TUMOUR MICROENVIRONMENT

Pregnancy-associated breast cancer and metastasis

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Abstract | Pregnancy-associated breast cancer, which has a poor prognosis, is often overlooked by clinicians and researchers alike. With the trend towards delayed child-bearing, an increase in the occurrence of breast cancer complicated by pregnancy is anticipated. The mechanisms that have been proposed to account for this poor prognosis, including increased hormone exposure, might not contribute significantly to the observed increase in metastasis seen in these patients. Instead, the mammary microenvironment might become tumour-promoting after pregnancy because of the remodelling of the mammary gland to its pre-pregnant state. This remodelling, which is associated with pro-inflammatory and wound-healing mechanisms, is proposed to support tumour-cell dissemination. This hypothesis will be discussed.

Epidemiological studies from the 1970s showed that, overall, pregnancy is associated with a reduction in a woman's lifetime risk of developing breast cancer[1]. These early observations were subsequently confirmed and extended in large prospective studies[2,3]. The findings were so compelling that research was directed towards identifying the mechanism(s) of pregnancy-induced protection. Specifically, the objective was to develop therapeutic interventions to mimic this protection[4–6]. However, in the early 1980s, it was recognized that the protective effect of pregnancy was neither immediate nor constant. So, the simple notion that pregnancy reduces the risk for developing breast cancer has advanced — pregnancy might both suppress and promote breast cancer.

Increase in breast cancer following pregnancy

In a 1982 paper by Janerich and Hoff, it was reported that, before the age of 40, any woman who has ever been pregnant (parous woman) is actually at an increased risk for breast cancer compared with any woman who has never been pregnant (nulliparous woman)[7]. Only after the age of 40 did a crossover in risk occur — parous women were now at lower risk for breast cancer than nulliparous women. The authors concluded that pregnancy must cause a period of promotion before it eventually produces its protective effect[7]. The transient increase in breast cancer risk following a pregnancy has also been referred to as the 'dual' effect. For example, in one study, pregnancy was found to be protective overall, but parous women with breast cancer were diagnosed on average a full 5.2 years earlier than nulliparous women with breast cancer[8]. This transient increase in breast cancer following pregnancy has been found to peak 6 years post-partum and to persist for approximately 10 years following parturition[9]. Diagnosis within this period is referred to as pregnancy-associated breast cancer[9–13]. However, because most breast cancers are diagnosed in older women, the cumulative effect of pregnancy is to lower overall risk.

Degree of protection conferred is age-dependent

As significant epidemiological efforts were directed towards understanding the relationship between pregnancy and breast cancer risk, it became evident that even the long-term protective effect of pregnancy was not constant, but varied depending on the mother's age at the time of first pregnancy. In an early study involving more than 16,000 women, a 3.5% increase in the risk of breast cancer was identified for every year that child-bearing was delayed[14]. So, a new mother at 35 years of age was found to have a 50% increased risk of developing breast cancer compared with a young mother of 20 (REF. 14). The age of approximately 35 years was identified as a crucial point, with full-term pregnancy before age 35 conferring some degree of protection, and pregnancy after this age being associated with a permanent increase in breast cancer risk[14].

Results from subsequent studies have been variable with respect to whether late age at the time of first pregnancy confers any long-term protection. In a prospective study of 1 million Norwegian women, the protective effect of parity was found to be particularly strong

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At a glance

- The protective effect of pregnancy is neither immediate nor constant — a transient increase in breast cancer risk occurs with pregnancy in women of all ages. The risk of pregnancy-associated breast cancer is greatest in older first-time mothers.
- Pregnancy-associated breast cancer has a higher mortality rate because metastasis is common. Possible reasons for breast cancer metastasis with pregnancy include the promotional effects of pregnancy-associated hormones and a delay in diagnosis in recently-pregnant women. However, existing data indicate that these factors do not account for the high mortality associated with pregnancy-associated breast cancer.
- Following pregnancy and lactation, the mammary gland regresses to its pre-pregnant state by a tissue remodelling process known as involution. The microenvironment of the involuting mammary gland shares attributes with inflammation and wound healing.
- Because pro-inflammatory tissue environments can promote tumorigenesis, the involuting mammary gland might be tumorigenic. Candidate mediators of tumour cell progression during gland involution include an influx of activated immune cells, increased concentrations of matrix metalloproteinases, remodelling of mammary extracellular matrix with subsequent release of tumour-promoting growth factors and matrix fragments with bioactivity, and loss of basement-membrane barrier function.
- Under normal conditions of involution, pro-inflammatory pathways are activated, but the balance of pro- to anti-inflammatory signals is tipped towards suppression of overt inflammation.
- The presence of occult disease at the time of gland involution is anticipated to aggravate the tumour-promoting microenvironment by tipping the balance towards overt inflammation. So, women with occult disease at the time of pregnancy might be at an increased risk of tumour cell dissemination during mammary gland involution.
- As the average age of child-bearing continues to rise, the incidence of pregnancy-associated breast cancer and its associated high mortality rate are also anticipated to rise.

