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The Susan G. Komen for the Cure(r) Tissue Bank at the IU Simon Cancer Center: A Unique Resource for Defining the "Molecular Histology"of the Breast

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Abstract

"Molecular histology" of the breast may be conceptualized as encompassing the normative ranges of histological structure and marker expression in normal breast tissues in relation to a woman's age and life experiences. Studies of molecular histology can aid our understanding of early events in breast carcinogenesis and provide data for comparison with diseased breast tissues. Until recently, lack of epidemiologically annotated, optimally prepared normal breast tissues obtained from healthy women presented a barrier to breast cancer research. The Komen Tissue Bank at Indiana University is a unique biorepository that was developed to overcome this limitation. The Bank enrolls healthy donors who provide questionnaire data, blood, and up to four breast biopsies, which are prepared as both formalin fixed paraffin embedded and frozen tissues. The resource is accessible to researchers worldwide through a proposal submission, review, and approval process. As of November 2010, the Bank had collected specimens and information from 1,174 donors. In this review, we discuss the importance of studying normal breast tissues, assess the strengths and limitations of studying normal tissues obtained from different sources, and summarize the features of the Komen Tissue Bank. As research projects are completed, results will be posted on the Bank's website.

Overview

Breast cancer accounted for approximately 200,000 incident cases and 40,000 deaths in the United States in 2009 (1). Mammographic screening and improved treatments have contributed to reductions in breast cancer mortality (2, 3), but progress towards improving outcomes for biologically aggressive cancers remains limited. Many lethal cancers occur prior to initiation of screening, elude mammographic detection or fail to completely respond to available therapies (4-6). Accordingly, developing better means of risk assessment, detection, and prevention of aggressive breast cancers is an important goal.

Most mechanistic and biomarker research in humans has taken a “backward looking” approach; markers and mechanisms are identified in cancer, and secondarily tested at earlier stages of carcinogenesis. Though productive, this approach is limited by effects of the carcinogenic process itself, which results in many changes (e.g. passenger mutations, genetic instability) that are neither causal nor early. These complexities might be avoided by taking a “forward looking” strategy in which the effects of risk factors on morphology and molecular markers in normal breast are assessed.

Investigations in animal models have revealed substantial insights into the transition between physiology and early carcinogenesis, but vast inter-species differences in biology have made translation of these findings to women challenging. Until recently, the lack of optimally prepared normal human breast tissues annotated with risk factor data has precluded observational studies of these changes in women. Thus, the “molecular histology” of the normal human breast, conceptualized as

encompassing the range of morphologic and molecular characteristics throughout the lifespan, is largely undefined, and therefore limits our ability to discern and characterize the earliest stages of carcinogenesis. Given that many such changes are probably reversible, identifying these processes may offer opportunities for successful prevention. To enable studies of breast molecular histology in humans, Indiana University in collaboration with Susan G. Komen for the Cure® has developed a novel specimen bank of annotated normal breast tissues (komentissuebank.iu.edu). In this commentary, we present the rationale for developing the Susan G. Komen for the Cure® Tissue Bank at the IU Simon Cancer Center ("Komen Tissue Bank"), highlight its potential value in breast research, and describe its characteristics.

What is "normal breast"?

"Normal breast," as defined by physical examination, radiological methods, histological study, or molecular analysis, varies widely in its implications. A normal physical examination roughly corresponds to the absence of a mass, a normal appearing nipple areola-complex and unremarkable skin, but offers little information about cellular content, apart from relative adiposity. Radiological methods are useful for identifying masses, asymmetries, and calcifications, and for estimating the percentage of fibroglandular tissue (mammographic density), but neither distinction of epithelium from stroma nor characterization of benign epithelium is easily achievable routinely. Finally, microscopic identification of unremarkable terminal duct lobular units (TDLUs), the source of nearly all breast cancer, may only partially satisfy criteria for normal. Studies have shown that TDLUs near breast cancers may share some molecular alterations with their associated cancers such as loss of heterozygosity and altered

expression of *p53* or *HER2 (7)*, DNA methylation of CpG islands (8) and altered mRNA expression profiles (9) that resemble cancer. Similarly, "normal" tissues associated with breast cancer may demonstrate expression profiles consistent with wound responses, which are specific for molecular subtypes and augur a poor prognosis (10).

