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TRANSFORMING GROWTH FACTOR-β IN BREAST MILK FROM WOMEN WITH BREAST BIOPSIES

by

Sidra Mahmood

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This paper was prepared

under the direction of

Professor Jeffrey Knight

and Professor Kathleen Arcaro

for eight credits.

To:

My parents who have endless trust and faith in me and everything that I do.

No words are enough to express my gratitude to you two except, “I love you.”

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ABSTRACT

About 1 in 8 (12%) American women will develop invasive breast cancer during their lifetime, and around 40,000 of them are estimated to die from it in 2012 alone (American Cancer Society, 2012). Diagnosis of breast cancer in young women is already difficult (Mintzer et al., 2002) and is further complicated when a woman gets pregnant or is breastfeeding because her breasts get denser, making clinical examination and mammography difficult to interpret (Schedin, 2006). As opposed to painful and invasive methods of tissue acquisition such as biopsies, human milk can be used to measure certain biomarkers in the breast, such as methylation, as previously demonstrated by the Arcaro lab (Browne et al., 2011). Transforming growth factor-β (TGF-β) is a naturally-secreted protein in breast milk (Saito et al., 1993). It is a multifunctional cytokine that is believed to play a role in cancer progression (Akhurst and Derynck, 2001), especially in pregnancy-associated breast cancer (Flanders and Wakefield, 2009).

In our observational study, we investigated the expression levels of TGF-β2, one of the three isomers of TGF-β, in breast milk from both breasts of 182 women who were scheduled to receive a biopsy each. They either had breast biopsies prior to or during their pregnancies, or after childbirth. The aim of our project was to examine the relationship between trends in TGF-β expression across different variables such as biopsied versus non-biopsied breast, biopsy category, the mother's age at first birth and her age at the time of milk donation, parity (number of live births), baby's age at the time of milk donation etc. Our results show that TGF-β2 levels vary greatly among our sample population, and none of the variables we examined (except being Caucasian) were significant upon regression. A larger sample size is needed to make further conclusions and perhaps other isoforms of TGF-β need to be analyzed to determine the role of TGF-β in this population.

INTRODUCTION

Breast cancer

Overview

Breast cancer is a malignant tumor that is initiated in the breast. A malignant tumor is made up of cancer cells that can grow into (invade) peripheral tissues or spread (metastasize) to distant parts of the body. Breast cancer is the most common cancer that affects women. About 1 in 8 (12%) American women will develop invasive breast cancer during their lifetime (American Cancer Society, 2012), whereas the incidence for men developing breast cancer is less than 1% (Ravandi-Kashani and Hayes, 1998). According to the estimates of American Cancer Society, about 226,870 new cases of invasive breast cancer will be diagnosed in American women in 2012 and about 39,510 women will die from it in 2012. Currently breast cancer is the second leading cause of cancer death in women after lung cancer. However, death rates have been steadily declining since 1990 due to earlier detection through screening and increased awareness, and improved treatment. On this day, there are more than 2.6 million breast cancer survivors in the United States (women who are still being treated and those who have completed treatment) (American Cancer Society, 2012).

Anatomically, the female breast is made up of primarily fatty tissue and contains 15-20 lobes, each made up of groups of lobules, the milk producing glands in the breast (Figure 1). Each lobule consists of grape-like structures of acini (also called alveoli) that are hollow sacks that produce and hold milk. The lobules are arranged around the ducts (tubes) that transport milk from the breast to the nipple during lactation (Slowic, 2010).

Hence, breast cancer can be categorized into two main types (A.D.A.M., 2011a):

1. *Ductal carcinoma* begins in the ducts;
2. *Lobular carcinoma* begins in lobules of the breast.

Out of all the invasive breast cancer cases, 50-75% of women develop ductal carcinoma whereas 10-15% of them develop lobular carcinoma (Susan G. Komen for the Cure, 2012). In rare cases, breast cancer can also start in other cell types (American Cancer Society, 2012).

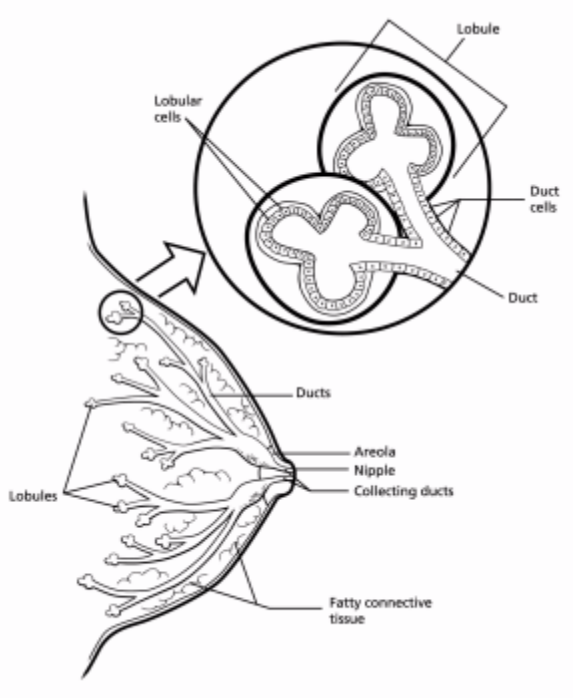

Figure 1. A normal female breast. The female breast is made up mainly of *lobules* (milk-producing glands), *ducts* (tiny tubes that carry the milk from the lobules to the nipple), and *stroma* (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Source: (American Cancer Society, 2012)

However, most breast lumps are not cancerous and are defined as *benign*. They are abnormal growths that do not spread beyond the breast or to other organs. In most cases benign lesions are not life-threatening but in some cases they may predispose the women to a higher risk of developing breast cancer. In such circumstances, these lumps may need to be pathologically analyzed to be declared non-cancerous (American Cancer Society, 2012).

Non-cancerous conditions can be broadly divided into three categories (American Cancer Society, 2011):

1. *Non-proliferative lesions* that include fibrosis, cysts, mild hyperplasia, adenosis (non-sclerosing), simple fibroadenoma, phyllodes tumor (benign), a single (solitary) papilloma, granular cell tumor, fat necrosis, mastitis, duct ectasia, and benign lumps or tumors (lipoma, hamartoma, hemangioma, hematoma, and neurofibroma). These lesions do not contribute to the overgrowth of breast tissue and are not likely to affect breast cancer risk. Even if they do, the effect is minimal.
2. *Proliferative lesions without atypia* consist of ductal hyperplasia (without atypia), complex fibroadenoma, sclerosing adenosis, multiple papillomas or papillomatosis, and radial scar. They are related to the growth of cells in the ducts of lobules of the breast tissue and can increase the risk of developing breast cancer subtly (1½ to 2 times the typical risk).
3. *Proliferative lesions with atypia* or *precancerous lesions* that consist of atypical ductal hyperplasia, atypical lobular hyperplasia, and lobular carcinoma in situ. These lesions are a result of excess growth of cells in the lobules or ducts of the breast tissue and can raise the risk of developing breast cancer to 4 to 5 times more than normal.

Breast cancer in young women

Breast cancer is primarily a disease of older women, with 75% of the cases diagnosed in women over the age of 50 years old (Mintzer et al., 2002). Only 2.7% of all breast carcinoma cases occur in women aged 35 years old or younger,

and 0.6% in women younger than 30. Yet, breast cancer is the main cause of cancer-related deaths in young women (Di Nubila et al., 2006). Young women are more likely to be diagnosed with a higher stage of breast cancer in comparison to their older counterparts and even at the same stages, they tend to have worse outcomes (Axelrod et al., 2008). Tumors in young women are likely to be of a higher grade, be hormone-insensitive and have a higher proliferative rate (Vollmer, 1996). They also have a higher incidence of lymphovascular invasion (Axelrod et al., 2008).

It may be more difficult to diagnose breast cancer in young women because their breasts tend to be more nodular and fluctuate in between the menstrual cycles (Mintzer et al., 2002). Lumps due to fibrocystic changes (fibrosis is the formation of scar-like (fibrous) tissue, and cysts are fluid-filled sacs (American Cancer Society, 2012)) are very common in this particular age group. Fibrocystic changes can cause swelling and pain, and in some cases, even a nipple discharge (some common symptoms of breast cancer) before the menstrual period. Hence, the physicians usually have a low suspicion rate for malignancy in younger women. In one study of 30 British women aged 35 years or younger, clinical examination was unpredictable in predicting malignancy with a sensitivity of only 37% (Ashley et al., 1989).

Since mammography screening is not recommended for women under the age of 40, it is not very common for breast cancers in these women to be detected through a mammogram (Mintzer et al., 2002) (a mammogram is an x-ray image

that is used to differentiate between malignant and cancerous tumors (A.D.A.M., 2010). It is noteworthy to mention that that the accuracy of mammography is also lower in young women because their breasts tend to be more dense as opposed to older women (Mintzer et al., 2002).The study by Ashley et al. (1989) also demonstrated that only 55% of mammograms of women with cancer confirmed malignancy whereas 22% of them could not be determined, hence raising total suspicion of malignancy to 77%. However, fine needle aspiration cytology (Figure 2) had the greatest accuracy with 78% definitely malignant cases and 15% suspicious, making the overall percentage to 93%.

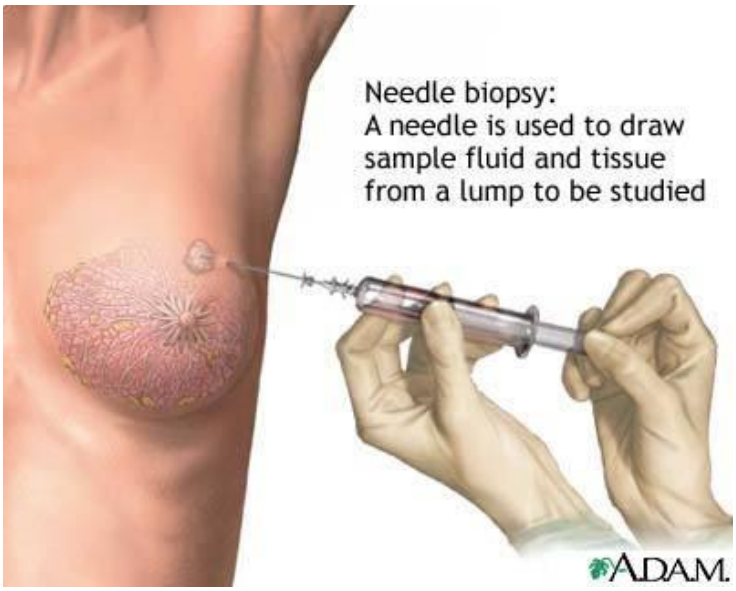

Figure 2. Needle biopsy of the breast. A needle biopsy is performed under local anesthesia. Simple aspirations are performed with a large gauge needle to attempt to draw fluid from lumps that are thought to be cysts. Fine needle biopsy uses a small gauge needle to make multiple passes through a lump, drawing out tissue and fluid. Withdrawn fluid and tissue is further evaluated to determine if there are cancerous cells present (A.D.A.M., 2011b).

Pregnancy-associated breast cancer

Breast cancer is the second most common pregnancy-linked malignancy after cervical cancer (Psyrri and Burtness, 2005). Less than 4% of breast cancers in the US are diagnosed simultaneously with pregnancy or lactation, but among women younger than 40 years of age, the incidence is approximately 15% (Psyrri and Burtness, 2005). Pregnancy-associated breast cancer (PABC) is defined as breast cancer diagnosed during pregnancy or within the first year after delivery (Pommier and Fields, 1995), while some have gone ahead to suggest up to 6 years postpartum (Albrektsen et al., 2005). The most common type of PABC is invasive ductal carcinoma (75-90%), followed by invasive lobular carcinoma (Middleton et al., 2003), and in rare cases, inflammatory carcinoma (around 4%) (Navrozoglou et al., 2008). A woman is predisposed to a higher risk of developing PABC if she has her first pregnancy at the age of 30 or later. Hence, as more women choose to postpone child-bearing until their mid to late-thirties, the occurrence of PABC is more likely to increase in the present world (Albrektsen, Heuch and Kvale, 1995).

As mentioned earlier, diagnosis of breast cancer in young women is difficult (Mintzer et al., 2002). It is further complicated when a woman becomes pregnant or is breastfeeding because both of these conditions increase the density of the breast, making clinical examination and mammography difficult to interpret eventually leading to a poor prognosis (Schedin, 2006). A poor prognosis is also associated with putative mechanisms such as the increase of gestational hormones

like estrogen, insulin like growth factor 1 (IGF1) and progesterone – hormones that are positively correlated with breast cancer progression especially in hormone-responsive tumor cells (Henderson and Bernstein, 1991).

However, there is a unique-to-only-pregnancy and a dramatic physiological change that takes place in the mammary gland after pregnancy that might increase the risk of breast cancer progression. This change is called mammary gland involution (Schedin, 2006) during which the mammary gland undergoes a highly coordinated tissue remodeling process (involution) that returns it to condition similar to that of the virgin gland (Flanders and Wakefield, 2009).

Mammary gland involution

Under non-pregnant and non-lactating conditions, the mammary gland is made up of networks of epithelial ducts that empty into the main lactiferous ducts (Masso-Welch et al., 2000). The milk-producing lobular component of the gland is rudimentary and is embedded in a mesenchymally-derived fat pad, the dominant tissue in the gland. During pregnancy, and in preparation for lactation, this epithelium undergoes massive proliferation and differentiation in response to gestational hormones. It expands to fill the gland and replaces the fat pad with milk-producing lobuloalveoli (Schedin, 2006). In rodent studies it has been observed that with the cessation of milk secretion, the alveolar cells undergo apoptosis (Strange et al., 1992) until the stage where this process becomes irreversible. The proteolytic breakdown of the basement membrane causes a

destruction of the alveoli (Lund et al., 1996) and the residual milk proteins and apoptotic cells are cleared by phagocytes (Monks et al., 2002). Finally, the stromal adipose tissue regenerates with remodeling of the extracellular matrix (ECM) and lipid accumulation (Flanders and Wakefield, 2009), and the mammary gland returns to its rudimentary, prepregnant state (Schedin et al., 2007) (Figure 3). The regression of these lubuloalveoli is most relevant to PABC according to Schedin et al. (2006) who propose that the tissue remodeling response during involution may change the mammary gland stroma into one that promotes tumor cell growth and dissemination.

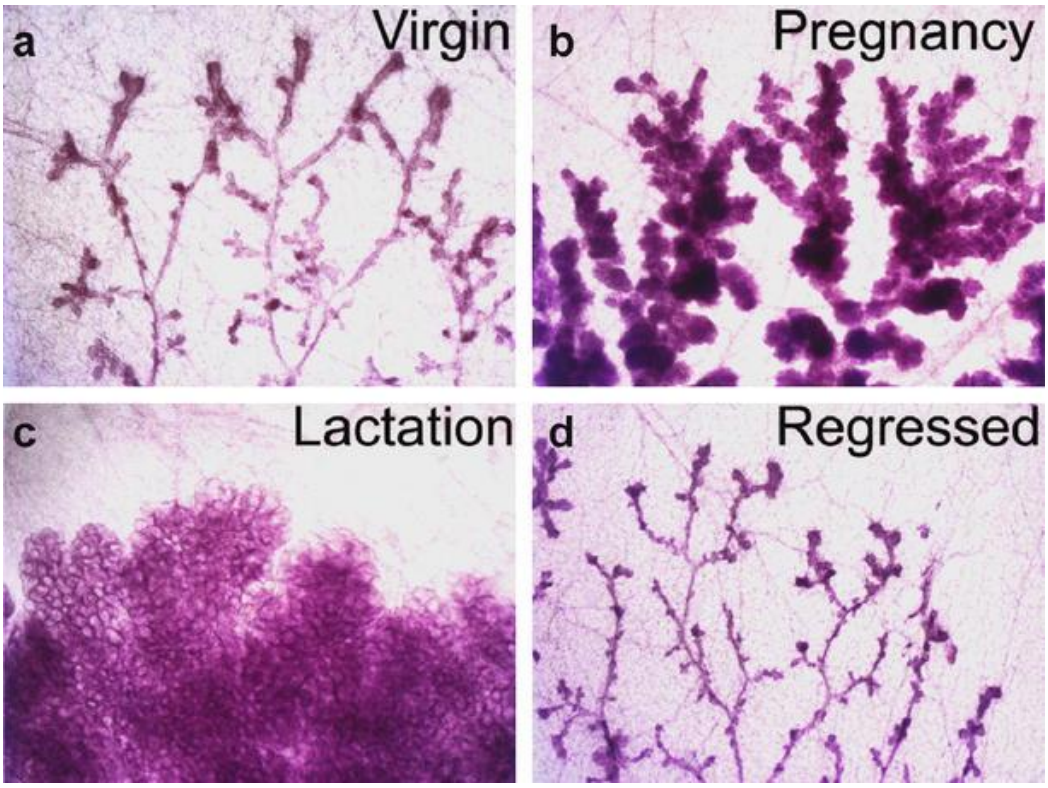

Figure 3. Morphological changes in mammary gland associated with reproductive state in the female Sprague–Dawley rat. Representative whole mount images of MG#5 from a virgin/nulliparous, b late-stage pregnant, **c** lactating day 7, **d** fully regressed/parous rats, original magnification 40x (Schedin et al., 2007).

