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Obesity-Induced Inflammation in Human Mammary Tissue: A Potential Microenvironment Favorable to the Development of Postmenopausal Breast Cancer Via the Wnt Signaling Pathway

A Thesis Presented

by

AGATHE ROUBERT

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

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Nutrition

Obesity-Induced Inflammation in Human Mammary Tissue: A Potential Microenvironment Favorable to the Development of Postmenopausal Breast Cancer Via the Wnt Signaling Pathway

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ABSTRACT

OBESITY-INDUCED INFLAMMATION IN HUMAN MAMMARY TISSUE: A POTENTIAL MICROENVIRONMENT FAVORABLE TO THE DEVELOPMENT OF POSTMENOPAUSAL BREAST CANCER VIA THE WNT SIGNALING PATHWAY

SEPTEMBER 2015

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Directed by: Dr. Zhenhua Liu

In the United States, over one third of adult women are obese, and one in eight women will be diagnosed with breast cancer in their lifetime. Obesity has been shown to be a risk factor for postmenopausal breast cancer and is associated with increased aggressiveness and poor prognosis regardless of menopausal status. However, the mechanisms involved in the relationship between obesity and breast cancer are still not fully understood. Wnt signaling is often elevated in breast tumors (~60%) and is suspected to play a key role in cancer development. It has been shown that inflammatory cytokines, such as TNF-α, IL-1β, IFNγ, are potential mediators in the regulation of Wnt-signaling. We hypothesize that the low-grade inflammatory state associated with obesity is present in human mammary tissue, stimulates Wnt activity, and thereby leads to the development of breast cancer. In this project, we propose to 1) characterize the inflammatory cytokine profile, including IFN-γ, IL-1β, IL-2, IL-6, IL-8, and TNF-α, in the mammary tissue of normal weight, overweight, and obese

postmenopausal women using a high performance electrochemiluminescence immunoassay; 2) determine the influences of the obesity-induced pro-inflammatory cytokines on Wnt-signaling by examining gene expression of seven Wnt-signaling target genes using real-time PCR; and 3) define the causality between TNF-α, one of the mot critical inflammatory cytokines, and Wnt signaling by measuring the gene expression of the Wnt targets in samples from normal to overweight and obese postmenopausal women treated with anti-TNF-α antibody or TNF-α recombinant protein respectively. We expect to define a novel mechanism that obesity mediates the development of postmenopausal breast via inflammation-driven Wnt signaling.

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CHAPTER 1

INTRODUCTION

There is mounting evidence that suggests that Wnt may play a critical role in the link between obesity-induced inflammation and the development of breast cancer, but the exact mechanisms involved are still unknown. The relationship between proinflammatory cytokines in the breasts of obese women, Wnt activity, and breast cancer risk has not yet been investigated. We aim to characterize the inflammatory cytokine profile and level of Wnt signaling activity in the breasts of obese women, and determine whether TNF-α neutralization results in reduced Wnt activation. Upon completion of the study, we should have gained new knowledge on the relationship between obesity and Wnt activity in the breasts, and the results may uncover a new area worth targeting by therapeutics for the treatment of breast cancer.

It is now well established that obesity is a risk factor for the development of postmenopausal breast cancer (Carmichael, 2006), and excess adiposity is associated with increased tumor size and poor prognosis regardless of menopausal status (Berdaz et al., 2004). The Million Women Study revealed that an increase of 10 units in BMI is associated with a relative risk of development of postmenopausal breast cancer of 1.40 (Reeves et al., 2007). The EPIC study revealed that weight gain during adulthood is also a risk factor for the development of post-menopausal breast cancer (Lahmann et al., 2005). Among non-users of hormone replacement therapy (HRT), the relative risk of breast cancer increased by 22 and 53% in women gaining 2-15kg and >15kg, respectively, compared with women with stable weight. With 36.1% of American women being obese and 65.8% being overweight or obese (Ogden et al., 2014), the relationship between obesity and breast cancer is a source of concern, and could

explain why 1 in 8 American women (12.3%) will be diagnosed with breast cancer in their lifetime (National Cancer Institute, 2015).

Because the majority of breast tumors express hormone receptors (estrogen and/or progesterone) (Howlader et al., 2013), sex hormones and especially estrogen, whose production is elevated in obese postmenopausal women, are often regarded as key factors in breast tumor development. However, the chronic inflammatory state associated with obesity has grown into a prime suspect to explain the link between obesity and breast cancer development (Rose & Vona-Davis, 2013). Indeed, elevated levels of macrophages and crown-like structures (CLS), characteristic of obesity, are found in the fat tissue of obese individuals and result in an increased production of pro-inflammatory cytokines by inflamed adipocytes (McArdle et al., 2013). CLS have been observed in the breast tissue of obese women with breast cancers (Morris et al., 2011), and those cytokines are suspected to activate several pro-inflammatory and pro-tumorigenic pathways (Gilbert & Slingerland, 2013).

The Wnt signaling pathway, which results in the translocation of β-catenin in the nucleus and the activation of target genes involved in cell proliferation, polarity, apoptosis and differentiation, is commonly upregulated in breast cancer (Howe & Brown, 2004). Activated β-catenin correlates with increased *cyclinD1* expression, a Wnt target and oncogene, and is associated with poor prognosis (Lin et al., 2000). Some evidence show that Wnt signaling may be activated by pro-inflammatory cytokines. Our laboratory previously showed that the elevated levels of TNF-α present in the colon of obese mice modulate Wnt activity (Liu et al., 2012). Other studies have shown that IL-6 and TNF-α both influence the Wnt pathway (Cawthorn et al., 2007; Gustafon & Smith, 2006; Isakson et al., 2009). Wnt signaling may thus

be an intermediate in the interaction between obesity-induced inflammation and the production of cytokines, and the development of breast cancer.

CHAPTER 2

LITERATURE REVIEW

A. Obesity-induced inflammation and pro-inflammatory cytokines

It is now well accepted that long term over-nutrition acts as a chronic stressor, and obesity is a state of chronic low-grade inflammation, characterized by the presence of several pro-inflammatory factors. Levels of C-reactive protein (CRP), an acute-phase protein and unspecific marker for inflammation, are elevated in obese patients and correlate with the amount of adipose tissue present in the body (Ramos-Nino, 2013). This inflammatory environment is suspected to promote the development of many obesity-associated chronic diseases, including several types of cancers. Tumor initiation, promotion and progression are encouraged by overexpression of inflammatory mediators. Ramos-Nino (2013) also reports that anti-inflammatory drugs have been shown to improve cancer incidence and progression, thus further supporting the hypothesis of a link between inflammation and cancer development.

Although adipose tissues are an important source of several pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, the interferon (IFN) family, etc. (Clément & Vignes, 2009), not all adipocytes have the same phenotype. Obesity is characterized by the presence of excess white adipose tissue (WAT), especially in the visceral area (central obesity) which is more predictive of obesity-associated comorbidity and mortality than subcutaneous adiposity (Ramos-Nino, 2013), and is characterized by a more inflammatory phenotype (Balistreri, Caruso, & Candore, 2010) and increased number of macrophages in adipose tissue (van Kruijsdijk, van der Wall, & Visseren, 2009). The

ATTICA study in Greece revealed that central obesity was associated with elevated serum levels of CRP, TNF-α and IL-6 (53%, 30%, and 42% higher than participants with normal fat distribution respectively), and that waist and waist-to-hip ratio, indicators of visceral adiposity, were better measurements than BMI, especially in women (Panagiotakos et al., 2005). Park, Park and Yu (2005) found similar results and therefore declare the accumulation of visceral fat a key factor in the up-regulation of obesity-associated inflammation.

The exact mechanisms linking obesity, stress and inflammation have not been fully understood yet, but over-nutrition triggers a chronic adaptive response that further promotes the development of an inflammatory state. A vicious cycle is established between the stressor and the prolongation of the adaptive response, which both contribute to the development and maintenance of the chronic inflammatory conditions characteristic of obesity. In healthy adipose tissue adipocyte expansion occurs with minimal inflammation via the effective recruitment of adipose progenitors and proper extra cellular matrix remodeling (Sun, Kusminski & Scherer, 2011). However, in metabolically-impaired obese individuals, excessive expansion of adipocytes results in hypertrophy, hypoxia, massive inflammation and fibrosis, and the release of many proinflammatory and mitogenic factors that can have dramatic consequences on the development of chronic diseases, including cancers (Hefetz-Sela & Scherer, 2013). Lipolysis increases and free fatty acids (FFAs) are released, which can activate inflammatory pathways, but WAT also have endocrine functions which stimulate the inflammatory response (McArdle et al., 2013).

Expansion of WAT results in the increased secretion of inflammatory adipokines by adipocytes, including IL-6, IL-1β, TNF-α and leptin, and decreased secretion of anti-inflammatory adiponectin (McArdle et al., 2013). The altered

expression of those adipokines has been associated with several metabolic disorders. Adipocytes also secrete monocyte chemoattractant protein (MCP)-1 which promotes monocytes infiltration and adhesion molecules which also attract monocytes. Monocytes can then differentiate into macrophages, which then secrete cytokines, thus participating in the establishment of inflammatory conditions. Macrophage infiltration and polarization seems to be a driving factor in the promotion of inflammation. While in normal conditions adipose tissues have anti-inflammatory functions via the action of M2 macrophages and the secretion of anti-inflammatory cytokines, in obese patients we generally observe a change in the cytokine profile with the release of iNOS (nitric oxide synthase) and pro-inflammatory cytokines by more activated M1 macrophages (Kraakman et al., 2014). WAT expansion and inflammation, and the resulting production of cytokines by adipocytes and resident macrophages, lead to the recruitment of monocytes which differentiate into more M1 macrophages and result in the further production of inflammatory cytokines.

