5%	165	14.0%	
Taxane	23	5.8%	117	9.9%	
Anthracycline and taxane	110	27.5%	362	30.7%	
Platinum	4	1%	1	0.1%	
Platinum and taxane	0	0%	1	0.1%	
Paclitaxel, trastuzumab and carboplatin	1	0.3%	1	0.1%	
Unspecified	188	47.0%	534	45.2%	
No	64	13.8%	211	15.2%	
5 Year Overall Survival (%) (n=395 / n=1230)	298	75.4%	1023	83.2%	0.000**
Survival total (%)	332	71.6%	1028	73.9%	0.085**

# ALND=axillary lymph node dissection

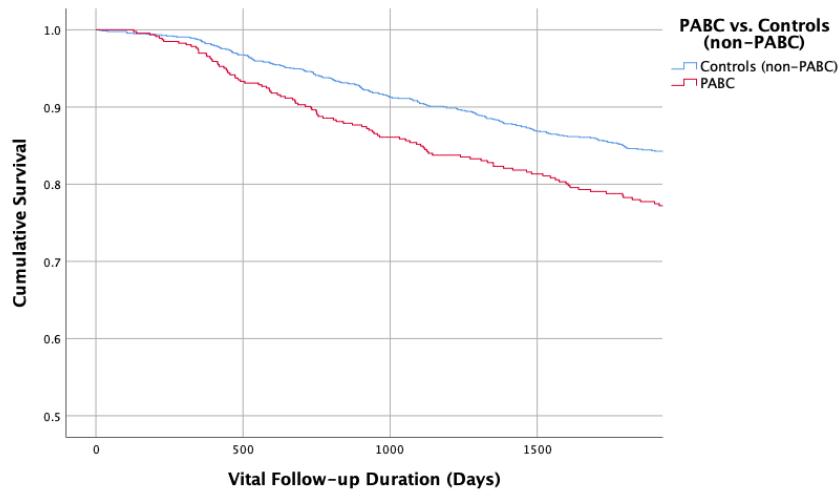
\* the histopathological data (ER, PR, HER2) were described in our previous paper [29].

### Survival analysis

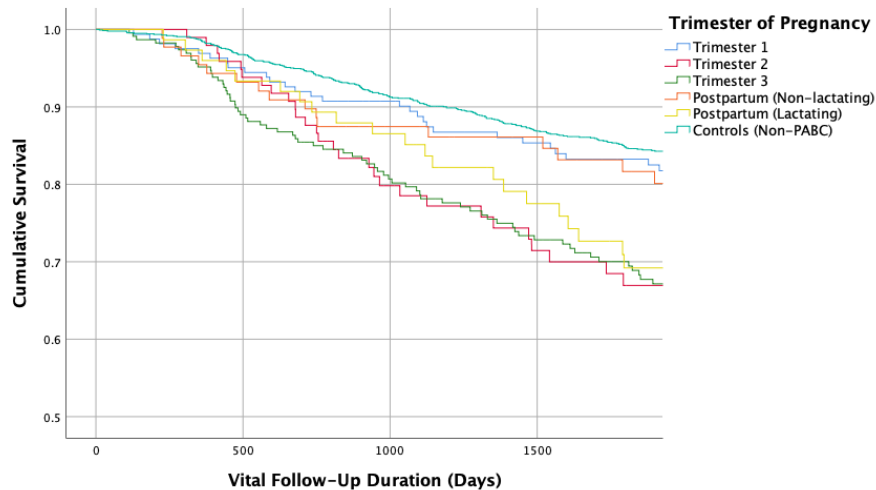
Figure 2 shows that five-year OS of the PABC patients was significantly worse at 75.4% compared with 83.2% for the non-PABC patients ( $p=0.000$ ). When evaluating subgroups (Figure 3), five-year OS of the first trimester (81.3%) and postpartum non-lactating patients (77.9%) was significantly better (and comparable to controls) than OS of second- and third trimester patients, and postpartum lactating patients (60.0%, 64.9%, and 65.6%, respectively ( $p=0.003$ ).

In multivariate analysis, age at diagnosis, ER and HER-2 status, grade and chemotherapy did not contribute to survival estimation and were therefore excluded from the multivariate model (Table 4). The final Cox regression model in the PABC cohort thereby included trimester of diagnosis, year of diagnosis, PR-status, stage at diagnosis and surgery (Table 5). Although HRs of patients diagnosed in the second and third trimesters were  $> 1$ , indicating a higher risk for mortality when compared to the first trimester, these differences were only significant for the second trimester patients ( $p=0.050$ ) after multivariate correction. Patients diagnosed in 1995 or before showed

a significantly higher risk of mortality (HR 3.818,  $p=0.001$ ) than patients diagnosed in 2015 or thereafter. Advanced overall tumor stage at diagnosis and forgoing surgery also indicated a significantly higher risk of death, while differences between the surgical methods were not significant.



**Figure 2.** Kaplan-Meier curves for 5-year overall survival for PABC versus control non-PABC patients, revealing the worse prognosis of PABC patients as a group ( $p=0.000$ , logrank test).



**Figure 3.** Kaplan-Meier curves for 5-year overall survival for PABC patients (subdivided by gestational trimester and postpartum status) versus control non-PABC patients.

Patients diagnosed in the first trimester of pregnancy fared best, closely followed by postpartum non-lactating PABC patients and controls, while second and third trimester and postpartum non-lactating PABC patients had a much worse prognosis.

**Table 4.** Results of univariate analysis in a nationwide cohort of pregnancy associated breast cancer patients

	Alive (n) %		Deceased		P-value
Total number of patients	452	66.8%	210	31.7%	
Pregnancy trimester at diagnosis					0.192
Trimester 1	118	72.8%	44	27.2%	
Trimester 2	64	66.0%	33	34.0%	
Trimester 3	144	63.2%	84	36.8%	
Postpartum non-lactating	67	76.1%	21	23.9%	
Postpartum lactating	50	66.7%	25	33.3%	
Unknown	9	75%	3	25%	
Age at diagnosis					0.809
18-25 years	4	50%	4	50%	
26-30 years	82	68.9%	37	31.1%	
31-35 years	199	67.5%	96	32.5%	
36-40 years	135	69.9%	58	30.1%	
>40 years	32	68.1%	15	31.9%	
Year of diagnosis					0.000
≤1995	32	46.4%	37	53.6%	
1996-2000	40	48.8%	42	51.2%	
2001-2005	79	65.3%	42	34.7%	
2006-2010	95	69.9%	41	30.1%	
2011-2015	108	74.5%	37	25.5%	
>2015	98	89.9%	11	10.1%	
Stage at diagnosis					0.000
1	109	84.5%	20	15.5%	
2	261	75.2%	86	24.8%	
3	72	55.4%	58	44.6%	
4	9	16.7%	45	83.3%	
Unknown	1	50%	1	50%	
Bloom Richardson grade					0.166
1	7	70%	3	30%	
2	83	76.1%	26	23.9%	
3	357	67.0%	176	33.0%	
Unknown	5	50%	5	44.4%	
Estrogen receptor status					0.344
Positive (+)	182	69.7%	79	30.3%	
Negative (-)	241	68.5%	111	31.5%	
Unknown	29	59.2%	20	40.8%	
Progesterone receptor status					0.070
Positive (+)	163	71.8%	64	28.2%	
Negative (-)	254	68.1%	119	31.9%	
Unknown	35	56.5%	27	43.5%	
Human epidermal growth factor receptor 2					0.024
Positive (+)	99	74.4%	34	25.6%	
Negative (-)	299	68.7%	136	31.3%	
Unknown	54	57.4%	40	42.6%	
Surgery					0.000
Mastectomy with ALND#	122	57.8%	89	42.2%	
Mastectomy without ALND	124	84.9%	22	15.1%	
Lumpectomy with ALND	55	58.5%	39	41.5%	
Lumpectomy without ALND	142	84.0%	27	16.0%	
Surgical method not specified	3	37.5%	5	62.5%	
No surgery	6	17.6%	28	82.4%	

**Table 4.** Continued

	Alive (n) %		Deceased		P-value
Total number of patients	452	66.8%	210	31.7%	
Chemotherapy					0.000
Anthracycline	101	79.5%	26	20.5%	
Taxane	27	62.8%	16	37.2%	
Anthracycline and taxane	109	83.2%	22	16.8%	
Platinum-based	13	86.7%	2	13.3%	
Unspecified	141	57.3%	105	42.7%	
No chemotherapy	61	61%	39	39%	

# ALND=axillary lymph node dissection

**Table 5.** Final step of the multivariate model using Cox regression.

	Number of patients	HR	p-value
Trimester of diagnosis			
Trimester 1 ( <i>Reference</i> )	158	NA	NA
Trimester 2	97	1.601	0.050
Trimester 3	223	1.200	0.359
Postpartum non-lactating	83	0.867	0.607
Postpartum lactating	72	0.934	0.799
Year of diagnosis			
≤1995	56	3.818	0.001
1996-2000	76	2.048	0.059
2001-2005	119	1.840	0.096
2006-2010	132	1.256	0.522
2011-2015	142	1.316	0.439
>2015 ( <i>Reference</i> )	108	NA	NA
Progesterone Receptor (PR) status			
Positive (+) ( <i>Reference</i> )	220	NA	NA
Negative (-)	364	1.588	0.005
Unknown	49	0.887	0.669
Stage at diagnosis			
1 ( <i>Reference</i> )	123	NA	NA
2	332	1.982	0.010
3	127	4.848	0.000
4	51	13.508	0.000
Surgery			
No surgery ( <i>Reference</i> )	32	NA	NA
Mastectomy with ALND	206	0.456	0.007
Mastectomy without ALND	144	0.310	0.001
Lumpectomy with ALND	88	0.530	0.042
Lumpectomy without ALND	163	0.325	0.001

NA = Not Applicable

## DISCUSSION

Given the need for a better understanding of the different PABC entities and the importance of the potential influence of gestational trimester and the post-delivery period (lactation vs. non-lactation), as an independent prognostic factor for worse patient outcome, we investigated the clinical course and outcomes of PABC patients according to multiple subgroups.

In our previous study, this patient cohort showed statistically significant histopathological differences between the gestational trimesters; second and third trimester breast tumors being significantly more often ER-negative and PR-negative and of higher grade than first trimester breast tumors [28]. In line with these previous observations, data of this study show a statistically significantly inferior outcome of PABC patients diagnosed in the second and third trimesters, when compared to the first trimester. In addition, lactating postpartum patients also had a poor survival, comparable to the second and third trimester patients. These differences partially persist after multivariate analysis, with a significantly higher hazard for second trimester patients when compared to first trimester patients. This indicates that the overall poorer survival for PABC patients as a group (Figure 2) can probably be explained by the poor survival of second and third trimester and lactating postpartum patients, since prognosis of the controls in the present study was similar to that of first trimester and postpartum non-lactating patients. The better prognosis of recently diagnosed (2015 or thereafter) patients compared to patients diagnosed decades before (<1995) is likely due to advances in systemic treatment (chemotherapy and targeted therapy after delivery (e.g. HER-2 blockade)) and increasingly less reluctance to the administration of chemotherapy during the second and third trimester of pregnancy, based on the occurrence of reassuring data on the safety for the unborn child.

Our data supports earlier findings of a more advanced T and N stage, higher histologic grade and hormone receptor negativity in PABC tumors, as compared to non-PABC tumors [12-18]. Yet, evidence of an increased risk of death among women with PABC compared to non-PABC women remains controversial, as mentioned earlier. Multiple case-control studies have attempted to answer this question [12, 14-18, 21-27, 31]. Some studies found no impact on survival when corrected for pathologic features [21-27, 31], while others showed conflicting results between different subgroups of women with PABC, such as an improved survival for non-lactating postpartum patients as compared to pregnant or lactating patients [32] and a shorter DFS and OS of patients with stage IIIA breast cancer diagnosed during pregnancy [33]. Others however suggested a worse outcome for the overall PABC population [12,15,16,34,35].

Putative factors for the observed worse prognosis in PABC patients most likely includes a delayed diagnosis (physiological breast changes associated with advanced pregnancy mask the detection of breast masses; illustrated by larger tumor sizes at diagnosis) or modified cancer treatment to assure the birth of a healthy infant, especially in PABC patients with a diagnosis before 2006 (when patients received treatment of lower intensity due to conflicting interests between cancer treatment and teratogenic effects on the fetus). Also, the pregnant state with advanced immunosuppression, increased vascularization and increased hormonal exposure on the highly stromalized mammary epithelium have been postulated as contributing factors.