Mammary gland involution and tumorigenesis

Involution activates cellular apoptosis, matrix remodeling, inflammation and angiogenesis – processes that are involved in the wound healing response and inflammation – and is believed to contribute to the aggressive nature of PABC (Schedin, 2006). Wound healing and inflammatory microenvironments are pro-oncogenic (Coussens and Werb, 2002) explaining why PABC occurs during pregnancy or within the first 12 months postpartum. In support of Schedin et al.'s (2006) hypothesis, McDaniel et al. (2006) demonstrated that ECM isolated from nulliparous rats promoted ductal organization of normal mammary epithelial cells (MEC) in three-dimensional culture and suppressed invasion of mammary tumor (MDA-MB-231) cells. On the other hand, ECM isolated from involuting glands did not support ductal development in normal cells but promoted invasiveness in tumor cells. Moreover, when the involuting matrix was mixed with MDA-MB-231 cells and injected into the mammary fat pad of nude mice, metastases to lung, liver, and kidney increased along with a twofold increase in tumor vascular endothelial growth factor (VEGF) expression and angiogenesis (McDaniel et al., 2006); and thus, cancer progression.

Mammary gland involution in preclinical breast cancer models has also been suggested to promote metastases through the production of bioactive proteolytic ECM fragments that activate tumor cell motility and invasion (Figure 4). These include cytokine-secreting immune cells, matrix metalloproteinases and tumor-promoting peptides such as fibronectin and laminin (Schedin, 2006).

Involution also mimics the wound-healing process with a massive cell death of secretory epithelium resulting in apoptotic debris that needs to be cleared to restore the pre-pregnant glandular architecture (Schedin et al., 2007). Fibroblasts secrete proteases that degrade ECM proteins resulting in a bioactive matrix that has tumor growth-, motility- and invasion-promoting properties. These properties are created by the active immune cells, primarily macrophages and neutrophils, and by cytokines such as interleukin-1 (IL-1), IL-6, tumor-necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Sauter et al., 2007; Schedin, 2006). This is where the relevancy of TGF-β in PABC comes into play (Wakefield, Yang and Dukhanina, 2000).

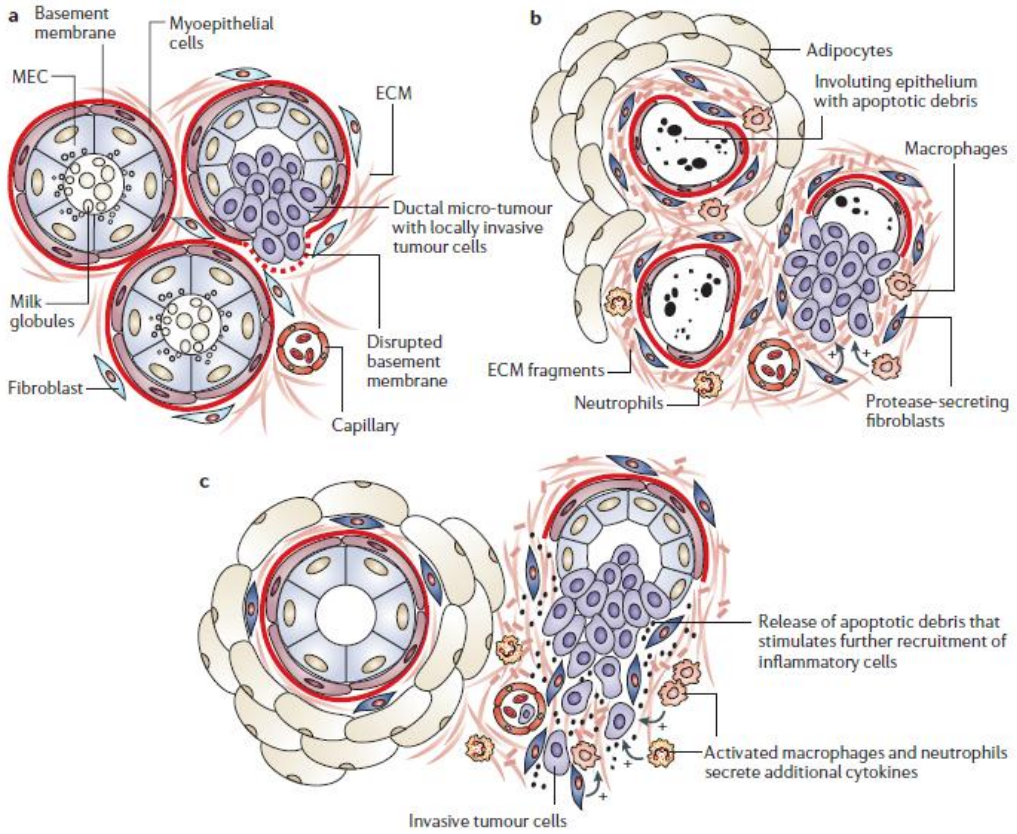

Figure 4. A model depicting tumor cell promotion during mammary involution. a. Acini from a lactating gland with an invasive micro-lesion. During lactation, secretory mammary epithelial cells (MEC) are surrounded by myoepithelial cells and intact basement membrane. The acini are embedded in a stroma containing fibroblasts, vasculature and ECM. A small ductal tumor with locally invasive tumor cells is shown in the figure. b**.** The matrix-proteinase-dependent stage of involution has properties of the wound-healing environment. The secretory epithelium undergoes apoptosis and is replaced by the adipocytes in the gland. As these epithelial cells are cleared and the ECM proteins are degraded by the fibroblast-secreted proteases, there is an abundance of immune cells such as macrophages, in the matrix. These factors aid the tumor in its progression and invasion. c. Involution-associated changes in the microenvironment are amplified by the presence of tumor cells, switching the finely-tuned inflammatory balance towards overt inflammation. The bioactive matrix induces the tumor cells to activate metalloproteinases that cause further matrix degradation. The matrix loss allows the highly immunogenic apoptotic debris to leak into interstitial space, resulting in a sustained influx of inflammatory cells that release additional cytokines. Previously quiescent tumor cells are now activated by the cytokine-rich microenvironment and can now invade to local vasculature and lymphatics (Schedin, 2006).

Transforming growth factor-β (TGF-β)

TGF-β background

Transforming growth factor-βs (TGF-βs) belong to a group of multifunctional cytokines that is collectively called 'the TGF-β superfamily.' The superfamily consists of over 100 distinct proteins and ligands, including activins, inhibins, nodals, bone morphogenetic proteins (BMPs) and growth and differentiation factors (Chin et al., 2004) that regulate a number of diverse biological responses in development, maintenance of cellular homeostasis and injury (Massague and Gomis, 2006). Out of a total of five homologous isoforms of TGF-β (Saito et al., 1993), mammals express three (TGF-βs 1, 2 and 3) that share 70% sequence identity in the biologically active C-terminal region with each other (Flanders and Wakefield, 2009). All three of them bind to the same receptor complex, the type II receptor (TβRII) and activate the same signaling pathway (Figure 5). It is secreted in an inactive, latent form that is made up of a 25kDa mature peptide in a non-covalent association with the N-terminal (Wakefield, Yang and Dukhanina, 2000). Most cell types express one or more isoforms of TGF-β and almost all cells express TGF-β receptors (Flanders and Wakefield, 2009).

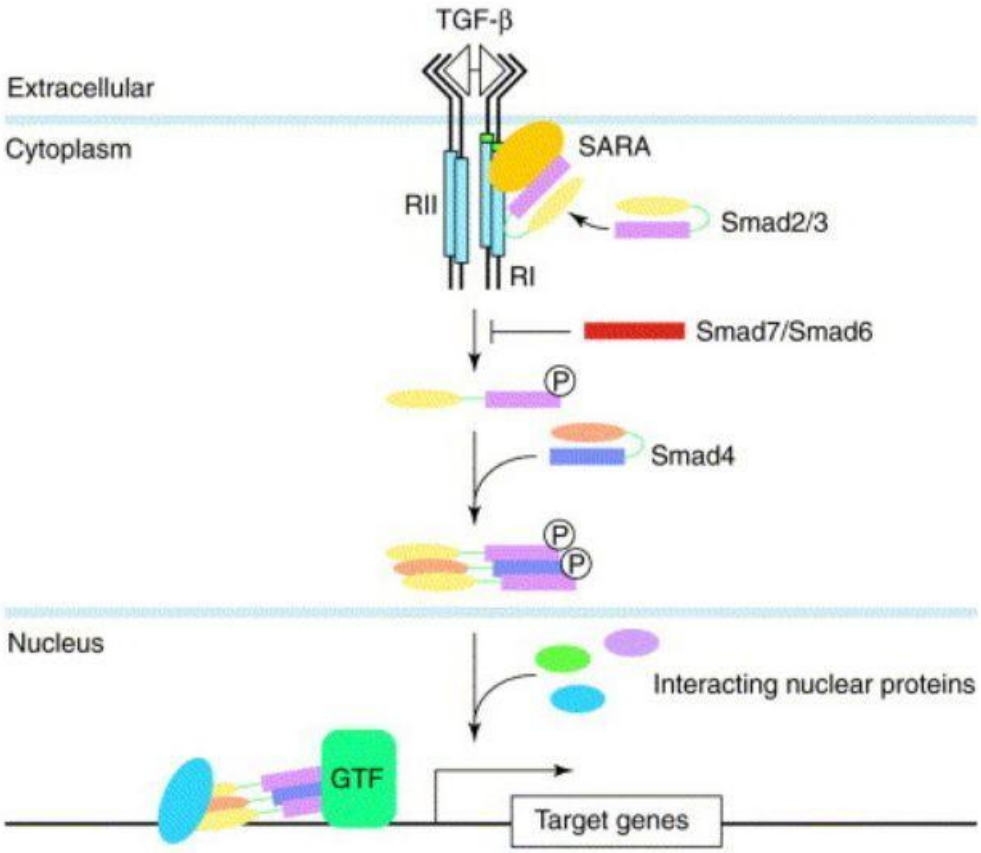

Figure 5. The TGF-β signaling pathway. TGF-β ligands bind to the active serine-threonine kinase transmembrane receptopr (TβRII) that complexes with and phosphorylates the type I receptor (TβRI). Consequently, TβRI phosphorylates the receptor-activated Smad proteins (Smads 2 and/or 3) allowing them to form an oligomeric complex with Smad4, that accumulates in the nucleus. This complex interacts with Smad binding elements in the DNA and with other transcription and chromatin remodeling factors to regulate target gene expression (Flanders and Wakefield, 2009). Source: (Akhurst and Derynck, 2001)

TGF-β was discovered by scientists studying platelet derived growth factors (PDGF) and epidermal growth factors (EGF and TGF-α) in 1983 (Anzano et al., 1983). It was named as TGF-β due to its ability to "transform" rat fibroblasts (Roberts et al., 1983). TGF-β has a variety of biological functions. However, its regulatory effects on several cell types is noteworthy (Kretzschmar, Doody and Massague, 1997) especially its regulatory role in cell replication and differentiation, bone formation, angiogenesis, hematopoiesis, cell cycle progression and cellular migration (Sporn and Roberts, 1992) – all processes that are active in wound healing and tissue development (Chin et al., 2004), and also in involution and the initiation of PABC as we discussed earlier.

Expression of TGF-β in the mammary gland

The role of TGF-β has been extensively studied in the rodent mammary gland. Robinson et al. (Robinson et al., 1991) demonstrated the expression of TGF-β in parenchyma-free fat pads and mammary glands from virgin and pregnant mice using Northern blot and in situ hybridization analysis. They showed that all three isoforms of TGF-β are present during most stages of mammary development but they are regulated differently. They also showed that TGF-β2 and TGF-β3 transcript levels increase during pregnancy whereas TGF-β1 transcripts do not, TGF-β3 transcripts are more intensely expressed in the ducts as

opposed to the alveoli in the pregnant animal, and that TGF-β2 transcript levels increased dramatically during pregnancy (Figure 6).

However, the mRNA expression of all three isoforms greatly reduces as determined via immunostaining during lactation (Robinson, Roberts and Daniel, 1993). TGF-βs inhibit the synthesis of milk casein proteins, as detected through Western blot in explant cultures of the pregnant mammary gland in response to lactogenic hormones (Robinson, Roberts and Daniel, 1993) and hence the relatively high levels of TGF-β during pregnancy may inhibit milk production with the sudden fall in the TGF-β mRNA levels at parturition to allow for milk secretion (Flanders and Wakefield, 2009).

It is during involution when TGF-β plays various roles in the mammary gland. The expression of TGF-βs 1 and 2 mRNA returns from the low levels observed in lactation back to that of the virgin gland, whereas TGF-β3 mRNA increases dramatically during involution, with a maximum 5-6-fold increase above that in the virgin gland and 3-fold increase compared to pregnancy (Faure et al., 2000). Most of it is expressed in the lobular-alveolar cells at day-1 post-weaning (Nguyen and Pollard, 2000) and in the myoepithelial cells at 4-days post-weaning as demonstrated by immunohistochemistry (Faure et al., 2000). Milk stasis is postulated to play a role in the dramatic increase in TGF-β3 expression at weaning. When teats were sealed in mice, TGF-β3 mRNA was expressed in the sealed gland as opposed to the contralateral feeding gland in the same mouse (Nguyen and Pollard, 2000). Weaning causes a decrease in the pituitary hormones

such as prolactin and oxytocin, but in the absence of suckling, exogenous delivery of these two hormones did not change TGF-β3 mRNA levels, suggesting that milk stasis and not changes in lactogenic hormones induces TGF-β3 expression upon weaning (Flanders and Wakefield, 2009).