Although adipocytes are the main components, WAT contain many other cells which all participate in the maintenance and promotion of the chronic inflammatory state (McArdle et al., 2013). Pre-adipocytes normally differentiate into adipocytes during adipogenesis, but in obese individuals we observe that inflammatory cytokines IL-6, IL-1β, TNF-α and IFN-γ impair preadipocytes maturation and their number increases. Pre-adipocytes also secrete inflammatory mediators including IL-6 and MCP-1 at even greater levels than mature adipocytes, and fibroblast growth factor which acts with TNF-α and IFN-γ to recruit monocytes and neutrophils, which can then differentiate into macrophages. Preadipocytes produce proinflammatory cytokines to promote their development, and these cytokines attract monocytes which are converted to macrophages with the help of high leptin levels and thus also

contribute to the production of proinflammatory cytokines, which further impairs preadipocyte to adipocyte maturation (Gilbert & Slingerland, 2013). Furthermore, adiponectin, an anti-inflammatory protein with antiangiogenic and antiproliferative properties that promotes the maturation of preadipocytes and is secreted by mature adipocytes, is downregulated in obese individuals. While mature adipocytes secrete both inflammatory leptin and anti-inflammatory adiponectin, preadipocytes secrete primarily leptin and in obese individuals leptin expression is upregulated while adiponectin levels negatively correlate with body fat. Other cytokines associated with obesity also interfere with preadipocytes maturation. Simons et al. (2005) showed that exposure to IL-1β, TNF-α, IFN-γ, and TGF-β inhibited adipogenesis and completely blocked adiponectin secretion (although interestingly IL-6, a pro-inflammatory cytokine and hallmark of obesity-induced inflammation, had no effect). IL-1β and TNF-α also significantly increased leptin production. They further showed that IL-1β, TNF-α, and IFN-γ induced preadipocyte proliferation and inhibited adipocyte formation by preventing growth arrest and promoting re-entrance in the S phase. Additionally, pre-adipocyte factor-1 (Pref-1), a protein that prevents adipogenesis, increases with the number of macrophages in metabolically altered WAT (McArdle et al., 2013). Preadipocytes thus also contribute to the inflammatory conditions and some believe that the disruption of adipogenesis, the inability of adipocytes to properly expand to store the excess energy consumed, might significantly contribute to the metabolic conditions associated with obesity (Christodoulides et al., 2008).

In the stroma-vascular fraction, many cell types are present including macrophages and cells involved in the immune response, which are increased in obesity and have a phenotype that further promotes the inflammatory condition. Macrophages are believed to play a critical role in adipocyte inflammation, but other

immune cells also seem to be influenced by obesity. Regulatory T-cells (T_{reg}), which secrete anti-inflammatory cytokines, inhibit macrophage migration and promote the M2 macrophage phenotypes, are down-regulated during weight gain, while pro-inflammatory T helper 1 (T_H1) cells are upregulated (McArdle et al., 2013). IFN-γ is secreted by T_H1 and stimulates the switch from M2 to M1 macrophages, and thus leads to the further production of pro-inflammatory cytokines IL-1, IL-6 and TNF-α. The secretion of inflammatory cytokines by expanding adipocytes is suspected to play an important role in the development and maintenance of the obesity-induced inflammation (Karalis et al., 2009). In chronic over-nutrition, the WAT mass continues to increase, and the inflammatory conditions are maintained (Figure 1).

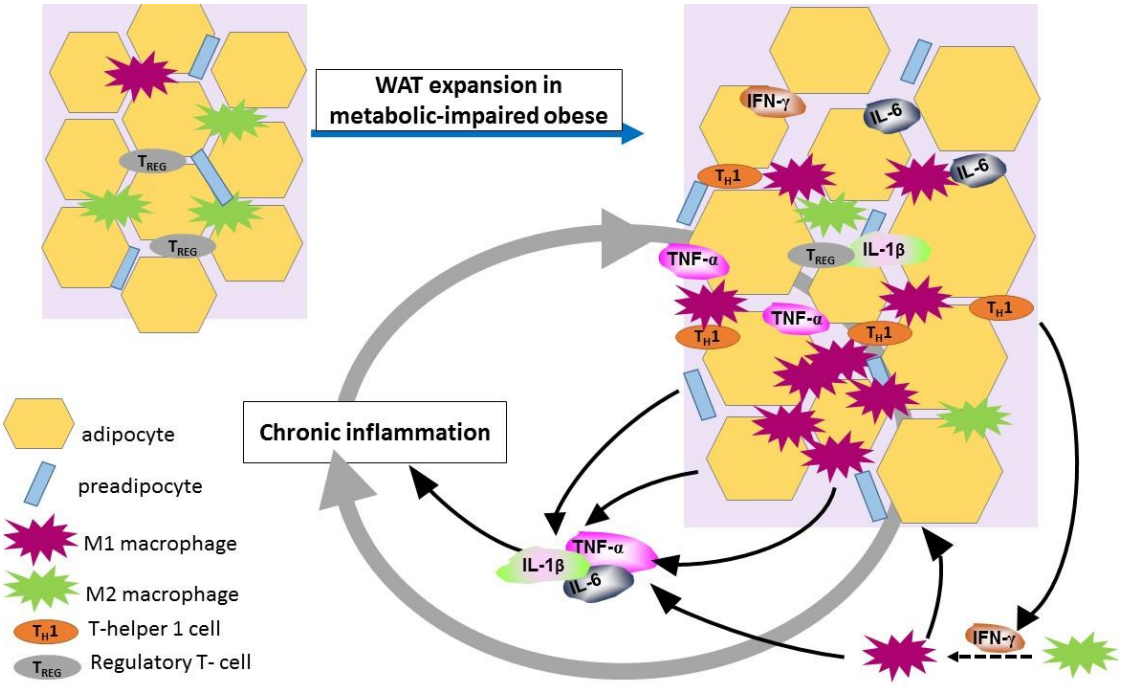

Figure 1: Metabolic changes and inflammation in white adipose tissue of obese individuals.

TNF-α is one of the most studied proinflammatory cytokines. Hotamisligil, Shargill, and Spiegelman were the first to demonstrate in 1993 that most TNF-α

mRNA was expressed by the adipocytes (in opposition to nonadipose cells, or the stromal-vascular fraction) and that obese adipose tissue secrete about twice as much TNF- α as lean tissue (Hotamisligil, Shargill, & Spiegelman, 1993). It was first believed that TNF-α, a macrophage-derived factor that could induce necrosis in tumor cells and is involved in the adaptive response of the immune system, could be used as an anticancer agent, but it has now been shown to promote carcinogenesis and cancer progression (van Kruijsdijk, van der Wall, & Visseren, 2009). It plays a key role in the normal response to infection, but dysregulation of its production can be harmful (Bradley, 2008). Isakson et al. (2009) showed that exposure of preadipocytes to TNF-α completely prevented their normal differentiation into adipose cells, and promoted a partial transdifferentiation of the preadipocytes to assume a macrophage-like phenotype, evidenced by elevated gene expression of chemokines and cytokines, including IL-1β, TNF-α, IL-6 and IL-8, thus participating in the maintenance of the inflammatory condition. It also seems to prevent the transcription of anti-inflammatory adiponectin (Tilg & Moschen, 2006). The presence of TNF-α has been shown to activate several inflammatory pathways, including the NF-κB pathway, which results in the expression of many inflammation-related genes and has been associated with tumor growth, metastasis, and survival (Prieto-Hontoria et al., 2011), and the Wnt signaling pathway, involved in several cancers (Oguma et al., 2008; Liu et al., 2012).

IL-1β also participates in the anti-adipogenic action of macrophages (Gagnon et al., 2013), and, along with other members of the IL-1 family, also activates the NF-κB pathway (Korkaya et al., 2011). It also stimulates other cytokines, including IL-6 and IL-8, and promotes angiogenesis, tumor growth, and metastasis. It participates in the angiogenic switch, during which the activity of anti-angiogenic factors decreases

while pro-angiogenic factors are activated (Voronov, Carmi & Apte, 2014). In tumors, it can recruit myeloid cells from the bone marrow and stimulate secretion of pro-inflammatory and pro-angiogenic molecules, it prevents myeloid cells maturation thus maintaining them in a pro-invasive and immunosuppressive state, it promotes the production of VEGF (vascular endothelial growth factor) and pro-angiogenic factors by endothelial cells, and it increases the invasiveness of malignant cells. In the absence of IL-1β, inflammation and invasiveness are diminished and myeloid inflammatory cells mature into anti-tumor M2 macrophages.