Beyond the recognition that definitions of normal breast differ by technique of assessment, it is also clear that singular definitions of normal lack broad biological relevance, given the remarkable changes that occur over a woman's lifespan or even a single menstrual cycle. Normative ranges of histology and marker expression ("molecular histology"), rather than rigid universal definitions, are needed to capture the inter-individual variation that results from the interplay of aging, genetics, and environmental and lifestyle factors (Figure 1). In fact, such characteristics may be population specific, reflecting the differences in the frequency of breast cancer risk factors and cancer incidence rates. While data suggest that age and other breast cancer risk factors influence the molecular histology of the breast (11-13), we also speculate that molecular histology modifies the effect of risk factors.

Observational data suggests that the state of the breast at the time of an exposure influences the net effect of that exposure with regard to carcinogenesis. This may partly account for why many breast cancer risk factors demonstrate "qualitative age interactions", reducing cancer risk at some ages while increasing risk at others (14). Similarly, "quantitative age interactions", reflecting a difference in magnitude of an effect (e.g. "window of vulnerability"), may also result from the action of identical risk factors on different normal histological substrates. This view is supported by animal studies showing that prior pregnancy or treatment with exogenous agents that mimic pregnancy

confers resistance to carcinogenic challenges and the observation that radiation exposure prior to a first pregnancy increases risk among women more than later exposure (reviewed in (15)). These observations are also consistent with the view espoused by Boyd et al that high mammographic density at early ages is a key determinant of breast cancer risk (16). Thus, the effect of carcinogenic exposures may vary by the amount and state of the tissue substrate upon which they act.

Terminal duct lobular unit (TDLU): The functional unit of normal breast

The terminal duct lobular unit (TDLU) is the basic milk producing structure within the breast and represents the anatomic source of nearly all breast cancer precursors and cancers (15). TDLUs are not present at birth; the prepubertal breasts of both girls and boys consist mainly of small ducts embedded in fibrous tissue. At puberty, ducts lengthen and branch, and then develop a cap of small acinar units that is enveloped by specialized stroma, constituting the terminal duct lobular unit (TDLU) (17). Data from a large Danish cohort found that high birth weight, greater height or lower body mass index at age 14 years, and peak growth at an early age increase risk for adult breast cancer, suggesting that the pace or degree of breast development is related to breast cancer risk (18). Furthermore, it is notable that breast development begins a year earlier among African American compared to Caucasian girls (19), and that rates of early onset breast cancer are higher among African American women (20).

Ducts and acini are lined by an inner layer of luminal cells surrounded by an outer layer of myoepithelial cells that are bound by a basement membrane. The TDLU is a highly dynamic structure, which undergoes cyclic changes during the menstrual

cycle (21) and progressive changes with aging (22). During pregnancy, TDLUs first expand and later differentiate into milk secreting structures, which persist until weaning and postpartum involution.

Pregnancy and lactation permanently alter the molecular histology of the breast. In rodent models, parity alters the molecular histology of the breast, rendering it relatively resistant to carcinogenic challenges. Limited data also suggests that parity and other factors related to breast cancer risk permanently alter the molecular histology of the normal breast among women and reduce susceptibility to malignant transformation (reviewed in (15) (17)). Specifically, the transcriptome of normal breast of nulliparous and parous women differs for genes involved in a diverse range of processes (15). Although pregnancy-related changes and postpartum involution seem to permanently alter breast biology and confer resistance to cancer, the breast maintains sufficient plasticity to support the development of milk production at subsequent pregnancies.

Postpartum involution and remodeling is an active process that may exert a promotional effect on breast cancer development (23). Data suggests that breast cancers that are detected in mothers after birth are particularly aggressive and may include etiologically and biologically distinctive tumors such as “basal cancer”. In the years immediately following birth, breast cancer risk is increased. The long-term effects of parity vary by age at first live birth (17). A first live birth at younger ages is associated with long-term reduced breast cancer risk, whereas at older ages a first birth is related to increased risk. In addition, new data suggests that parity may not reduce risk for basal breast cancers (24).