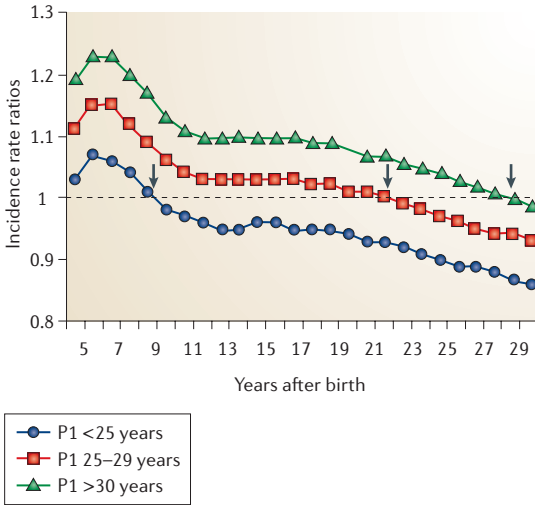

Figure 1 | **Evidence for a transient increase in breast cancer risk following pregnancy.** This figure shows the predicted incidence-rate ratio of breast cancer for women of parity according to time since first birth (P1), in subgroups of 'age at first birth' (for nulliparous women, the relative-rate ratio = 1.0). In this cohort of 22,890 women with breast cancer, a transient increase in risk is seen up to 10-years post-pregnancy (see REF. 9). In young mothers, the crossover to protection occurs approximately 10 years after first pregnancy (indicated by the arrow). For mothers aged 25–29 years, crossover to protection occured around 22 years after pregnancy (indicated by the arrow), and for mothers 30 years and older, the crossover occured approximately 30 years after pregnancy (indicated by the arrow). Data compiled from incidence-rate ratio charts provided by G. Albrektsen, University of Bergen, Norway using a no-interaction model.

among women with first birth before the age of 20, and was weaker, but not absent, among those who delayed child-bearing to their 30s (REF. 3). Newer results from a prospective study of 1.7 million Norwegian women, which identified 22,890 women with breast cancer, found that the adverse effects of an older first pregnancy persisted until the seventh decade of life[9]. This study demonstrated that late age at first pregnancy delayed the crossover effect for decades, but did not entirely eliminate the long-term protective effect of pregnancy (FIG. 1). By contrast, data from 58,000 participants of the Nurses' Health Study showed that women with a first birth at 35 years or older had a persistent increase in the risk of developing breast cancer compared with nulliparous women[15]. That is, women with late-age first pregnancy never crossed over from being at increased to decreased risk compared with nulliparous women.

Increased risk for older first-time mothers

Not surprisingly, it has been found that the transient increase in breast cancer risk seen immediately following pregnancy is more pronounced in women with late-age first birth compared with early-age first birth[9,10]. Older first-time mothers are more likely to be diagnosed with pregnancy-associated breast cancer than younger mothers[9,10].

Pregnancy-associated breast cancer mortality

Pregnancy-associated breast cancer, regardless of the age of the mother, is associated with a significantly poorer prognosis. This is because women with pregnancy-associated breast cancer are more likely to die from metastases. For example, parous women were found to be at a 40% increased risk of dying from breast

Relative risk
An epidemiological measure of treatment effect in an intervention study (clinical trial) or exposure association in a non-experimental observational study. The relative risk is the ratio of risk in an exposed group (that is, recent birth group) to the risk in an unexposed, control group (no recent birth).

Table 1 | **Survival rates***

Survival	Pregnancy-associated breast cancer	Non-pregnancy-associated breast cancer
5-year	52%	80%
10-year	44%	69%

*Data taken from REF. 16.

cancer during the first 5 years after diagnosis (relative risk (RR) = 1.4) compared with women who had never given birth[11]. In another study, the 5-year survival for pregnancy-associated breast cancer patients was only 52%, significantly less then the 80% survival for age-matched patients who had not had a recent pregnancy[16] (TABLE 1). In a more recent study, the relationship between survival and the duration of time that occurred between birth and breast cancer diagnosis was evaluated in more than 3,700 women with pregnancy-associated breast cancer[17]. These pregnancy-associated breast cancer cases were subdivided into diagnosis within 12 months, between 13 and 48 months, and 48 months after pregnancy. An inverse relationship was identified, with survival being significantly poorer in women who were diagnosed within the first year of giving birth[17] (TABLE 2). In summary, these studies clearly demonstrate that women with pregnancy-associated breast cancer are more likely to die from their disease than all women of similar ages with breast cancer who have not recently given birth. Simply stated, women with pregnancy-associated breast cancer have a higher incidence of metastatic disease than matched non-pregnancy-associated breast cancer cases.

Magnitude of the problem

The probability that a woman will be diagnosed with breast cancer increases exponentially with age. For example, if risk is set to 1 for women in their 20s, then risk increases more than 10-fold for women in their 30s, more than 100-fold for women in their 50s and more than 1,000-fold for women in their 80s. It is common for these breast cancer statistics to be graphically displayed as either cumulative breast cancer risk (FIG. 2a) or as cases of breast cancer per 100,000 women in a particular age group (FIG. 2b). These data clearly identify a woman's age as being the dominant risk factor for breast cancer. However, one unintended consequence of these particular statistical displays is that breast cancer seems to be rare in younger women. Therefore, one might conclude that pregnancy-associated breast cancer affects only a small fraction of the total number of women diagnosed with breast cancer. However, because the distribution of women in the population decreases with increasing age, approximately the same number of women in their 40s and 50s are diagnosed with breast cancer as women in their 60s and 70s (FIG. 2c), which is about 85,000 per year. Similarly, although the cumulative breast cancer risk for women in their 30s and 40s is very low (FIG. 2a), more than 45,000 women in these age groups are expected to be diagnosed with breast cancer in the coming year (FIG. 2c).

When one considers that there has been a steady increase in the age of child-bearing mothers in the United States because women are electing to have children later (FIG. 2d), then the number of breast cancer cases that might be pregnancy-associated is seen to be a significant fraction of the total cases. If 35 years of age is set as being a conservative upper limit for reproduction, then it can be estimated that 30,000 cases of breast cancer diagnosed in 2005 might be complicated by a recent pregnancy. As the average age of child-bearing continues to rise, morbidity and mortality owing to pregnancy-associated breast cancer are likely to increase further[18].

Table 2 | **Poor survival of patients diagnosed within 1 year of giving birth***

	Diagnosis within 12 months after birth	Diagnosis between 13–48 months after birth	Diagnosis 48 months after birth	Age-matched nulliparous women
15-year survival	38%	51%	60%	65%

*Data taken from REF. 17.