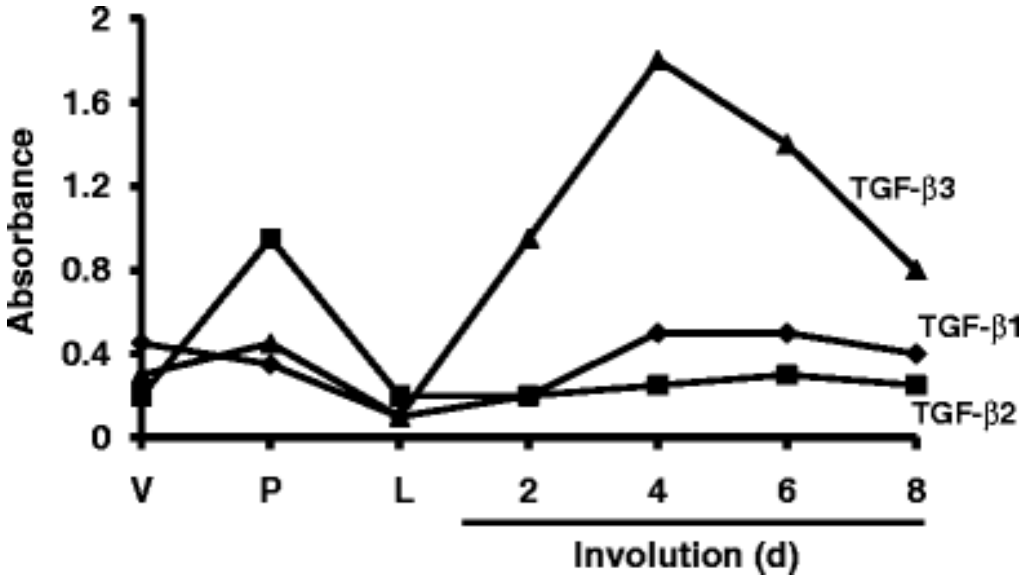

Figure 6. Quantitation of TGF-β mRNA levels in the mammary gland from a virgin mouse (V), pregnant mouse at 15 d gestation (P), lactating mouse (L), and days 2, 4, 6, and 8 of involution.(♦ TGF-β1, ■ TGF-β2, ▲, TGF-β3). Source: (Faure et al., 2000)

TGF-βs are very active in the wound-healing and inflammation aspects of involution. These biological roles of TGF-β in involution are primarily explained by studies conducted by Nguyen and Pollard (2000). In wild-type C57B1/6 mice, expression of TGF-β preceded the peak of alveolar apoptosis in the mammary gland after pup removal (Faure et al., 2000). Since TGF-βs induce apoptosis in target cells in vitro (Sanchez-Capelo, 2005), it was suggested that TGF-β3 is critical for inducing epithelial cell apoptosis during involution. Moreover, when mammary glands from TGF-β3 null mice were transplanted in wild-type hosts that were sacrificed one day after giving birth, a 70% reduction in apoptosis took place in the TGF-β3 null mice as compared to the corresponding wild-type glands, suggesting that endogenous TGF-β3 plays a major role in the initial stage of alveolar apoptosis (Nguyen and Pollard, 2000).

The later stages of involution revolve around the wound-healing response with the influx of immune cells, loss of basement membrane, removal of dead cells and tissue remodeling (Lund et al., 1996; Schedin et al., 2007). Northern blot and microarray studies have identified genes expressed during the later stage of involution (Clarkson et al., 2004; Stein et al., 2004), many of which are involved in the wound-healing cascade and are TGF-β targets and/or regulators (Schedin et al., 2007). In wound-healing, TGF-βs have been shown to be chemoattractants for immune cells such as neutrophils (Reibman et al., 1991) and monocytes (Parekh et al., 1994; Wahl et al., 1987), and thus their role as anti-inflammatory mediators (Flanders and Wakefield, 2009). TGF-βs also play an active role in matrix

remodeling since they are chemotactic for fibroblasts (Cordeiro et al., 2000; Postlethwaite et al., 1987), especially TGF-β3 that has been shown to induce motogenesis in dermal fibroblasts in 3-dimentional matrices (Schor et al., 2006). TGF-βs can increase differentiation of quiescent fibroblasts into activated fibroblasts that produce ECM proteins (Serini and Gabbiana, 1996) and help the involuting gland to return to its virgin state.

Role of TGF-β in breast cancer

Because of the active role of TGF-β in involution (Robinson et al., 1991) and due to the direct link of the involuting gland with tumorigenesis (Schedin et al., 2007), it is proposed that TGF-β plays an active role in breast cancer, especially pregnancy-associated breast cancer (Schedin, 2006). TGF-β has been proposed to play a tumor-suppressive role in the pre-malignant stage of breast cancer (Massagué, 2008) and a tumor-promoting role in the malignant stages of breast cancer (Massague and Gomis, 2006) because of its effects on wound-healing and matrix remodeling (Schedin, 2006). However, more studies are needed to determine the exact morphological changes of TGF-β's double-edged sword role in breast cancer.

TGF-β in human breast milk

TGF-β has been identified in human breast milk (Bottcher et al., 2000). Human milk contains TGF-β1, TGF-β2 and other isoforms at both mRNA and

protein levels with TGF-β2 being the major isoform (95%) (Kalliomäki et al., 1999). The immunoactive factors in breast milk may influence the development and maturation of the mucosal immune system of the infant (Ando et al., 2007) and growing evidence suggests that TGF-β, a multifunctional polypeptide, may be a key immunoregulatory factor for the establishment of this response, by promoting IgA production as well as induction of oral tolerance (Kalliomäki et al., 1999; Oddy et al., 2003; Ogawa et al., 2004; Rautava et al., 2011).

Project Objectives

Methods of tissue acquisition such as breast biopsies do not survey the whole breast including the ducts and the lobes, and hence, limit the prediction of cancer risk in entirety. Even though biopsies are more conclusive, they tend to have adverse physiological effects on the patient. The breast may be sore for several days and in some women infection may occur as well. Bruising is quite common and if surgical incisions are made, the women may need to take over-the-counter painkillers (A.D.A.M., 2011b).

TGF-β is naturally secreted in breast milk (Saito et al., 1993), an excellent source of cytokines (Rautava, Kalliomaki and Isolauri, 2002) as well as exfoliated epithelial cells (Browne et al., 2011). Hence, we decided to use breast milk as part of an assay that could help us determine the levels of TGF-β in the breast.

Since TGF-β2 (Figure7) is the most abundant isoform of TGF-β in human milk, we decided to examine it in this study in hopes that future work could be performed on other isoforms of TGF-β as well. In our observational study, we investigated the expression levels of TGF-β2 in breast milk from both breasts of 182 women who were scheduled to receive a biopsy or had already had a breast biopsy at time of milk collection. They either had breast biopsies prior to or during their pregnancies, or after childbirth. The aim of our project was to examine the relationship between trends in TGF-β2 expression across biopsied vs. non-biopsied breast and the biopsy categories.

Even though I did not work directly with the questionnaire data, we wanted to analyze the TGF-β2 levels in light of other variables that could have affected the variability in the TGF-β2 levels. The main variables that we selected to be examined alongside our two main variables of interest were:

- mother's age at the time of milk donation;
- mother's age at first birth;
- parity (number of live births including the baby that was being nursed at the time of milk donation);
- mother's body mass index (BMI);
- family history of breast cancer; and
- baby's age at the time of milk donation.

Our study was mainly observational, however, based on the active role of TGF-β in cancer, we hypothesized that TGF-β2 expression would be higher in biopsied vs. non-biopsied breast, and that it would be the highest in biopsy category 4 (cancerous lesions) as opposed to the rest of the three categories.

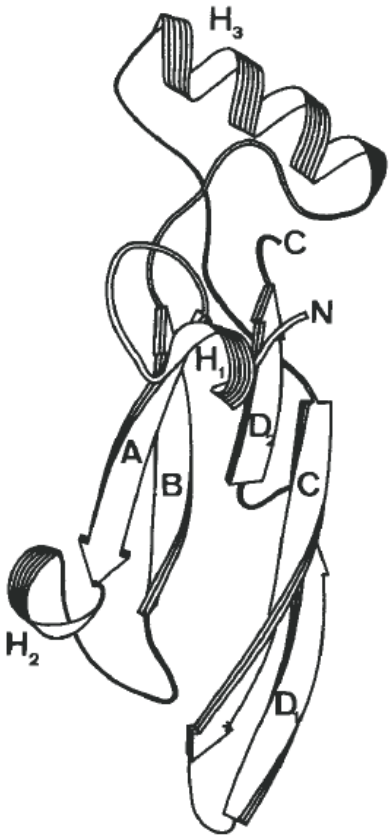

Figure 7. The crystalized structure of human recombinant TGF-β2. TGF-β2 monomer is a flat, elongated and slightly bent molecule with an overall size of 60 x 20 x 15$Å^3$. It consists of a long α-helix with its axis perpendicular to the β-sheet which is separated from two antiparallel pairs of β-strands. The amino-terminal short α-helix on the outside of the monomer is followed by an exposed loop that is fixed to the core through a disulfide bridge. The other three disulfide bonds connect the different β-strands with each other. Image borrowed from (Schlunegger and Grutter, 1992).

MATERIALS AND METHODS

Study population

Subject recruitment and eligibility

This study was approved by the Institutional Review Boards of the University of Massachusetts and the Congressionally Directed Medical Research Program. Dr. Arcaro and her team recruited a group of lactating women who either had a breast biopsy or were scheduled to receive a breast biopsy through the study "Molecular Biomarkers for Assessing Breast-Cancer Risk." Dr. Arcaro advertised the study on websites and contacted mammography centers, lactation consultants and breast cancer and breast feeding organizations across the US to spread the word. Lactating women of ages 18 and above who either had a breast biopsy or were scheduled to have one were eligible to participate provided that they were free from debilitating mental and physical sickness. Participants had to be willing to complete a questionnaire, submit a copy of their breast biopsy pathology report, and donate a sample of milk from one or both of their breasts. The questionnaire called for details such as their age, their age at their first birth, their baby's age at the time of milk donation, their weight and height, their ethnicity etc. (Browne et al., 2011).

Collection of breast milk samples

Eligible women were each sent an informed consent form and a reproductive history and health questionnaire by post or email. Consented women each received a "breast milk collection box" containing four 100mL glass bottles, an ice pack, a prepaid return label and directions for filling the bottles and returning the collection box. All the four bottles were legibly labeled: two bottles for frozen milk (not used in my project) and two bottles for fresh milk from both left and right breasts. The participating women stored the ice pack and the two bottles of their frozen milk in their freezers at least one night prior to returning their boxes to the Arcaro lab. On the morning they dispatched the boxes, they either hand-expressed or pumped all the milk from each breast and transferred it to the respective bottles ("left fresh" and "right fresh"). Each woman then placed these two bottles in the collection box along with the two frozen milk bottles and ice pack, and called the express mail carrier for immediate pick-up and delivery.

Processing the breast milk

Milk samples were processed as soon as they were delivered to the laboratory, for the most part within 24 hours of being expressed. Frozen milk samples were thawed and transferred to acid-rinsed amber bottles and archived at -20ºC for future studies. On the other, 5mL of fresh milk from each breast was transferred in glass vials and was also archived at -20ºC. The remaining milk was diluted with phosphate buffered saline (PBS) in the ratio of 1:1 and centrifuged at

1000G for 10 minutes in 50 mL glass centrifuge tubes. The supernatant was poured in 250 mL acid-rinsed amber bottles while the cell pellets were transferred to polypropylene tubes, and then washed twice for 5 minutes at 500G in sterile PBS. The glass tubes were washed with half of the original volume of the sterile PBS and this rinse was added to the 250 mL acid-rinsed bottles. The diluted milk was stored at -20ºC and was used for the current study.

Classification of biopsy reports

Once the women had donated milk samples from each breast, they were asked to contact their Health Care Provider (HCP) and request a copy of their pathology reports. In some cases, the HCP personally spoke with Dr. Arcaro before releasing the report. Dr. Arcaro's team reviewed each biopsy report and used the American Cancer Society's classification scheme to classify biopsy results into one of the four categories (American Cancer Society, 2011):

1. *Non-proliferative lesions* include fibrosis, cysts, mild hyperplasia, adenosis (non-sclerosing), simple fibroadenoma, phyllodes tumor (benign), a single (solitary) papilloma, granular cell tumor, fat necrosis, mastitis, duct ectasia, and benign lumps or tumors (lipoma, hamartoma, hemangioma, hematoma, and neurofibroma).
2. *Proliferative lesions without atypia* consist of ductal hyperplasia (without atypia), complex fibroadenoma, sclerosing adenosis, multiple papillomas or papillomatosis, and radial scar.

3. *Proliferative lesions with atypia* or *precancerous lesions* that consist of atypical ductal hyperplasia, atypical lobular hyperplasia, and lobular carcinoma in situ.
4. *Malignant* or *cancerous lesions* that include ductal carcinoma in situ and invasive carcinomas.

Selection of milk samples

Dr. Arcaro has an archived database of milk samples from 250 women who donated milk for the "Molecular Biomarkers for Assessing Breast-Cancer Risk" study (Browne et al., 2011). Out of these 250 women, 208 of them submitted their pathology reports of biopsies. Since for the purpose of our study, we needed women who had a known categorization of their breast lumps, we preselected these women from the database. Then we narrowed them down by the four biopsy categories as described in the previous section and came up with a total of 185 women. Out of these 185 women we could not locate milk for 2 women in the refrigerators in the lab while one woman did not have sufficient milk for her right breast and hence, we dropped her from the study. Hence, the final number of women for this study was 182.

Aliquoting milk samples

We thawed the amber jars overnight in 3 batches over the course of 3 days. The jars were removed from -20ºC in the evening and five 500µL aliquots

were removed from each jar the next morning and transferred to individual polypropene tubes. We made these aliquots to avoid the repeated freeze-thaw cycle of the big amber jars. The polypropene tubes were immediately transferred to -20ºC and the ones labeled for TGF-β2 were removed every time we conducted the experiments for this study.

Assessment of TGF-β2

The protein expression levels of TGF-β2 in our samples were determined by the enzyme linked immunosorbant assay (ELISA). We used the *Quantikine Human TGF-β2 Immunoassay* kit (R&D Systems DB250) to perform our ELISA experiments. The kit is a 4.5 hour solid-phase ELISA designed to measure activated TGF-β2 in cell culture supernates, serum and plasma, and is capable of measuring TGF-β2 up to a concentration of 2000pg/ml accurately. The kit was stored at 8ºC and the reagents were removed when we needed them. We followed the manufacturer's instructions while undertaking the following steps:

Activation reagent preparation

In order to activate the latest TGF-β2 to the immunoreactive form, we prepared the followed solutions for acid activation and neutralization.

- 1 N HCl (100mL) – 8.33mL of 12 N HCl were slowly added to 91.67mL deionized water and were mixed well.
- 1.2 N NaOH/0.5 M HEPES (100mL) – To 75mL of deionized water, we slowly added 12mL 10 N NaOH and mixed them well. 11.9g of HEPES were added to this solution and the final volume was brought to 100mL with deionized water.

We made these two reagents before the very first experiment and then stored them in glass bottles. Aliquots were made for consecutive experiments.

Standard preparation

The TGF-β2 standard comes in 4ng/vial and is made up of lyophilized recombinant human TGF-β2 in a buffered protein base with preservatives. We reconstituted it with Calibrator Diluent RD51 (buffered protein base with preservatives) and allowed the standard to sit at least for 15 minutes with gentle agitation before making the dilutions. The reconstitution produces a stock solution of 2000pg/mL which we used to produce a dilution series (Figure 8). We pipetted 500µL of Calibrator Diluent RD51 in each tube and then transferred 500µL of the standard in the first tube to produce a 1000pg/mL TGF-β2 solution. This tube was then used to make consecutive dilutions up to 62.5pg/mL and it was ensured that the solutions were mixed thoroughly before making the next transfer. Calibrator Diluent RD51 serves as the zero standard (0pg/mL). Fresh standards were used for each day of experiments.

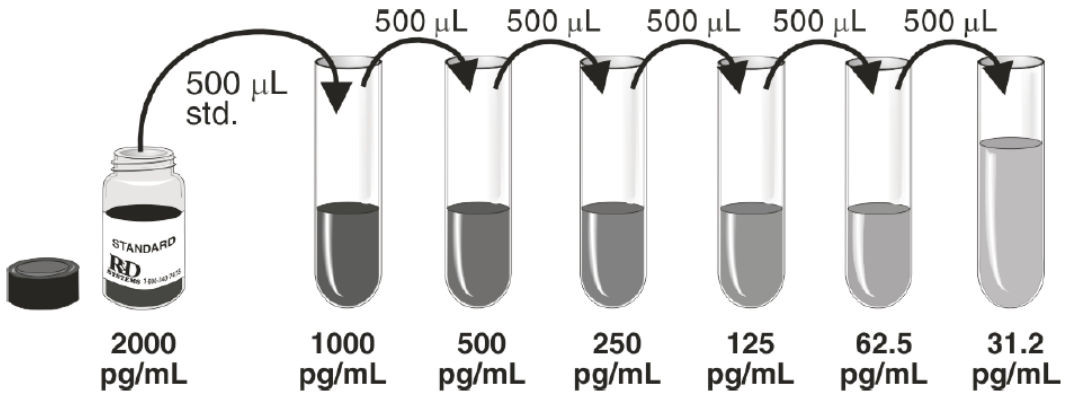

Figure 8. Schematic of TGF-β2 standard dilutions.