In general, most of these studies were hampered by small sample sizes (containing < 50 PABC patients). Yet, a larger population-based cohort study by Rodriguez et al. (n = 797) did find a small but significant effect on outcome after multivariate analyses, with a 14% increased risk of death [15]. Furthermore, Johansson et al. demonstrated differences in mortality rates between PABC (n=317) and non-PABC among women (n=2,965) >35 years and among women with PABC diagnosed within one year postpartum [12]. The outcome that age appears to act as the principle driver to the increased mortality rate in PABC patients is in contrast to our study, in which no significant difference was found between the mortality rates of PABC and non-PABC age groups, suggesting that factors other than age mainly determine the significantly impaired survival observed in PABC patients.

Mortality of PABC subgroups (especially during the different gestational trimesters and the postpartum period) was only analyzed in two small case cohort studies. Dimitrakakis et al. indicated substantial heterogeneity regarding OS among a small group of 39 PABC patients, with significantly shorter OS for PABC diagnosed in the third trimester (HR 4.47, p=0.006) [36]. Boudy et al. could not demonstrate differences in survival between patients diagnosed during the first vs. second and third gestational trimesters in a small PABC study on 51 patients [23]. A meta-analysis by Azim et al. found PABC to be independently associated with a poorer outcome, especially for patients diagnosed shortly postpartum [37].

In general, our data and previously described results are difficult to compare due to the varying definitions of PABC from study to study, including a breast cancer diagnosis up to five years post-delivery as PABC, and the absence of large population studies with sub differentiation between the gestational trimesters in antepartum PABC patients. In our large population based study with a restricted definition of PABC, the patient groups with the worst prognosis were those diagnosed during the second and third trimesters, in addition to postpartum lactating patients diagnosed within a maximum of six months after pregnancy. As to the origin of the observed worse outcome for PABC a number of questions still remains to be answered. We do observed a predominant triple negative

histopathological profile of the tumor concomitant with a high percentage of grade III tumors. Studies unravelling the molecular signature of the various trimester-associated and postpartum tumors should provide further insight into the mechanisms behind PABC. Underlying hormonal influences on the highly stromalized mammary epithelium and the altered immune response during pregnancy have been proposed as contributing factors.

As for the unfavorable influence of lactation on prognosis it is difficult to speculate about the causal factors. Several studies [2,32,38] demonstrates similarly worse survival outcomes for lactating women diagnosed with breast cancer, when compared to non-lactating PABC and non-pregnant young breast cancer patients, even when adjusted for age and extent of disease. In view of the short interval between pregnancy and remodeling of the mammary gland during lactation, it seems likely that the increased estrogen levels will act synergistically with the pro-inflammatory environment to promote oncogenesis. This process is possibly enhanced by the influence of prolactin [39,40], since prolactin and its receptors have also been implicated as promoters of tumor cell growth and progression, especially in an estrogen high environment. Future functional studies with e.g. organoids should include the effect of lactation.

Our study has both advantages and limitations in comparison with prior studies on PABC. Our study is a large, population based retrospective evaluation including almost all Dutch breast cancer cases diagnosed during pregnancy or within six months after delivery over a defined period of time. Only for 82 patients crucial data coupling information was unavailable in the pathological or clinical databases.

One of the limitations is that not all risk factors of interest (such as HER-2 expression) were available in the data, since many patients were diagnosed in the pre-trastuzumab era. However, our prognostic factor modeling and survival analysis of the PABC subgroups was not focused on these features. Further, our method of case-selection (screening of pathology reports for keywords) might have resulted in a lower number of postpartum patients. And, although over the years physicians gained confidence in administering systemic chemotherapy in the second and third trimester, we cannot exclude that there are still a number of patients that have received treatment of lower intensity due to conflicting interests between cancer treatment and maintaining pregnancy.

Finally, we currently have insufficient data on *BRCA* status of our patients, which would have been important because such patients have an increased risk of additional cancers and second breast cancers. Prospective registries should consider gathering these data.



In conclusion, in the largest cohort study to date we found that breast cancer diagnosed within the second and third trimesters of pregnancy seems to be a clinically and biologically distinct entity, with unfavorable clinicopathologic characteristics and outcomes when compared to first trimester pregnancy and non-PABC patients. Furthermore, in postpartum PABC patients, lactation seems to have an unfavorable influence on prognosis. These novel findings add to the existing literature, and imply that PABC should not be considered a homogeneous group of breast cancer, and be redefined according to trimester of diagnosis and lactational status for postpartum patients. Additional molecular studies on tumor and surrounding stroma hopefully will provide us with tools to deliver treatment targeted on the specifics of the different PABC leading to a better outcome for these young women.

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# Genomic copy number alterations as biomarkers for triple negative pregnancy-associated breast cancer

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## ABSTRACT

### Background

PABC, commonly defined as breast cancer diagnosed during or  $\leq 1$  year after pregnancy, accounts for 7% of all breast cancers in women  $\leq 45$  years. Compared to age-matched non-PABC patients, PABC is characterized by a particularly aggressive histopathologic profile with poorly differentiated and estrogen- and progesterone receptor negative tumors and associated high mortality rates. This study assessed the genomic background of triple-negative PABC tumors by detection of copy number alterations (CNAs).

### Methods

MLPA was used to compare CNAs in breast cancer-associated chromosomal loci between triple-negative PABC- and subtype-matched non-PABC patients. Both CNA patterns were evaluated by cluster analysis; associations between individual gene CNAs, pathological characteristics and survival were explored.

### Results

Triple-negative PABC tumors exhibited unique CNAs compared to non-PABC tumors, including enrichment for *TOP2A* copy number loss, an independent predictor of worse overall survival (HR 8.96,  $p=0.020$ ). Cluster analysis based on CNA profiles identified a triple-negative PABC-subgroup with a particularly poor prognosis, characterized by chromosome 8p copy number loss. Individual gene CNAs analysis revealed that *FGFR1* copy number loss on chromosome 8p11.23 was an independent predictor of poor outcome in multivariate analysis (HR 3.59,  $p=0.053$ ) and predicted the development of distant metastases ( $p=0.048$ ).

### Conclusion

This study provides novel insights into the biology of triple-negative PABC tumors suggesting that CNAs, particularly 8p loss and *TOP2A* loss, are involved in the development of breast cancer during pregnancy. *FGFR1* loss and *TOP2A* loss seem to be promising new biomarkers that independently identify subgroups of PABC patients with poor prognosis. These genomic biomarkers may provide clues for personalized therapy.



## INTRODUCTION

Breast cancer is the most common malignancy diagnosed during pregnancy and its incidence is rising notably due to the present-day trend of delayed childbearing, the increase of young-onset breast cancer and the introduction of non-invasive prenatal testing (NIPT) in obstetrical care (resulting in the accidental identification of several asymptomatic pregnant patients in developed countries) [1-4].

Pregnancy-associated breast cancer (PABC), generally defined as breast cancer diagnosed anytime during gestation, lactation or within one year after delivery, represents a heterogeneous disease with fundamental histological and clinical variation between patients. Every year, one in 3,000 to 10,000 pregnant women are diagnosed with breast cancer, representing only 0.2 – 3.8% of overall breast cancer cases [2,5-7].

The molecular nature of PABC remains an underexplored field, and considerable controversy exists regarding the influence of pregnancy on breast cancer prognosis [8-16]. PABC is generally believed to exhibit particularly aggressive behavior and its poor outcome is largely attributed to unfavorable tumor characteristics: advanced tumor (T) stage at diagnosis, lymph node involvement, high histologic grade, negative estrogen receptor (ER) and progesterone receptor (PR) status, and human epidermal growth factor receptor-2 (HER-2) amplification and overexpression [13,14,17,18]. To date, little progress has been made in unraveling the molecular mechanisms of the aggressive pathological characteristics of PABC tumors. A deeper understanding of the molecular makeup of PABC may not only help explain its aggressive biological attributes, but may also provide individualized biomarkers and potential targets for new cancer therapies.

Somatic copy number alterations (CNAs) are part of the molecular makeup of breast cancer [19]. Multiple studies have reported an association between CNAs and specific tumor characteristics such as histologic grade, risk of recurrence, and metastasis [20-26]. CNAs have been reported to be of independent prognostic, even after adjustment for stage, histologic grade, TP53 status, histologic subtype and total aneuploidy [20].

In our previous large population based study, triple negative breast cancer (TNBC: ER negative, PR negative, absence of HER-2 overexpression) was the most frequently observed subtype in PABC compared to age-matched non-PABC tumors [27], in line with other case control studies [14,18, 28, 29].

To assess whether this frequently observed subtype in PABC bears a unique molecular signature, we compared the genomic background of triple negative PABC and control non-pregnant breast cancer patients by detection of specific DNA copy number alterations.

Associations between individual gene CNAs, clinicopathological characteristics and survival were explored.

## MATERIALS AND METHODS

### Patient selection

Using our Dutch nationwide population based ‘PABC cohort’ of women  $\leq 45$  years of age ( $n = 744$ ), with a first diagnosis of invasive breast cancer (BC) during a first pregnancy or within six months after delivery [27], we extracted PABC patients with a triple negative receptor status ( $n = 283$ ). Of these patients, breast tumor specimens have been requested from Dutch laboratories using the Dutch nationwide network and registry of histo- and cytopathology (PALGA) [30]. Only patients with full relevant clinical information about their outcome and available formalin-fixed paraffin-embedded material of their pregnancy associated breast tumor could be included for this molecular analysis ( $n = 31$ ). As controls, triple negative and poorly differentiated tumors of 23 randomly selected premenopausal non-PABC patients (defined as first diagnosis of invasive BC without any sign of pregnancy in the patient history), were identified from the archives of the Department of Pathology at the University Medical Center Utrecht, The Netherlands.

All data from the PALGA database are pseudonymized by a trusted third party (ZorgTTP, Houten, The Netherlands). Consent was given by all Dutch laboratories for the storage of their data by PALGA, and for scientific use of these data. Use of anonymous or coded ‘left over’ material for scientific purposes does not require informed consent according to our institutional medical ethical review board and according to Dutch legislation [30-32].

### DNA extraction and multiplex ligation-dependent probe amplification

Hematoxylin-eosin stained slides were reviewed by an experienced pathologist (PJvD) to confirm and mark the presence of malignancy in tumor samples. Areas with lymphocytic infiltrate or ductal carcinoma *in situ* were avoided. The ratio of tumor cells compared to other cell types in the infiltrative tumor was determined and expressed as a percentage of the total number of cells. After deparaffinization in xylene, DNA was extracted from the marked tumor area on five 10- $\mu$ m unstained sections. Areas were scraped off with a scalpel and specimens were heated at 90°C for 15 minutes in 200  $\mu$ L lysis buffer (lysis buffer: 50mM Tris-HCl buffer, pH 8.0, 0.5% Tween 20). Then, 20  $\mu$ L proteinase K solution (10 mg/ml; Roche, Almere, The Netherlands) was added, and the sample was incubated at 56°C overnight (~16 hours) for lysis of the tissues. Inactivation of proteinase K was achieved by heating the sample for 15 minutes at 80°C. The crude lysate was centrifuged for 10 minutes at 14,000 rpm, and 5  $\mu$ L (50-100 ng) from the supernatant was used for each multiplex ligation-dependent probe amplification (MPLA) reaction according to the manufacturers’

instructions, using the Po78-D2 breast tumor kit (MRC Holland, Amsterdam, The Netherlands) as before [33]. This probe mix contains 55 MLPA probes, including in total 41 probes for the following breast cancer associated chromosomal regions: 6q25 (*ESR1*), 7p11 (*EGFR*), 8p12-p11 (*ZNF703*, *FGFR1*, *ADAM9*, *IKBKB*), 8q13-q24 (*PRDM14*, *MTDH*, *MYC*), 11q13 (*CCND1*, *EMSY*), 16q22 (*CDH1*), 17q11-q25 (*CPD*, *MED1*, *ERBB2*, *CDC6*, *TOP2A*, *MAPT*, *PPM1D*, *BIRC5*), 19q12 (*CCNE1*) and 20q13 (*AURKA*). In addition, 14 reference probes are included which target copy number stable regions in various tumor types including breast cancer.