Putative mechanisms that drive poor prognosis

One explanation for why pregnancy-associated breast cancer has a poor prognosis is that diagnosis is delayed in new mothers[19,20]. Pregnancy and breastfeeding increase breast density, making clinical examinations and mammography more difficult to interpret. An alternative hypothesis is that the gestational hormones are responsible for the poor prognosis of pregnancy-associated breast cancer. Pregnancy is characterized by significantly increased concentrations of oestrogen, insulin-like growth factor 1 (IGF1) and progesterone — hormones that are intimately associated with breast cancer aetiology and progression[21,22]. Therefore, gestational hormones are anticipated to increase risk and negatively affect prognosis by virtue of their growth-promoting effects on hormone-responsive breast tumour cells[23,24]. In both cell culture and xenograft models of human breast cancer, it is clear that the increased concentrations of hormones during pregnancy can increase tumour cell proliferation and tumour size[25,26]. Furthermore, pharmacological inhibition of oestrogen-receptor- and IGF-signalling inhibits human breast tumour cell growth in xenograft models[25–27]. In a potentially related observation, the pathological pregnancy condition pre-eclampsia has been shown to correlate with a twofold-reduced risk of pregnancy-associated breast cancer[28]. Pre-eclampsia is a complex and poorly understood condition involving placental breakdown, which is characterized by decreased concentrations of systemic oestrogen and IGF1 (REF. 29). It has been suggested that pre-eclampsia lowers the risk for pregnancy-associated breast cancer by reducing the promotional effects of these gestational hormones[29].

A prediction of both of these hypotheses — delayed diagnosis and promotional effects of gestational hormones

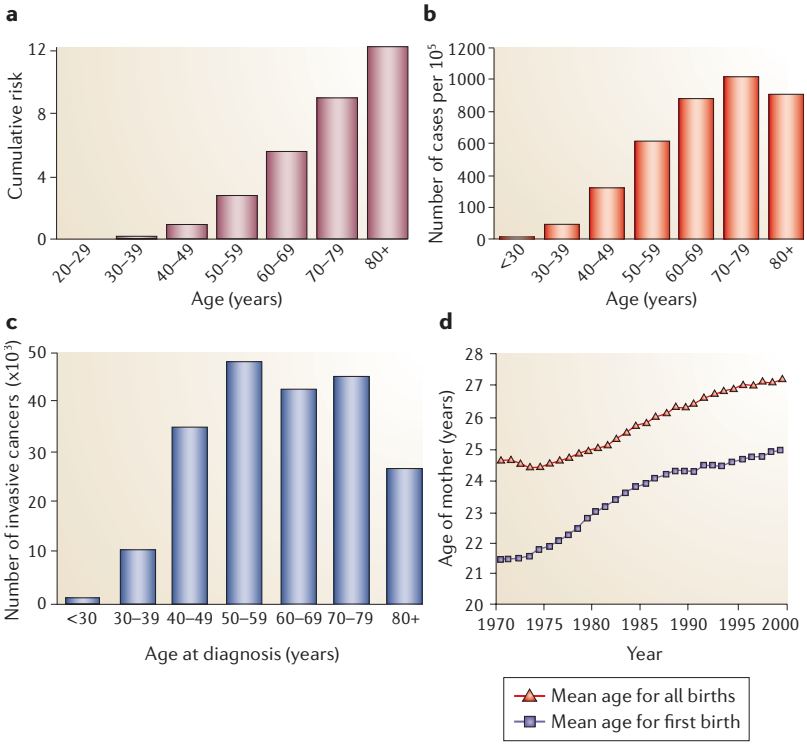

Figure 2 | **Breast cancer and pregnancy-trends statistics. a** | The probability of developing breast cancer according to age demonstrates that risk increases exponentially with age. **b** | The number of breast cancers per 100,000 women by age group also demonstrates that risk increases sharply with age. **c** | The number of actual cases of invasive breast cancer by age group shows that more women in their fifth decade of life are diagnosed with invasive breast cancer than any other age group. **d** | 30-year birth trends in the United States depict a steady increase in the mean age of first-time mothers and for all mothers.

— is that pregnancy-associated breast tumours would be diagnosed at a larger size and later stage. This has not been consistently demonstrated[11,17,18]. Furthermore, when adjustments for stage and grade of tumours are performed, pregnancy at the time of diagnosis does not seem to be an independent, negative prognostic marker. This is because, to date, the poor prognosis associated with breast cancer diagnosed during pregnancy is lost when tumour size and stage are taken into account. In other words, when tumour size and stage are controlled between groups, breast cancer that is diagnosed during pregnancy has not been found to have a poorer prognosis compared with non-parous cases[18,30]. As study sample sizes increase, risks that are specifically associated with breast cancer diagnosed during pregnancy might become evident. However, and importantly, a completed pregnancy 5 years or less before diagnosis has been demonstrated to be an independent, negative prognostic marker[11,17,18,30]. Cumulatively, these studies do not rule out an important role for pregnancy-associated hormones in breast cancer progression, but indicate that if hormones are the reason, then hormone exposure does not strongly relate to size or stage of tumour. As such, these studies implicate factors other than gestational hormones as being important for the high metastatic rate of breast tumours that are diagnosed shortly after pregnancy.

An additional hypothesis that has been put forward to explain the high mortality of pregnancy-associated breast cancer is that breast tumours with poor prognostic characteristics are selected for by pregnancy hormones. A prediction of this hypothesis is that pregnancy-associated breast cancers would express increased levels of validated markers for poor prognosis. There is, in fact, a paucity of data in this area. However, in one study prognostic markers were evaluated by immunohistochemical methods in more than 700 breast cancers[11]. The tumours from women who had recently given birth did not differ in size, stage, nodal status, oestrogen receptor status or ERBB2 (also known as HER2) expression[11]. However, in this study pregnancy-associated breast cancers were more likely to be progesterone-receptor-negative and p53-positive, and to have a higher histological grade. Progesterone-receptor negativity is a poor prognostic factor, in part because it is an independent predictor of sensitivity to the breast cancer drug tamoxifen[31]. In addition, progesterone-receptor negativity has been shown to confer an invasive phenotype to breast tumour cells in model systems[32,33]. Increased expression of p53 in pregnancy-associated breast cancer would also be consistent with pregnancy selecting for more aggressive tumour cells[34]. Additional studies are required to determine whether pregnancy-associated breast cancers do express a unique prognostic marker profile. Nonetheless, even after controlling for this broad panel of breast tumour markers, Daling *et al.* found that a birth within 2 years of diagnosis remained an independent predictor of mortality, and concluded that other, unknown factors that are related to a recent pregnancy have a significant impact on the course of the disease[11].