Protein activation

We removed the milk aliquots from -20ºC and left them at room temperature while we prepared the standards and labeled the polypropene tubes for sample activation. Usually the thawing process would take around half an hour to 45 minutes.

Once the samples were thawed, we first "spiked" an aliquot of our pooled sample. In a polystyrene tube, we transferred 100µL of the pooled sample and then added 100µL of the 1000pg/mL standard. This resulted in a spiked pool sample with extra 500pg/mL TGF-β2. We undertook this step in order to have a component of a known concentration as part of our control.

To 125µL of sample, we added 25µL of 1 N HCl, mixing it well and then incubated it for 10 minutes at room temperature. After the incubation, we added 25µL 1.2 N NaOH/0.5M HEPES and mixed the sample well. Finally 800µL of Calibrator Diluent RD51 was added to each sample and the samples were assayed right away. All these steps accounted for a dilution factor of 7.8.

Assay procedure

All reagents and samples were brought to room temperature before use. The TGF-β2 microplate, 96 well polystyrene microplate coated with a mouse monoclonal antibody against TGF-β2, was removed from its foil pouch. We then added RD1-17 (buffered protein base with preservatives) to each well and then transferred 100µL of the standards and activated samples per well in duplicates. A

usual plate layout will look like Figure 9 although the positions of pooled and spiked pooled samples were switched from one plate to another. The plate was covered with the adhesive strip provided in the kit and incubated for 2 hours at room temperature.

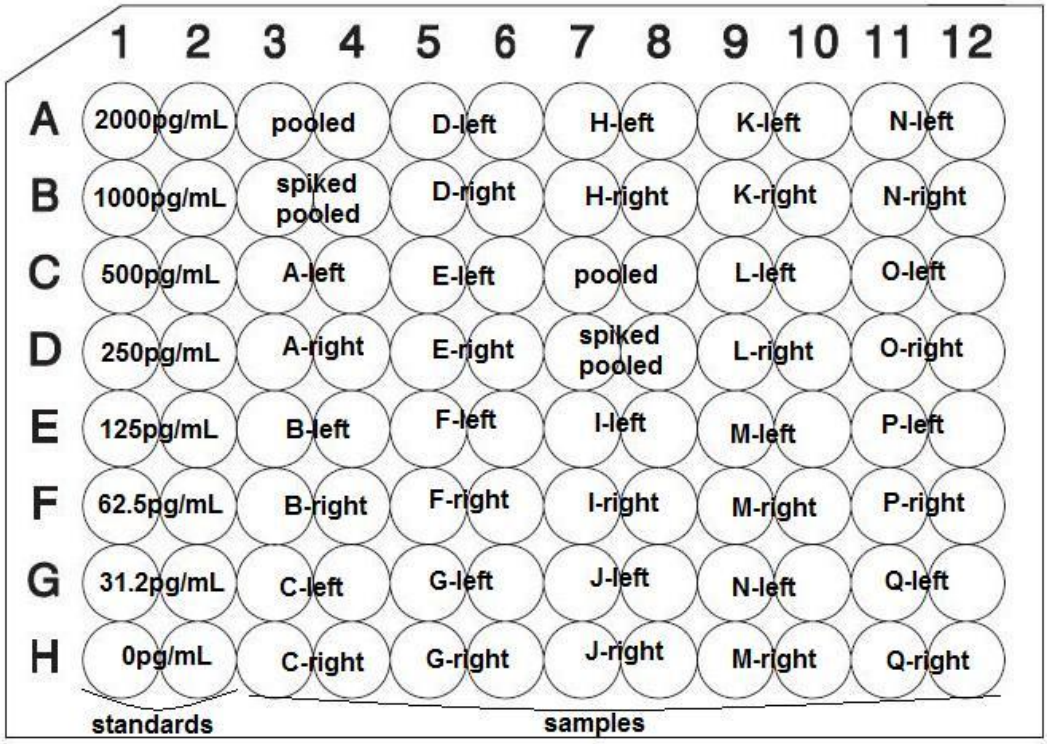

Figure 9. Schematic of TGF-β2 microplate layout for a typical ELISA run.

Before the 2 hour incubation was scheduled to end, we diluted 20mL of the Wash Buffer Concentrate (21mL/vial of 25-fold concentrated solution of buffered surfactant with preservatives) with distilled water to prepare 500mL of the of wash buffer. The wash buffer was used for multiple experiments and more was prepared when needed.

At the conclusion of the incubation period, we inverted the plate and blotted it against clean paper towels. Each well was filled with 400µL wash buffer using a multichannel pipettor. The microplate was inverted and then blotted on paper towels. The washing and blotting were repeated twice.

200µL of TGF-β2 conjugate (polyclonal antibody against TGF-β2 conjugated with horseradish peroxidase with preservatives) was added to each well and then the microplate was covered with a new adhesive strip. The plate was incubated for another 2 hours at room temperature.

Right before the incubation period ended, we prepared the substrate solution by mixing the following two solutions in equal volumes:

- 10mL of Color Reagent A – stabilized hydrogen peroxide
- 10mL of Color Reagent B – stabilized chromogen, specifically tetramethylbenzidine

Care was taken that the substrate was used within 15 minutes of preparation and that it was protected from light by covering the flask with tin foil.

Upon completion of the incubation, we repeated the washing step again making sure that no wash buffer remained at the end of the last wash. 200µL

substrate solution was added to each well and the microplate was covered with a new adhesive strip. We sealed the plate back in its foil pouch to protect it from light and then incubated it for 20 minutes at room temperature.

50μL of stop solution (2 N sulfuric acid) was added to each well, upon which the color in the wells changed from blue to yellow. If the color change was not uniform or the wells appeared green, the plate was put on a plate-shaker for around half a minute to ensure thorough mixing.

The optical density of each well was determined using a microplate reader set to 450nm. Another reading was taken at a wavelength of 570nm. The readings at 570nm were subtracted from the readings at 450nm and this subtraction ensured that optical imperfections were corrected in the plate. The plate was read within 30 minutes of the addition of stop solution.

Calculation of results

The duplicate readings of each standard were averaged and then the average zero standard optical density was subtracted from each of them to generate the standard curve. The mean absorbances for each standard were plotted on the y-axis against the TGF-β2 concentration on the x-axis and the best fit line was generated through the points on a log/log graph (Figure 10). A standard curve was generated every day the assay was run.

The O.D. of each sample was determined through the following equation:

$$\textbf{O.D.}_{\textbf{sample}} = \textbf{(absorbance at 450nm)}_{\textbf{sample}} - \textbf{(absorbance at 570nm)}_{\textbf{sample}} - \textbf{Average O.D.}_{\textbf{zero}}$$

Using the equation of the standard curve (Y = mX + C), the concentration of each sample was determined by plugging in the O.D. for each sample in place of Y.Because milk samples were diluted with PBS upon processing and storage (in most cases with a dilution factor of 2.5) and then later on upon activation (dilution factor of 7.8), the final concentration was obtained by multiplying these concentrations with a total dilution factor (10.3 in most cases).

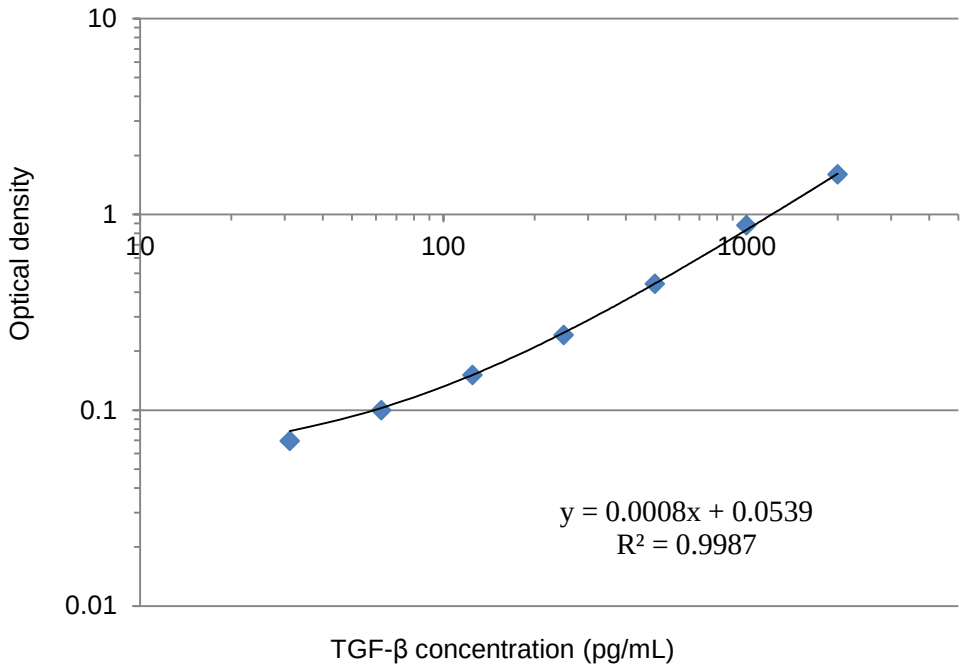

Figure 10. Standard curve for TGF-β2.

Assessment of total protein

The TGF-β2 levels in a milk sample are highly correlated with the amount of total protein it contains. Hence, in order to have more accurate deductions of TGF-β expression in our samples, we quantified the total protein in each sample using the well-known Bradford coomassie-binding, colorimetric method. When the coomassie die binds to protein in an acidic medium, its brown color immediately changes to blue with a shift in absorption maximum from 465m to 595nm. We specifically used the Thermo Scientific *Coomassie (Bradford) Protein Assay Kit* (Catalog #23200) and followed the manufacturer's instructions as below:

Preparation of diluted albumin (BSA) standards

Table 1 was used as a guide to prepare a set of protein standards. We diluted the contents of the albumin standard (BSA) ampule in PBS using clean polypropene tubes. We used PBS because it was the diluent we used to dilute our milk samples originally.

Table 1. Preparation of diluted albumin (BSA) standards.

Dilution Scheme for Micro Test Tube or Microplate Protocols (Working Range = 1–25µg/mL)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	2370µL	30µL of Stock	25µg/mL
B	4950µL	50µL of Stock	20µg/mL
C	3970µL	30µL of Stock	15µg/mL
D	2500µL	2500µL of vial B dilution	10µg/mL
E	2000µL	2000µL of vial D dilution	5µg/mL
F	1500µL	1500µL of vial E dilution	2.5µg/mL
G	5000µL	0	0µg/mL = Blank

Standard microplate procedure (working range = 100-1500µg/mL)

Through multiple troubleshooting experiments, we determined that a dilution of 1:10 per sample would generate an absorbance within the working range of the kit. Hence, we first diluted each milk sample in the ratio of 1:10 before beginning the Coomassie assay. In the meantime we poured some Coomassie reagent solution in a beaker after mixing it well (to disperse the dye-dye aggregates) and brought it to room temperature before use. We then pipetted 5µL of each standard or unknown sample into the microplate wells in duplicates with our plate layout looking very similar to that of Figure 9. Consequently, we poured the Coommassie reagent in a multichannel trough and added 250µL to each well, mixing it with a plate shaker for approximately 30 seconds. The plate was removed from the shaker and incubated for 10 minutes at room temperature.

We then measured the absorbance of the plate at 595nm using a plate reader. We used the SOFTpro Max software to generate a standard curve and

determine the concentrations of our samples. The average 595nm measurement of the blank replicates was subtracted from the absorbances of all other standard and sample replicates. A standard curve was generated by plotting the average blank-corrected measurement of each BSA standard against its concentration in µg/mL, and was used to determine the protein concentration in each unknown sample. The final concentration was further multiplied by a dilution factor of 12.5 (10 before the Coomassie assay and 2.5 from the original milk dilution) to obtain the total protein concentration in each sample. In some cases, if the absorption was too high for the measurable range, we further diluted the sample (in 1:20 ratio as opposed to 1:10) and repeated the assay again until the concentration could be determined through the standard curve.

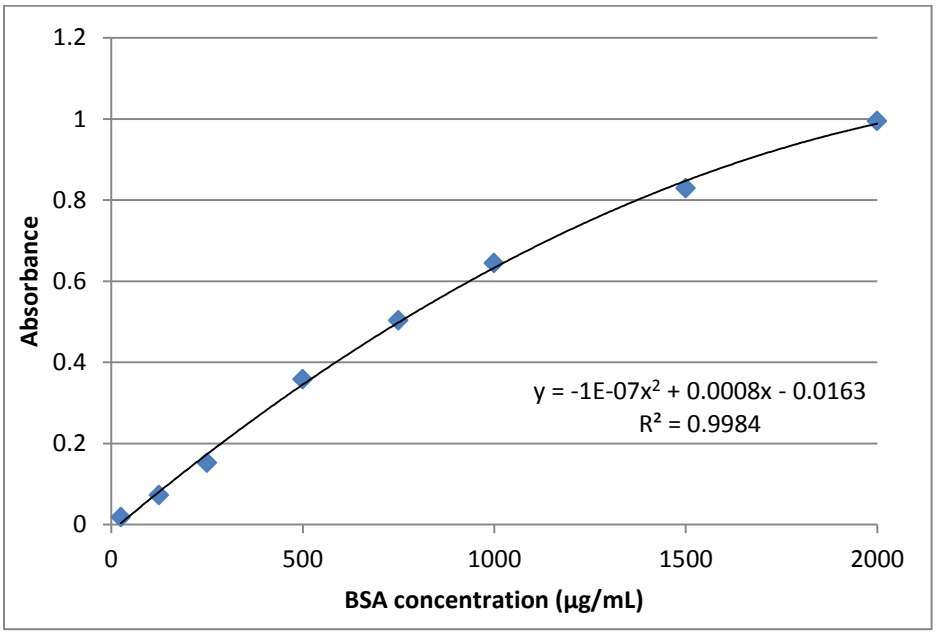

Figure 11. Standard curve for BSA using the Coomassie assay.

Data analysis

We report the TGF-β2 levels in two different ways: absolute concentration (pg/mL), and normalized levels (μg/g protein). Various studies (Bottcher et al., 2000; Hawkes, Bryan and Gibson, 2002; Kalliomäki et al., 1999) have reported TGF-β2 levels in absolute terms, and hence for the purpose of comparing our data with them, we retained the TGF-β2 concentrations as they were directly obtained via the ELISA assay. On the other hand, since scientifically it is more relevant to compare the TGF-β2 levels as a proportion of the total protein, the same data analysis was also conducted on normalized levels of TGF-β2 where the TGF-β2 concentration (pg/mL) was divided by the total protein concentration (μg/mL) to obtain the normalized levels of TGF-β2 (μg/g protein) in each sample.

The statistical analysis and most figures were generated via IBM SPSS Statistics version 19. Since each woman had more than two data points, we needed a function that would group all the errors for each woman together without affecting the P-value of each variable. Hence, for the linear regression of different variables affecting the levels of TGF-β2 in the biopsied breast, we used STATA version 11() that had an option to "cluster" our samples as individual groups of data points. The regression was performed by Dr. Douglas Anderton at UMass who generously demonstrated and explained the process to me while he conducted the analysis on STATA.