In contrast to postpartum involution, which is comparatively rapid and at least partly reversible, age related involution seems to evolve more slowly and contributes to the variable appearance and functional state of TDLUs among older women. However, as originally conceptualized by Pike et al (25), differences in risk factor exposures may slow or hasten "breast tissue aging" as compared with a woman's chronological age, and thereby influence cancer risk through a number of incompletely understood mechanisms (26). In the Mayo benign breast disease cohort, the identification of non-atrophic TDLUs was a marker of increased breast cancer risk (27). Allred et al (28) have suggested that hyperplastic TDLUs represent an early change in the development of cancer. Additional data from the Mayo study has shown that involution of TDLUs, a microscopic feature, is associated with lower mammographic density, a radiological characterization (29). Thus, high mammographic density, a strong breast cancer risk factor, may be associated with molecular histology, suggesting that macroscopic radiological features reflect microscopic and sub-microscopic changes in tissues.

Challenges to understanding the molecular histology of the breast

Approaches for studying breast tissues of young women are limited because neither imaging nor biopsy is commonly performed until screening is initiated, typically at age 40 years in the United States or 50 years elsewhere. Most strategies for studying "molecular histology" are suboptimal because well-preserved annotated tissues obtained from normal women have been lacking. Accordingly, various alternative sources of "low-risk" breast tissue have been studied, each of which has strengths and limitations (Table 1).

Prospective collection of fresh normal tissue, especially terminal duct lobular units, is difficult because normal epithelium, normal stroma and most examples of benign breast disease are indistinguishable grossly. Although fresh tissue has advantages for molecular analysis, researchers often use formalin fixed tissues, which presents limitations. Notably, analysis of normal appearing structures in formalin-fixed, paraffin-embedded tissue blocks identified by reviewing hematoxylin and eosin stained sections cut from such blocks may be flawed because such structures are part of specimens that were removed for a clinical indication (e.g. a radiographic finding or palpated abnormality), and thus may reflect molecular changes characteristic of the associated disease state. This concern also applies to mammoplasty tissues. Pathologic review of reduction mammoplasty specimens has demonstrated that 88% contain benign breast disease, including 17% with proliferative changes, whereas in the Komen Tissue Bank, 35% of tissues show benign changes and only 3.3% demonstrate proliferative changes, suggesting possible differences between these groups of women (30).

Currently, most suspicious lesions are assessed using radiologically-guided biopsies, which are typically small, available only as fixed tissue, and largely consumed in preparing diagnostic pathology sections. Limited data suggests that histologically normal tissue identified in surgical excisions already demonstrates molecular alterations, either reflecting early carcinogenic alterations surrounding the tumor (“field effect”) or modification secondary to the effects of the nearby cancer (“reverse causality”). These tissues may have value for comparison of cases, but are less useful for defining baseline normal parameters. Reduction mammoplasty specimens are

commonly studied as a source of non-diseased tissue, but this approach belies the reality that these women often have extremely large fatty breasts, suggesting that neither the patients nor their organs are normal. A recent review of 516 mammoplasty specimens found that 18% of women had proliferative lesions, 5% had DCIS and 3% had lobular neoplasia, further calling into question the normalcy of these samples (31). Finally, even biopsies that do not demonstrate clinically significant pathology were prompted by clinical findings, and may be at least subtly abnormal (e.g. radiologic calcification or asymmetry).

Analysis of postmortem breast tissues has been explored, including a novel historical effort in which consecutive forensic procedures were subjected to detailed sampling (32). This work yielded seminal information about the morphology of the breast, including appearances characteristic of different phases of the menstrual cycle (21) and aging, the contrasting prevalence of benign breast lesions among women of different ethnic backgrounds, and the composition of tissues associated with radiological density. However, access to postmortem tissues is often limited or delayed by the need to locate and consent next of kin; as a result, tissue degradation limits the value of such specimens, especially for molecular studies. Medical postmortem studies, even using rapid autopsy techniques, are generally restricted to hospitalized women with chronic illnesses. Nonetheless, the ability to perform extensive sampling of the entire body may allow new insights about breast biology and pathophysiology from a systemic perspective.