These epidemiology-based studies indicate that an event associated with pregnancy, but independent of the potential growth-promoting effects of gestational hormones, is crucial for breast cancer metastasis. The consistency of the data that demonstrates increased risk owing to a recently completed pregnancy, rather than pregnancy *per se*, identifies the window of time immediately following pregnancy as being implicated in breast cancer progression. In other words, an event subsequent to pregnancy might be crucial for determining metastasis. The question that remains is what biological events are unique to the period of time immediately following pregnancy that could account for this increase in risk?

There is, in fact, one dramatic change in mammary gland physiology following pregnancy that might increase the risk of breast cancer progression, and this is mammary gland involution. Soon after birth, if the mother does not nurse, or following lactation if she does, the fully differentiated gland regresses to its pre-pregnant state. This regression process is referred to as involution and occurs because of the initiation of an innate tissue-remodelling programme. Evidence indicates that mammary gland involution uses some of the same tissue-remodelling programmes that are activated during wound healing and inflammation. Importantly, wound healing and inflammatory microenvironments are pro-oncogenic, with inflammation-associated cancers having poor prognoses compared with cancers that do not have an inflammatory component[35–37].

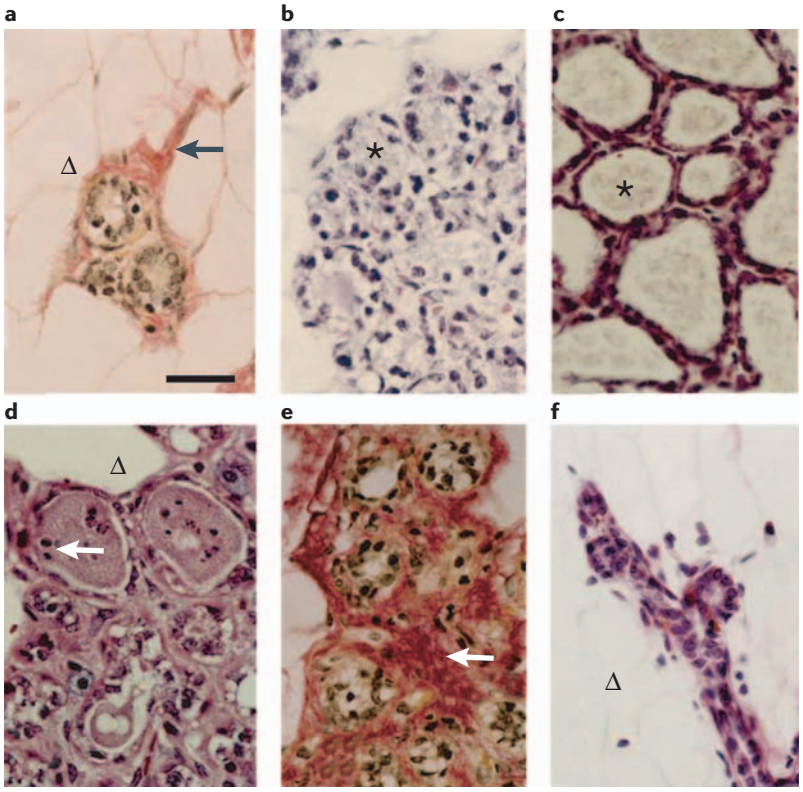

Figure 3 | **The lactation–involution cycle in the rat mammary gland.** The following series of histological sections of the mammary gland chart the progression from a pre-pregnant state, through pregnancy to the cessation of milk production and involution of the gland. This series demonstrates the remarkable tissue remodelling that occurs during physiological gland remodelling. **a** | Shows a section from a nulliparous mammary gland with small epithelial alveoli embedded in the fat pad mesenchyme. An adipocyte is indicated by Δ and Sirus-red-stained collagen-rich inter- and intra-lobular stromal sheath is shown by the arrow. Scale bar = 50 μm. **b** | A haemotoxylin and eosin (H&E)-stained section of mammary gland showing dramatic alveolar expansion, which is characteristic of a mid-pregnant rat. The asterisk indicates a single alveolus. **c** | This section shows a H&E-stained section of lactating mammary gland composed of large lobuloalveoli with milk-filled lumen (indicated by the asterisk). Adipocytes are absent and the intralobular stromal component is reduced. **d** | A H&E-stained mammary gland 4-days post-weaning is filled with collapsed alveoli and apoptotic bodies (indicated by the arrow). Reappearance of adipocytes is evident (indicated by Δ). **e** | A Sirus-red-stained 6-day post-weaning mammary gland showing an expansion of both intra- and inter-lobular collagen-rich stroma (indicated by the arrow). Collapsed alveoli and apoptotic bodies are present. **f** | This section shows a fully regressed parous mammary gland at 2-months post-weaning. The epithelium is primarily ductal and the adipocyte-rich mammary fat pad is restored (indicated by Δ).

So, an alternative mechanism to account for the transient increase in metastatic breast cancer following pregnancy is that the pro-inflammatory changes that occur during involution promote tumour cell dissemination.