RESULTS

Population demographics

Our sample population was not as diverse as we ideally would have hoped for. Most of our sample population consisted of 162 Caucasians (89.01%) whereas the rest comprised of 3 African Americans (1.65%), 4 Pacific Islanders/Asians (2.205), 12 Hispanics (6.59%) and 1 other (0.55%) (Figure 12). Table 2 shows the demographics for the variables that could have been confounding factors for the observed levels of TGF-β. The mean age of the women was 34.1±4.4 years while the mean age at the time of their first births was 30.3±4.7 years. On average women had 2±1 live births (including the baby that was being nursed at the time of donation) with an average BMI of 24.3±4.2. The maximum number of the women's first relatives (mother and/or sisters) affected by breast cancer was 2 and the lowest number was 0. Lastly, the average age of the baby at the time of milk donation was 262±218 days.

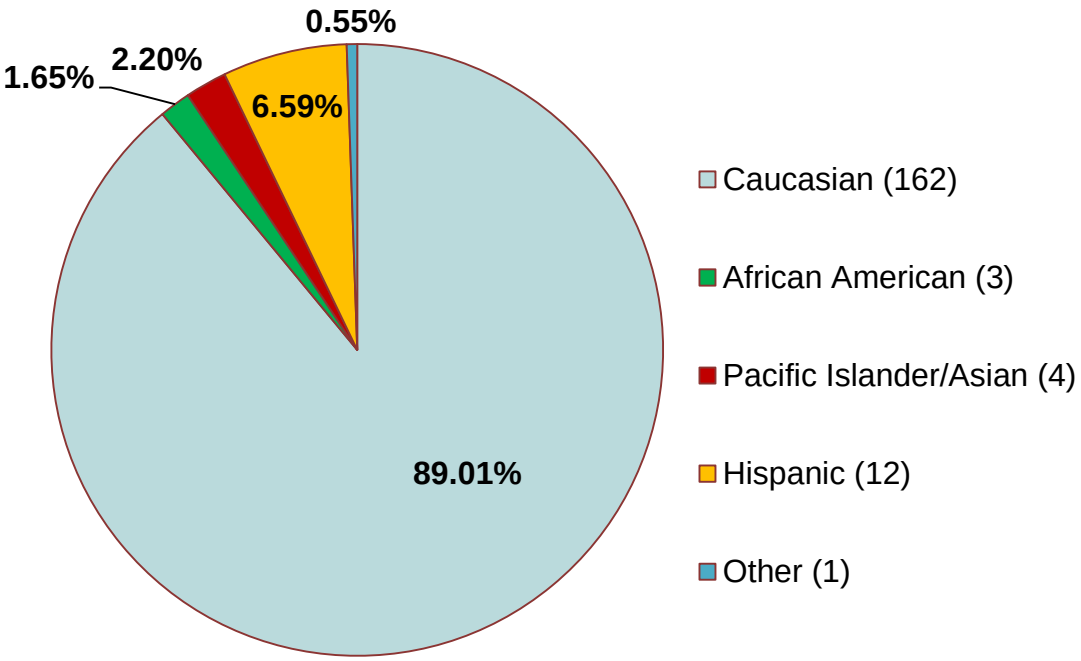

Figure 12. Population distribution by ethnicity. The pie chart shows the percentages of each ethnic group whereas the legend includes the description of each pie as well as the absolute numbers of women corresponding to each group in brackets.

Table 2. Demographics of the sample population by 6 different variables. All women did not have information for each variable; hence the different number of observations for each entry.

Variable	Observations	Mean	S.D.	Minimum	Maximum
mother's age (years)	182	34.1	4.4	25	52
mother's age at first birth (years)	182	30.3	4.7	18	52
number of live births per woman	182	2.0	1.0	1	5
mother's family history of breast cancer	158	0.32	0.48	0	2
mother's body mass index (BMI)	178	24.3	4.2	17.2	39.3
baby's age (days)	181	262	218	7	1,440

TGF-β2 concentration distribution

In order to get a general distribution of the TGF-β2 concentrations, we plotted scatterplots for the non-normalized and normalized TGF-β2 concentrations for all samples i.e. readings from both breasts for each woman (Figure 13 and 15). The non-normalized concentration ranged between 0-64,369 pg/mL whereas the normalized one ranged between 0-7,175 ng/g of total protein. We observed that since most values were so condensed near the baseline, a log transformation would be viable (Figure 14 and 16). However, when we performed the rest of our statistical analysis especially the regression, we realized that the log transformed data did not yield any different conclusions that the non-log transformed data. Hence, for the purpose of ease and comparison with other studies, we decided to work with the non-log transformed data.

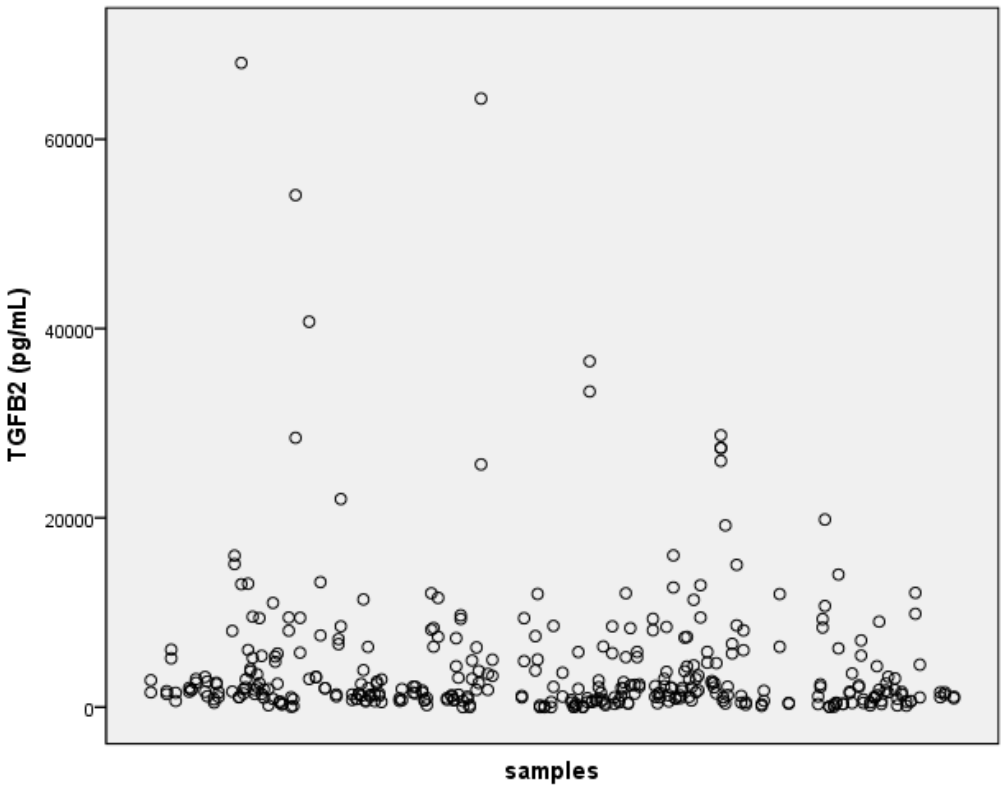

Figure 13. Scatterplot of non-normalized TGF-β2 concentrations for all milk samples (n=364). The highest TGF-β2 concentration observed was 64,369 pg/mL and the lowest concentration was 0 pg/mL.

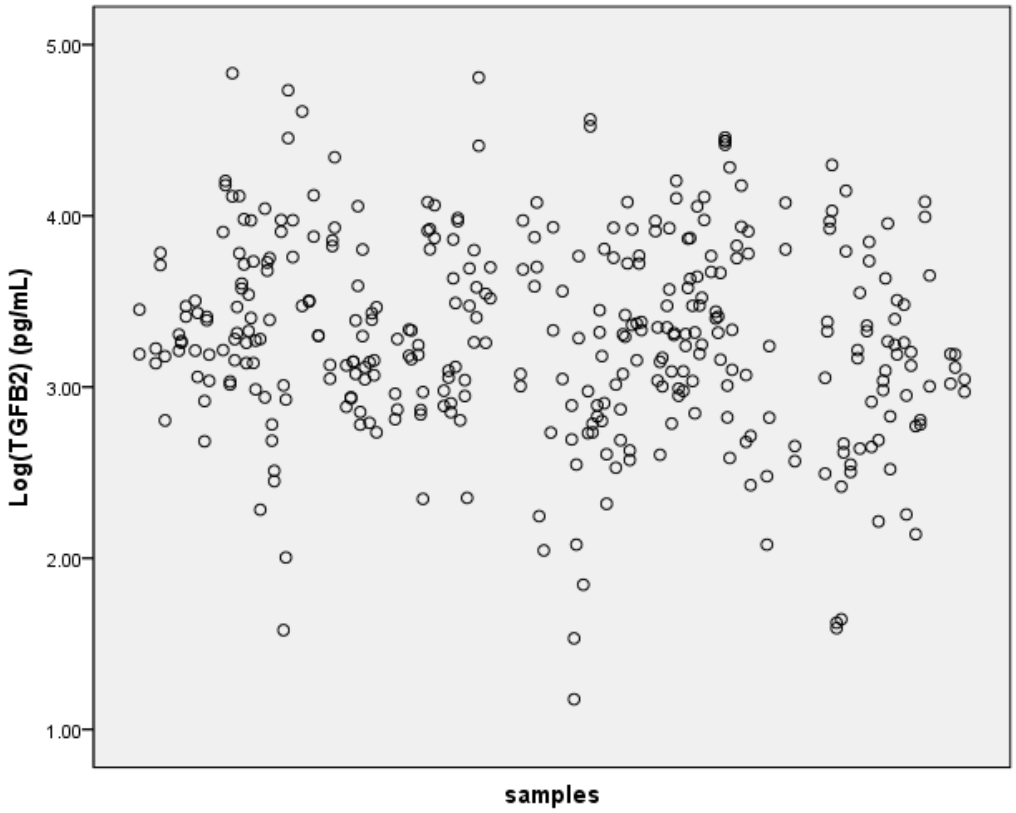

Figure 14. Scatterplot of log-transformed non-normalized TGF-β2 concentrations for all samples (n=366).

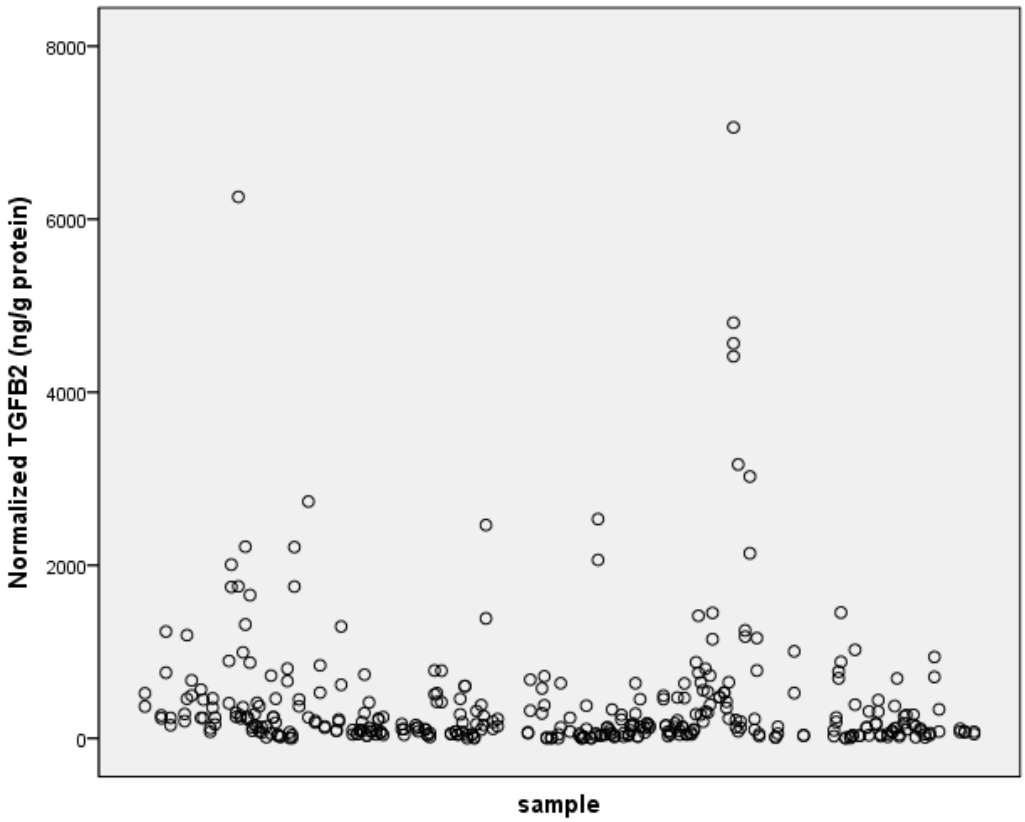

Figure 15. Scatterplot for normalized TGF-β2 concentrations for all samples (n=366). The highest data point was 7,175 ng/g protein while the lowest being 0 ng/g protein.

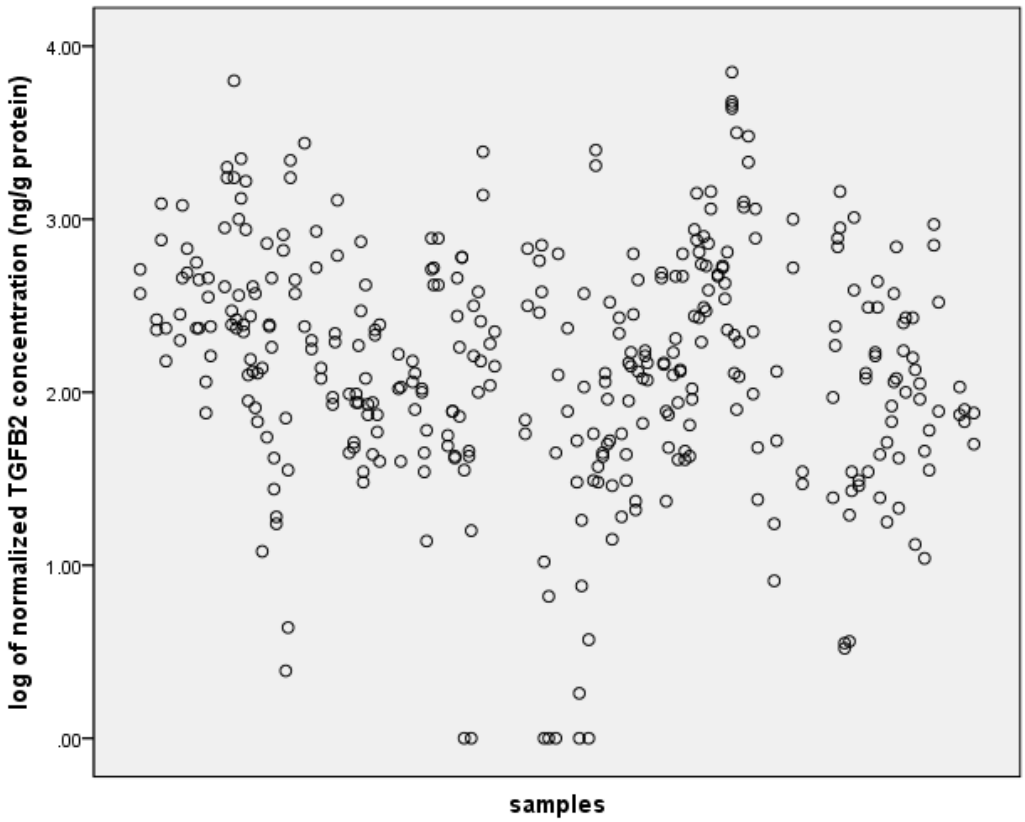

Figure 16. Scatterplot of log-transformed normalized TGF-β2 concentrations for all samples (n=366).