All tests were performed in duplicate on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI3730 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genemapper (Applied Biosystems) and Coffalyser NET software (MRC-Holland). Six negative reference samples (two blood and four formalin-fixed paraffin embedded normal breast tissue specimens) were taken along in each MLPA run to normalize MLPA ratios. For genes with more than one probe present in the kit, the arithmetic mean of all the probe peaks of this gene in duplicate was calculated. A mean probe ratio value below 0.7 was defined as loss, a value between 0.7 and 1.3 was defined as normal, 1.3–2.0 as gain/low-level amplification, and values >2.0 were defined as high-level amplification, as established previously [34].

## Statistics

CNA data was summarized and plotted using GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, California USA). The web tool ClustVis was used to visualize CNA patterns and create heatmaps after unsupervised hierarchical cluster analysis using Ward's linkage algorithm with Euclidean distance metrics [35].

Statistical analysis was performed using IBM SPSS statistics for Windows version 26.0.0.1. Differences in number of CNAs between PABC and non-PABC patients, and between PABC subgroups (clusters) were evaluated by independent samples t-test and ANOVA with post-hoc Tukey HSD test, respectively. Differences between categorical variables were examined by chi-square statistics or Fisher Exact test when indicated. Individual significance level was set at  $p < 0.05$ . Bonferroni-Holm Correction was applied for multiple comparisons. Overall survival curves were constructed using the Kaplan–Meier method and the log-rank test was used to test for significance. Multivariate survival analysis was done using a backward Cox proportional hazards model. Characteristics with a p-value < 0.10 in univariate analysis and potential confounders were included.

## RESULTS

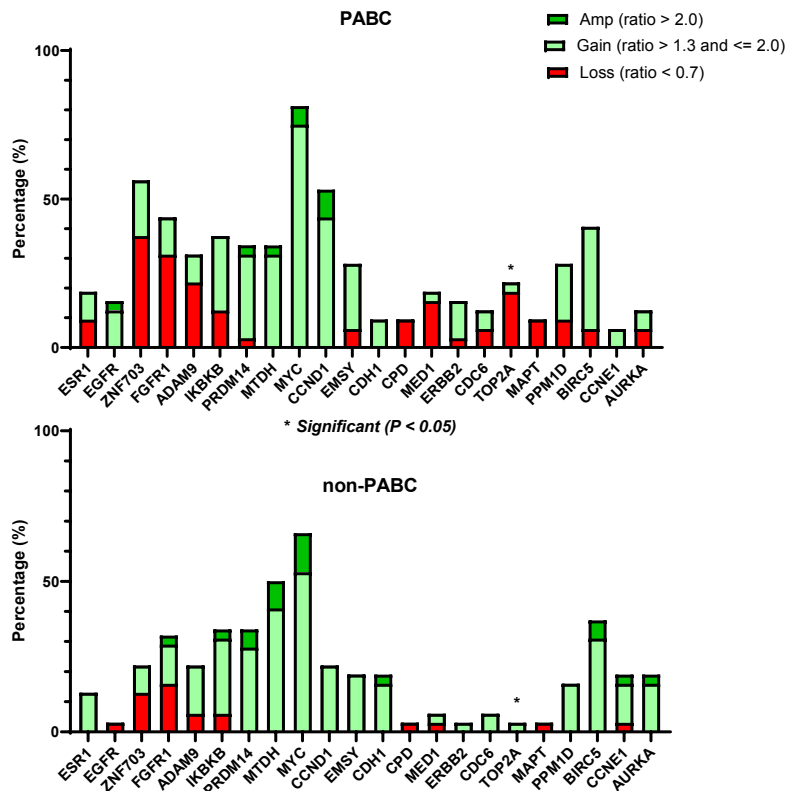
Table 1 compares the clinicopathologic characteristics of the selected PABC and non-PABC patients. All non-PABC and all but one PABC tumors were poorly differentiated (grade III) according to the modified Bloom-Richardson Scarff grading system [36]. Mean age of PABC and non-PABC patients was 33 (range 23 – 42) and 40 (range 29 – 48) years, respectively. The mean tumor percentage of the microscopic slides of PABC and non-PABC patients was 70.3% (SD  $\pm$ 12.2%) and 70.9% (SD  $\pm$ 11.6%) respectively, whilst the median tumor percentage was identical in both groups (70%, IQR 60-80%).

**Table 1.** Clinicopathologic characteristics of pregnancy associated breast cancer (PABC) and non-PABC cohorts in this study.

		PABC	non-PABC	significance
		n=31	n=23	
<b>Age</b>	Range	23-42	29-48	P<0,0001
	Mean	33,3	40,3	
	Stdev	3,58	6,02	
<b>cT</b> N=29 and 22	1	20,6	45,4	p=0,110
	2	58,6	40,9	
	3	17,2	13,6	
	4	3,4	0	
	mm range (avg)	15-100 (34)	11-75 (27)	
<b>cN</b> N=29 and 21	0	79,3	71,4	p=0,216
	1	20,7	23,8	
	2	0	4,8	
	3	0	0	
<b>cM</b> N=29 and 20	0	96,6	100	p=1,000
	1	3,4	0	
<b>Surgery type</b> N=29 and 23	MST + OKD	24,1	39,1	p=0,029
	MST - OKD	44,8	8,7	
	LMP + OKD	13,8	13	
	LMP - OKD	17,2	39,1	
<b>Mortality</b> N=29 and 22	yes	37,9	27,3	p=0,424
	no	62,1	72,7	
<b>Follow-up (days)</b>	Range	324-7367	420-5960	p=0,106
	Mean	2744	3504	
	Stdev	2189	1703	
<b>Stage</b> N=25	1	20	-	
	2	64	-	
	3	12	-	
	4	4	-	
<b>Trimester</b> N=28	1	17,9	-	
	2	14,3	-	
	3	46,4	-	
	postpartum	21,4	-	

### TOP2A copy number loss is more frequent in triple negative PABC compared to non-PABC

In general, PABC triple negative tumors showed significantly more losses ( $p = 0.046$ ) and tended to show fewer high-level amplifications than non-PABC triple negative tumors. Table 2 compares the frequencies of individual gene CNAs between PABC and non-PABC cohorts, and shows mean and median MLPA copy number ratios per gene. *TOP2A* loss was frequent in PABC (19%) while it was not observed in non-PABC patients ( $p = 0.03$ ; non-significant after correction for multiple comparisons). For all 21 other genes, no significant differences were observed. Figure 1 depicts observed frequencies of losses, gains and amplifications in PABC and non-PABC patients. *MYC* was the most frequently gained/amplified gene (81% and 66% of PABC and non-PABC patients, respectively). No *ESR1* and *ERBB2* (*HER2*) high-level amplifications were observed.



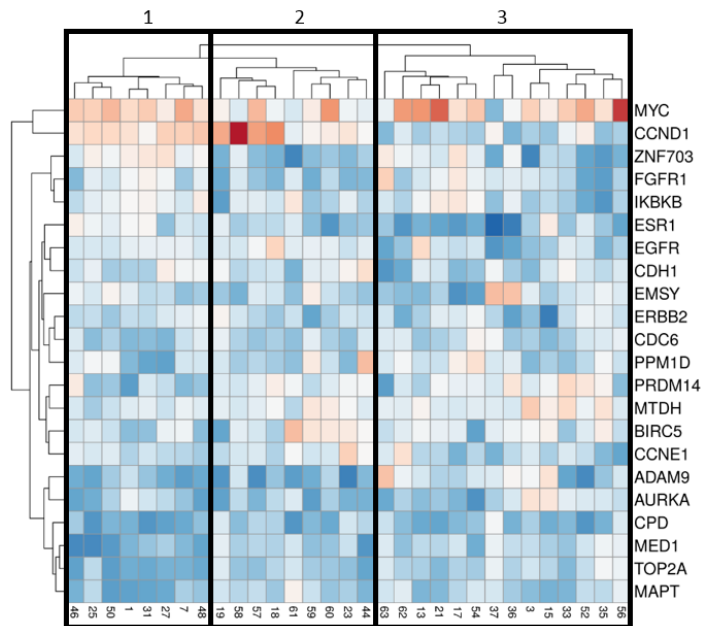
**Figure 1.** Copy number alteration (amplification, gain and loss) frequencies of 22 breast-cancer related genes in pregnancy associated breast cancer (PABC) and non-PABC.

**Table 2.** Gene-specific frequencies of copy number alterations by MLPA in pregnancy-associated breast cancer (PABC) and non-PABC patients. Mean and median MLPA copy number ratio, including standard deviation (stdev) and interquartile range (iqr), as well as the results of inter-group statistical comparison, are also given.

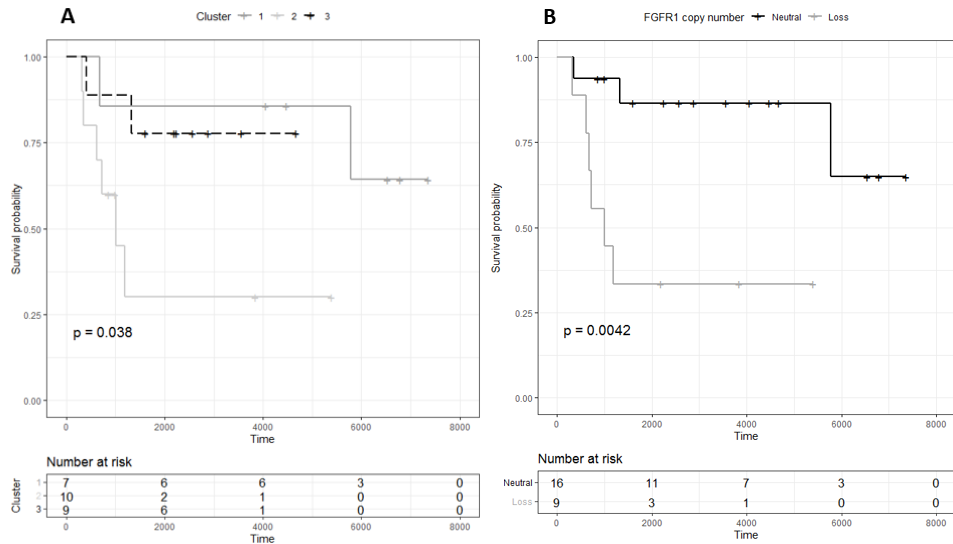
Gene	Chr	PABC (N=31)							non-PABC (n=23)							chi-square statistics	
		mean	stdev	median	iqr	loss	gain	HL amp	mean	stdev	median	iqr	loss	gain	HL amp	Lvs N	GA vs N
ESR1	6q	0.99	0.26	0.92	0.28	9%	9%	0%	1.10	0.20	1.09	0.31	0%	13%	0%	0.25	0.69
EGFR	7p	1.12	0.32	1.05	0.19	0%	13%	3%	0.95	0.16	0.96	0.21	3%	0%	0%	0.46	0.13
ZNF703	8p	1.06	0.33	1.16	0.42	38%	19%	0%	1.07	0.25	1.05	0.22	13%	9%	0%	0.36	0.45
FGFR1	8p	1.04	0.29	1.04	0.33	31%	13%	0%	1.12	0.43	1.02	0.41	16%	13%	3%	0.77	1.00
ADAM9	8p	0.95	0.33	0.93	0.40	22%	9%	0%	1.04	0.27	1.00	0.39	6%	16%	0%	0.45	0.44
IKBKB	8p	1.10	0.25	1.07	0.27	13%	25%	0%	1.22	0.34	1.12	0.45	6%	25%	3%	1.00	0.54
PRDM14	8q	1.17	0.30	1.12	0.40	3%	28%	3%	1.37	0.45	1.21	0.29	0%	28%	6%	1.00	0.49
MTDH	8q	1.26	0.26	1.23	0.25	0%	31%	3%	1.46	0.36	1.42	0.45	0%	41%	9%	no loss	0.08
MYC	8q	1.57	0.34	1.63	0.51	0%	75%	6%	1.81	1.22	1.59	0.69	0%	53%	13%	no loss	0.98
CCND1	11q	1.44	0.65	1.27	0.46	0%	44%	9%	1.21	0.25	1.16	0.32	0%	22%	0%	no loss	0.27
EMSY	11q	1.04	0.24	0.98	0.23	6%	22%	0%	1.16	0.21	1.14	0.28	0%	19%	0%	0.50	0.87
CDH1	16q	1.08	0.17	1.09	0.21	0%	9%	0%	1.18	0.36	1.17	0.30	0%	16%	3%	no loss	0.26
CPD	17q	0.87	0.15	0.88	0.17	9%	0%	0%	0.93	0.12	0.96	0.16	3%	0%	0%	0.63	no GA
MED1	17q	0.94	0.22	0.91	0.27	16%	3%	0%	0.97	0.15	0.98	0.14	3%	3%	0%	0.23	1.00
ERBB2	17q	1.02	0.22	1.01	0.34	3%	13%	0%	0.99	0.18	0.95	0.16	0%	3%	0%	1.00	0.37
CDC6	17q	1.01	0.19	1.01	0.26	6%	6%	0%	1.08	0.23	1.03	0.17	0%	6%	0%	0.50	1.00
TOP2A	17q	0.93	0.22	0.92	0.31	19%	3%	0%	1.01	0.17	1.06	0.23	0%	3%	0%	0.03	1.00
MAPT	17q	0.92	0.18	0.92	0.28	9%	0%	0%	0.90	0.11	0.89	0.08	3%	0%	0%	1.00	no GA
PPM1D	17q	1.07	0.27	1.09	0.34	9%	19%	0%	1.07	0.27	0.96	0.22	0%	16%	0%	0.25	1.00
BIRC5	17q	1.15	0.26	1.16	0.34	6%	34%	0%	1.32	0.36	1.25	0.28	0%	31%	6%	0.51	0.51
CCNE1	19q	1.06	0.17	1.01	0.19	0%	6%	0%	1.16	0.35	1.08	0.20	3%	13%	3%	0.40	0.22
AURKA	20q	0.97	0.27	0.96	0.23	6%	6%	0%	1.22	0.57	1.15	0.34	0%	16%	3%	0.50	0.12