Ductal lavage and nipple aspiration represent potential means of obtaining normal epithelial cells and fluid; however, yields are often low, stroma is not represented

and architectural context is lost. Only structures that communicate with the duct system can be sampled and the inevitable occurrence of fibrocystic changes with aging therefore often renders aspects of the breast inaccessible. Random fine needle aspiration represents another cytologic sampling technique, which may suffer from limitations similar to lavage, especially in non-expert hands. Finally, analysis of cells and fluid comprising breast milk represents a promising approach for understanding postpartum breast biology through analysis of fresh cells and fluid. Using immunomagnetic methods to isolate breast epithelial cells, Wong et al have demonstrated DNA methylation of tumor suppressor genes in breast epithelium of healthy young women (33). However, analysis of milk is limited to studying the postpartum breast, and methods for collection and fractionation of milk have not been completely developed and tested.

Amassing experience demonstrates that multiple factors such as medications, hypoxia, and devascularization occurring both intra- and postoperatively may affect molecular analyses (34). Most surgeries are preceded by biopsies that induce wounds and fat necrosis, which may alter not only the sampled tissues but also the systemic milieu. Ideally, normal tissues should be collected through non-operative procedures and frozen immediately.

What can be learned from studying normal breast?

The full value of research on normal breast tissue remains incompletely defined. A few applications are noted below.

- Determining the molecular histological footprint of non-genetic and genetic risk factors as a means of assessing the impact of these exposures;
- Identifying mechanisms that mediate the effects of risk factors and protective factors, with the goal of developing prevention strategies that inhibit the former and mimic the latter;
- Assessing levels of candidate biomarkers for risk assessment or early detection in normal tissue to assess their specificity for cancer and its precursors and determine optimal cut points for sensitivity and specificity;
- Providing evidence for causal effects of circulating biomarkers by demonstrating changes in molecular histology that would suggest increased risk (e.g. increased proliferation, decreased apoptosis);
- Assessing the expression of molecular targets that represent candidates for therapeutic or preventive interventions;
- Understanding the breast stroma and its interaction with TDLUs;
- Furthering the understanding of "stem cells" including number, distribution, and genomic integrity.

The Susan G. Komen for the Cure® Tissue Bank at the IU Simon Cancer Center ("Komen Tissue Bank")

In 1997, the National Cancer Institute convened a meeting of basic and clinical researchers from academia, industry and government, and representatives of the

patient advocate community for the purpose of identifying barriers to progress in the treatment and prevention of breast cancer. Thirteen deficiencies were identified, the first of which was as follows:

"*Our limited understanding of the biology and developmental genetics of the normal mammary gland is a barrier to progress. ...it is now clear that a more complete understanding of the normal mammary gland at each stage of development—from infancy through adulthood—will be a critical underpinning of continued advances in detecting, preventing, and treating breast cancer.*"

The Komen Tissue Bank is a unique biorepository established expressly to acquire healthy breast tissue from volunteer donors. Approximately five tissue collection events are held each year, most of them at the IU Simon Cancer Center in Indianapolis, IN. Tissue samples are obtained from approximately 100 women at each event. Donors have been recruited primarily by word of mouth and newsletters, as well as by occasional coverage in news media. Donors interested in future donation events can contact Dr. Anna Maria Storniolo at the Tissue Bank.

The collections are performed under the approval of the Indiana University Institutional Review Board, even when they occur at other locations. The donors provide a broad consent, allowing the tissue to be used for unspecified future breast cancer research. Virtually all donors have consented to future contact as necessary. Abiding by HIPAA mandate that the donors' personal identifying information not be revealed, the aforementioned contact will be done by tissue bank personnel.

The tissue and blood samples, as well as the clinical annotation, are available to researchers world-wide, via a proposal application process which is described in detail

on the Komen Tissue Bank website. Proposals are reviewed by an independent panel that assesses the quality of the science, and also ensures non-duplication of effort and appropriate utilization of such a limited resource.

As of November 2010, the Bank has acquired breast tissue from 1174 unique donors. Self-described African-Americans comprise 5.2% of donors. The Bank's specimens are annotated with details of the donors' reproductive, medical and family histories, and current medication usage. These tissue specimens are a significantly limited resource. To mitigate this limitation, the KTB has produced 33 epithelial and 36 stromal cell lines from the tissue specimens; 8 of the epithelial and 10 of the stromal lines were derived from the breast tissue of African-American women. The cell lines have been characterized using karyotyping, interphase FISH mapping, immunohistochemistry and flow cytometry. A subset of the cell lines have been assayed for single nucleotide polymorphisms (SNPs), DNA copy number variation (i.e., greater than or less than diploid), and gene expression.