Expression of TGF-β2 in biopsied vs. non-biopsied breasts

We had hypothesized that TGF-β2 levels will be higher in the biopsied breast as opposed to the non-biopsied breast; however, our observations served to be on the contrary (Figure 17 and Table 3). The levels of non-normalized TGF-β show that there was more TGF-β detected in non-biopsied breast (5,030±9,632 pg/mL) in comparison to the biopsied breast (3,935±5,449 pg/mL). The differences in the normalized TGF-β2 levels were even more subtle (Figure 18 and Table 4). The TGF-β2 levels were slightly higher in the non-biopsied breast (421±703 ng/g protein) as compared to the biopsied breast (398±846 ng/g protein).

As displayed, the standard deviations were so high for both of these categories that none of these observed differences were significant. Not to mention that ANOVA could not be conducted on this variable alone because there were other variables (Table 2) that affected the TGF-β2 concentrations as well.

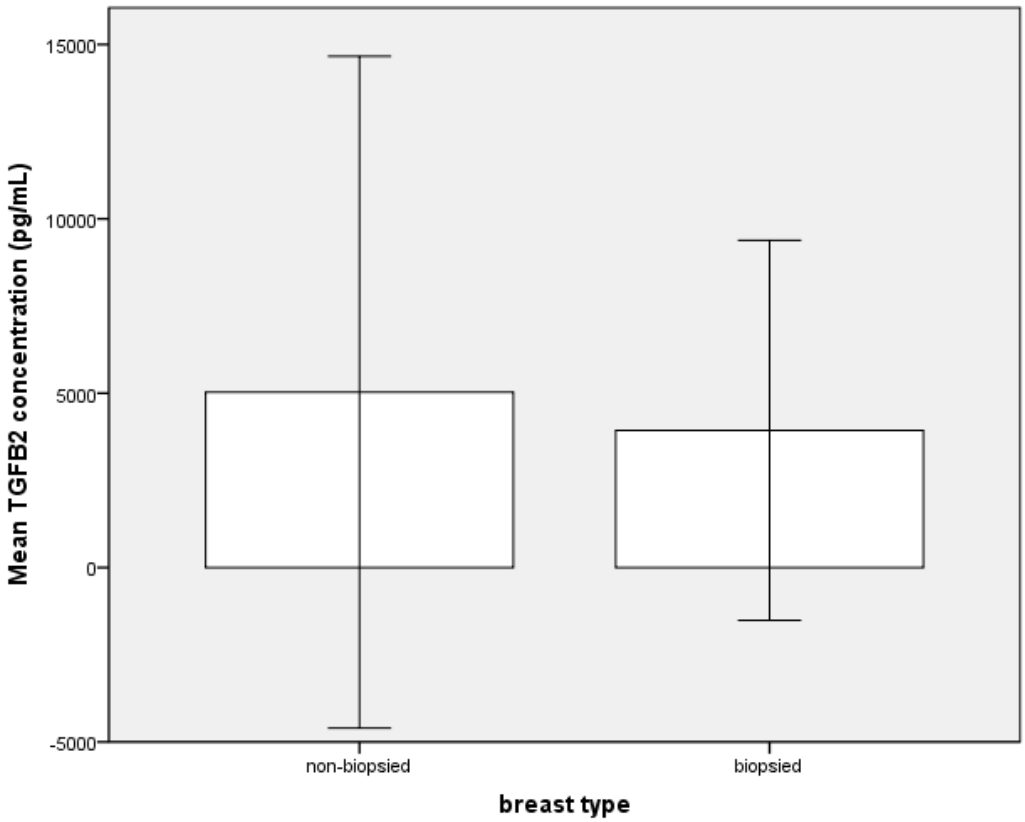

Figure 17. Average levels of TGF-β2 concentration (pg/mL) for non-biopsied vs. biopsied breast. The error bars represent ±1SD.

Table 3. Non-normalized TGF-β2 levels (pg/mL) for non-biopsied and biopsied breasts for all women. A few women had biopsies in both breasts, hence distributing N unequally.

		TGF-β2 concentration (pg/mL)		
breast type	**N**	**Mean ± S.D.**	**Minimum**	**Maximum**
non-biopsied	176	5,030 ± 9,633	0	64,369
biopsied	188	3,935 ± 5,447	0	34,938

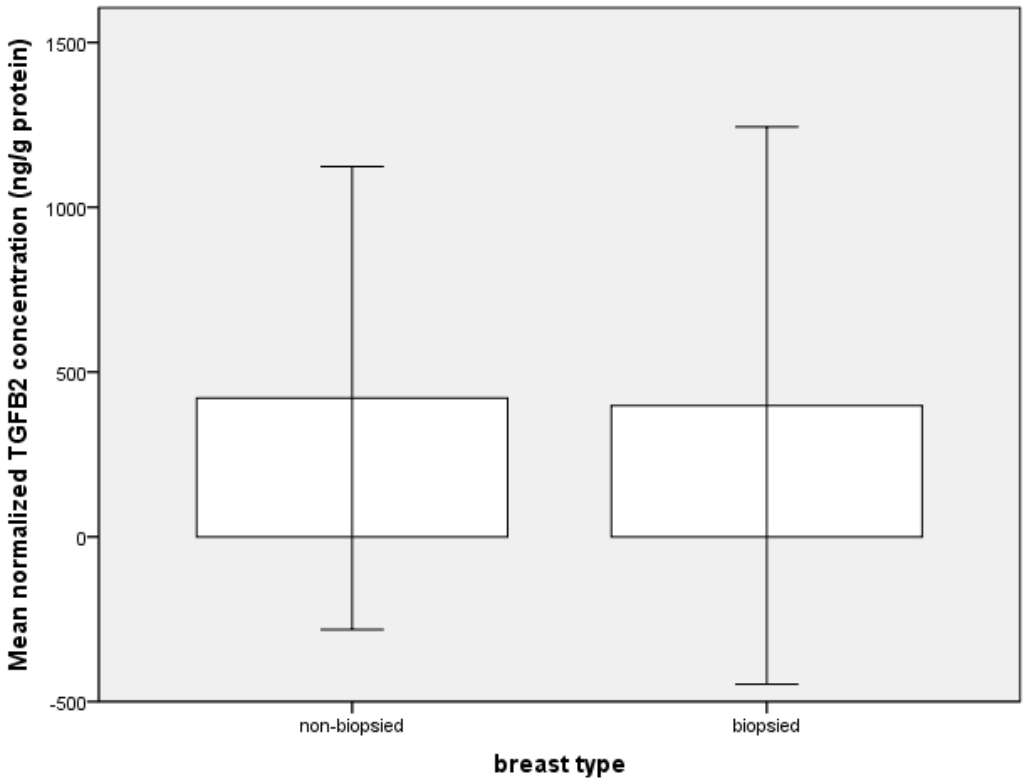

Figure 18. Average levels of normalized TGF-β2 concentration (ng/g protein) for non-biopsied vs. biopsied breast. The error bars respresent ±1SD.

Table 4. Normalized TGF-β2 levels (ng/g protein) for non-biopsied and biopsied breasts for all women.

		normalized TGF-β2 concentration (ng/g protein)		
Breast type	**N**	**Mean ± S.D.**	**Minimum**	**Maximum**
non-biopsied	176	421 ± 702	0	5,918
biopsied	188	398 ± 846	0	7,175

Expression of TGF-β2 across biopsy categories

We first compared the TGF-β2 levels for both breasts across the four biopsy categories. TGF-β2 levels were lowest (4,131±7,588 pg/mL) for biopsy category 1 (non-proliferative lesions) in the non-normalized data (Figure 19 and Table 5) whereas for the normalized data (Figure 20 and Table 6), they were the lowest (312±470 ng/g protein) for biopsy category 2 (proliferative lesions without atypia). TGF-β2 levels were highest for biopsy category 4 (cancerous lesions) for both normalized (9,971±10,960 pg/mL) and normalized (1,601±2,256 ng/g protein) data.

We also wanted to see if the TGF-β2 levels followed a progression from category 1 to 4 in the biopsied breasts specifically. However, the trends in the data were similar to those when the data was analyzed for both breasts. TGF-β2 levels were lowest for category 1 in non-normalized data (Figure 21 and Table 7) and category 2 in normalized data (Figure 22 and Table 8). The TGF-β2 levels were highest for category 4 in both types of data as well.

Because the distribution of data points was so uneven in the four categories, regression analysis for the four individual categories could not be performed to evaluate the significance of differences in each biopsy category.

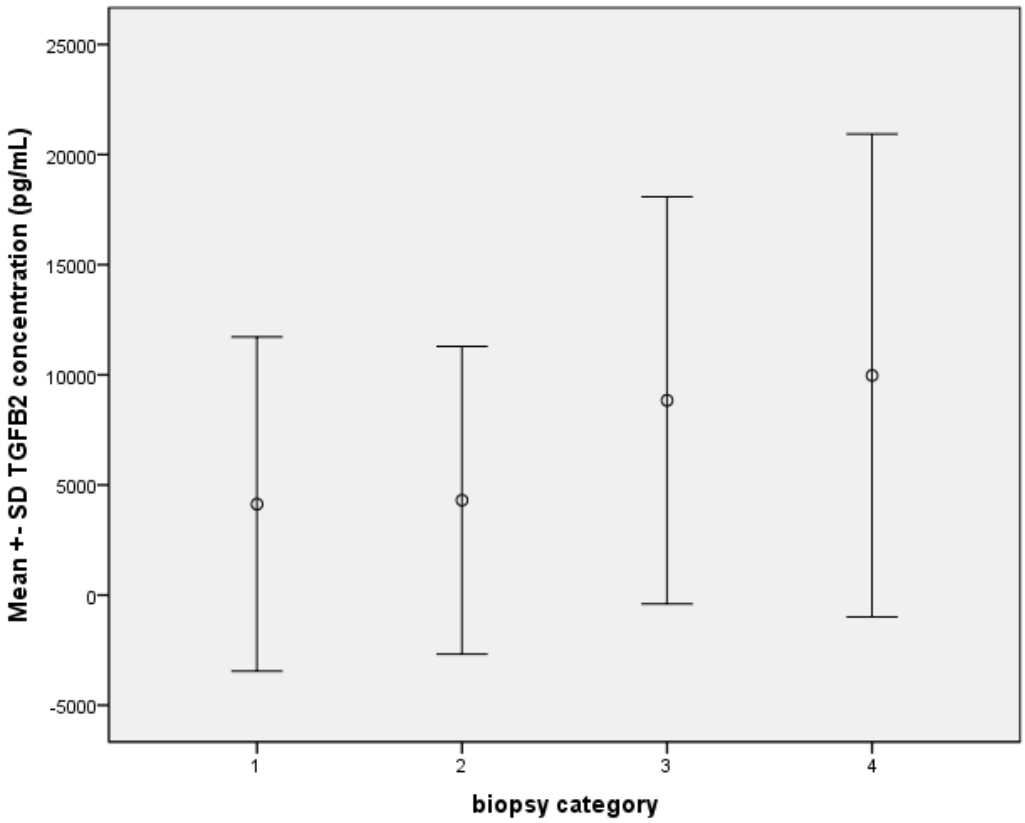

Figure 19. Mean±SD of TGF-β2 concentrations (pg/mL) across four biopsy categories for both breasts of the sample population (n=364).
1 = non-proliferative lesions (n=292), 2 = proliferative lesions without atypia(n=52), 3 = proliferative lesions with atypia (n=4), and 4 = cancerous lesions(n=16).

Table 5. Non-normalized TGF-β2 concentrations (pg/mL) across four biopsy categories for both breasts in the sample population (n=364).

	TGF-β2 concentration (pg/mL)		
biopsy category	**Mean**	**S.D.**	**N**
1	4,131	7,588	292
2	4,307	6,984	52
3	8,842	9,238	4
4	9,971	10,960	16
			364

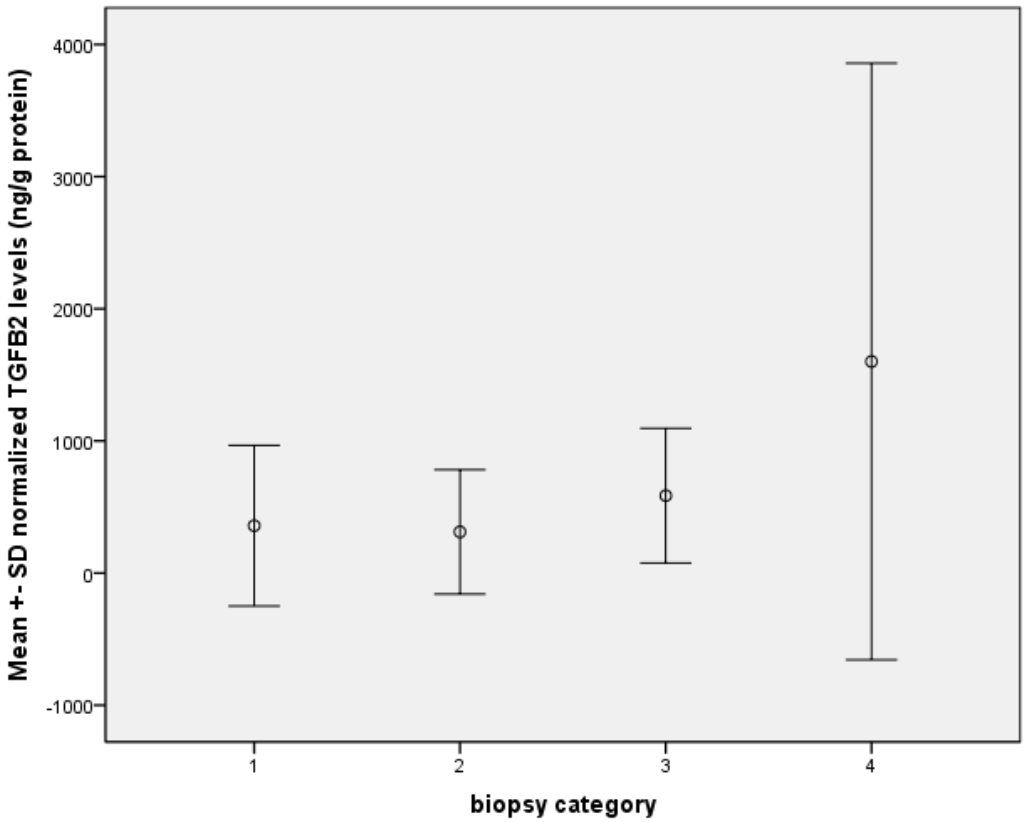

Figure 20. Mean±SD of normalized TGF-β2 levels (ng/g protein) across four biopsy categories for both breasts of the sample population (n=364).
1 = non-proliferative lesions (n=292), 2 = proliferative lesions without atypia(n=52), 3 = proliferative lesions with atypia (n=4), and 4 = cancerous lesions(n=16).