IL-6 is a hallmark of obesity-induced inflammation and many inflammation-associated diseases, including cancer, however recently studies have found that IL-6 may actually have a homeostatic, anti-inflammatory role during obesity-associated inflammation (Mauer, Denson & Brüning, 2015). IL-6 may increase insulin-stimulated glucose disposal in healthy humans rather than promoting type-2 diabetes, increase energy expenditure, and be involved in the maintenance of M2 macrophages in adipose tissue. However, in cancerogenesis, the presence of IL-6 stimulates tumor growth, progression, and relapse. M1 inflammatory macrophages have been shown to express high amounts of several inflammatory cytokines including IL-6, which has been shown to play an essential role for cancer development in several tissues including the breast. IL-6 is elevated by both obesity and cancer development, and studies in mice have shown that obesity may enhance tumor formation by increasing IL-6 production and STAT3 activation, and that IL-6 deficiency protects against obesity-induced tumor burden. IL-6 promotes tumorigenesis, angiogenesis and metastasis, and is associated with poor patient outcome (Korkaya, Liu & Wicha, 2011). It creates a procarcinogenic, tumorigenic microenvironment by STAT3 activation, which also activates NF-κB and leads to the production of additional IL-6

and IL-8. There is thus a positive feedback loop that further stimulates tumor development and metastasis. Similarly to TNF-α, it has also been shown to suppress adiponectin transcription and translation (Tilg & Moschen, 2006). Walter et al. (2009) proved that adipose stromal cells (ASCs) greatly stimulated the migration and invasion of breast cancer cells via the stimulatory actions of IL-6.

Several pro-inflammatory cytokines are thus stimulated by obesity, via inflammation, and seem to be playing a key role in the development of obesity-associated conditions. IL-8 is also secreted by many cells present in the WAT, including mesenchymal, macrophages, and immune cells, and is associated with poor cancer prognosis (Korkaya, Liu & Wicha, 2011). As mentioned earlier IFN-γ is also involved in the immune response to obesity and the establishment of inflammatory conditions. Because inflammatory cytokines are elevated not only in the adipose tissue but also in the serum of obese individuals, they are suspected to be able to circulate throughout the body and affect the inflammatory status of other tissues, including the breasts where they have been found to be elevated in cancer cases (Gilbert & Slingerland, 2013). Although the relationship between BMI, cytokines and breast cancer has not been fully investigated yet, inflammatory cytokines seem to have the potential of being key players in the development of breast tumors.

B. Inflammation and breast cancer

There is now abundant epidemiological evidence that adiposity is a risk factor for the development of breast cancer, especially in post-menopausal women, but the mechanisms involved are not fully understood. Estrogen is considered a key factor in the development of breast cancer. Indeed, the large majority (about 80%) of breast

cancer cases that develop after menopause are tumors that express the estrogen receptor (Rose & Vona-Davis, 2014). After menopause, the production of estrogen by the ovaries stops, and fat tissue become the main producer of estrogen. Thus, obese women are exposed to higher levels of estrogen, which are suspected to increase their risk of developing hormone-responsive tumors. Insulin resistance and hyperinsulinemia are also suspected to play an important role by causing decreased levels of sex-hormone binding globulin (SHBG) and therefore increasing the amount of circulating free estrogen (Khan et al., 2013). However, other elements, not necessarily mutually exclusive, are most certainly also at play in the development of breast cancer, and inflammation is becoming a prime suspect.

Breast cancer has not been associated with inflammation until recently, and inflammation might still not be one of the driving forces in the development of mammary tumors in lean women, but evidence now suggests that it plays a critical role in the development and progression of breast cancer in obese women (Santander et al., 2015). As discussed previously, obesity, and more specifically visceral obesity, is associated with inflammation of the adipocytes, while subcutaneous fat is less metabolically active. Because of its location breast adipose tissue (bAT) is considered subcutaneous fat, but Santander et al. (2015) argue that inflammatory changes are likely to occur in bAT as well. Indeed, bAT take up a larger volume than other subcutaneous adipose tissues, which may allow them to become hypertrophic and hyperplastic, two characteristics of inflamed visceral adipocytes. The presence of several cell types in the breast, including adipocytes, preadipocytes, fibroblasts, macrophages, etc., may also facilitate cross-talk between them and the production of pro-inflammatory molecules. Santander et al. (2015) showed that the cross-talk between adipocytes, mammary tumor and macrophages in obese mice results in the

expression of numerous cytokines, chemokines, and growth factors with chemotactic, pro-inflammatory, pro-angiogenic and tumor-promoting activities, in the recruitment of macrophages via chemokine CCL2 (MCP-1), and in the down-regulation of lipolysis, all of which promote inflammation. They thus showed that breast adipocytes are metabolically similar to visceral adipocytes.

The cytokines mentioned previously are highly expressed in adipose tissue of obese individuals, but their serum levels are also elevated, which raises the concern that they may circulate throughout the body and have inflammatory effects in other tissues as well (Gilbert & Slingerland, 2013). They may also be locally produced by macrophages within specific tissue. For example, Liu et al. (2012) showed that diet induced obesity (DIO) resulted in elevated expression of TNF-α, IL-1β and IL-18 (by 72%, 44% and 41% respectively) in the colon of obese mice. Cytokines are expressed in primary human breast cancers, and although no studies have been performed yet to investigate the link between body weight and cytokine expression in breast cancers, it is suspected that they contribute to the pro-inflammatory conditions needed for breast tumor development and activate cancer-related signaling pathways (Gilbert & Slingerland, 2013).

Subbaramaiah et al. (2011) showed that obesity leads to inflammation of mammary tissue in mice, as evidenced by infiltration and activation of macrophages in the tissues, and that the resulting elevated levels of inflammatory mediators (i.e. TNF-α, IL-1β, and Cox-2) were accompanied by increased levels of aromatase mRNA and activity. They attribute the link between proinflammatory gene expression and increased aromatase expression to the activation of the NF-κB pathway in the mammary gland. Pro-inflammatory cytokines have also been linked to increased CYP19 transcription, the gene responsible for cytochrome P450 aromatase, the

enzyme that catalyzes the synthesis of estrogen from androgens. The effects of inflammation on breast tumor development thus needs to be explored in addition to estrogen and aromatase. Indeed, Subbaramaiah et al. (2011) also found that macrophages aggregate in crown-like structures (CLS) around the adipocytes in both visceral fat and the mammary gland, a phenotype characteristic of obesity-induced inflammation that promotes adipocytes necrosis. Formation of CLS was also observed in the breast tissue of obese women with breast cancer (Morris et al., 2011).

Evidence now shows that the interaction between the tumor and the adipocytes surrounding it, referred to as cancer-associated adipocytes (CAA), is bidirectional and helps promote the establishment of a pro-tumorigenic vicious cycle (Hefetz-Sela & Scherer, 2013). The tumor alters the phenotypes of the CAA, and the adipocytes also modify the tumors' characteristics. Santander et al. (2015) showed that bAT inflammation in the tumor microenvironment correlates with body weight – obese mice have the highest number of macrophages and CLS, overweight mice have fewer, and lean mice have the least – and that the same correlation is observed in the bAT distal from the tumor. However, bAT in the tumor microenvironment have higher inflammation than distal ones, indicating that not only inflammation promotes tumor development, but contact with the tumor also promotes inflammation of the adipocytes. Dirat et al. (2011) also showed that invasive cancer cells modify the phenotype of their surrounding tissues, especially adipocytes. The inflammatory cytokines discussed previously may participate in the exchange between adipocytes and tumors. Walter et al. (2009) suspect that IL-6 stimulates breast tumor cell migration and invasion. Adipose stromal cells (ASC) from bAT (and also from visceral adipose tissues) stimulated breast tumor cells migration both *in vitro* and *in vivo*. While ASC most likely secrete several cytokines and growth factors that could

enhance the migratory capacity of breast cancer cells, IL-6 was shown to have great influence since its depletion significantly reduced ASC-induced migration and invasion. Furthermore, breast tumor cells cocultivated with mature adipocytes exhibited an enhanced invasive phenotype, and adipocytes isolated from tumor samples exhibit overexpression of several inflammatory markers, including IL-6 (but not IL-1β or TNF-α) (Dirat et al., 2011). Higher levels of IL-6 were associated with larger tumor size and lymph nodes involvement. CAAs seem to promote tumor growth and survival. IL-8 expression has also been shown to be elevated in metastatic tumors compared to non-metastatic tumors and is thus suspected to participate in cancer migration and invasion (De Larco et al., 2001). IL-8 is induced by other inflammatory cytokines (IL-1β and TNF-α), and the effect of these two cytokines on IL-8 expression is about 180 times greater in metastatic cancer cell lines. Moreover, metastatic cancer cell lines had basal levels of IL-8 expression equal to or higher than non-metastatic cancer cell lines even after treatment with IL-1β or TNF-α, thus further confirming the link between IL-8 and metastatic potential.

There is most likely more than just one pathway responsible for the link between obesity and breast cancer, but pro-inflammatory cytokines produced by adipocytes are suspected to play a central role. By influencing aromatase expression they may contribute to the initiation of breast cancer, especially ER-positive breast tumors, but their inflammation-promoting activity is likely to also have a strong influence on cancer progression and may explain why obesity is associated with poor prognosis.