Volunteer tissue donors are asked to complete a questionnaire detailing demographics and most established breast cancer risk factors. Following a blood draw, donors undergo a tissue acquisition procedure in which up to four cores of breast tissue are obtained. Cores of tissue of the upper outer quadrant of either breast are acquired using 10-gauge needles and immediately processed as snap frozen tissues or as formalin-fixed paraffin-embedded tissues. The standardization of the procedures ensures rapid, consistent and optimal handling of the tissues.

Though the Komen Tissue Bank provides clear advantages over some of the other sources of "normal" breast tissue, it too has some shortcomings. The ideal normal breast tissue bank would include thoroughly annotated specimens obtained from a representative population-based sample of women who have provided fresh breast tissue sufficient to fully characterize their breasts bilaterally. Further, repeated sampling and extended follow-up of a large cohort of such women could provide a comprehensive picture of breast carcinogenesis from initiation to progression. However, developing such a resource is challenging. The Komen Tissue Bank represents a step forward by obtaining multiple breast core biopsies from women who provide a risk factor questionnaire and blood. However, core biopsies are also limited in terms of their size and representativeness of the breast. Some samples consist entirely of fat, and therefore, are not useful for evaluating epithelium or non-fatty stroma. Finally, biopsies performed at one time point do not reveal the evolution of dynamic processes (e.g. proliferation, involution, neoplasia, etc.) and without follow-up one cannot guarantee that a subject does not have prevalent occult cancer or a precancerous lesion.

Selected characteristics of the first 521 enrolled volunteers without a personal history of cancer are shown in Table 2. Subjects are predominantly White non-Hispanic women who are somewhat better educated than the U.S. population overall. The women range in age from 18 to 83 years and the majority are premenopausal. Most women are parous, including about 50% of the premenopausal subjects. About 25% of participants report having a first-degree relative with breast or ovarian cancer. The mean body mass index of subjects is 28 Kg/m^2 indicating a large proportion of women

are overweight or obese, similar to the general U.S. population. About 16% of the women have had a history of prior breast biopsies.

The resource has been established to provide broad access to the research community, predicated on approval based upon scientific merit, feasibility and external funding as required. Upon completion, results of analyses will be made available on the Komen Tissue Bank's virtual tissue bank website.

Some of the initial projects will relate patient characteristics, non-genetic breast cancer risk factors, and relationships of susceptibility loci to characterization of the morphology of terminal duct lobular units, cellular composition, assessment of markers of proliferation, apoptosis and hormone receptors, and mRNA profiling.

Conclusion

The Komen Tissue Bank has been established to address the research need for "normal" breast tissue specimens. These specimens, paired with detailed clinical annotation, will serve to characterize the spectrum of normal. The molecular histology of the breast - from early adulthood to menopause and across different races and ethnicities - can now be defined. This knowledge will allow further investigations into the molecular changes leading to carcinogenesis. The Komen Tissue Bank represents a critical tool in the ongoing efforts to find new strategies for the treatment and prevention of breast cancer.

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Figure Legends

1. Three images of terminal duct lobular units (TDLUs); progression from A-C demonstrate a reduction in number of acini with replacement by fibrous stroma, consistent with a process of involution.

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Table 1: Comparison of different specimens for studying normal breast

Specimen Type	Strengths	Limitations
Surgical resection for cancer, cancer precursors	Tissue is abundant and available	1) "Normal" appearing tissues may demonstrate field effects or changes secondary to nearby cancer
		2) Prior biopsy and anesthesia may alter molecular histology
Reduction mammoplasty	Tissue is abundant and available	1) Biased sample (e.g. young age, obesity)
		2) Non-standardized acquisition and processing
Postmortem examination	1) Available	1) Chronic disease and medications may affect tissue
	2) Can relate breast tissue to other tissue samples and clinical history	2) Autolysis limiting for molecular studies
Ductal lavage, nipple aspirate, random fine needle aspirate	Fresh samples	1) Modest cellularity with limited stroma and tissue architecture
		2) Special expertise required
		3) Typically, high risk women
Milk	Available fresh	1) Only postpartum women
		2) Lacks architectural context
		3) Optimization of collection and fractionation ongoing
The Komen Tissue Bank	1) Fresh samples	1) Samples represent "one moment in time"
	2) Medical / demographic annotation	2) Limited amount of tissue
	3) Derived cell lines	3) Variable sample quality
	4) Matched with blood and blood components	