Table 6. Normalized TGF-β2 levels (ng/g protein) across four biopsy categories for both breasts in the sample population (n=364).

	normalized TGF-β2 levels (ng/g protein)		
biopsy category	**Mean**	**S.D.**	**N**
1	359	608	292
2	312	470	52
3	586	510	4
4	1,601	2,257	16
			364

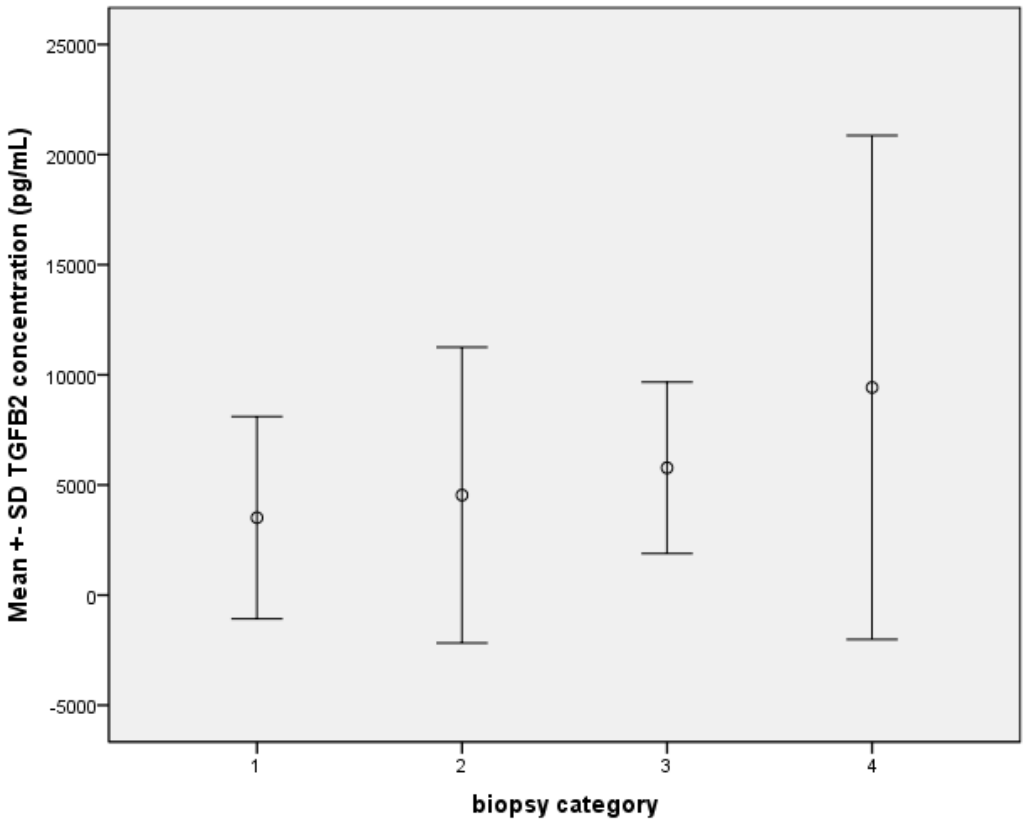

Figure 21. Mean±SD of TGF-β2 concentrations (pg/mL) across four biopsy categories for the biopsied breast only (n=188).
1 = non-proliferative lesions (n=151), 2 = proliferative lesions without atypia (n=27), 3 = proliferative lesions with atypia (n=2) , and 4 = cancerous lesions (n=2).

Table 7. Non-normalized TGF-β2 concentrations (pg/mL) across four biopsy categories for the biopsied breast only (n=188).

	TGF-β2 concentration (pg/mL)		
biopsy category	**Mean**	**S.D.**	**N**
1	3,512	4,591	151
2	4,540	6,713	27
3	5,781	3,893	2
4	9,427	11,437	8
			188

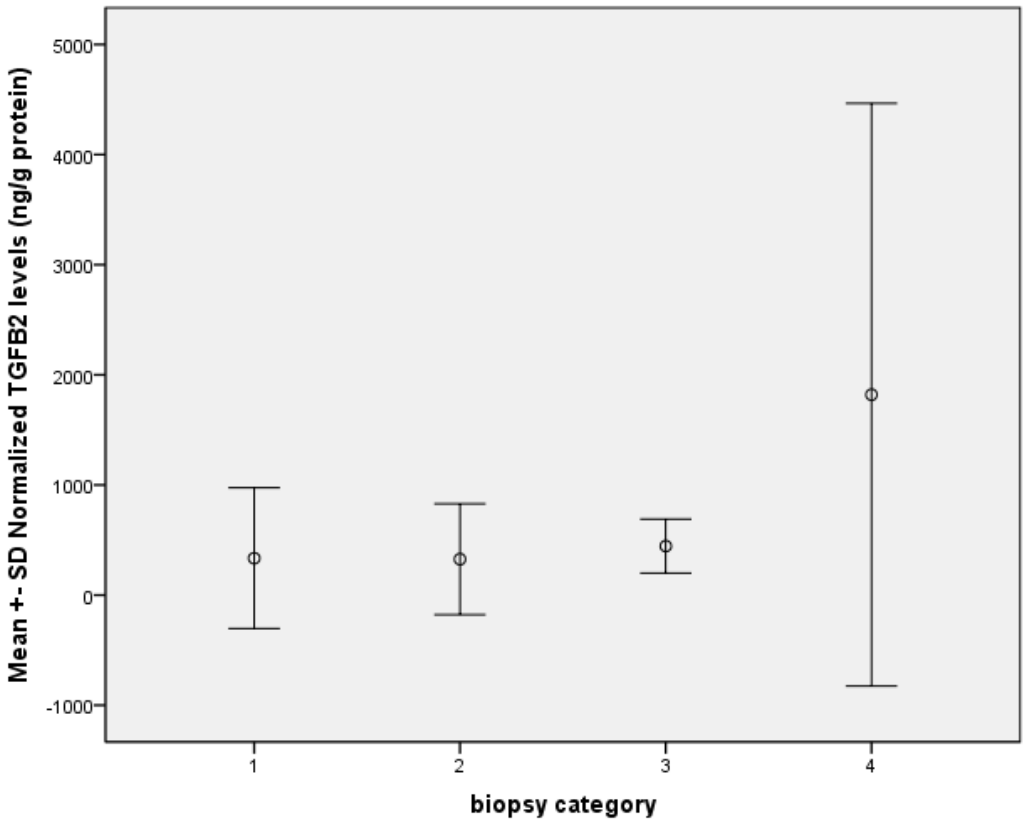

Figure 22. Mean±SD of normalized TGF-β2 levels (ng/g protein) across four biopsy categories for the biopsied breast only (n=188). 1 = non-proliferative lesions (n=151), 2 = proliferative lesions without atypia (n=27), 3 = proliferative lesions with atypia (n=2) , and 4 = cancerous lesions (n=2).

Table 8. Normalized TGF-β2 levels (ng/g protein) across four biopsy categories for the biopsied breast only (n=188).

	normalized TGF-β2 levels (ng/g protein)		
biopsy category	**Mean**	**S.D.**	**N**
1	335	639	151
2	326	504	27
3	445	245	2
4	1,819	2,645	8
			188

Variables affecting expression of TGF-β2

Last but not the least, with the help of Dr. Anderton we were able to conduct regression analysis for differences in biopsied breast while controlling for other covariates. Since we were only determining statistical significance, for the purpose of this paper we will only include the normalized data (Table 9) since it more accurately depicts the changes in TGF-β2 levels (as a proportion of total protein) in each sample. Moreover, the regression conducted on non-normalized data did not yield any different results; hence, it gave us another reason to not include it in this paper.

We clustered different readings for each woman and then performed the regression in Table 9. According to the table, TGF-β2 levels fell from non-biopsied to biopsied breast as we discussed earlier. Secondly, as the age of women increased, TGF-β2 levels fell again (Figure 23), however, they rose with the rising age at first birth (Figure 24). Baby's age had a positive correlation too with the levels of TGF-β2 i.e. the older the baby was at the time of milk donation, the higher the TGF-β2 levels were (Figure 25). More live births also showed the same correlation (Figure 26) while being Caucasian raised the women's chances of having higher TGF-β2 levels (Figure 27). Increased family history yielded lower TGF-β2 levels while increase in BMI (Figure 28) led to an increase of TGF-β2 expression. Out of all of these variables Caucasian was a significant contributor to the differences in TGF-β2 levels, $F(8,174)=1.29$, $p<0.05$.

Table 9. Linear regression for differences in TGF-β2 levels (ng/g protein) in biopsied breast while controlling for other covariates (n=352). [F(8,174)=1.29]

Variable	Coefficient	Standard Error*	t	P>|t|	95% Confidence Interval	
Biopsied breast	-51.4	42.4	-1.21	0.227	-135	32.2
Age	-54.3	35.3	-1.54	0.125	-124	15.3
Age at first birth	73.2	40.7	1.80	0.074	-7.1	153
Baby's age	0.19	0.21	0.93	0.356	-0.22	0.61
# of live births	298	173	1.72	0.086	43.1	639
Caucasian	204	88.2	2.31	**0.022**	29.9	378
Family history	-10.7	132	-0.08	0.935	272	250
BMI	10.7	13.5	0.80	0.426	-158	37.3

* Standard error adjusted for 175 clusters in sample population

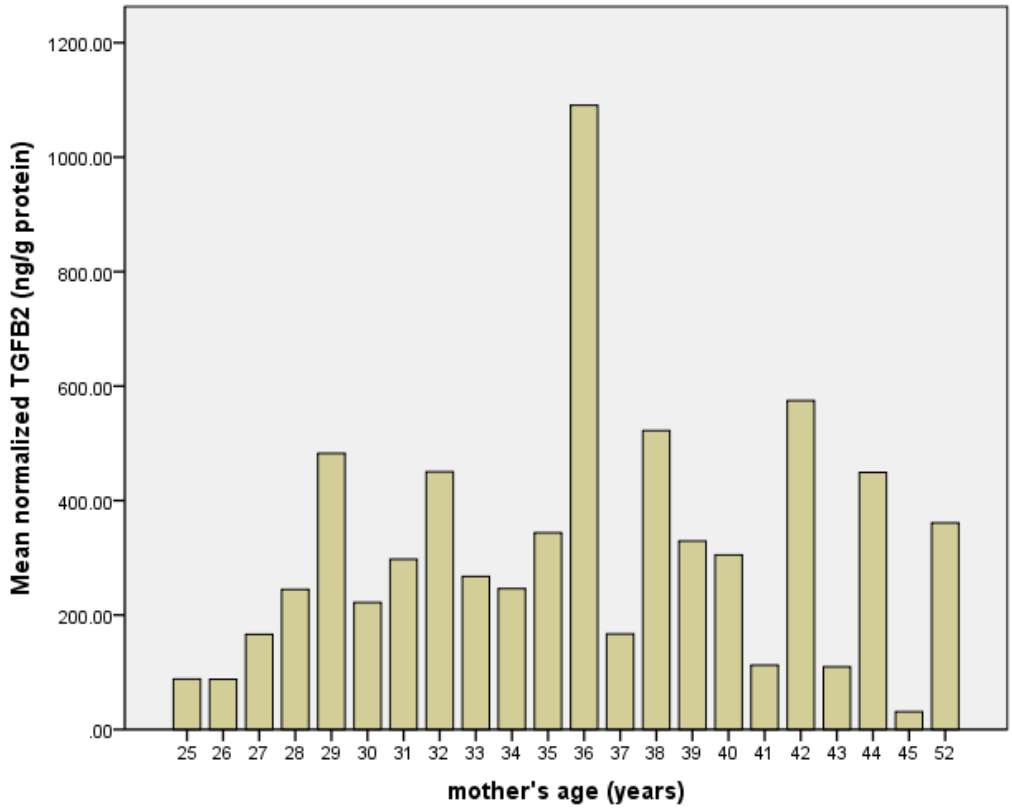

Figure 23. Distribution of normalized TGF-β2 levels by mother's age at the time of milk donation.

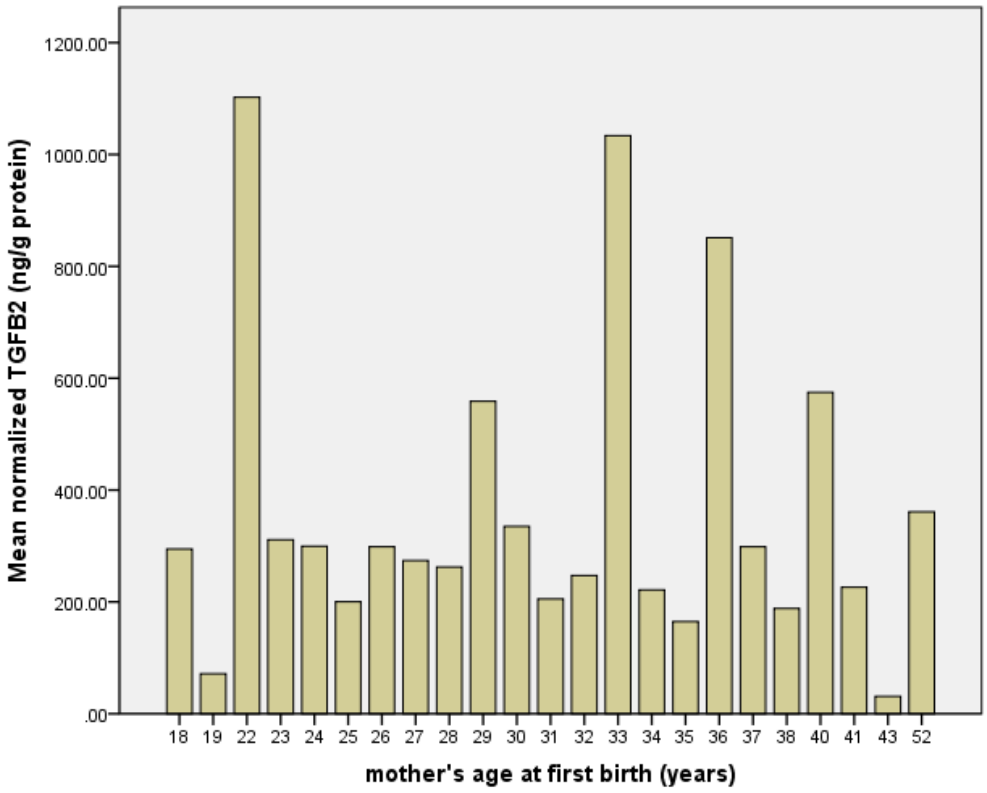

Figure 24. Distribution of normalized TGF-β2 by mother's age at first birth.

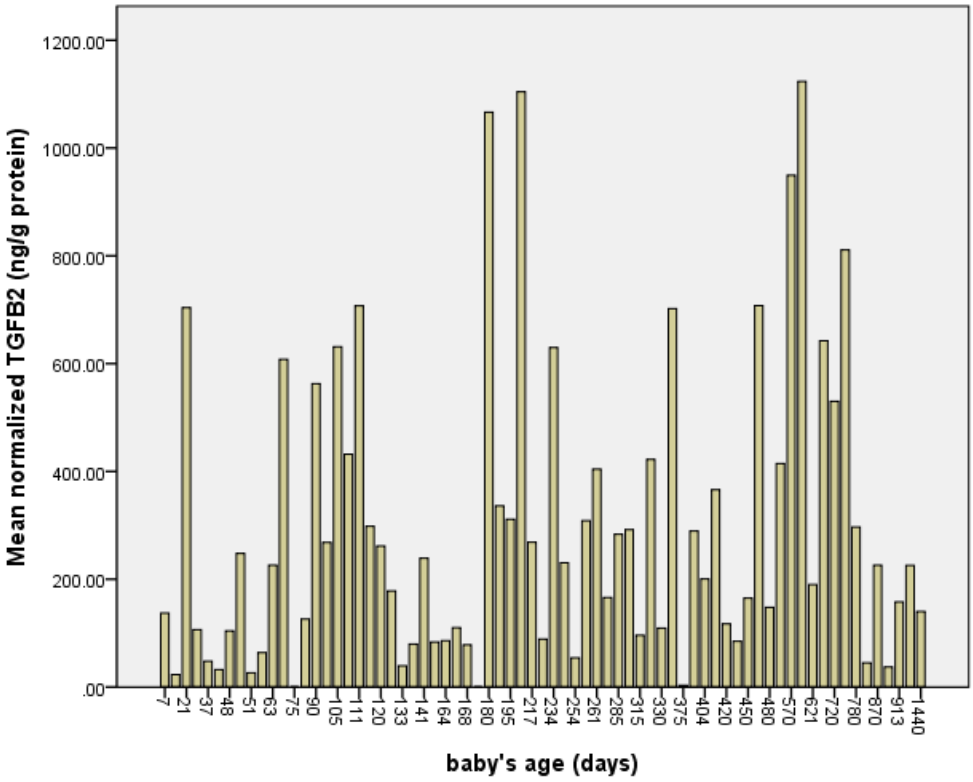

Figure 25. Distribution of normalized TGF-β2 levels by baby's age at the time of milk donation.