C. Wnt signaling and Cancer

Several inflammatory pathways have been investigated to explain the relationship between adiposity, inflammation and inflammatory cytokines, and cancer development, and the Wnt/β-catenin signaling pathway has recently drawn interest. The Wnt pathway is involved in the regulation of a number of mechanisms during embryonic development and the homeostasis of many adult tissues (Tree, 2013). It is involved in cell proliferation, cell polarity, and cell identity and therefore its dysregulation has been linked to several disorders, including some types of cancers. There are one canonical and two non-canonical Wnt pathways, but the latter are less well understood and the canonical Wnt pathway, or Wnt/β-catenin pathway, is the most studied for its link with cancer. The link between Wnt signaling and breast cancer was established in 1982 when Roel Nusse and Harold Varmus, from the University of California, San Francisco, showed that mammary gland tumorigenesis was induced by a virus that caused a loss-of-function mutation of a gene they named Int1 (Klaus & Birchmeier, 2008).

The canonical Wnt pathway is described as a two-state model, either on or off, which controls the level of accumulation of β-catenin in the nucleus (Tree, 2013). When the pathway is not activated, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), part of the cytoplasmic destruction complex along APC (adenomatous polyposis coli) and Axin, sequentially phosphorylate β-catenin which can then be recognized by β-Trcp, an E3 ubiquitine ligase, and degraded. Cytoplasmic levels of β-catenin thus remain low, which prevents β-catenin translocation to the nucleus. In the on state the Wnt/β-catenin pathway is activated by Wnt proteins, present in the extracellular space, that bind to two receptors: a Frizzled (Fz) receptor, a seven pass transmembrane protein, and a low-density lipoprotein related receptor

(LRP5 or 6), a single pass transmembrane protein. There are 19 Wnt proteins and 10 Fz receptors in mammals, but not all proteins can bind to all receptors. The formation of the tripartite complex leads to the recruitment of the cytoplasmic protein Dishevelled (Dsh) which causes the phosphorylation of LRP and the recruitment of parts of the destruction complex, resulting in its deactivation. β-catenin thus cannot be degraded and accumulates in the cytoplasm and eventually translocates to the nucleus. There it displaces the inhibitor Groucho and forms a complex with the DNA binding proteins TCF/LEF, which results in the transcription of Wnt target genes, some of whom regulate cell faith (Figure 2). The Wnt pathway remains turned off in the absence of Wnt proteins or in the presence of inhibitors, including SFRP, DKK and WIF.

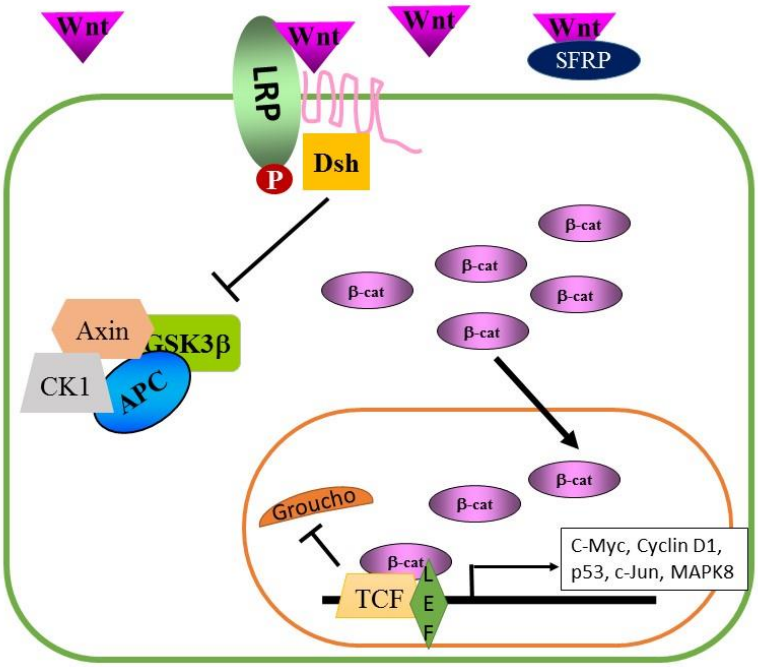

Figure 2: Scheme of Wnt signaling.

Wnt signaling is involved in the regulation of tissue development and homeostasis in many organs, and therefore its dysregulation can lead to tumor growth

and development. Abnormal Wnt activity has been observed in several types of cancers. It can be activated via a mutation in APC, β-catenin or other elements of the Wnt pathway, as it is the case in over 90% of colorectal tumors (Luu et al., 2004), but in breast cancers mutations are rare. However, accumulation of β-catenin in the cytoplasm and/or nucleus is observed in up to 60% of breast tumors, while it is not detected in normal breast tissue (Howe & Brown, 2004). Moreover, cytoplasmic and nuclear β-catenin accumulation and the accompanying increase in the expression of target genes are associated with poor prognosis.

Overexpression of some Wnt proteins has been reported in several studies, and the expression of Wnt inhibitors, including WIF1, DKK1 and sFRPs, may also be impaired. Suzuki et al. (2008) reported methylation of Wnt antagonists in a number of breast cancer cell lines as well as in primary breast tumors. They found that sFRP1, sFRP2, sFRP5 and DKK1 were commonly absent or downregulated in many breast cancer cell lines while they were all expressed in normal tissue. They also observed methylation of sFRP1, sFRP2 and sFRP5 in the large majority of cell lines tested (in 7, 11 and 10 cell lines respectively out of 11 tested), while methylation of DKK1 was detected in only 3 cell lines. These antagonists were also methylated in primary breast tumors, although a little less commonly. Finally, they showed that sFRPs suppress breast cancer cells growth, suggesting that sFRPs, and potentially DKK1, may have tumor suppressor functions in breast cancer. Matsuda et al. (2008) also provided evidence that SFRP1 may regulate Wnt activity in breast cancer cells and play a role in cancer progression. SFRP1 was shown to prevent Wnt-induced migration of breast cancer cells and to lead to the formation of fewer metastases. The antagonist inhibits Wnt signaling in breast cancer cell lines, evidenced by decreased levels of three

Dishevelled proteins and lower levels of total and active β-catenin, and results in decreased growth and proliferation.

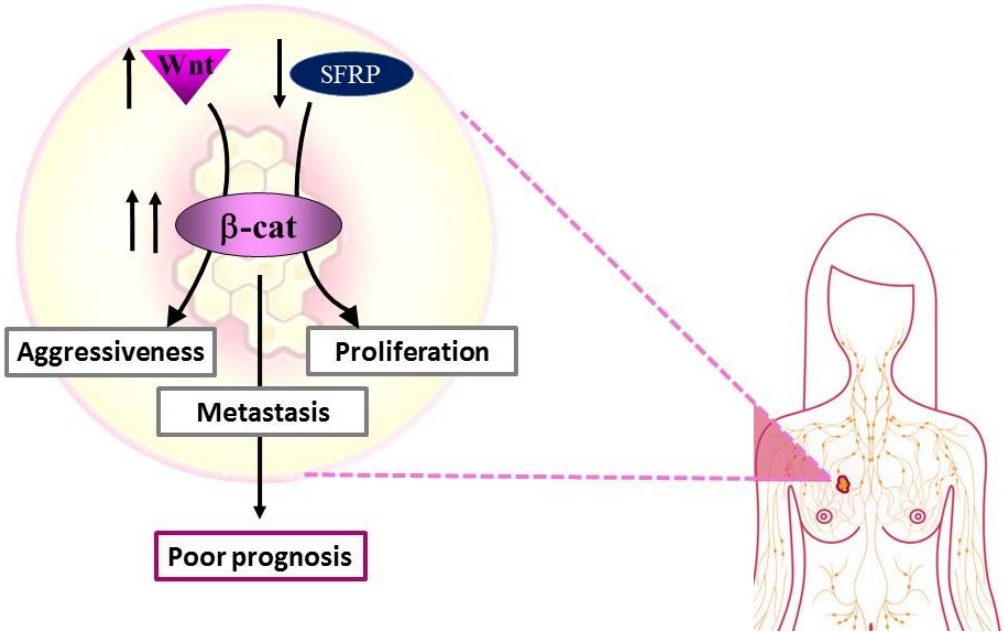

Figure 3: Wnt signaling and breast cancer development.

Howe and Brown (2004) concluded from their review that overexpression of Wnt1 in mouse mammary tissue resulted in hyperplasia rather than carcinoma, indicating that Wnt1 could contribute to the establishment of a premalignant state. However, Wnt signaling may also contribute to epithelial to mesenchymal transition (EMT) and thus promote migration and increase aggressiveness of cancers, and subsequently stimulate mesenchymal to epithelial transition (MET) to help the establishment of the secondary tumor at the site of metastasis (Neth et al., 2007). Supporting the idea of the role of Wnt in tumor invasion, colonic tumor cells located at the invasive front of the tumor and migrating cells show elevated nuclear β-catenin while cells located within the tumor have membranous β-catenin expression comparable to normal cells (Fodde & Brablez, 2007).