An overview of mammary gland involution

In the non-pregnant, non-lactating state, the mammary gland consists of networks of epithelial ducts that empty into the main lactiferous ducts[38]. The milk-producing lobular component of the gland is rudimentary, but poised to respond to gestational hormones. This immature glandular epithelium is embedded in a mesenchymally-derived fat pad, which, at the histological level, is the dominant tissue present. With pregnancy, and in preparation for lactation, the epithelium undergoes remarkable proliferation and differentiation. The epithelium expands to fill the gland, replacing the fat pad with milk-producing lobuloalveoli. For the purposes of this Review, the events associated with regression of these milk-producing lobuloalveoli following pregnancy are the most relevant.

With cessation of milk secretion, the mammary gland resorbs the elaborate milk-producing lobuloalveoli generated during pregnancy and returns to its rudimentary, ductal state[39,40] (FIG. 3). The scale and rapidity of this tissue remodelling is unique to the mammary gland, and even exceeds that which occurs in most pathological conditions, such as in wound healing[41]. However, in contrast to the tissue remodelling that accompanies wound healing and inflammation, mammary gland involution is physiological, orderly and developmentally programmed. As such, mammary gland involution has been regarded as a non-inflammatory remodelling process[40,42,43].

Mammary gland involution in rodent models

Mammary gland involution has been extensively investigated in rodent models. Early on, it was hypothesized that the massive alveolar cell death and subsequent tissue remodelling of involution was mediated by the loss of cell adhesion to the underlying basement membrane. In ultrastructural and histological studies, evidence indicated that the basement membrane is indeed targeted for limited proteolysis during involution[41,44,45]. Subsequent studies identified proteolytic cleavage products of the basement-membrane proteins entactin, fibronectin and laminin 5 (REFS 40,46,47). Given that basement-membrane integrity is essential for mammary gland morphogenesis and the expression of milk proteins[48], the hypothesis that loss of basement-membrane integrity could trigger involution was investigated. *In vitro* and *in vivo* studies confirmed that mammary epithelial cells die when integrin attachments to the extracellular matrix (ECM) are disrupted[49–54]. Cumulatively, these and other studies indicated that ECM-degrading proteinases, which are secreted by mesenchymal cells, led to basement-membrane degradation, epithelial cell detachment and subsequent death of the unwanted secretory mammary epithelium[46,52,53,55,56].

Consistent with this hypothesis, a protease-dependent phase of mammary gland involution has been characterized[45,55,57]. During this phase of involution, tissue plasminogen activator and the matrix metalloproteinases MMP2, MMP3, MMP9 and MMP14 (which is also known as membrane-type MMP1) are dramatically upregulated[41,52,56,57]. In addition, concurrent with the peak in MMP activity, lobuloalveolar architecture collapse and tissue remodelling are at their maximum. However, the simple model that MMPs primarily contribute to tissue remodelling through dissolution of the basement membrane has been re-assessed. For example, although MMP3 is dramatically upregulated during mammary gland involution, mice that overexpress tissue inhibitor of metalloproteinases 1 (TIMP1), or mice that carry a targeted mutation in *Mmp3*, do not, as one would have

anticipated, have a delay in apoptotic cell death of mammary epithelial cells[58]. Furthermore, the concept that the main function of MMPs is to degrade ECM proteins has been questioned[59]. In a recent and comprehensive review on the physiological roles of MMPs, it has been argued that the primary function of MMPs *in vivo* is to regulate inflammation and repair processes[59]. In support of this hypothesis, otherwise phenotypically normal MMP-deficient mice show various immune-deficiencies when challenged by injury or infection[59]. The emerging view is that MMPs function primarily on cytokines, chemokines and other immunomodulators, resulting in a potentiation of immune function[59]. During mammary gland involution, MMPs might also be functioning as modulators of immune function.

Role for inflammation in gland involution

Evidence indicates that mammary gland involution might have an inflammatory component. Recent studies that detail the mRNA transcript changes in murine mammary glands that are undergoing involution have identified evidence for a wound-healing response[60–62]. Quite surprisingly, molecular markers for both the innate and adaptive immune responses have been identified within the involuting gland and provide indirect evidence for neutrophil, macrophage, T-cell and B-cell activation[60–62]. For example, a transient increase in the expression of components of the death-receptor pathway of apoptosis was observed within the first 24-hours after weaning-induced involution[61]. Specifically, upregulation of the four death-receptor ligands — tumour-necrosis factor-α (TNFα), Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL, also known as TNF–ligand-superfamily, member 10 (TNFSF10)) and TNF-related weak inducer of apoptosis (TWEAK, also known as TNFSF12) — was apparent[61,63]. The role of these death-receptor ligands was originally described in T-cell-mediated killing of infected cells and indicates a mechanistic overlap between immune-mediated cell-killing and apoptosis of mammary epithelial cells during involution. The increased expression of signal transducer and activator of transcription 3 (STAT3), whose function was also first described in relation to inflammation, provides further evidence that events during involution share some similarities with inflammation-mediated tissue remodelling[61,62,64]. STAT3 is a known inducer of the acute-phase response, and activated STAT3 can lead to increased expression levels of interferon. Secreted interferon can recruit innate and adaptive immune cells to the site of infection[65]. The influx of these cells is required for efficient microbial removal and wound healing. Consistent with a conserved role for STAT3 during mammary gland involution, there is evidence in some bovine and murine models for an influx of neutrophils, macrophages and plasma cells into the involuting gland[38,43,62]. However, there is also evidence that mammary gland involution can proceed in the absence of inflammatory cells (P. Henson, unpublished observations).

In an independent analysis of gene expression profiles through the pregnancy–involution cycle, 145 genes were found to be specific to involution[62]. These genes were not upregulated in the mammary gland during the tissue remodelling phase that accompanies pregnancy. As tissue remodelling associated with the expansive phase of pregnancy has not been associated with breast cancer risk, whereas the window of time associated with mammary gland involution has, these 145 involution-specific genes might be important in pregnancy-associated breast cancer. Of these genes, 12 encoded proteins that are involved in the acute-phase response, including the lipopolysaccharide (LPS) receptor CD14 and LPS-binding protein (LBP)[62]. Furthermore, 49 of these upregulated transcripts encoded immunoglobulins, demonstrating the presence of plasma cells within the involuting mammary gland — an observation that had been previously reported[60]. The role of plasma cells during mammary gland involution is currently unknown. Importantly, genes that are known to suppress inflammation, such as *IL1RN* (interleukin-1-receptor antagonist), which is an inhibitor of IL-1-receptor signalling, were also identified in these microarray analysis experiments[61,62]. Cumulatively, these observations indicate that under normal conditions of involution, pro-inflammatory pathways are activated, but the balance of pro- to anti-inflammatory signals is tipped towards the suppression of overt inflammation.