### Cluster analysis identifies triple negative PABC subgroup with poor outcome

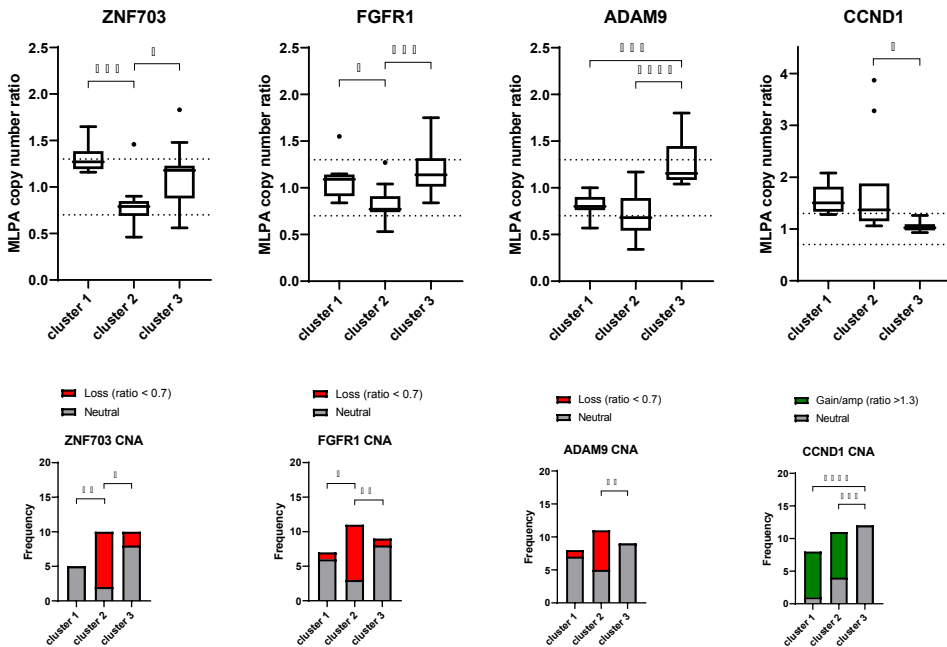
Unsupervised hierarchical cluster analysis of PABC and non-PABC patients based on CNA profiles revealed no clear distinction between both groups (Supplementary Figure 1). Clustering within PABC patients, however, revealed 3 major clusters (Figure 2) based on significant CNA differences between chromosomal regions 6q (*ESR1*), 8p (*ZNF703*, *FGFR1*, *ADAM9*), 11q (*CCND1*), 17q (*CPD*, *MED1*, *CDC6*, *TOP2A*, *MAPT*) and 20q (*AURKA*). Supplementary Table 1 provides an overview of the different clusters. One of these three clusters consisted of patients showing a far worse survival compared to the other triple negative PABC patients ( $p=0.038$ ; Figure 3), and was characterized by more 8p loss (*ZNF703*, *FGFR1* and *ADAM9*) compared to the other two clusters (Figure 4). No significant differences in gestational trimester, age at BC diagnosis or cTNM stage were observed between clusters.



**Figure 2.** Unsupervised hierarchical cluster analysis of triple negative pregnancy associated breast cancer (PABC) patients based on somatic copy number alteration patterns of 22 breast cancer related genes. Cluster 2 was associated with significantly worse prognosis compared to cluster 1 and 3.



**Figure 3.** Kaplan Meier survival plots comparing outcome (A) in three pregnancy associated breast cancer (PABC) copy number alteration-classified subgroups identified by unsupervised hierarchical cluster analysis, and (B) patients with (ratio < 0.7) and without *FGFR1* copy number loss by multiplex ligation-dependent probe amplification.



**Figure 4.** Differences in *ZNF703*, *FGFR1*, *ADAM9* and *CCND1* copy number between three triple negative pregnancy associated breast cancer (PABC) subgroups identified by unsupervised hierarchical cluster analysis (clusters 1, 2 and 3). Boxplots extend from the 25th to 75th percentiles. Whiskers and outliers



were identified by the Tukey method. Cumulative number of patients with neutral copy number, loss and gain/amplification per cluster are shown in the bottom row.

\*  $p < 0.05$ ; \*\*  $p > 0.01$ ; \*\*\*  $p < 0.001$

### FGFR1 and TOP2A copy number loss are independent prognosticators in triple negative PABC

CNAs individually associated with poor overall survival were *ESR1* loss ( $n = 3$  events,  $p = 0.025$ ), *FGFR1* loss ( $n = 9$  events,  $p = 0.0042$ ; Figure 3), *ADAM9* loss ( $n = 7$  events,  $p = 0.037$ ) and *CCNE1* gain ( $n = 2$  events,  $p = 0.021$ ). Patients presenting with tumors harboring *FGFR1* loss developed more frequently distant metastases (67% vs. 25% if copy number neutral,  $p = 0.048$ ). Tumors harboring *MYC* gain or amplification were less likely to develop lymph node metastases (9% vs. 57% if copy number neutral,  $p = 0.018$ ). *TOP2A* loss, *ESR1* loss, and *FGFR1* loss were independent predictors of overall survival (OS) in Cox regression analysis including cT and cN (HR 8.960 (95% CI 1.407-57.079),  $p = 0.020$ ; HR 10.589 (95% CI 1.046-107.2108),  $p = 0.046$ ; and HR 3.586 (95% CI 0.981-13.103),  $p = 0.053$ , respectively). Of these 3 CNAs, only *FGFR1* loss and *TOP2A* loss remained in the model when entered together (HR 4.408,  $p = 0.073$  and HR 7.100,  $p = 0.056$  respectively).

### Associations found in PABC are not seen in non-PABC

In the non-PABC group, no significant associations with survival were observed for *FGFR1* or any other interrogated gene, although *AURKA* gain/amp ( $p = 0.066$ ) and *EMSY* gain/amp ( $p = 0.056$ ) tended to predict worse survival. Unsupervised cluster analysis of non-PABC patients also revealed three clusters based on significant CNA differences between chromosomal regions 8p (ZNF703, *FGFR1*, *ADAM9*) and 17q (*TOP2A*, *MAPT* and *BIRC5*). All three patient clusters however had a similar survival ( $p = 0.463$ ).

## DISCUSSION

To investigate the underlying mechanisms resulting in the aggressive clinical behavior of PABC, we aimed to identify specific gene CNAs characterizing triple negative PABC, by conducting a comparative analysis of a cohort of triple negative PABC patients and subtype-matched non-PABC patients (with a diagnosis of invasive breast cancer  $\leq 45$  years of age). We have shown that triple negative PABC tumors exhibit enrichment for copy number losses by MLPA in general and some unique CNAs, including the enrichment for *TOP2A* copy number loss. In addition, *MYC* was the most frequently gained/amplified gene in PABC [37].

Cluster analysis based on CNA profiles identified a triple negative PABC subgroup with a particularly poor prognosis, characterized by chromosome 8p copy number loss. Further

analysis of individual gene CNAs revealed that *FGFR1* copy number loss on chromosome 8p11.23 was the best prognosticator residing in this chromosomal region. *FGFR1* loss was an independent predictor of worse overall survival in multivariate analysis and predicted the development of distant metastases.

In line with our observations, other studies have previously described 8p copy number loss as a frequent event in various cancer types including breast cancer, suggesting that this region harbors one or more tumor suppressor genes. Loss of 8p has been linked to advanced tumor stage, high grade, high proliferation index, negative ER and PR status, early-onset breast cancer, poor survival rates and shortened response to oncologic systemic treatment [38-40]. Cai et al. examined the effect of a chromosome 8p 2-35 Mb targeted deletion, which was insufficient to transform MCF10A cells, but altered the fatty acid and ceramide metabolism leading to increased invasiveness and enhanced autophagy [41]. Their results provided evidence to suggest that screening for 8p loss in breast tumors may serve as a selection strategy for treatment with microtubule inhibitors (confers resistance), statins (confers resistance), and/or autophagy inhibitors (confers sensitivity). This strategy may thus be of particular interest in a PABC context.

Besides *FGFR1*, *TOP2A* copy number loss on 17q21.2, *ESR1* loss on chromosome 6q25.1 and *CCNE1* gain on 19q12 were identified as biomarkers for poor outcome in triple negative PABC patients. *TOP2A* loss, enriched in PABC tumors and covered by three independent MLPA probes, was an independent predictor of poor overall survival alongside *FGFR1* loss.

*TOP2A* encodes the topoisomerase II $\alpha$  protein, an intracellular target of anthracyclines. Several studies have therefore suggested that anthracycline-containing therapy might be most effective in patients whose tumors carry amplified *TOP2A* [42-44]. Interestingly, *TOP2A* gene deletion might also confer increased sensitivity to anthracyclines [42,45-47] suggesting a potential benefit of anthracycline-containing chemotherapy in triple negative PABC patients.

*ESR1* encodes the estrogen receptor alpha and, as expected since it usually leads to ER alpha overexpression, did not show amplifications in both triple negative cohorts. Yet, we did observe several *ESR1* losses in PABC tumors (9%; covered by two MLPA probes). Activating mutations in *ESR1* are recurrent mechanisms of acquired resistance to aromatase inhibitors in ER-positive tumors, but *ESR1* allelic losses have only rarely been described [48]. Laenkholm et al. reported that a large fraction of ER negative tumors showed *ESR1* deletion (55%) by FISH [49]. They also noted an elevated number of deletions in cohorts with a higher number of ER negative patients in the DBCG trial 89D. Thus *ESR1* deletions may contribute to the ER alpha negative status of these cancers.

Cyclin E1 (CCNE1) plays a critical role in cell cycle regulation, DNA replication, chromosome segregation, and G1 to S-phase transition [50]. CCNE1 overexpression and gene amplification have both been associated with poor prognosis in triple negative breast cancer [51-53] as well as epithelial ovarian cancer [54]. In ovarian cancer, the near mutual exclusivity of homologous recombination pathway mutations and CCNE1 amplification generally results in resistance to platinum-based cytotoxic chemotherapies and ineffective Poly (Adenosine Diphosphate (ADP)-Ribose Polymerase (PARP) inhibition [54].

CCNE1 amplified tumors can cause faster mitotic exit, an increased rate of mitotic slippage and resistance to anti-mitotic chemotherapies such as taxanes. Breast tumor cells engineered to overexpress cyclin E have been shown to have an increased sensitivity to cisplatin and paclitaxel combinations [55,56]. Promising targeted strategies using CDK2 inhibitors and WEE1 kinase inhibitors are currently being examined in ongoing biomarker driven clinical trials.