D. Inflammation and Wnt signaling

A few studies have also investigated the link between obesity-induced inflammation and Wnt signaling activity, and although it has not been fully explored yet it has led some teams of scientists to wonder whether Wnt could act as an intermediate between obesity-induced inflammation and cancer development. Gauger et al. (2014) have investigated the effects of high fat diet (HFD) and diet-induced obesity (DIO) on Wnt signaling. They showed that Wnt4 was increased by both loss of the antagonist SFRP1 and HFD independently, and in the absence of SFRP1 ($Sfrp1^{-/-}$ mice), HFD and DIO resulted in increased Wnt activation, as evidenced by increased expression of the oncogenic Wnt target *Myc*, and upregulation of active β-catenin. SFRP5 also seems to play a key role in Wnt activity in obese individuals. Ouchi et al. (2010) showed that the Wnt antagonist acts as an anti-inflammatory adipokine. SFRP5 is primarily secreted by white adipose tissue, but its expression is reduced in obese animals (ob/ob mice and Zucker diabetic fatty rats) as well as in mice fed a high fat-high sugar (HF/HS) diet. SFRP5 is known to inhibit Wnt5a and Wnt11, and while Wnt11 could not be detected, Wnt5a and Wnt5a to sFRP5 ratio were increased in obesity. SFRP5-knocked out mice on the HF/HS diet saw significant impairment in glucose tolerance and insulin sensitivity, and showed signs of increased inflammatory response (increased macrophage content and increased levels of TNF-α, IL-6, and MCP-1). Addition or restauration of sFRP5 reversed those effects. Since Wnt antagonists are commonly downregulated in breast cancer (Suzuki et al., 2008), these results suggest the potential for Wnt activation in obese women, which could contribute to the promotion and progression of breast cancer in those individuals. Indeed, DIO and sFRP1 loss resulted in decreased expression of the tumor suppressor p53 and the absence of the Wnt inhibitor sFRP1 led to resistance to

anoikis (cell death due to loss of attachment), which is critical for cell survival during metastasis (Gauger et al., 2014).

As mentioned previously, impaired adipogenesis may be one of the contributing factors to the link between obesity and inflammation, and Wnt signaling is suspected to play an important role in the regulation of adipocytes maturation and the mediation of adipose cell cross-communication (Christodoulides et al., 2008). Indeed, studies in cell lines and in mice showed that activation of Wnt via increased levels of Wnt proteins (Wnt1 and Wnt10b) or inhibition of β-catenin phosphorylation prevented preadipocytes differentiation. Conversely, addition of recombinant inhibitors (sFRP1 or sFRP2) or increased expression of Axin or dnTCF7L2 (TCF4) led to spontaneous differentiation. DKK1, another Wnt antagonist that binds to LRP5/6 co-receptors, is expressed during differentiation of human preadipocytes and blocks Wnt signaling to promote adipogenesis.

The cytokines discussed previously might exert their anti-adipogenic functions via the activation of Wnt signaling. Indeed, the inhibition of Wnt (via knockdown of β-catenin) lessened the inhibitory effects of TNF-α on adipogenesis (Cawthorn et al., 2007). Gustafson and Smith (2006) showed that IL-6 and TNF-α prevented the degradation of β-catenin during adipocytes differentiation thus preventing preadipocytes maturation. IL-6 did not alter levels of Wnt proteins but rather allowed cytoplasmic and nuclear levels of β-catenin to remain elevated. TNF-α had the same, even stronger, effects, and did increase Wnt10b and Pref-1 expression, and also increased the expression of *CyclinD1*, a target gene. Wnt10b is known to prevent adipocyte differentiation and is secreted by preadipocytes. Neither the effects of IL-6 nor of TNF-α were reduced by the presence of the Wnt antagonist sFRP2. The two cytokines also maintained high expression of LRP6 and Dsh, and decreased the

expression of Axin (which is normally reduced in Wnt signaling). Isakson et al. (2009) also found that addition of both Wnt3a and TNF-α individually inhibit preadipocyte differentiation, and that Wnt10b was increased in preadipocytes in the presence of TNF-α. It thus seems that inflammatory cytokines alter adipocytes maturation through the Wnt signaling pathway. Also supporting the idea of a link between inflammation and Wnt activation, Ouchi et al. (2010) found that in visceral adipose tissue of obese individuals, the presence of CLS is associated with decreased levels of the antagonist sFRP5, as well as increased TNF-α. However, they believe that sFRP5 acts via the noncanonical Wnt-pathway, based on evidence of increased phosphorylation of c-Jun N-terminal kinase (JNK), while the transcription of *cyclinD1* or *WISP2*, targets of the canonical pathway, was not influenced by sFRP5.

The location of tumor cells with β-catenin accumulation within the tumor mass suggests that although mutations in APC or β-catenin may initiate Wnt activity, other factors, such as growth factors or inflammatory cytokines, may play a key role in nuclear β-catenin accumulation (Fodde & Brablez, 2007). As mentioned previously, cells located at the invasive front showed elevated nuclear β-catenin while cells within the tumor had normal levels. Tumor cells located at the invasive front produce extracellular matrix and cytokines and growth factors, which allows them to interact with parenchymal cells and thus promotes their proliferative and invasive capacity. Some pro-inflammatory cytokines may be able to activate Wnt signaling and thus promote cancer development. Macrophage-derived IL-1β has been shown to be a strong inducer of Wnt activity in colon tumor cells (Kaler, Augenlicht & Klampfer, 2009a). IL-1β was sufficient and necessary to activate Wnt signaling, and its neutralization prevented macrophages from inducing Wnt activity and clonogenic growth of tumor cells, thus reducing macrophages-induced tumorigenesis. Treatment

of colon cancer cells with IL-1β resulted in increased phosphorylation of GSK3β and increased levels of the target gene *c-myc*. However, while Kaler et al. (2009b) found that IL-1β was a more potent inducer of Wnt activity via TCF4/β-catenin transcription than TNF-α, Oguma et al. (2008) and Liu et al. (2012) both found that TNF-α was able to initiate Wnt signaling. Oguma et al. (2008) found that in gastric tumors TNF-α was able to activate Wnt/β-catenin signaling via increased phosphorylation of GSK3β. Exposure of gastric cancer cell lines to activated macrophages resulted in increased Wnt activity. Treatment with TNF-α alone produced similar results while blockage of either of its two receptors suppressed TNF-α-induced Wnt signaling. Contrary to what was found in colon cells by Kaler et al. (2009b), Oguma et al. (2008) found that Wnt activity was not promoted by IL-1β, nor by IL-6 or IL-11. They suggest that phosphorylation of GSK3β is due to the activation of the Akt pathway by TNF-α. Liu et al. (2012) also report that TNF-α, which is elevated in the colon of obese mice, is accompanied by increased GSK3β phosphorylation, accumulation of β-catenin, and increased expression of the Wnt target *c-myc*.

Although not clearly defined yet, a role for Wnt in cell proliferation thus seems very likely. It could partially explain the fact that while the relationship between obesity and breast cancer risk varies by menopausal status – obesity increases breast cancer incidence in post-menopausal women but has no influence or may be protective before menopause – high BMI has been linked with increased mortality and aggressiveness regardless of menopausal status (Carmichael, 2006). This may indicate that obesity-induced inflammation acts primarily on the proliferative front rather than at the tumor initiation stage, and that other factors, including sex hormones which do vary by menopause status, may regulate tumor initiation. Moreover, obesity is a risk factor for the development of triple-negative

breast cancer, which is not influenced by sex hormones, in premenopausal women as well (Turkoz et al., 2013). Supporting the tumor-promoting role of Wnt, Wnt2 and Wnt5a were found to be upregulated in the progression from adenoma to carcinoma in colon cancer (Smith et al., 1999).

E. Conclusion

The link between obesity and breast cancer is well established, but the mechanisms involved have not been fully elucidated. Obesity is associated with abnormal expansion of WAT, which have been shown to promote the production of inflammatory cytokines by M1 macrophages. Obesity-induced inflammation results in increased levels of those cytokines in the serum of obese individuals, and preliminary evidence show that they may also be present locally in various tissues, including the breast. Those cytokines have also been found to be over-expressed in breast tumors and are suspected to participate in tumor promotion via the activation of inflammatory pathways. However, very few studies have investigated the link between obesity, inflammation and cytokines in the breast, and breast cancer. Wnt activity is suspected to be elevated in women with breast cancer, evidenced by accumulation of β-catenin. Cytokines have been shown to modulate Wnt activity in several cancer cell lines and animal models, but the relationship between pro-inflammatory cytokines and Wnt activity in human breast tissues has not been investigated. Being able to establish a causal relationship between cytokines and Wnt signaling in the breast would greatly enhance our understanding of the relationship between obesity and breast cancer, and could potentially uncover a new area of investigation for the development of treatment options.

CHAPTER 3

PURPOSE OF THE STUDY

Numerous studies have established that chronic over-nutrition acts as a stressor and the resulting obesity-induced inflammation is suspected to play a critical role in the development of breast cancer, but the mechanisms involved have not yet been fully understood. Inflammatory cytokines, important participants in the immune response to inflammation, have been shown to be elevated in the serum as well as adipose tissue of obese individuals, and a few studies suggest that they may be present in local tissues, including the breasts, as well. However, very little research has been conducted on the link between body weight, the presence of inflammatory cytokines in breast tissue, and elevated breast cancer risk in humans. Inflammatory cytokines are suspected to play a critical role in the activation of several tumorigenic pathways. Wnt signaling is believed to be increased in many breast cancer cases (60%), but the effects of obesity-induced inflammatory cytokines on the activation of Wnt-signaling in mammary tissue is unknown. We therefore propose to characterize the inflammatory cytokine profile and determine how Wnt-signaling responds to obesity-induced inflammation in the breasts of obese postmenopausal women. We also propose to investigate whether TNF-α, one of the most critical inflammatory cytokines, is a strong inducer of Wnt activity by ex vivo treatment of mammary tissues with anti-TNF-α antibody or TNF-α recombinant protein.