The morphological similarities between tissue remodelling associated with wound healing and tissue remodelling associated with mammary gland involution are noteworthy. Wound healing eliminates damaged epithelium, whereas involution eliminates unwanted secretory epithelial cells. Furthermore, during wound healing, mesenchymal cells are stimulated to proliferate, upregulate expression of immunomodulators (including TNFα, hepatocyte growth factor (HGF) and transforming growth factor-β (TGFβ)), remove existing ECM and deposit provisional-matrix that is rich in fibrous collagen[39,56,61,62]. All these processes facilitate wound closure and remodelling of the damaged tissue. Similarly, during involution the mesenchymal compartment is stimulated because the gland is repopulated with mesenchymally derived adipocytes, which is concurrent with epithelial regression[58]. Also, similar to wound healing, stimulation of the mammary mesenchymal compartment is associated with expression of TNFα, HGF and TGFβ, remodelling of the existing matrix and deposition of fibrillar collagen[56]. In addition, during wound healing, the potent immunomodulator TGFβ is thought to be responsible for the opposite effects that tissue damage has on the epithelial and stromal compartments — inhibitory to epithelial growth and stimulatory to mesenchymal growth. Similarly, TGFβ might have similar functions during mammary gland involution, as TGFβ3 expression is significantly increased during involution[61,62,66,67].

Overall, the characterization of the tissue microenvironment during mammary gland involution demonstrates that the stromal compartment of the physiologically normal involuting mammary gland shares attributes of wound-healing stroma. Consequently, a model is emerging that implies that

Innate immune response
Innate immunity is the first line of defence against injury and is mediated by the myeloid lineage cells, primarily the dendritic cells, neutrophils and monocytes. The trigger of innate immunity can be conserved microbial molecules, such as LPS, or chemokines and cytokines. The result of activating the innate immune response is killing of microbes and recruitment of additional immune cells through cytokine mediators.

Adaptive immune response
Adaptive immunity — also known as acquired immunity — is the second line of defence mediated by cells of the lymphoid lineage, the T cells and B cells. Adaptive immunity is subordinate to innate immunity because activation relies on innate immune cells for antigen presentation.

Acute-phase response
The acute-phase response is the systemic inflammatory component of innate immunity and comprises the non-specific physiological and biochemical response to tissue damage. The acute phase response was first described in the liver, which, in response to circulating cytokines released at the site of pathology, rapidly synthesizes a number of immunomodulatory proteins, including C-reactive protein, amyloid A protein, proteinase inhibitors, and coagulation and complement proteins.

the remodelling of the gland to its pre-pregnant state is dependent on some inflammation-based pathways. Importantly, tumour cell proliferation, motility and invasiveness can be stimulated by inflammation-associated stroma through various mechanisms. These mechanisms include tumour cell activation by inflammatory chemokines and cytokines, and growth factor release from proteolysed matrix. These mechanisms also include the angiogenic, chemotactic and growth-promoting activities that are caused by the presence of cryptic ECM fragments. The question of how a pro-inflammatory microenvironment contributes to cancer promotion and progression has been the focus of several outstanding reviews[35–37,68,69], and therefore will not be covered further here. With respect to breast cancer, the role of inflammation is poorly understood. Both wound healing and mastitis have been reported to increase the risk of breast cancer[70,71]. Nonetheless, it is currently not possible to reach any conclusions on the role of pathologically induced inflammation in breast cancer.

It is important to note that the putative inflammation cascade proposed to be driving physiological mammary gland involution must be highly regulated in magnitude and duration, as involution does not normally progress to overt inflammation. Therefore, the question remains: is the pro-inflammatory tissue microenvironment during mammary gland involution sufficient to activate tumour cells? If the answer is yes, this would provide a plausible mechanism to account for the high metastatic rate of pregnancy-associated breast cancer.

Involution and breast cancer model systems

To begin to investigate whether the mammary microenvironment becomes tumour-promotional during gland involution, ECM has been isolated from quiescent mammary glands of nulliparous rats and from actively involuting glands of parous rats. These endogenous mammary ECM preparations were evaluated for their ability to differentially modulate the metastatic behaviour of human breast cancer cells. Mammary matrix isolated from actively involuting glands was found to promote *in vitro* motility and invasion of the highly metastatic MDA-MB-435 and MDA-MB-231 human breast tumour cell lines[56,72,73]. Conversely, mammary matrix isolated from nulliparous rats was found to suppress tumour cell motility and invasion[56,72,73]. Furthermore, when MDA-MB-231 cells were pre-mixed with matrix isolated from actively involuting mammary glands and then injected into the mammary fat pad of immunocompromised mice, metastases to lung, liver and kidney were significantly increased compared with mice in which the tumour cells had been pre-mixed with mammary matrix isolated from nulliparous rats[73]. These data provide evidence that the mammary microenvironments of the nulliparous and actively involuting glands differ in their ability to support metastatic attributes of tumour cells and indicate that tissue remodelling during involution does, in fact, create a microenvironment that is permissive for tumour cell dissemination.