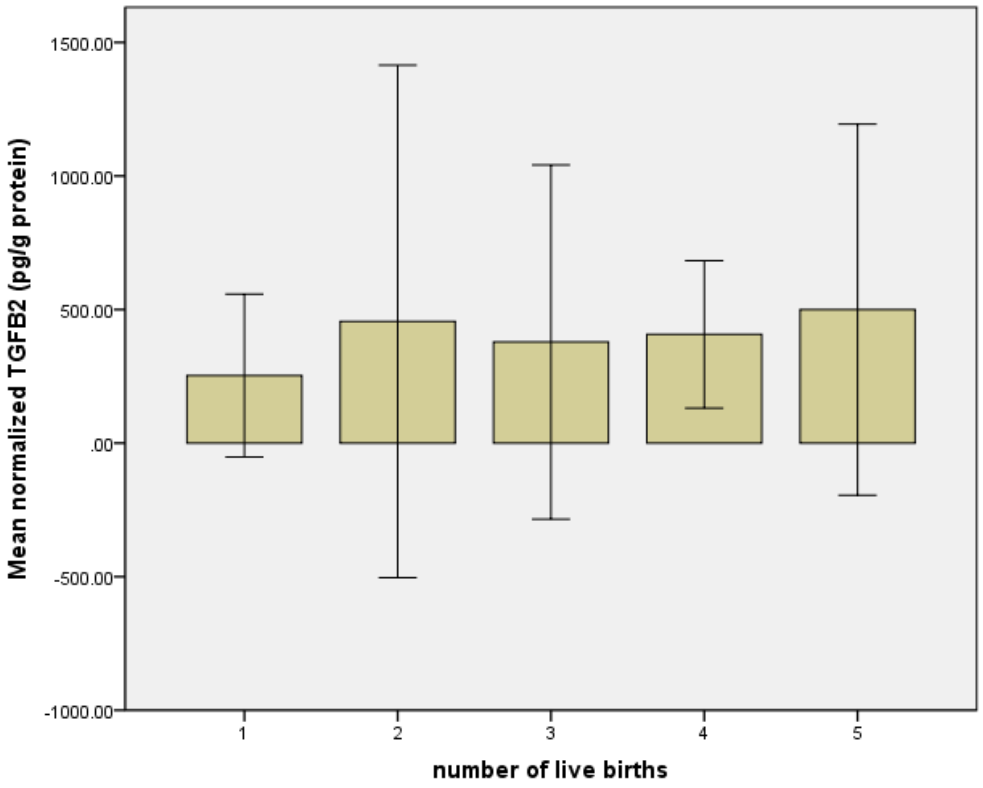

Figure 26. Distribution of normalized TGF-β2 levels by the number of live births given by a mother.

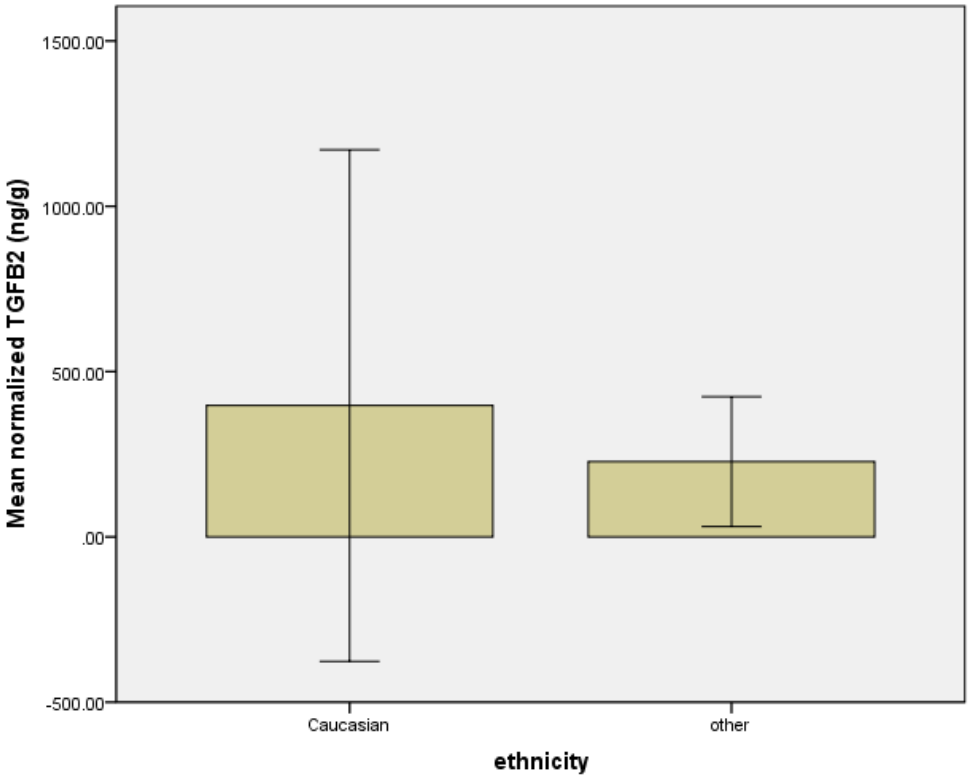

Figure 27. Levels of TGF-β2 in Caucasian and non-Caucasian mothers.

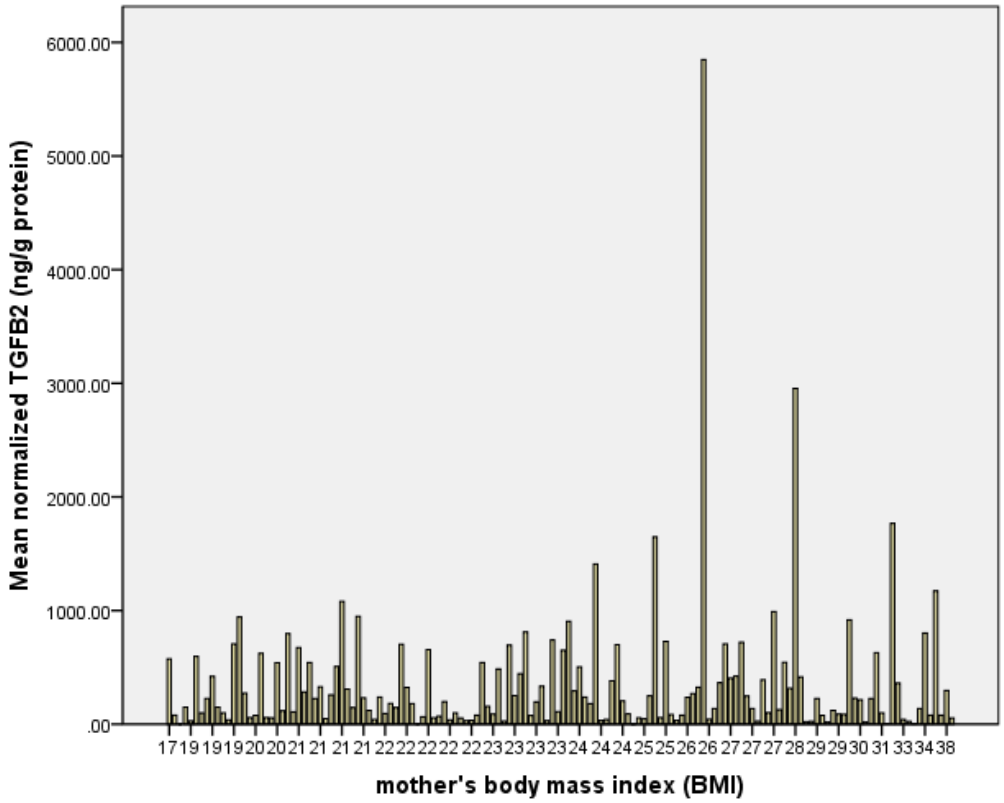

Figure 28. Distribution of TGF-β2 levels by the mother's body mass index (BMI) at the time of milk donation.

DISCUSSION

Multiple studies as reviewed by Oddy et al. (2010) have examined TGF-β in breast milk due to its immunological, atopic and developmental roles in newborn infants and young children. A handful of studies such as the one done by Sauter et al. (2007) have also examined levels of cytokines in nipple aspirate fluid and nipple discharge collected prior to excisional breast biopsies in women who were not necessarily pregnant. However, our project is a novel project at many levels because our study is the first study to examine levels in TGF-β2 in breast milk under the umbrella of breast cancer. The Arcaro lab has been involved in examining expression levels of proteins TGF-β2 in relation to methylation in epithelial cells in breast milk (Browne et al., 2011; Qin et al., 2012; Sauter et al., 2012), but this is our first study that has used such a large sample size and has focused only on the TGF-β2 ligand itself.

Initially when we began the study, we did not really have a specific range of TGF-β2 concentrations narrowed down. One of the highest ranges of TGF-β2 concentration was reported by Hawkes et al. (2002) as 281 – 57,935 pg/mL in one of their population subgroups. Bottcher et al. (2000) reported a median concentration 786 pg/mL (<250 – 11,696 pg/mL) of TGF-β2 in milk after one month of birth in a sample of 49 women. Another study performed by Kalliomäki et al. (1999) reported a mean concentration of 1,644 (593 – 2,697) pg/mL in 47

women at 3 months after giving birth. As we discussed earlier, TGF-β has an immunoprotective role in infants. The study performed by Rautava et al. (2002) compared levels of TGF-β2 in women receiving probiotics (n=30) with women who did not (n=32) at the time when their babies were 3 months old. Women who received probiotics had higher (2,855 pg/mL [95% CI, 1624-4146]) TGF-β2 levels than women who received placebo (1,340 pg/mL [95% CI, 978-1702]). Keeping in mind these wide ranges, our data did correspond to the multiple findings as the range for our non-normalized TGF-β2 concentrations was also between 0 - 64,369 pg/mL. It's surprising how most studies performed up to date did not perform total protein analysis which limits us to compare our normalized data with the majority of them.

Higher expression of TGF-β2 in non-biopsied breasts

We hypothesized that TGF-β2 would be higher in biopsied breast as opposed to non-biopsied breast, but we saw quite opposite of that in our study. More TGF-β2 was detected in non-biopsied breast (5,030±9,632 pg/mL) in comparison to the biopsied breast (3,935±5,449 pg/mL) in the non-normalized data and the results followed the same trend in the normalized data: 421±703 ng/g protein in the non-biopsied breast in comparison to 398±846 ng/g protein in biopsied breast. The regression analysis did not conclude these results as significant as well.

In a previous study that has been submitted for publication, the Arcaro lab analyzed TGF-β2 levels in milk in 5 women who had cancer in one of their breasts (Sauter et al., 2012). The non-normalized data showed that TGF-β2 levels were higher for these women in the breast with the cancer/biopsy (mean 23,210 pg/mL) when compared to the non-cancerous/non-biopsied breast (mean 19,620).

Since TGF-β2 has a tumor-suppressive role (Massagué, 2008), it could be postulated as the reason why TGF-β2 levels are higher in the non-biopsied breast. Perhaps the high level of TGF-β2 is preventing any possible advent of cancerous initiation in the non-biopsied breast.

Cancerous lesions had the highest expression of TGF-β

We had predicted to see the highest TGF-β2 expression levels in biopsy category 4 and we did observe higher levels in both the non-normalized and normalized data. However, in absolute terms, TGF-β2 levels were lower for the breasts with cancerous lesions (9,427 pg/mL) than the results reported by Sauter et al. (2012) when they used five samples instead (23,210 pg/mL). Even though our sample size was not very different from theirs (8 compared to 5 women), individual variability of the samples themselves could have added to the differences in the observed values. Plus, this time we had such an uneven distribution between the observations within each breast category, that statistical significance could not be determined to verify our results.

These results are explained by the postulation that as the lump progresses towards breast cancer development, the TGF-β2 levels will rise due to the active role of TGF-β2 in cancer progression and metastases (Wakefield, Yang and Dukhanina, 2000; Massagué, 2008).

Other variables affecting expression of TGF-β2

We have already discussed the effects of biopsy on our data in the previous sections; however, there were interesting confounding factors that affected our results. All except one variable (Caucasian) were not significant but for the purpose of discussion, we will discuss what the regression analysis suggested.

Mother's age: The older the woman is, the higher her risk of breast cancer (Colditz and Rosner, 2000). With increasing age of mothers, we observed lower levels of TGF-β2. Considering that the standard error was so high (35.3 years) especially when the ages of our sample population varied between 25 and 52 years old, our results were not significant.

Mother's age at first birth: More importantly, women who have their first pregnancy at the age of 30 or higher have a higher risk of developing breast cancer than those who are younger (Kelsey, Gammon and John, 1993). Interestingly, our data suggests the same as well because as the woman's age at first birth increased, TGF-β2 levels rose as well. Qin et al. (2012) (in collaboration with Dr. Arcaro) measured TGF-β2 levels for women at different ages at first full term pregnancy (FFTP). They observed that TFG-β2 expression rose from baseline (within 10 days of the initiation of lactation) to weaning in women under the age of 26 years old. However, TGF-β2 expression decreased from baseline until 2 months but increased after that until weaning in women

between the ages of 26-34 years. TGF-β2 levels were higher at all stages for women above the age of 34 years old. However, the distribution of our data is quite wide and it's difficult to suggest the exact categorization of TGF-β2 according to mother's age at first birth.

Baby's age: On the other hand, we wanted to determine if length of nursing i.e. baby's age influenced TGF-β2 levels in breast milk because previous studies have shown that longer duration of nursing is associated with lower breast cancer risk (Daniilidis et al., 2010). Similar to Qin et al. (2012) we also observed a rise in TGF-β2 levels with the length of nursing. Hawkes et al. (1999) measured levels of TGF-β2 for 3 months after delivery. They observed that TGF-β2 levels from week 4 or 28 days (1,902±238 pg/mL) to week 12 or 84 days (2,020±496 pg/mL). We observe similar results with TGF-β2 levels lower at day 28 and then higher on day 84. However, our data is very spread out because the youngest baby in our sample was 7 days old as opposed to the oldest one being almost 4 years old (1,440 days). Thus, our data had large variability and hence, the absence of significance.

Number of live births: The risk of breast cancer declines with the number of children born. Women who have given birth to five or more children have half of the risk of women who have not given birth (Lambe et al., 1996). Our regression analysis demonstrates the same relationship as well. Higher births represent

higher levels of TGF-β2 and perhaps this indicates a tumor suppressive role of TGF-β2.

Caucasian vs. non-Caucasian: Our data indicates that being a Caucasian is associated with higher levels of TGF-β2 in breast milk. However, since the majority of the women in our sample are Caucasian (162 out of 182) it is possible that this statistically significant result is due to chance.

Family history: Interestingly, women in our study had lower levels of TGF-β2 as the number of women with breast cancer in their family increased. Having a family history of breast cancer predisposes a woman to a higher breast cancer risk (Colditz and Rosner, 2000). The lower levels of TGF-β2 associated with a family history of breast cancer could reflect the loss of the tumor-suppressive role of TGF-β2.

Body mass index (BMI): It has been suggested that obesity in premenopausal women is associated with reduced risk of breast cancer (Huang et al., 1997). Obesity upregulates the expression of certain growth factors such as insulin growth factor (IGF) (Morimoto et al., 2002) explaining why we witness higher levels of TGF-β2 in our sample population as well. Hence, the higher TGF-β2 expression levels in women with a higher BMI in our study could be associated with a reduced risk of breast cancer.

CONCLUSION

TGF-β2 levels in our study were influenced by several variables that competed with one another. Even though we witnessed higher levels of TGF-β2 in cancerous lesions, a greater sample size for each variable is needed for us to draw further conclusion about the relationship of TGF-β2 in milk and breast cancer. Since TGF-β1 and TGF-β3 play such a crucial role in breast involution and the onset of PABC (Flanders and Wakefield, 2009), future studies should analyze them to determine the collective role of TGF-βs in breast cancer in this population as well as others.

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