<u>Hypothesis 1</u>: The levels of inflammatory cytokines are elevated in the breast tissue of obese postmenopausal women

Specific Aim 1: Characterize the inflammatory cytokine profile in the mammary tissue of lean, overweight, and obese postmenopausal women using a high-performance, electrochemiluminescence immunoassay (Meso Scale Discovery System®). A panel of 6 inflammatory cytokines, IFN-γ, IL-1β, IL-2, IL-6, IL-8, and TNF-α, was selected.

Hypothesis 2: Wnt signaling activity is elevated in the breast tissue of obese postmenopausal women

Specific Aim 2: Measure Wnt activity in the mammary tissue of lean, overweight, and obese postmenopausal women using real-time PCR to measure the expression of 7 Wnt target genes: *c-Myc, Cyclin D1, Axin2, COX2, P53, C-Jun,* and *MAPK8*.

Hypothesis 3: Wnt activity in the breasts is regulated by proinflammatory cytokines and the blockade or addition of TNF-α will respectively decrease or increase Wnt activity

Specific Aim 3: Measure Wnt activity in the mammary tissue of obese postmenopausal women treated with anti-TNF-α antibody and in the tissue of lean women treated with recombinant TNF-α protein using real-time PCR to measure the expression of 7 Wnt target genes: *c-Myc, Cyclin D1, Axin2, COX2, P53, C-Jun,* and *MAPK8.*

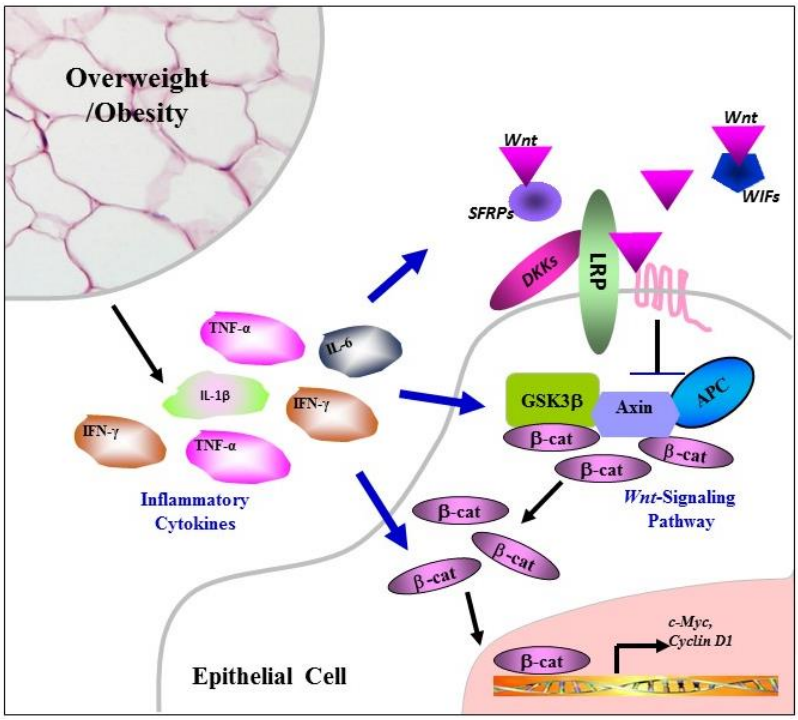

Figure 4: The scheme of the hypothesis.

CHAPTER 4

MATERIALS & METHODS

Our experiment investigates the relationship between body mass index (BMI) and the concentration of inflammatory cytokines in the breast tissue of obese women, and their influence on Wnt-signaling activity. The inflammatory cytokines profile in the breast tissue of normal weight, overweight and obese women were first evaluated using a high-performance electrochemiluminescence immunoassay. The gene expression of Wnt target genes was measured in the same women using real-time PCR to evaluate the correlation between BMI and Wnt activity. The effects of addition or inhibition of the pro-inflammatory cytokine TNF-α on Wnt activity was then investigated by measuring the level of expression of Wnt target genes in breast tissue samples treated with either anti-TNF-α antibody or TNF-α recombinant protein in order to be able to establish a causal relationship between inflammatory cytokines and Wnt activation.

A. Breast tissue samples

Two batches of breast tissues were obtained from Baystate Medical Center in Springfield, MA.

- **Batch 1** consists of 31 tumor-free breast samples from women, mostly postmeopausal, who underwent elective breast reduction surgery. The average BMI is 32.2kg/m², ranging from 21 to 48kg/m². Age and BMI of the individuals are summarized in Table 2 in the Appendices.
- **Batch 2** consists of 11 pairs of samples from 5 postmenopausal obese women (BMI ranging from 30.4 to 50.1kg/m²) and 6 postmenopausal normal weight

or slightly overweight women (23.8 to 26.6kg/m²) who underwent either elective surgery or mastectomy to remove a tumor. For each woman, one sample remained untreated as the control and one sample underwent treatment. Samples from obese women were treated with anti-TNF-α antibody, while samples from lean women were treated with TNF-α recombinant protein. Age, BMI, tumor condition, and treatment are summarized in Table 3 in the Appendices.

Each sample was divided in 3 aliquots. All tissue are preserved at -80°C for the duration of the project.

B. Pro-inflammatory cytokines profile

Tissue samples (one aliquot of Batch 1) were homogenized in a Complete Lysis buffer (10-20mg of tissue per 400µL of buffer), and protein concentrations were determined using a commercially available BCA kit and a microplate reader (SpectraMax M2, by Molecular Devices) following the protocol outline in the Appendices. Proteins samples were diluted to the lowest concentration obtained (300µg/mL). α

Concentrations of the pro-inflammatory cytokines IFN-γ, IL-1β, IL-2, IL-6, IL-8 and TNF-α were measured using Meso Scale Discovery System®, a high-performance electrochemiluminescence immunoassay, following the company's protocol which is summarized in the Appendices.

Pearson correlations were calculated to evaluate the relationships between BMI and the protein levels of each cytokine (expressed in ng of cytokine/mg of total

protein). The samples were then separated into 2 groups, BMI > 30 and BMI < 30, and a t-test was performed to compare the average concentration of each cytokine in obese vs. leaner women. P-values of less than 0.05 were used as the cut-off for significance.

C. Wnt signaling pathway activity

RNA was isolated from 1 aliquot of each sample of both Batch 1 and Batch 2 using TRIzol® Reagent (Ambion® RNA, by Life Technologies™) and following the company's protocol outlined in the Appendices.

cDNA was synthesized from the RNA samples using QuantiTect® Reverse Transcription (Qiagen®) and following the company protocol.

DNA primers for the target genes of interest (*c-Myc*, *Cyclin D1*, *Axin2*, *COX2*, *P53*, *C-Jun*, and *MAPK8*) were designed using The Massachusetts General Hospital's PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and were ordered from Invitrogen™ (Invitrogen™ Custom DNA Oligos, Life Technologies®). A list of the primers used can be found in Table 4 in the Appendices.

The expression of the genes of interest was measured by real-time PCR (Applied Biosystems ViiA™ 7 RT-PCR System, by Life Technologies®). Gene expression was evaluated with ΔCt, using β-actin as the house-keeping gene. ΔCt is defined as $Ct_{(target)} - Ct_{(\beta\text{-actin})}$. The protocol used for real-time PCR can be found in the Appendices.

a) Wnt activity and BMI

Gene expression of 7 Wnt targets (*c-Myc*, *Cyclin D1*, *Axin2*, *COX2*, *P53, C-Jun*, and *MAPK8*) was measured in all 31 samples from Batch 1.

Pearson correlations were calculated to evaluate the relationships between BMI and gene expression (ΔCt values). We also performed a t-test to compare the target genes expression in women with BMI > 30 vs. women with BMI < 30. Finally, Pearson's correlations were calculated to investigate the relationship between each cytokine and each target gene for which a correlation with BMI was found (IL-6, IL-1β and TNF-α, and *CyclinD1*, *Axin2* and *MAPK8*). P-values of less than 0.05 were used as the cut-off for significance.

b) Effects of TNF-α on Wnt activity

Gene expression of 7 Wnt targets (*c-Myc*, *Cyclin D1*, *Axin2*, *COX2*, *P53, C-Jun*, and *MAPK8*) will be measured in the 11 controls and corresponding treated samples from Batch 2.

The fold-increase or -decrease in each gene expression was calculated as fold-change = $2^{-\Delta\Delta Ct}$, with $\Delta\Delta Ct = \Delta Ct_{(treatment)} - \Delta Ct_{(control)}$. A paired t-test was performed to evaluate the change in gene expression after treatment, and a p-value < 0.05 was used as the cut-off for significance.