ECM mediators

To investigate components in the involuting matrix that might be responsible for promoting tumour cell metastasis, the endogenous mammary ECM preparations have been partially characterized. ECM isolated from actively involuting glands had increased levels of proteolytic cleavage fragments of fibronectin and high levels of MMP9 activity[52,56,72,73]. *In vitro* treatment of both non-tumorigenic and tumorigenic mammary epithelial cells with fibronectin fragments resulted in the induction of MMP9 activity and promoted invasion by mammary tumour cells[52,72]. These observations indicate a positive-feedback loop between matrix degradation and MMP expression during involution, which would be anticipated to facilitate remodelling of the gland to its pre-pregnant state[52,72]. Evidence for such

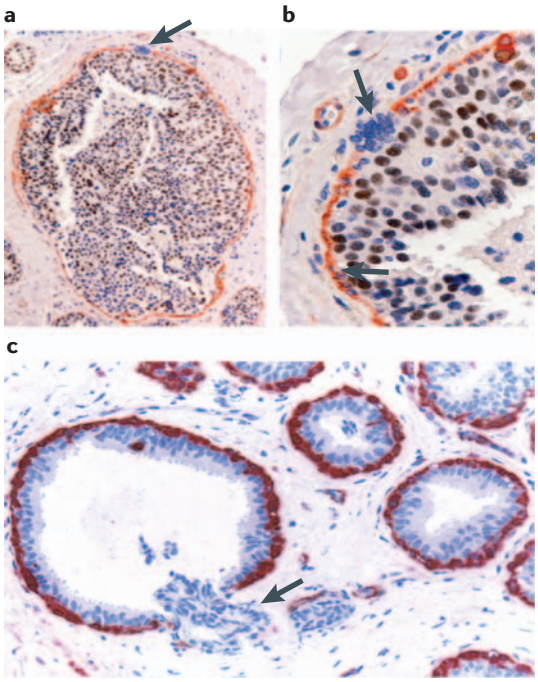

Figure 4 | **Micro-invasive lesions in human breast biopsies demonstrate focal disruption in the myoepithelial cell layer and loss of oestrogen-receptor expression in the micro-invasive tumour cells.** A human breast biopsy paraffin section was double-immunoassayed for the myoepithelial cell marker smooth-muscle actin (red), and oestrogen receptor (dark brown). Arrows identify a focal disruption in the myoepithelial cell layer and overlying oestrogen-receptor-negative cell clusters. Photomicrographs were obtained at 100X magnification (**a**) and 400X magnification (**b**). Photomicrograph **c** (100X magnification) shows evidence for the loss of basement-membrane barrier function at sites of local micro-invasion. A human breast biopsy paraffin section was immunoassayed for both smooth-muscle actin and the basement-membrane marker collagen IV using the same chromophore. Disruption in both the basement membrane and myoepithelial cell layer is evident at the site of micro-invasion, and the epithelial cells are in direct contact with the stroma (indicated by the arrow). Photomicrographs are adapted with permission from REF. 76 © (2004) Elsevier Science.

a positive-feedback loop between matrix proteolysis and MMP production was first described in a rabbit synovial fibroblast model for wound healing. In this study, fibronectin fragments, but not intact fibronectin, induced MMP activity[74]. These investigators suggested that fibronectin fragments are natural inducers of MMP activity and subsequent tissue remodelling during wound healing, and data from the involution model indicates that fibronectin fragments have this role during mammary gland involution as well[52,56]. In addition, the basement-membrane protein laminin 5 is targeted for proteolysis. This liberates an epidermal growth factor (EGF)-like fragment that stimulates downstream mitogen-activated protein kinase (MAPK) signalling, MMP2 expression and breast tumour cell migration *in vitro*[75]. Cumulatively, these studies indicate