The abovementioned prognostic CNAs proved to be unique to PABC tumors as similar associations were not observed in the breast tumors diagnosed outside pregnancy of postpartum period. This reinforces the notion that PABC represents an even more distinctive entity of breast cancer than previously reported, requiring its own biomarkers and therapeutic approaches.

Even though several studies on the genomic profile of PABC have been conducted [57], this analysis is novel as it focuses specifically on the triple-negative PABC subtype and correlates the clinicopathological features of the disease and outcomes with the CNAs. Although MLPA analysis alone cannot determine whether triple-negative PABC is defective in homologous recombination, recent genomic analysis has revealed that a significant portion of TNBC is characterized by abundant chromosomal structural variants and CNAs due to homologous recombination deficiency [58].

Some limitations of this study to be noted. Although MLPA is a multiplex technique that can assess multiple relevant CNAs simultaneously, we have only examined a limited number of genes here. PABC and non-PABC cohorts were relatively small but perfectly matched for triple negative subtype, and still provided prognostically significance. These new genetic insights can serve as a starting point for further more extensive copy number analyses by next generation sequencing in our entire PABC cohort, after obtaining the formalin-fixed paraffin-embedded (FFPE) tumor material of the remaining patients. In addition, age-matching was not perfect as PABC patients were on average slightly younger (33 years) than non-PABC patients (40 years) upon final analysis. Age was, however, not significantly associated with any of the interrogated variables, so we do not believe that this has played an important role here.

In conclusion, this study provides important new insights into the biology of triple negative PABC and suggests that several copy number alterations, particularly 8p loss, *TOP2A* loss, *ESR1* loss and *CCNE1* gain are implicated in tumor progression during pregnancy. *FGFR1* loss and *TOP2A* loss are promising new biomarkers that independently identify a subgroup of triple negative poor prognosis PABC patients that require personalized cancer treatment. In addition, this study provides unprecedented therapeutic clues for further studies to pursue in a larger PABC population.

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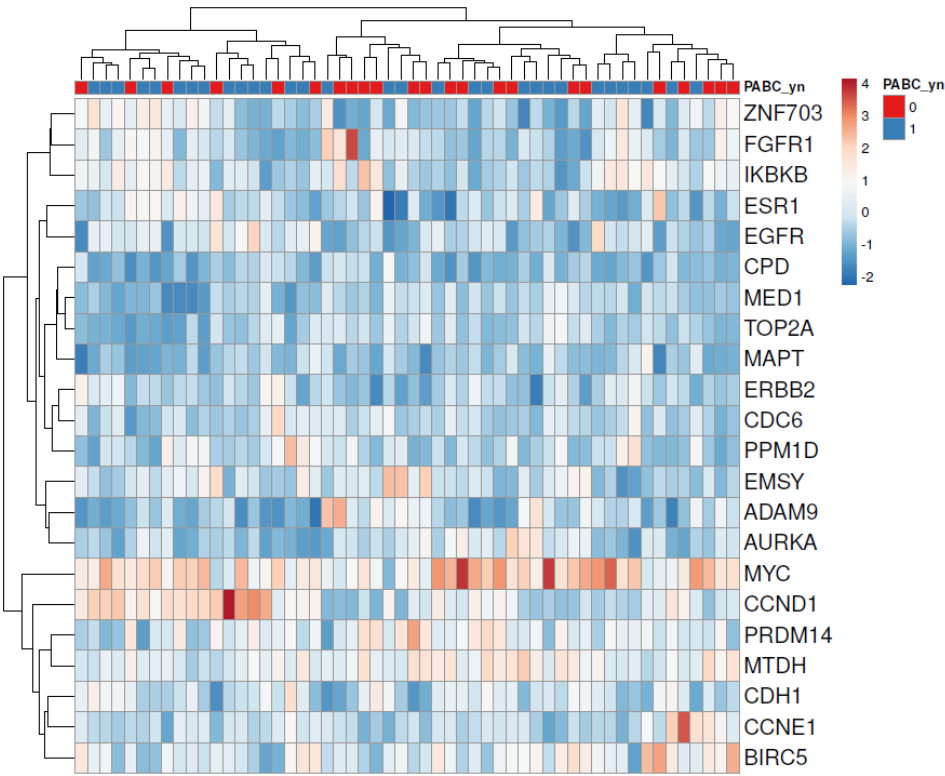


## SUPPLEMENTARY FILES

**Supplementary Table 1.** Differences between three triple negative pregnancy associated breast cancer (PABC) subgroups identified by unsupervised hierarchical cluster analysis of somatic copy number alterations determined by multiplex ligation-dependent probe amplification (clusters 1, 2 and 3). Differences in absolute MLPA copy number ratio were determined by ANOVA with Tukey posthoc test. Differences in CNA frequency between clusters were determined by Chi square test. NS = non-significant

Gene	chr arm	MLPA copy number ratio differences				CNA frequency differences			
		1 vs 2	1 vs 3	2 vs 3	direction	1 vs 2	1 vs 3	2 vs 3	direction
ESR1	6q	0.018	NS	NS	1>2	NS	NS	NS	-
ZNF703	8p	0.001	NS	0.021	1/3>2	0.007	NS	0.023	loss 2>1/3
FGFR1	8p	0.039	NS	0.001	1/3>2	0.028	NS	0.005	loss 2>1/3
ADAM9	8p	NS	0.0003	0.000006	3>1/2	NS	NS	0.006	loss 2>3
CCND1	11q	NS	NS	0.015	2>3	NS	0.0001	0.001	GA 1/2>3
CPD	17q	NS	0.002	0.019	3>1/2	NS	NS	NS	-
MED1	17q	NS	0.00005	0.003	3>1/2	NS	NS	NS	-
CDC6	17q	NS	NS	0.017	3>2	NS	NS	NS	-
TOP2A	17q	NS	0.00004	0.001	3>1/2	NS	NS	NS	-
MAPT	17q	NS	0.001	0.013	3>1/2	NS	NS	NS	-
AURKA	22q	NS	NS	0.024	3>2	NS	NS	NS	-

\* 0.05  
 \*\* <=0.01  
 \*\*\* <=0.001  
 \*\*\*\* <=0.0001



**Supplementary Figure 1.** Unsupervised hierarchical cluster analysis of pregnancy associated breast cancer (PABC; blue) and non-PABC patients (red) based on somatic copy number alterations of 22 breast cancer related genes.



07



## Summarized discussion

## Future perspectives



## SUMMARIZING DISCUSSION

Within the last decade, there has been considerable progress in managing breast cancer that develops during pregnancy. The increased knowledge on the safety of available treatment options for pregnant women, and perhaps even more importantly for their offspring, has led to more adequate breast cancer treatment during pregnancy [1-5]. In contrast, little progress has been made in unravelling the complex biology and pathophysiology of pregnancy-associated breast cancer (PABC). The molecular nature of PABC currently remains an unknown field, while literature provides considerable controversy regarding the influence of pregnancy on breast carcinogenesis [6-11] and prognosis [12-22]. This indicates the need for clear definitions, more insights into clinicopathologic profiles of young women with breast cancer during pregnancy, during lactation, and outside of pregnancy, as well as exploring the role of potential subgroups within PABC.

Research within this thesis was conducted to create a comprehensive overview of the clinicopathologic characteristics and outcomes of PABC patients, subdivided according to gestational trimesters and (for postpartum patients) lactational status at diagnosis, as compared to (age-)matched non-pregnant young breast cancer patients. In addition, molecular differences between PABC subgroups were identified. These molecular differences may serve as a starting point for additional biomarker research into individualized early-stage diagnosis and treatment. Altogether, this detailed PABC fingerprint may serve as a fundament for further in-depth research into PABC carcinogenesis, and thereby, potentially provide a way forward to well-defined personalized care of this unique breast cancer subtype.

### Defining the histopathologic profile of PABC

The lack of a comprehensive understanding of the interaction between the hormonal environment during and after pregnancy (e.g. remodeling of the mammary gland) and the initiation of tumorigenesis of breast cells, indicates the need for more insights into the development of PABC. This starts with establishing a clear histo- and clinicopathologic profile of PABC patients, obtained from a sufficiently large study population. This is especially relevant since currently available data are usually derived from small patient cohorts and varying histopathologic profiles have been reported.

In **Chapter 2** we show the results of a large nationwide study in which we compared the histopathologic profile of 741 PABC patients to an age-matched cohort of 741 non-pregnant breast cancer patients. A particularly aggressive histopathologic profile was observed for PABC patients, as their tumors were significantly more often of higher histologic grade, estrogen receptor (ER) and progesterone receptor (PR) negative and

human epidermal growth factor receptor-2 (HER-2) positive. Furthermore, a higher incidence of triple-negative tumors in PABC patients was observed.

Although younger age at breast cancer diagnosis is associated with more aggressive clinicopathologic features, this study showed an even more aggressive profile of breast cancer diagnosed during pregnancy or within six months after delivery, implicating that PABC may biologically be a different breast cancer entity than breast cancer diagnosed in non-pregnant young women. Our results are in line with previous studies, though in smaller PABC cohorts [16,17,23-27], which reported comparable proportions of ER- and PR negative tumors in PABC patients (between 50 and 60%), as well as comparable proportions of tumors of higher histologic grade.

These findings render interesting clues for further studies to unravel the molecular and genetic background of PABC. For example, the high proportion of ER- and PR-negative tumors in PABC patients seems counter-intuitive at first sight, as estrogen and progesterone levels are generally high during pregnancy. It has however been shown that, while lacking hormone-receptor expression, tumors arising in xenograft models of PABC require systemic estrogen for their formation, and increasing the levels of these hormones promote the initiation and progression of ER negative cancers (i.e. the tumor cells do not express estrogen receptors themselves). In addition, these PABC xenografts tumors exhibit a highly stromalized histologic phenotype [11]. Together with the fact that PABC cell lines do not seem to proliferate *in vitro* (i.e. without their stroma) in response to estrogen, this suggests that PABC tumorigenesis may be driven by hormonal influences on the host stroma, rather than on the mammary epithelial cells.

In addition, other factors could play a role, such as immune changes in pregnancy and in the first months postpartum, consisting of a combination of cellular immunosuppression, immune tolerance, and enhanced inflammatory responses related to mammary gland involution and molecular changes leading to neo-antigens.[28]. Next, epigenetic changes, that by themselves affect gene regulation, has been suggested to contribute to PABC tumorigenesis as well [12].

### Defining the histopathologic profile of PABC subgroups

Next to demonstrating the unique histopathologic profile of PABC we defined different breast cancer subgroups during pregnancy and during the first months after delivery (i.e.  $\leq 6$  months postpartum). This is in contrast to previous histopathologic analyses which were performed in PABC patients (up to five years after delivery), without any differentiation between gestational age at diagnosis and lactation status [16,17,23-27,29]. Therefore, currently no distinctions between pregnant and post-partum PABC patients are made, although it is claimed that two-third of PABC patients are diagnosed

postpartum (up to 12 months after delivery) [30,31]. One could however argue that breast cancer during pregnancy differs from cancers arising following delivery, especially after six months post-partum, since hormonal levels then likely have changed [25]. In addition, postpartum breast tumors in lactating patients potentially have a higher dependency on prolactin rather than estrogen or progesterone. In **Chapter 3** we therefore focused on the histopathologic characteristics between the three gestational trimesters and lactational status within the post-delivery period. For which three important circumstances were analyzed: i) Theoretically, it would be possible that breast cancers diagnosed in the first gestational trimester have initiated before pregnancy ii) Due to upregulation of biological processes (e.g. elevating levels of estrogen, progesterone and insulin-like growth factor-1 (IGF-1) during pregnancy, tumorigenesis can be promoted at the different gestational stages. iii) Postpartum breast cancers may be influenced by epigenetic changes following delivery.

The results of this analysis demonstrated a higher proportion of grade III tumors within the second and third gestational trimesters, as compared to the first trimester. For postpartum PABC patients an even higher percentage of grade III tumors was observed, which was independent of lactational status. Overall, significantly higher percentages of ER- and PR-negative tumors at advanced gestational trimesters were observed. In lactating patients, the fraction of ER- and PR-negative tumors increased even further, in contrast to the non-lactating patients. For HER-2 no significant differences were observed between gestational trimesters, but in lactating patients a remarkable decline in HER-2 positivity was found.