Table 2: Frequencies and distributions for select characteristics and known breast cancer risk factors in the first 521 participants of the Komen Tissue Bank

	All women		**Menopausal Status[a]**				
	(n=521)		Pre-menopausal (n=351)		Post-menopausal (n=159)		
Age, mean (SD)	39.9 (15.1)		32.5 (10.2)		56.2 (11.0)		
Age, min-max	18.4 - 83.1		18.4 - 55.8		27.4 - 83.1		
	n	%	n	%	n	%	*P*-value[*]
Education level							<0.0001
High school/GED/or less	155	29.75	118	33.62	34	21.38	
Vocational/Tech school or associates degree	74	14.20	36	10.26	36	22.64	
College degree	172	33.01	129	36.75	39	24.53	
Graduate/Professional Degree	113	21.69	65	18.52	46	28.93	
Race							0.16
White	446	85.60	301	85.75	136	85.53	
Black	30	5.76	15	4.27	14	8.81	
Asian	3	0.58	2	0.57	1	0.63	
American Indian/Alaskan Native	5	0.96	4	1.14	1	0.63	
Other/missing	37	7.10	29	8.26	7	4.40	
Ethnicity							0.03
Hispanic	56	10.75	45	12.82	10	6.29	
Non-Hispanic	462	88.68	306	87.18	147	92.45	
Age at menarche							0.47
≤11	103	19.77	69	19.66	32	20.13	
12	152	29.17	105	29.91	42	26.42	
13	157	30.13	110	31.34	44	27.67	
≥ 14	107	20.54	67	19.09	39	24.53	
Body mass index, kg/m^2							<0.0001
<25	212	40.69	165	47.01	42	26.42	
25-29	142	27.26	94	26.78	45	28.30	
≥30	159	30.52	88	25.07	68	42.77	

No. of full-term births							<0.0001
nulliparous	247	47.41	215	61.25	27	16.98	
1	60	11.52	34	9.69	25	15.72	
2	118	22.65	58	16.52	57	35.85	
≥ 3	91	17.47	42	11.97	47	29.56	
Age at first full-term birth among parous women (n=269)							0.01
< 20	40	14.87	25	18.66	13	10.08	
20-24	71	26.39	29	21.64	41	31.78	
25-29	83	30.86	35	26.12	47	36.43	
≥ 30	70	26.02	43	32.09	25	19.38	
Breastfeeding among parous women (n=269)							0.22
Never	66	24.54	27	20.15	38	29.46	
< 24 mo.	157	58.36	83	61.94	71	55.04	
≥ 24 mo.	46	17.10	24	17.91	20	15.50	
Oral contraceptive use							
Non-current user			229	65.24			
Current user			122	34.76			
Hormone replacement therapy use							
Never					74	46.54	
Current/recent use					21	13.21	
Past use/Former					64	40.25	
Family history of breast or ovarian cancer in first degree relatives							0.01
No	388	74.47	272	77.49	106	66.67	
Yes	133	25.53	79	22.51	53	33.33	
Ever had a breast biopsy							<0.0001
Never	430	82.53	313	89.17	106	66.67	
Ever							
Yes, 1	63	12.09	27	7.69	35	22.01	
Yes, 2+	20	3.84	5	1.42	15	9.43	

Ever had a screening mammogram							<0.0001
No	229	43.95	216	61.54	10	6.29	
Yes	290	55.66	133*	37.89	149	93.71	
Smoking							<0.0001
Never	357	68.52	263	74.93	90	56.60	
Former	116	22.26	60	17.09	53	33.33	
Current	39	7.49	22	6.27	14	8.81	

[a] Women were considered pre-menopausal if they reported having had a period within 12 months from the questionnaire date or reported taking oral contraceptives. Women were considered post-menopausal if they reported having their last period more then 12 months from the questionnaire date, reported having a hysterectomy, or reported both ovaries removed.