CHAPTER 5

RESULTS

A. Pro-inflammatory cytokines profile

In order to evaluate the inflammatory status of the breasts of normal weight (n=5), overweight (n=7) and obese (n=19) women, levels of 6 pro-inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-6, IL-8 and TNF-α) were measured using Meso Scale Discovery System®. Pearson's correlations were calculated to evaluate the relationship between BMI and protein concentrations of inflammatory cytokines (in ng/mg of total protein) in the breasts of women who underwent breast reduction surgery.

Significant correlations were found between BMI and protein levels of IL-1β, IL-6 and TNF-α (p-values were 0.010, <0.001, 0.028 respectively; Figure 5). However, the levels of IL-8, IFN-γ, or IL-2 do not seem to be influenced by body weight (Figure 6). IL-1β, IL-6 and TNF-α are the cytokines the most often cited in relation to obesity and consistently found elevated in the serum of obese individuals, so it seems consistent that they would be the ones most elevated in the breast of women with high BMIs.

We then divided the women into two groups: one obese group (BMI>30; n=16) and one normal and overweight group (BMI<30; n=9). We combined lean and overweight women because of our small sample size. We looked at the difference between the average concentrations of each cytokine between the two groups. The levels of IL-6 and TNF-α were significantly higher (p-values were 0.011 and 0.014 respectively) in the obese group compared to the leaner group (Figure 7). The increase

in IL-1β was borderline non-significant (p=0.065), and the other cytokines did not show any difference between the two groups.

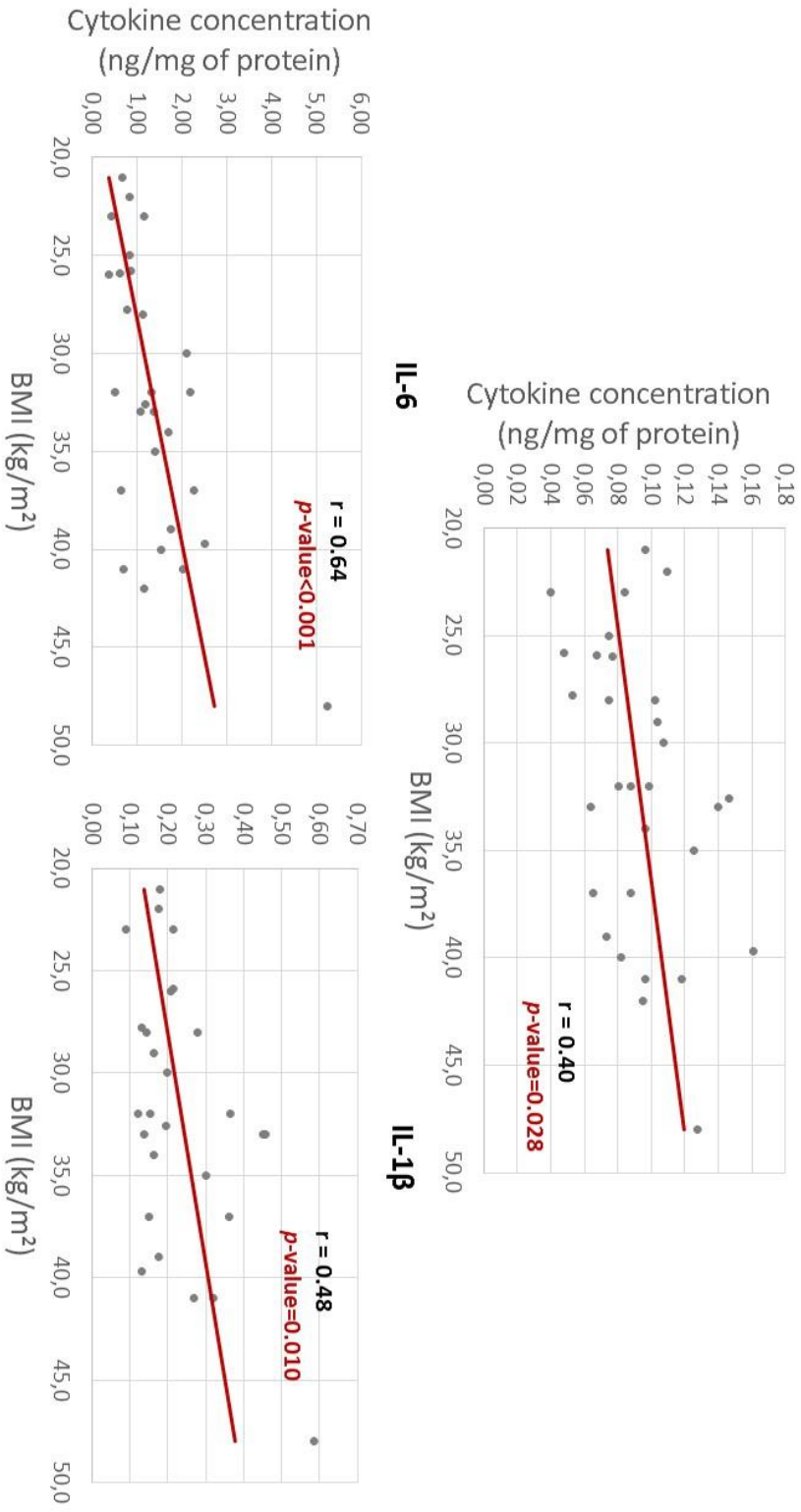

Figure 5: Levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in human mammary tissue are positively correlated with BMI.

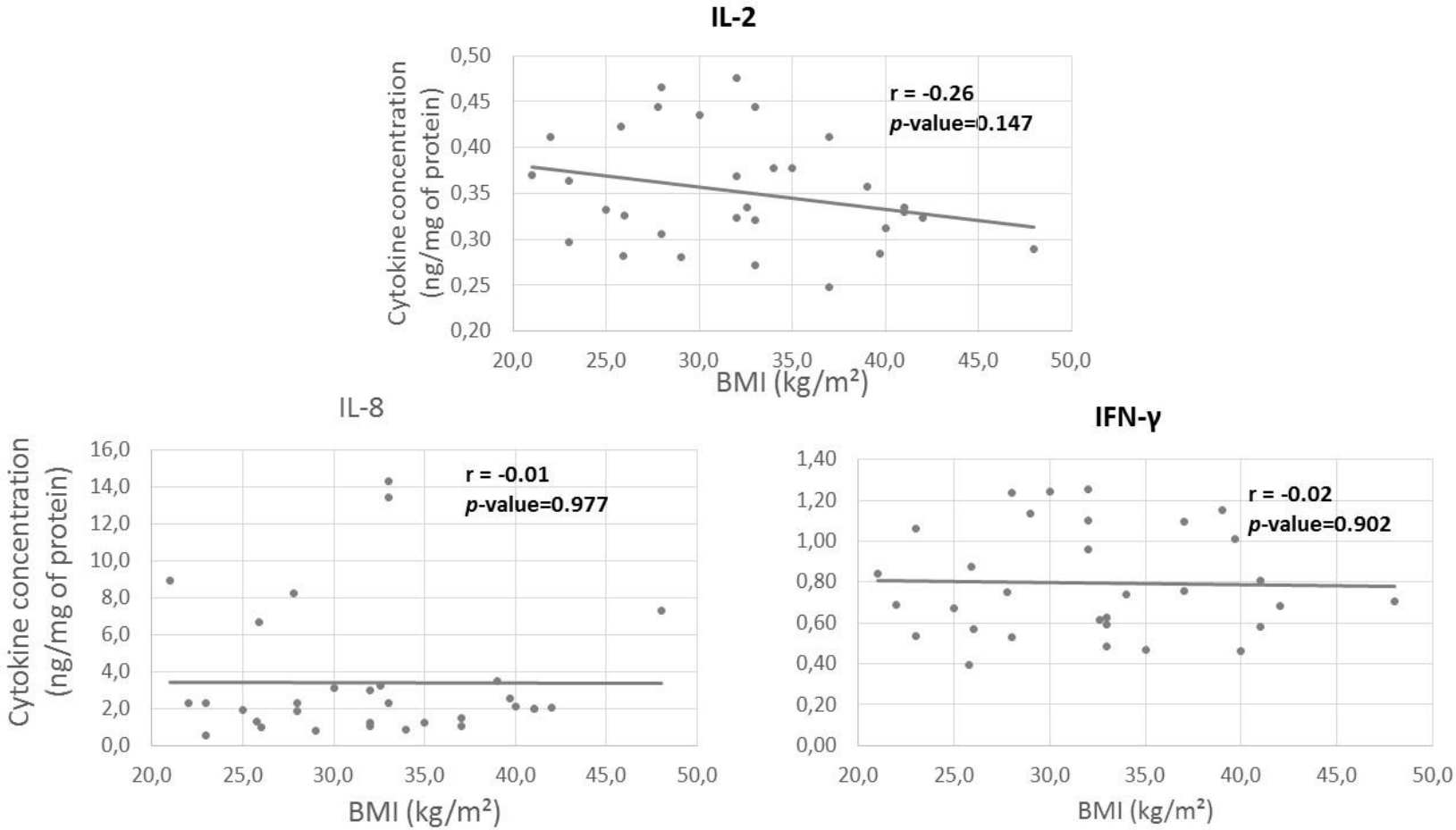

Figure 6: No association between the levels of pro-inflammatory cytokines IL-2, IL-8 and IFN-γ in human mammary tissue is observed in this study.