In conclusion, PABC is inconsistently defined as either breast cancer diagnosed exclusively during pregnancy, or combined with breast cancer diagnosed within six months to five years after delivery, and sometimes even longer. Although pregnancy and the postpartum period are intertwined, persistent linking of this two entities leads to conflicting results on the relationship between pregnancy, prior pregnancy (i.e. postpartum women) and breast cancer outcome. For example, the longer after the delivery date breast cancer is diagnosed, the less clear the association with pregnancy may become. In addition, these cases may be largely underreported as well, since the likelihood of linking breast cancer to the previous pregnancy (months or years before) decreases. Therefore, with regard to the histopathologic differences as shown in **Chapter 3**, breast cancer diagnosed during pregnancy (BCdP) may not necessarily be the same disease entity as PABC. Furthermore, even within pregnant- and within postpartum patients, different entities according to gestational trimester and lactational status seem to exist.

In order to close a gap in existing literature, **Chapter 4** provided a review of the receptor status landscape of solely BCdP, to further elucidate the seemingly



contradictory relationship between tumor growth in a high hormonal environment and the lack of ER- and PR-receptor expression of tumor cells. Interestingly, in this review we found higher percentages of ER/PR negativity in BCdP patients than published before in large studies that also included postpartum breast cancer patients. Additionally, this review evidenced, besides the predominantly ER-and PR negative tumors, also a high incidence of HER2 positivity, which is in accordance with data from our own pregnancy-associated breast cancer cohort. Therefore, we suggest to consider BCdP a separate entity, distinct from breast cancer diagnosed postpartum, which, according to recent insights, can extend to 5-10 years after delivery. Finally, the potential carcinogenetic differences according to gestational trimester and lactation status undoubtedly indicate the need for further research into carcinogenesis.

### Prognosis of PABC

Overall, parity and lactation are associated with long-term protective effects against breast cancer [20, 32-34]. However, in a small group of young fertile women, a unique form of breast cancer occurs during pregnancy or the postpartum period, with particularly poor prognostic tumor characteristics, consisting of an advanced tumor (T) stage at diagnosis, nodal involvement, high histologic grade (i.e. poor differentiation), negative estrogen receptor (ER) and progesterone receptor (PR) status and HER-2 amplification and overexpression [12,13,16-18,35].

The period of increased risk, in which parous women in comparison to those who are nulliparous, have a higher incidence of breast cancer, has been estimated to extend up to 15 years after the last parturition [29]. However, current literature renders a mixed view on whether this aggressive breast cancer entity diagnosed during pregnancy or the postpartum period results in a worse prognosis than breast cancer diagnosed outside pregnancy. In addition, whether pregnancy itself negatively influences prognosis remains the subject of debate.

The available results are most likely conflicting as they focused on different populations: i) the outcome of the entire PABC population (without comparison of survival between subgroups), ii) the postpartum patients with a follow-up period up to ten years, iii) BCdP patients solely (without differentiation to gestational trimester at breast cancer diagnosis). A comparison of the available data was deemed too complex due to the small numbers of patients examined in each of the prior individual studies and due to the dissimilar reference populations. Given the need for a better understanding of the different PABC entities and the importance of the potential influence of gestational trimester and the post-delivery period (lactation vs. non-lactation), as independent indicator of worse patient outcome, we investigated the clinical course and outcomes of PABC patients according to multiple subgroups in **Chapter 5**.

In line with the previous observations described in **Chapter 3**, data of this large nationwide population based study showed a statistically significant inferior outcome of PABC patients diagnosed in the second or third trimester, when compared to a PABC diagnosis in the first trimester. In addition, patients who were included in the lactating postpartum group showed similarly poor survival rates.

These differences persisted after multivariable analysis (including, year of diagnosis, PR status, stage at diagnosis and surgery), with a significantly higher hazard ratio for second trimester patients as compared to first trimester PABC patients. Since the prognosis of the matched controls in the present study was similar to that of first trimester and postpartum non-lactating PABC patients, this indicates that the observed poorer overall survival for PABC patients as a group can probably be explained by the poor survival rates of second and third trimester patients, as well as lactating postpartum PABC patients.

Probable factors for the observed worse prognosis in PABC patients most likely include a delayed diagnosis (physiological breast changes associated with advanced pregnancy mask the detection of breast masses illustrated by larger tumor sizes at diagnosis [13,17] or modified (i.e. less intense) cancer treatment to assure the birth of a healthy infant, especially in PABC patients with a diagnosis before 2006 (when patients received treatment of lower intensity due to conflicting interests between cancer treatment and teratogenic effects on the fetus) [1,2,4,5]. Also, the pregnant state with advanced immunosuppression, increased vascularization and increased hormonal exposure on the highly stromalized mammary epithelium have been postulated as contributing factors [9,11,28,30,36].

Our observed survival rates of BCdP patients are not in agreement with those of Amant et al. [14] and Schedin et al., [36] who reported different survival rates for patients with primary breast cancer diagnosed during pregnancy compared to non-pregnant patients with breast cancer. Interestingly, Amant et al. showed similar 5-year OS rates (81%) for non-BCdP patients in line with our study results, and the 5-year OS rates of BCdP patients (78%) match exactly with the first trimester BCdP patients in our cohort, in contrast to the 5-year OS of patients diagnosed during the second and third gestational trimesters (OS 60.0% and 64.9% respectively). Possibly BCdP patients in the study cohort of Amant et al. predominantly concern breast cancer diagnosed during the first months of pregnancy. Although the study of Amant et al. contains smaller groups of non-BCdP patients (control group) and the median age of these patients was over the age of 40, which leads to expected decline in comparability. This study does provide BCdP patients matched for more prognostic factors (i.e. stage, HER2 status, type of chemotherapy and adjuvant targeted therapy and radiotherapy) than our study, which may explain the observed differences as well.

With regard to prognosis of the overall PABC-population, and more specifically postpartum patients, our OS rates are in agreement with the existing literature. In a large retrospective study of 619 patients, Callihan et al. demonstrated that breast cancer diagnosed within five-years postpartum renders a 2.8 times higher risk of metastasis and a 2.7 times higher risk of mortality, as compared to nulliparous cases [29]. Interestingly, the postpartum risk of recurrence and death was most pronounced after the second postpartum year. In addition, a meta-analysis of Azim et al., including 3,628 PABC cases with a diagnosis up to two years after pregnancy, showed a significantly higher risk of death (mostly driven by those who were diagnosed  $\geq 1$  year postpartum) in PABC patients, as compared to 37,100 non-PABC controls, suggesting worse survival outcomes during the postpartum period [37]. Unfortunately, within these studies, lactational status of patients was not provided.

Overall, these findings (from literature and our own studies) emphasize the importance of a better definition of both BCdP and postpartum PABC. For BCdP gestational trimesters at diagnosis may represent different BCdP entities. For PABC after delivery, two relevant samples are identified for future research: i) PABC for patients within 6 months after delivery (for a increased insight into the “pregnancy associatedness” and (short term) lactational status, and ii) PABC for patients in the more advanced postpartum stage, meaning diagnosis of breast cancer at least six months post delivery.

Interestingly, lactating postpartum patients in our study show a worse survival than non-lactating postpartum patients. This was unexpected since previous epidemiologic evidence showed a protective effect of breastfeeding against breast cancer in a meta-analysis of 50,302 women with invasive breast cancer and 96,973 controls, showing a decrease in lifetime risk of developing breast cancer (not necessarily PABC) of 4.3% for every 12 months of breastfeeding [32].

In line with our study however, several studies demonstrated similarly worse survival outcomes for lactating women diagnosed with breast cancer, even when adjusted for age and extent of disease [12,38,39]. This adverse outcome for lactating women persisted despite correction for nodal status, tumor size and age.

Overall, a possible explanation for the observed worse overall survival in lactating patients is that the protective effect of breastfeeding is an effect on the long term; the process of “physiologic” involution (as weaning occurs) will take months, which means that there is a period where not all oncogene-overexpressing cells have undergone full terminal differentiation nor have been detected and eliminated by the infiltrated immune cell system [30]. In addition, in view of the short interval between pregnancy and the remodeling of the mammary gland during lactation, it seems likely that the increased estrogen levels will act synergistically with the pro-inflammatory environment to promote oncogenesis. This process is possibly enhanced by the influence of prolactin

[9,40-42], since prolactin and its receptors have also been implicated as promoters of tumor cell growth and progression, especially in an estrogen high environment. Nevertheless, breastfeeding may contribute to less timely detection of breast masses and clinical (breast) examination; the delayed diagnosis allows more time for tumor growth, increasing the metastatic potential of the disease [37].

As to the origin of the observed worse outcome for second and third gestational trimesters and postpartum lactating PABC patients, a number of questions still remain to be answered. Studies unravelling the molecular signature of the various trimester-associated and postpartum tumors and their microenvironment should provide further insight into the mechanisms behind PABC, which may differ between gestational trimesters and between lactating and non-lactating women. With regard to the unfavorable influence of lactation on prognosis it is premature to speculate on possible causal factors. Functional studies with e.g. organoids should include the effect of lactation.

### Potential biomarkers

Finally, in **Chapter 6** we assessed whether the discriminatory clinicopathologic features of the PABC subgroups are also reflected by a (unique) molecular signature. In this study we analyzed the genomic profile of a selected group of 32 triple-negative (ER/PR/HER-2 negative) PABC patients, which were compared to 23 subtype-matched non-PABC patients ( $\leq 45$  years of age), by detection of copy number alternations (CNAs).

This hypothesis generating study demonstrated that triple-negative PABC tumors exhibit enrichment for copy number losses by multiplex ligation-dependent probe amplification (MLPA) in general, and that they exhibited some unique CNAs, including the enrichment for *TOP2A* copy number loss. Cluster analysis based on CNA profiles identified a unique subgroup with a particularly poor prognosis, characterized by chromosome 8p copy number loss. Analysis of individual gene CNAs revealed that *FGFR1* copy number loss on chromosome 8p11.23 was the best prognosticator. *FGFR1* loss appears to be an independent predictor for poor overall survival in multivariate analysis and predicted the development of distant metastases.

In line with our observations, other studies have previously described chromosome 8p copy number loss as a frequent event in various cancer types (including breast cancer) suggesting that this region harbors one or more tumor suppressor genes. Loss of 8p has been linked to advanced tumor stage, high histologic grade, high proliferation index, negative ER and PR status, early-onset breast cancer, poor survival rates and shortened response to oncologic systemic treatment [43-45]. Interestingly, tumors carry amplified *TOP2A* or *TOP2A* gene deletion could benefit from anthracycline-containing

chemotherapy, since *TOP2A* encodes the topoisomerase II $\alpha$  protein which serves as an intracellular target of anthracyclines [46-48].

The abovementioned prognostic CNAs in our study proved to be unique to triple negative BCdP tumors, as similar associations were not observed in the breast tumors diagnosed outside pregnancy or the postpartum period (within one year post-delivery). This reinforces our previous findings that BCdP represents a distinct entity of breast cancer. Furthermore, within triple negative BCdP the observed unique molecular signature, renders possible therapeutic implications, which need to be studied further.

Our findings provide important novel insights into the biology of triple-negative PABC tumors in which *FGFR1* loss and *TOP2A* loss seem to be promising new biomarkers that independently identify a subgroup of pregnant triple-negative breast cancer patients with poor prognosis. Therefore, this genomic biomarker analysis will serve as a starting point for further, and more extensive, copy number analyses by next generation sequencing within our entire PABC cohort. In addition, this study provides unprecedented therapeutic clues for further studies to pursue in a larger (triple negative) PABC population.

## Conclusion

In conclusion, this thesis established a unique clinicopathologic profile for breast cancer patients diagnosed during the different gestational trimesters and the early postpartum period. These novel findings imply that PABC needs to be redefined according to gestational trimester of diagnosis and lactational status for postpartum patients, allowing further studies to focus on these different breast cancer entities and their carcinogenesis. This will hopefully provide us with new tools to deliver targeted and personalized therapies, leading to a better outcome for these young breast cancer patients.

## FUTURE PERSPECTIVES

As result of this thesis, a unique clinicopathological profile for breast cancer patients diagnosed during the different gestational trimesters and the early postpartum period has been discovered. In view of our data, showing a worse outcome of PABC subgroups compared to non-PABC breast cancer patients, a search for better treatment modalities is warranted. This first requires a further elucidation of the molecular and immunological signature of the PABC subgroups to guide us to the most effective targeted therapies. Future (large population based) studies should focus on precision medicine for the PABC subgroups involving identification of the underlying molecular signatures and the clinical phenotype.