* χ^2 test to determine if risk factor distributions are significantly different between pre-menopausal and post-menopausal women

** Of these 91 women (69%) are age 40 and older and 13 (10%) have a history of breast cancer in a first degree relative and under age 40.

NOTE: Differences between cell counts in table and total number are due to missing questionnaire data.

Figure 1a

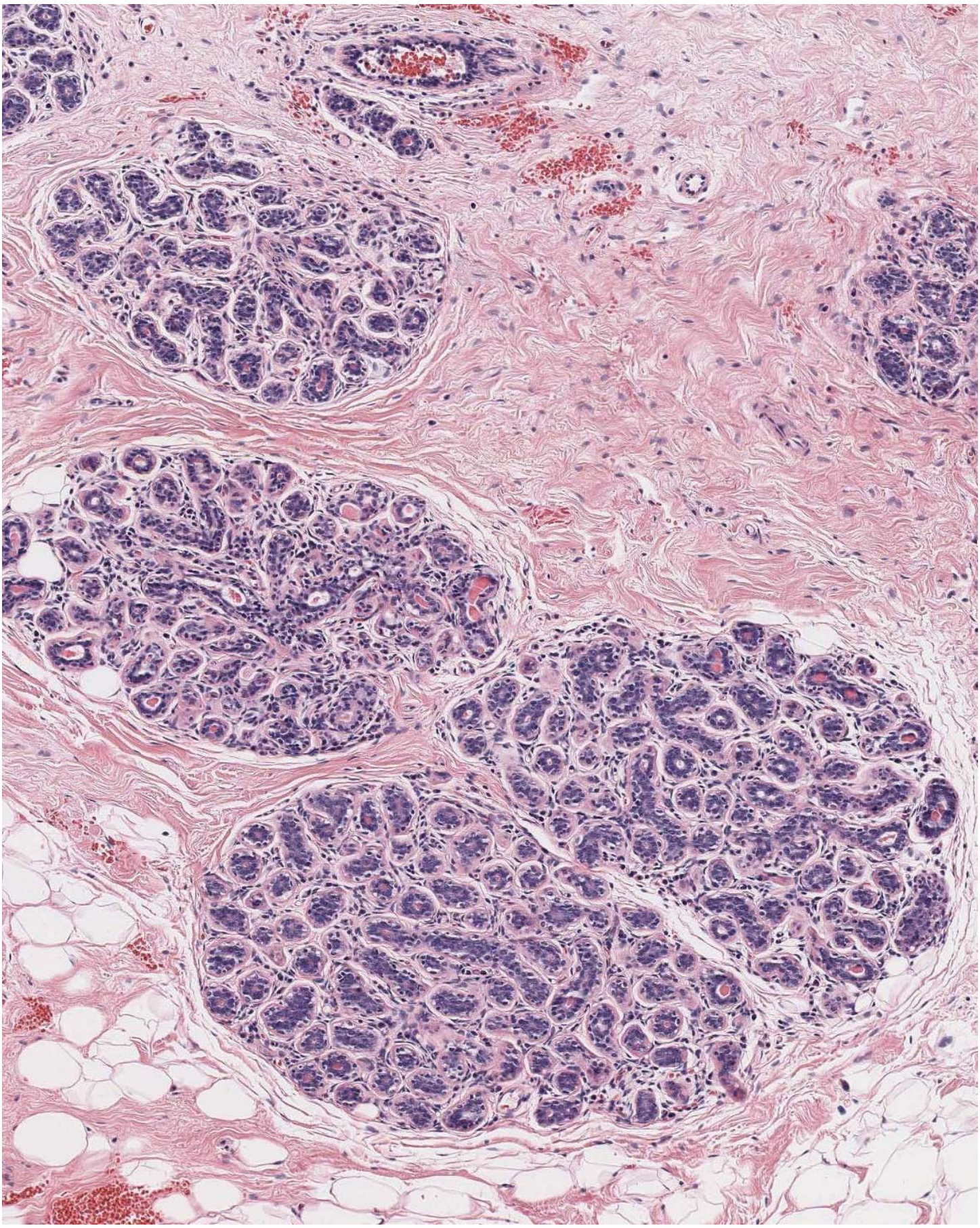

Figure 1b

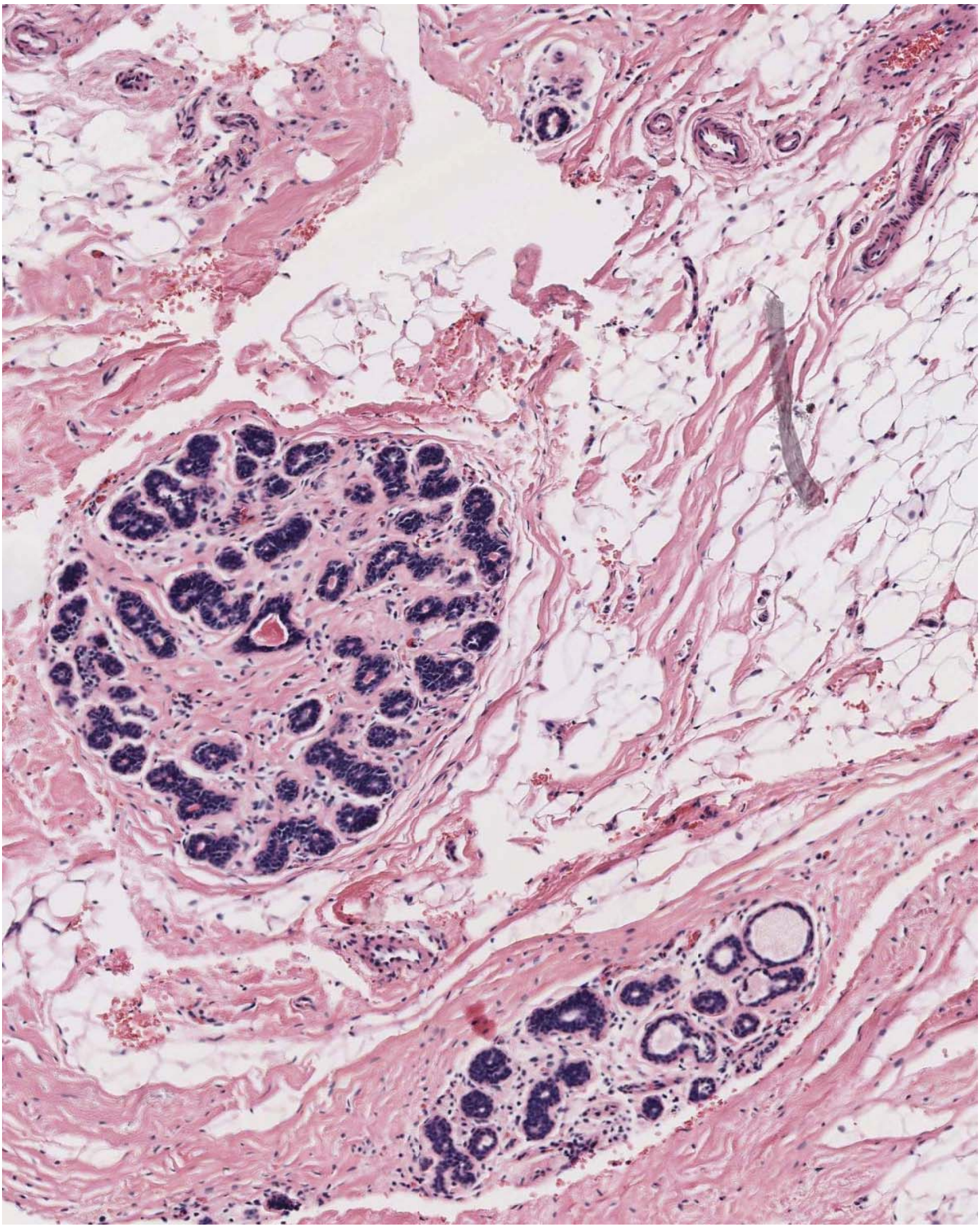

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Figure 1c