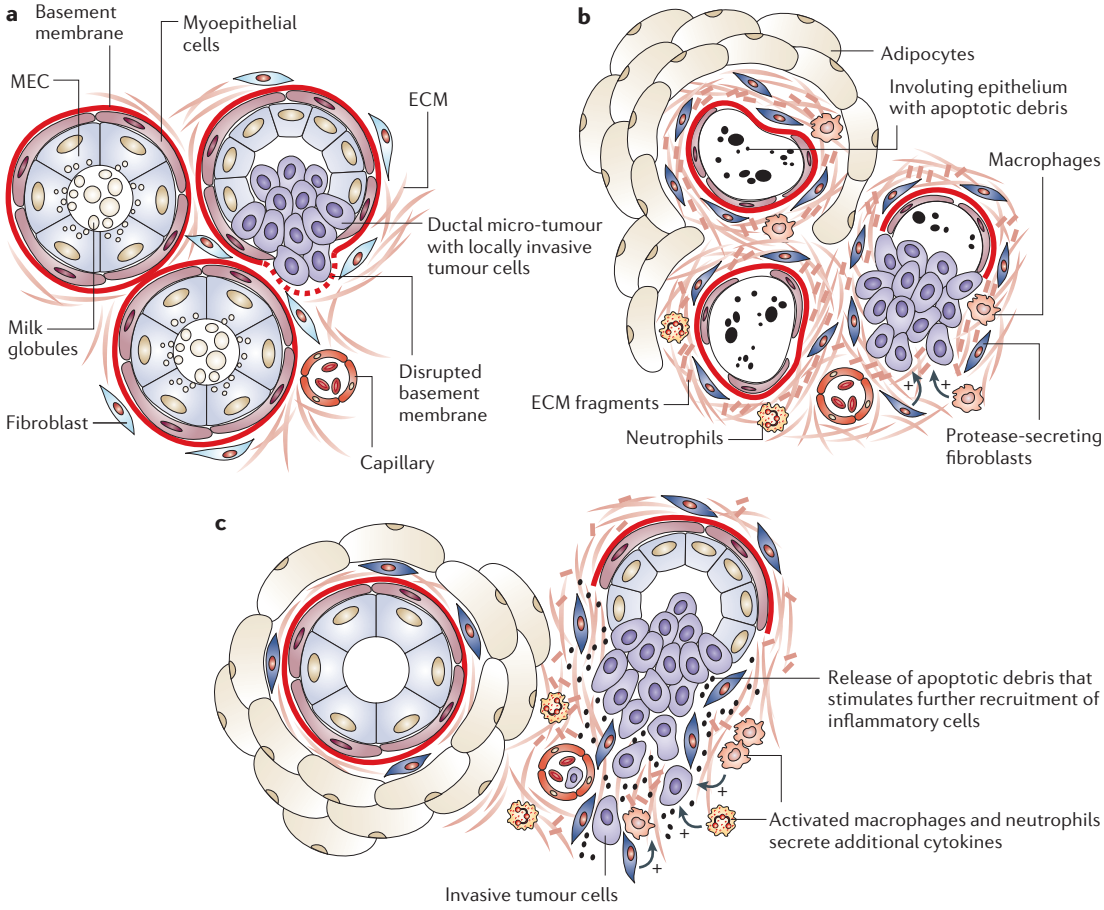

Figure 5 | **A model depicting tumour cell promotion during mammary involution. a** | Ascini from a lactating gland with an invasive micro-lesion. During lactation, secretory mammary epithelial cells (MEC) are surrounded by myoepithelial cells and an intact basement membrane. Individual ascini are embedded within a sparse intra- and inter-lobular stroma containing fibroblasts, vasculature and extracellular matrix (ECM). A small ductal tumour with locally invasive tumour cells is shown. Local disruption in the basement membrane at the site of the invading tumour cells is depicted as red dashes. **b** | The matrix-proteinase-dependent phase of involution has attributes of a wound-healing environment. With involution there is massive cell death of secretory epithelium, and the ascinar lumen fill with apoptotic debris. As the epithelial cells are lost, the gland is repopulated with adipocytes. The intra- and inter-lobular stroma is increased, in part due to increased deposition of fibrillar collagen. Fibroblasts secrete proteases (indicated by the arrow) that degrade ECM proteins, including fibronectin and laminin. This results in the release of bioactive matrix fragments that have tumour growth-, motility- and invasion-promoting activities. Immune cells, such as macrophages and neutrophils, are more abundant, probably owing to the chemoattractant properties of proteolysed matrix. **c** | Involution-associated changes in the microenvironment are amplified by the presence of the tumour cells, switching the finely-tuned inflammatory balance towards overt inflammation. Fibronectin and laminin 5 fragments induce tumour cells to activate matrix metalloproteinase 9 (MMP9) and MMP2, resulting in further matrix degradation. Furthermore, owing to the compromised basement membrane at the site of the tumour, there is leakage of highly immunogenic apoptotic debris from the ductal lumen into the interstitial space, resulting in a sustained influx of inflammatory cells. The recruited macrophages and neutrophils release additional cytokines such as colony-stimulating factor 1 (CSF1), tumour-necrosis factor-α (TNFα), interleukin-1 (IL-1), IL-6, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatic growth factor (HGF) and proteases (indicated by the arrows). Previously quiescent tumour cells are activated by the cytokine-rich microenvironment and become motile and invasive. The activated tumour cells traverse along the fibrillar collagen-rich interstitial matrix of the involuting gland to gain access to local vasculature and lymphatics.

that one mechanism by which mammary gland involution promotes tumour cell metastasis is through the production of bioactive proteolytic ECM fragments that activate tumour cell motility and invasion. So, candidate mediators of tumour cell progression following pregnancy include an influx of cytokine-secreting immune cells, increased concentrations of matrix metalloproteinases, remodelling of mammary ECM with subsequent release of cryptic tumour-promoting peptides such as fibronectin and laminin fragments, and loss of basement-membrane barrier function. It is important to emphasize that these provocative results, which implicate involution-associated stromal changes in mediating tumour-cell dissemination, have been obtained in preclinical models for human breast cancer. Validation with human breast tissue is required. Furthermore, such local changes are not being proposed as an all-encompassing explanation for an increase in the number of women diagnosed with metastatic disease, but rather involution is being highlighted as a potential therapeutic window for metastasis suppression.

Women at high risk for disease progression

Recently, it has been demonstrated that in patients with breast cancer, a subset of occult breast cancer lesions contain focal disruptions in the myoepithelial cell layer, resulting in direct contact between early-stage tumour cells and the mammary stromal microenvironment[76,77] (FIG. 4). Tumour cells that are in direct contact with the stroma are anticipated to be exceptionally vulnerable to the tumour-promoting environment of the involuting mammary gland. In addition, when the myoepithelial cell layer is compromised, the luminal contents of the involuting gland might be able to flow backwards into the interstitial tissue. This luminal material, which is rich in apoptotic alveolar cells and milk, is anticipated to be highly immunogenic. Therefore, the presence of such micro-invasive lesions at the time of involution is likely to tip the finely-tuned balance of anti-inflammatory to pro-inflammatory signals towards overt inflammation. Crosstalk between the tumour cells and this localized inflammatory environment would further activate the tumour cells. So, it is predicted that a subset of women — those with micro-invasive disease at time of pregnancy — would be at an exceptionally high risk of tumour cell metastasis following pregnancy. A model depicting this scenario is shown in FIG. 5.

Future directions

Recent studies, including those described in this Review, indicate that the composition of the mammary gland stroma is coordinated with and facilitates hormonally driven changes in mammary epithelial cell proliferation, differentiation and death[47,52,56,72,73,75,78,79]. The fact that the microenvironment of the normal mammary gland is highly dynamic indicates that it might be possible to target the mammary mesenchyme with preventive or therapeutic interventions. Indeed, the chemopreventive activity of difluoromethylornithine and retinoids correlates with mammary gland ECM remodelling, fibronectin proteolysis and epithelial cell loss[40,80]. These and other studies provide proof-of-principle that the mammary microenvironment can be targeted to inhibit breast tumour progression. Investigating whether anti-inflammatory agents, taken at the time of gland involution, suppress the tumour-promoting nature of the involuting mammary stroma is a logical next question to address. However, effective breast cancer screening in recently pregnant women is warranted immediately.

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Competing interests statement
The author declares no competing financial interests.

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