The International Network on Cancer, Infertility and Pregnancy (INCIP) started an international cohort of patients diagnosed with cancer – including breast cancer- during pregnancy to investigate the oncological management and maternal, neonatal and pediatric outcomes of these patients. This international cooperation specifically assesses the clinical associations between cancer type, treatment modality and outcomes. Extending these multicenter data with clinicopathological findings and further in-dept research could provide more detailed and unique genetic and molecular information on breast cancer diagnosed during pregnancy and postpartum in an even larger study population, with complete data; from clinicopathologic, molecular, and genetic data, to clinical data from patients identified at baseline by their treating physicians.

The development of a larger “real-world” database will facilitate advanced information on confounding factors in PABC carcinogenesis and potential effect modifiers such as age at first pregnancy, age at PABC diagnosis, previous pregnancies before the diagnosis of PABC, previous miscarriages or abortions, *in vitro* fertilization treatments, premature delivery, family history, breastfeeding and interval of breastfeeding.

These data should be supported by data from in-depth molecular research, to be performed on the malignant epithelial cells and tumor-associated stroma of PABC patients, and whole-exome sequencing to refine mutation patterns. Furthermore, the inflammatory environment of PABC subgroups should be investigated by detection of stromal and intra-tumoral tumor-infiltrating lymphocytes (TILs). In addition, potential biological mechanisms underlying associations between pregnancy characteristics and breast cancer should be investigated. This includes characterizing the potential interplay between prolactin and the high levels of estrogens during pregnancy, and determining whether genetic variability in prolactin-related genes is associated with an increased breast cancer risk in pregnant and lactating women. The potential antagonistic effects of human chorionic gonadotrophin (hCG) on tumor cell growth and progression also

deserves to be studied, through functional experiments with organoids.

Moreover, the role of mutations in the *BRCA1/2* genes in the development of PABC, and the application of targeted therapies like PARP inhibitors (pharmacological inhibitors of the enzyme poly ADP-ribose polymerase) should be explored. Lastly, the potential effects of tamoxifen (as a selective estrogen receptor modulator with reported benefits in 5-10% of ER-negative breast cancers) and immune checkpoint inhibitors as adjuvant therapy for PABC after pregnancy need to be evaluated.

Since the worldwide incidence of breast cancer continues to rise, the number of women that will be diagnosed with breast cancer during pregnancy will likely follow this increase. Regarding the often dismal outcome of breast cancer in this specific group of breast cancer patients, every effort should be taken to improve their prognosis. Understanding the differences between breast cancer diagnosed during the different gestational trimesters and during the different postpartum times would better permit the translation of informative data from basic science and epidemiologic studies into the most optimal clinical care and treatment of BC in these young women.

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08



**Summary in Dutch**  
**(Nederlandse samenvatting)**  
**Acknowledgments**  
**(Dankwoord)**  
**Curriculum Vitae**  
**List of publications**



## SUMMARY IN DUTCH (NEDERLANDSE SAMENVATTING)

Een diagnose van kanker tijdens de zwangerschap komt in toenemende mate voor, gedeeltelijk te verklaren op basis van de toenemende leeftijd van vrouwen die zwanger worden. Toch blijft kanker gediagnosticeerd tijdens de zwangerschap relatief zeldzaam; in 1 op de 1000 zwangerschappen wordt de diagnose gesteld, hetgeen  $\leq 0.1\%$  van alle kankers betreft. De meest voorkomende vorm van kanker tijdens de zwangerschap in de Westerse wereld is borstkanker, gevolgd door schildklierkanker, baarmoederhalskanker, melanoom en lymfeklierkanker.

Borstkanker gediagnosticeerd tijdens de zwangerschap of de postpartum periode wordt ook wel PABC (pregnancy-associated breast cancer) of zwangerschaps-geassocieerde borstkanker genoemd. De definitie van de duur van de postpartum periode varieert hierin sterk binnen de literatuur, variërend van 0,5 tot 5 jaar (of soms langer) na een bevalling. Deze variatie leidt tot conflicterende resultaten over de relatie tussen zwangerschap, voorafgaande zwangerschap en borstkanker uitkomsten. Daarnaast is het moeilijk om een direct verband tussen het ontstaan van borstkanker en de zwangerschap aan te tonen bij een langere postpartum periode, wanneer ook andere risicofactoren een prominentere rol gaan spelen.

In het afgelopen decennium is er aanzienlijke progressie gemaakt binnen de behandeling van borstkanker gediagnosticeerd tijdens de zwangerschap (PABC). Door de toegenomen kennis over de veiligheid van de diverse behandelingen voor zwangere vrouwen (en het nog ongeboren kind), zijn we er in geslaagd om voor deze specifieke vorm van borstkanker meer behandelingsopties ten tijde van de zwangerschap te genereren. Echter, over de complexe biologie en pathofysiologie van PABC is nog steeds weinig bekend; hierin vertoont de literatuur controversen over de invloed van zwangerschap en het ontstaan van borstkanker en de prognose van PABC. Daarnaast biedt de beperkte kennis over de moleculaire achtergrond van PABC op dit moment nog weinig aanknopingspunten voor ontwikkelingen van nieuwe gerichtere en gepersonaliseerde behandelingen. Met als uiteindelijk doel een zo effectief mogelijke behandeling van de borstkanker met een zo beperkt mogelijk aantal bijwerkingen (t.t.v. de zwangerschap) en zo min mogelijk schade voor de moeder en het nog ongeboren kind.

Het onderzoek in dit proefschrift richt zich op het verkrijgen van een uitgebreid overzicht over de klinische en pathologische karakteristieken en uitkomsten van PABC patiënten, specifiek onderverdeeld naar zwangerschapstrimester en postpartum status, ten tijde van de diagnose borstkanker. Daarbij worden vergelijkingen gemaakt met leeftijd-gematchte niet-zwangere borstkanker patiënten. Daarnaast worden moleculaire verschillen tussen de PABC subgroepen geïdentificeerd. Deze moleculaire analyses dienen als een startpunt voor aanvullend biomarker onderzoek naar geïndividualiseerde vroegtijdige diagnostisering en behandeling. Bij elkaar genomen kan dit unieke PABC

profiel worden gebruikt als een fundament voor verdiepend onderzoek naar de ontstaanswijze van PABC en de ontwikkeling van beter gedefinieerde gepersonaliseerde zorg binnen dit unieke borstkanker type.

### Definiëring van het histopathologische profiel van PABC

**Hoofdstuk 2** toont de resultaten van een groot nationaal patiëntencohort waarin het histopathologisch profiel van 741 PABC patiënten wordt vergeleken met een leeftijd-gematcht cohort niet-zwangere borstkanker patiënten. PABC wordt in deze studie gedefinieerd als de diagnose borstkanker gesteld tijdens de zwangerschap tot zes maanden postpartum.

Er wordt een bijzonder agressief histopathologisch profiel geobjectiveerd bij PABC patiënten; tumoren vertonen een significant hogere histologische gradering en oestrogeen receptor (ER) -en progesteron receptor (PR) negativiteit in combinatie met een HER-2 (human epidermal growth factor receptor-2) amplificatie of overexpressie. Daarnaast wordt een hogere incidentie gezien van triple-negatieve tumoren (d.w.z. ER, PR en HER-2 negatief) binnen de PABC patiënten.

Hoewel een jonge leeftijd ten tijde van de diagnose borstkanker is geassocieerd met agressievere klinische en histopathologische kenmerken, toont deze studie binnen PABC patiënten een zelfs nog agressiever borstkanker profiel. Hetgeen impliceert dat PABC biologisch een duidelijk andere borstkanker entiteit is dan borstkanker gediagnosticeerd bij jonge vrouwen die niet zwanger zijn of recent zwanger zijn geweest. Deze resultaten geven interessante aanwijzingen voor verder onderzoek naar het ontrafelen van de moleculaire en genetische achtergrond van PABC. Het is immers bijzonder te noemen dat een groot aandeel van de PABC tumoren ER-en PR negatief is, ten tijde van een zwangerschap waarin de oestrogeen en progesteron niveaus zeer hoog zijn. Mogelijk kan dit verklaard worden op basis van hormonale beïnvloeding van het omgevende stroma van de borst, leidend tot kankergroei, in plaats van beïnvloeding van de epitheliale cellen van borst zelf, hetgeen ook is aangetoond in *in vitro* onderzoek. Hieruit blijken PABC cellijnen niet te prolifereren in respons op oestrogenen in de afwezigheid van hun stroma.

Daarnaast is eerder aangetoond dat tumoren die ontstaan in xenograft modellen van PABC, hoewel ze geen hormoonreceptor-expressie hebben, systemisch oestrogeen nodig hebben voor hun formatie. Het verhogen van de niveaus van deze hormonen blijkt de initiatie en progressie van ER-negatieve tumoren te bevorderen (d.w.z. de tumorcellen zelf brengen geen oestrogenen tot expressie).

Ook andere factoren zouden een bijdrage kunnen leveren aan het ontstaan van



PABC, zoals immunologische veranderingen tijdens de zwangerschap en in de eerste maanden postpartum. Bestaande uit een combinatie van cellulaire immunosuppressie, immunotolerantie en verhoogde inflammatoire responsen gerelateerd aan borstklier involutie en moleculaire veranderingen leidend tot vorming van neo-antigenen. Ook epigenetische veranderingen, die genregulatie kunnen beïnvloeden, kunnen mogelijk bijdragen aan de ontwikkeling van PABC.

### Definiëring van het histopathologische profiel van PABC subgroepen

De verschillende en conflicterende resultaten in de literatuur over de klinische en histopathologische kenmerken van PABC en de prognose hiervan lijken voort te komen uit het niet onderscheiden van de resultaten bij vrouwen met de diagnose borstkanker ten tijde van hun zwangerschap en postpartum. Veelal worden zwangere en postpartumpatiënten als één en dezelfde groep beschouwd, waarin de postpartum patiënten (tot een postpartumperiode van twee jaar) oververtegenwoordigd blijken (vaak 2/3 van het totale aantal patiënten). Mogelijke specifieke invloeden van de verschillende hormonen (verhoogde niveaus van oestrogenen, progesteron en insulin-like growth factor I (IGF-1)) tijdens de zwangerschapstrimesters en postpartum en de mogelijke invloed van prolactine bij lacterende patiënten kunnen hierbij dus niet worden geobjectiveerd en geanalyseerd.

**Hoofdstuk 3** focust daarom op de verschillende histopathologische karakteristieken binnen de drie zwangerschapstrimesters en de lactatiestatus (postpartum). Deze studie toont een hoger aantal graad III borsttumoren in het 2<sup>e</sup> en 3<sup>e</sup> zwangerschapstrimester, vergeleken met het 1<sup>e</sup> trimester. Bij postpartum patiënten wordt eveneens een hoger percentage graad III tumoren geobserveerd, onafhankelijk van de lactatiestatus. Daarnaast worden significant meer ER-en PR negatieve tumoren gezien bij een vorderend zwangerschapstrimester. Bij lacterende patiënten is het aantal ER-en PR negatieve tumoren nog hoger, in tegenstelling tot niet-lacterende patiënten. Voor HER-2 werden geen significante verschillen zichtbaar tussen de verschillende zwangerschapstrimesters; bij lacterende patiënten werd echter wel een lager aantal HER-2 positieven geobjectiveerd.

Concluderend blijkt het histopathologisch profiel tussen de verschillende zwangerschapstrimesters en postpartum duidelijk te verschillen; hetgeen impliceert dat borstkanker gediagnosticeerd tijdens de zwangerschap (ook wel BCdP: breast cancer during pregnancy) niet als dezelfde ziekte entiteit mag worden gezien als PABC. Een onderscheid tussen zwangere en postpartum patiënten lijkt van groot belang, waarbij ook de opsplitsing naar zwangerschapstrimester en lactatiestatus een belangrijk aandeel moet gaan krijgen.

Om een beter inzicht te krijgen in de histopathologische kenmerken van BCdP in de bestaande literatuur wordt in **hoofdstuk 4** een overzicht getoond over het receptorstatus landschap van BCdP (waarbij de postpartum patiënten geëxcludeerd zijn), te meer om een goede vergelijking te kunnen maken met ons eerder opgestelde nationale patiëntencohort (waarin een groot aandeel van BCdP patiënten aanwezig is). Dit overzicht toont hogere percentages van ER-en PR negatieve tumoren specifiek bij BCdP patiënten, dan eerder gepubliceerde data bij PABC patiënten. Dit verschil is vooral te verklaren op basis van het hoge aantal geïnccludeerde postpartum patiënten in deze studies. Daarnaast toont dit onderzoek een hoger aantal HER-2 positieve tumoren bij BCdP patiënten, volledig in lijn met ons patiëntencohort. De resultaten uit dit overzicht ondersteunen dus opnieuw dat BCdP moet worden gezien als een aparte entiteit binnen de PABC populatie, en moet worden gesplitst van de postpartum PABC patiënten.

### Prognose van PABC

Over het algemeen zijn zwangerschap en het geven van borstvoeding (lactatie) geassocieerd met een langdurig beschermend effect tegen borstkanker. Toch kan er binnen een kleine groep van vruchtbare, jonge vrouwen een unieke vorm van borstkanker ontstaan tijdens de zwangerschap of de postpartum periode, welke gekenmerkt is door slechte prognostische tumorkarakteristieken, bestaande uit een gevorderd tumor (T) stadium bij diagnose, lymfekliermetastasen, hoge histologische gradering (d.w.z. slecht gedifferentieerd), negatieve oestrogeen receptor (ER) en progesteron receptor (PR) status en HER-2 amplificatie en overexpressie. De periode van een verhoogd risico op borstkanker voor zwangere vrouwen in vergelijking tot nullipara kan oplopen tot zelfs 15 jaar na de laatste bevalling.

De huidige beschikbare literatuur toont conflicterende informatie over de prognose van PABC, waarbij gefocust wordt op een verschillende en niet-vergelijkbare patiëntengroepen, bestaande uit:

- I) de uitkomsten van de volledige PABC populatie, zonder vergelijking van de overleving tussen de verschillende subgroepen (BCdP versus postpartum)
- II) de postpartum patiënten met een follow-up periode tot zelfs 10 jaar
- III) BCdP patiënten, zonder differentiatie naar zwangerschapstrimester op het moment van de borstkanker diagnose.

Daarnaast blijkt het vergelijken van de prognose binnen deze verschillende studies complex door de vaak kleine patiëntenaantallen per studie en de ongelijkwaardige referentiepopulaties.

Om een duidelijker antwoord te krijgen over de klinische uitkomsten van BCdP en de eerste zes maanden postpartum binnen een grote patientenpopulatie met een

gelijkwaardige referentiepopulatie, is ons grote patiëntencohort uitgebreid met klinische data.

**Hoofdstuk 5** toont de klinische data en uitkomsten van dit grote nationale PABC patiëntencohort, waarin een significant slechtere prognose wordt aangetoond bij patiënten die met borstkanker zijn gediagnosticeerd in het 2<sup>e</sup> en 3<sup>e</sup> trimester van hun zwangerschap, vergeleken met patiënten met een PABC diagnose in het 1<sup>e</sup> zwangerschapstrimester. Daarnaast blijken patiënten die borstvoeding hebben gegeven in de eerste zes maanden postpartum eenzelfde slechte prognose te hebben (in vergelijking met het 2<sup>e</sup> en 3<sup>e</sup> trimester). Deze verschillen persisteren na multivariabele analyse (bestaande uit jaar van diagnose, PR status, stadium van diagnose en chirurgische behandeling), met een significant verhoogde hazard ratio (HR) voor het 2<sup>e</sup> zwangerschapstrimester vergeleken met het 1<sup>e</sup> zwangerschapstrimester. De prognose van de gemaakte (niet-zwangere) controles was gelijk aan de patiënten gediagnosticeerd in het 1<sup>e</sup> trimester en in de postpartum patiënten die geen borstvoeding gaven (niet-lacterende groep). Het is hierdoor dus aannemelijk dat de slechte prognose van de gehele PABC groep (zwangere en postpartum patiënten) vooral te verklaren is o.b.v. de slechte overlevingscijfers van de 2<sup>e</sup> en 3<sup>e</sup> zwangerschapstrimesters, evenals de lacterende patiënten.

Mogelijke factoren die bijdragen aan deze slechte prognose kunnen zijn; het vertraagd stellen van de diagnose borstkanker (door fysiologische veranderingen van de borst ten tijde van een zwangerschap of lactatie) en door gemodificeerde borstkankerbehandelingen om de veiligheid van het kind te kunnen waarborgen (vooral bij een diagnose voor 2006). Daarnaast kan de toegenomen immunosuppressie ten tijde van zwangerschap, verhoogde vascularisatie en verhoogde hormonale blootstelling aan het stroma van de borst een bijdrage hebben geleverd aan de slechtere prognose.

Deze bevindingen onderstrepen het belang van het opstellen van een betere definitie voor BCdP en postpartum PABC patiënten. Waarbij ook binnen BCdP een onderscheid gemaakt moet gaan worden tussen een borstkankerdiagnose ten tijde van de verschillende zwangerschapstrimesters. Voor de postpartum populatie zal verder onderzoek moeten worden verricht naar I) postpartum patiënten tot zes maanden na de bevalling, opgesplitst in lacterende en niet-lacterende patiënten II) postpartum patiënten tot 2 jaar na de bevalling III) postpartum patiënten > 2 jaar na de bevalling IV) de specifieke invloed van lactatie.

### Potentiële biomarkers

Hoewel er duidelijke verschillen in klinische kenmerken en prognose zijn aangetoond binnen de verschillende zwangerschapstrimesters en postpartum (lacterend versus

niet-lacterend), blijven een ruim aantal vragen onbeantwoord. Vooral de onderliggende moleculaire signatuur van de verschillende subgroepen en hun micromilieu zal verder inzicht moeten geven in de mechanismen achter PABC, welke naar alle waarschijnlijkheid zal verschillen tussen de diverse subgroepen.

**Hoofdstuk 6** toont de genomische achtergrond van een kleine groep van triple-negatieve (ER/PR/HER-2 negatief) PABC patiënten, door middel van detectie van copy nummer veranderingen (CNAs; copy number alterations). MLPA (multiplex ligation-dependent probe amplification) technieken werden gebruikt om copy nummer veranderingen in borstkanker-geassocieerde chromosomale loci te vergelijken tussen triple-negatieve PABC- en niet-PABC patiënten. Beide CNA patronen werden geëvalueerd door cluster analyses en associaties tussen individuele gen CNAs, pathologische karakteristieken en overleving werden geëxploreerd.

Triple-negatieve PABC tumoren blijken unieke CNAs te tonen, in vergelijking met niet-PABC tumoren, bestaande uit verrijking voor *TOP2A* copy nummer verlies; een onafhankelijke voorspeller voor slechte overleving.

Clusteranalyse gebaseerd op CNA profielen identificeert daarnaast, binnen een triple-negatieve PABC-subgroep, een nog slechtere overleving, gekarakteriseerd door chromosoom 8p copy nummer verlies.

Individuele gen CNAs analyse laat daarnaast zien dat *FGFR1* copy nummer verlies op chromosoom 8p11.23 een onafhankelijke voorspeller is voor slechte uitkomsten en de ontwikkeling van afstandsmetastasen kan voorspellen.

De bevindingen van deze studie tonen belangrijke inzichten in de biologie van triple-negatieve PABC tumoren, waarbij CNAs (in het bijzonder 8p verlies en *TOP2A* verlies) betrokken blijken bij de ontwikkeling van zwangerschaps-geassocieerde borstkanker. *FGFR1* verlies en *TOP2A* verlies lijken veelbelovende nieuwe biomarkers te zijn die onafhankelijke subgroepen van PABC-patiënten met een slechte prognose kunnen identificeren. Deze genomische biomarkers zullen dienen als een startpunt voor verdere en uitgebreidere analyses naar copy nummers door het gebruik van next generation sequencing (NGS) binnen het gehele PABC cohort.

## Conclusie

Het onderzoek in dit proefschrift toont een uniek klinisch en histopathologisch profiel voor patiënten met borstkanker gediagnosticeerd tijdens de verschillende zwangerschapstrimesters en de vroege postpartum periode ( $\leq 6$  maanden postpartum). Deze nieuwe bevindingen impliceren dat de diagnose PABC geredefineerd moet worden, met een opsplitsing naar zwangerschapstrimester (ten tijde van de borstkankerdiagnose) en lactatiestatus voor postpartum patiënten. Nieuwe studies zullen zich moeten focussen op deze verschillende borstkanker entiteiten en hun carcinogenese. Dit zal hopelijk nieuwe aanknopingspunten opleveren voor gerichtere en gepersonaliseerde behandelingen, leidend tot betere uitkomsten voor deze jonge groep van borstkankerpatiënten.

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Lieve **Ruth en Joost**, niet alleen de liefde voor het ziekenhuis en onze patiënten delen we, maar ook de liefde voor eten, wijn en genieten van Franse en Italiaanse sferen. De kracht van onze vriendschap is dat we altijd weer snel de draad oppakken. Samen met onze lieve kinderen hebben we niet meer nodig! Ik kijk nu al uit naar een volgend diner of vakantie samen.

Lieve **Anniek en Irene**, mijn paranimfen. We hebben de afgelopen 20 jaar al heel veel gedeeld; daarom vind ik het erg speciaal dat jullie aan mijn zijde staan op dinsdag 29 maart. Dit voelt zeer vertrouwd, omdat ik weet dat ik niet alleen dan, maar ook op alle andere momenten op jullie kan rekenen! Ik koester de vele gezellige momenten op onze studentenkamers in Wittevrouwen, het dansen in het Pakhuis of het Heerenplein, de vele feestjes en borrels later in Amsterdam, de prachtige boottochten en onze gezamenlijke gastronomische passie. Jullie kunnen mij als geen ander weer “back to basic” brengen in tijden van Britt’s door denderende trein naar perfectie. Dank voor jullie altijd sprankelende aanwezigheid, gezelligheid en vertrouwen!

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en de mooie gesprekken in Nederland en het buitenland bij ondergaande zon onder het genot van een goed glas wijn.

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*Het leven is een opeenvolging van momenten,  
Je slaagt pas als je van elk moment kunt genieten!*

Britt Suelmann

## CURRICULUM VITAE



Britt Berendine Maria Suelmann werd geboren op 21 mei 1984 te Oldenzaal en groeide op in een warm gezin samen met haar zusje Lorette.

Na het behalen van het VWO-diploma in 2002 aan het Twents Carmel Lyceum te Oldenzaal, ging zij geneeskunde studeren aan de Universiteit van Utrecht. In 2009 behaalde zij haar arts examen, waarna zij startte als ANIOS Interne Geneeskunde in het Diaconessenhuis te Utrecht (onder leiding van dr. A.F. Muller).

Zij vervolgde haar carrière kort daarna (eveneens in het Diaconessenhuis) en startte met de opleiding tot internist, welke zij vanaf 2013 in het UMC Utrecht voorzette (opleiders dr. A.F. Muller en prof. dr. M.M.E. Schneider).

In 2014 startte zij haar differentiatie tot medisch oncoloog in het UMC Utrecht (onder leiding van prof. dr. P.O. Witteveen). Ten tijde van deze opleiding werd zij geïntroduceerd binnen de onderzoeksgroep van prof. dr. E. van der Wall en prof. P.J. van Diest en verrichtte zij onderzoek naar beeld- en bloed gestuurde behandelingen binnen het mammacarcinoom.

In 2017 behaalde zij haar titel tot internist-oncoloog en ging werken binnen de medische staf van de afdeling Medische Oncologie van het UMC Utrecht. Zij zette haar werkzaamheden binnen de mammacarcinoom onderzoeksgroep voort en startte haar PhD project gericht op zwangerschaps-geassocieerde borstkanker (PABC), in samenwerking met het NKI-AVL (prof. dr. S. Linn).

Resultaten afkomstig uit dit promotieonderzoek zijn gepresenteerd op diverse internationale congressen; San Antonio Breast Cancer Symposium (Texas, Amerika), ESMO (European Society of Medical Oncology) Breast Congress, ASCO (American Society of Clinical Oncology) Annual Meeting en het BOOG (Borstkanker onderzoeksgroep) Symposium.

Op dit moment is zij nog steeds werkzaam als internist-oncoloog (met een specifieke expertise voor uro-genitale oncologie en palliatieve zorg) binnen het UMC Utrecht. Britt woont samen met Rutger van der Klaauw in Hilversum, samen met hun twee kinderen: Mick en Anne-Sophie.

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