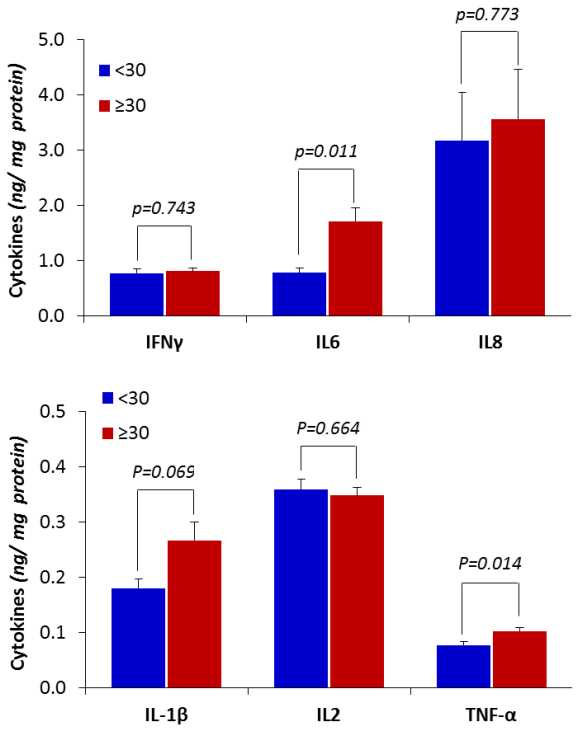

Figure 7: Levels of pro-inflammatory cytokines in obese women (BMI ≥ 30) vs. women with BMI less than 30.

Our results thus do suggest that there is a pro-inflammatory environment present in the breasts of obese women, evidenced by increasing levels of IL-1β, IL-6 and TNF-α as BMI increases, which could promote the development of breast tumors. Because both obesity and breast cancer have been individually suspected to be linked to Wnt signaling, we then investigated the correlation between BMI and Wnt activity in human mammary tissue.

B. Wnt-signaling activity and BMI

The correlation between Wnt activity and BMI was evaluated by measuring the levels of gene expression of 7 Wnt targets (*Myc, Jun, Cyclin D1, P53, Cox2, Axin2, MAPK8*) using real-time PCR. Because menopausal status influences breast

cancer risk and may impact Wnt activity, only post-menopausal women (>50 years old) were included in the analysis (n=25). Pearson's correlations were calculated to evaluate the relationship between gene expression of the 7 target genes (expressed using ΔC_T) and BMI.

We found a significant positive correlation between BMI and *CyclinD1* expression (p-value = 0.006; Figure 8a). As BMI increases ΔC_T decreases, indicating an increase in the gene expression. We also observed a positive trend between BMI and *Axin2* expression (Figure 8b). Although the correlation is not significant, it seems highly influenced by the data point with a BMI of 48kg/m², which acts as an outlier. Removal of this data point causes the p-value to drop to 0.028. The expression of the other targets was not significantly related to BMI (data not shown).

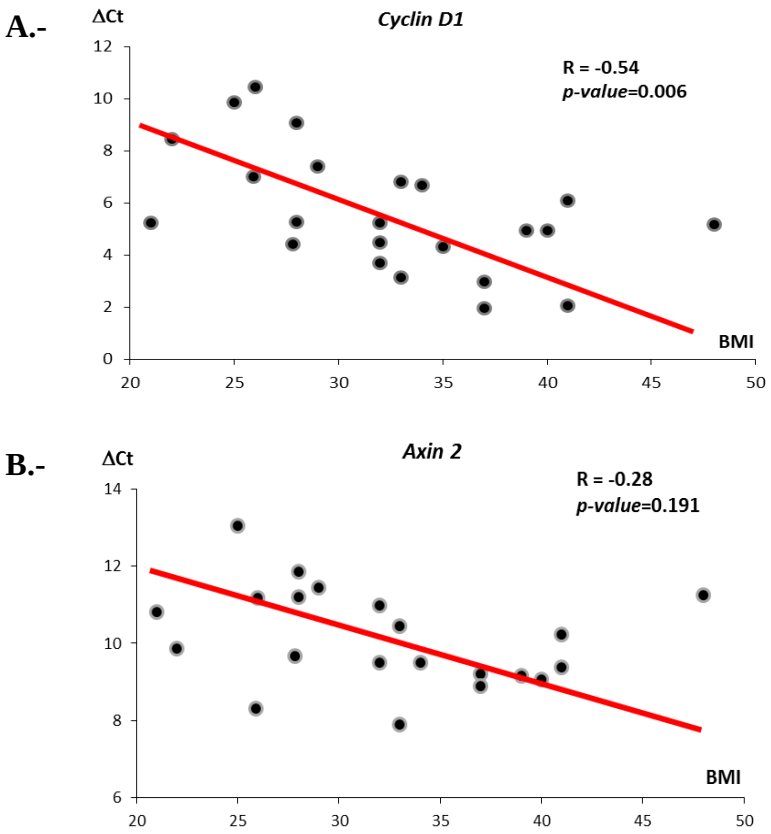

Figure 8: Correlation between BMI and Wnt targets *Cyclin D1* and *Axin2* expression. **A.-** *Cyclin D1* expression increases (ΔC_T decreases) with BMI. **B.-** *Axin2* expression tends to increase with BMI.

We then looked at the difference in gene expression between the two groups (BMI > 30 and BMI < 30) using a two sample t-test. The gene expressions of *CyclinD1* and *Axin2* are significantly higher (p-values are <0.001 and 0.030 respectively) in the obese group compared to the normal/overweight group (13-fold and 3-fold increases respectively; Figure 9). The gene expression of *MAPK8* is close to also being significantly increased in obese individuals (p=0.058). Although not significantly, we note that all the Wnt targets tend to be more expressed in obese individuals than in leaner women.

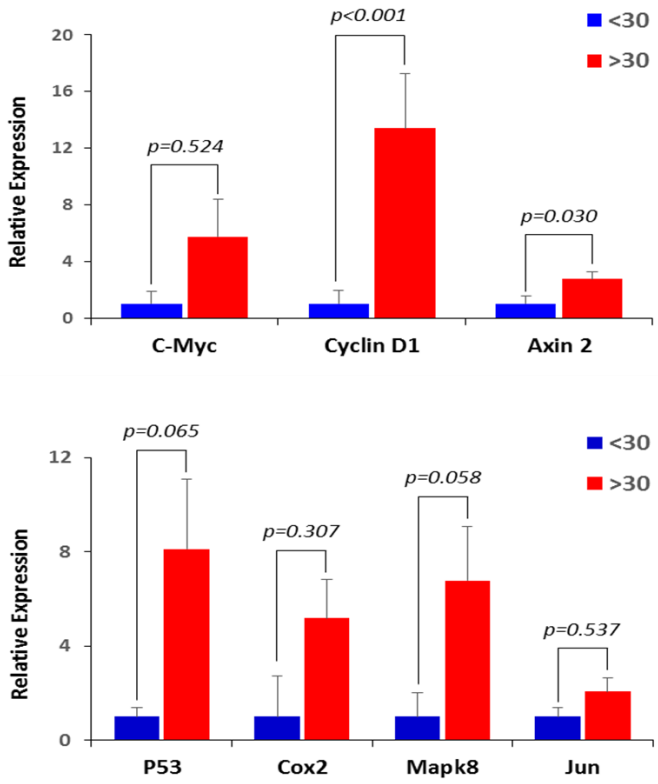

Figure 9: Expression of Wnt targets in women with BMI ≥ 30 vs. leaner women (BMI < 30).

As mentioned previously, a few studies have linked Wnt signaling and obesity before, and our laboratory has shown that cytokines, and especially TNF-α, may regulate Wnt activity (Liu et al., 2012). We therefore think that the elevated levels of

cytokines in women with high BMIs described previously may explain the increase in Wnt activity. We therefore looked at the correlation between *CyclinD1*, *Axin2* and *MAPK8* (*JNK1*) with each cytokine found elevated in the breasts of obese women (IL-1β, IL-6 and TNF-α) and found that IL-6 was positively correlated with *CyclinD1* and *Axin2* (p-values are 0.028 and 0.048 respectively) and TNF-α was significantly correlated with *JNK1* expression (p = 0.046; Figure 10). These results may indicate that inflammatory cytokines play a role in the increased Wnt activity observed in obese women.

C. Effects of TNF-α on Wnt activity

To evaluate whether there is a causal relationship between elevated cytokines and Wnt activity, we compared the expression of the seven Wnt targets in the breast tissue of menopausal women after ex-vivo treatment with either anti-TNF-α antibody or TNF-α recombinant proteins with non-treated controls.

We found that the expression of *CyclinD1* was significantly decreased by anti-TNF-α antibody treatment, and increased by addition of TNF-α recombinant protein (over 3-fold decrease and increase in comparison to untreated controls; p-values are 0.031 and 0.023). Anti-TNF-α treatment also decreased the expression of *Cox2* 4-fold (p = 0.017), and TNF-α recombinant protein decreased the expression of tumor suppressor *p53* 2-fold (p = 0.014) and tended to increase *Axin2* expression as well (p = 0.082). The relative expressions of each gene in each treatment group are summarized in Table 1 below.

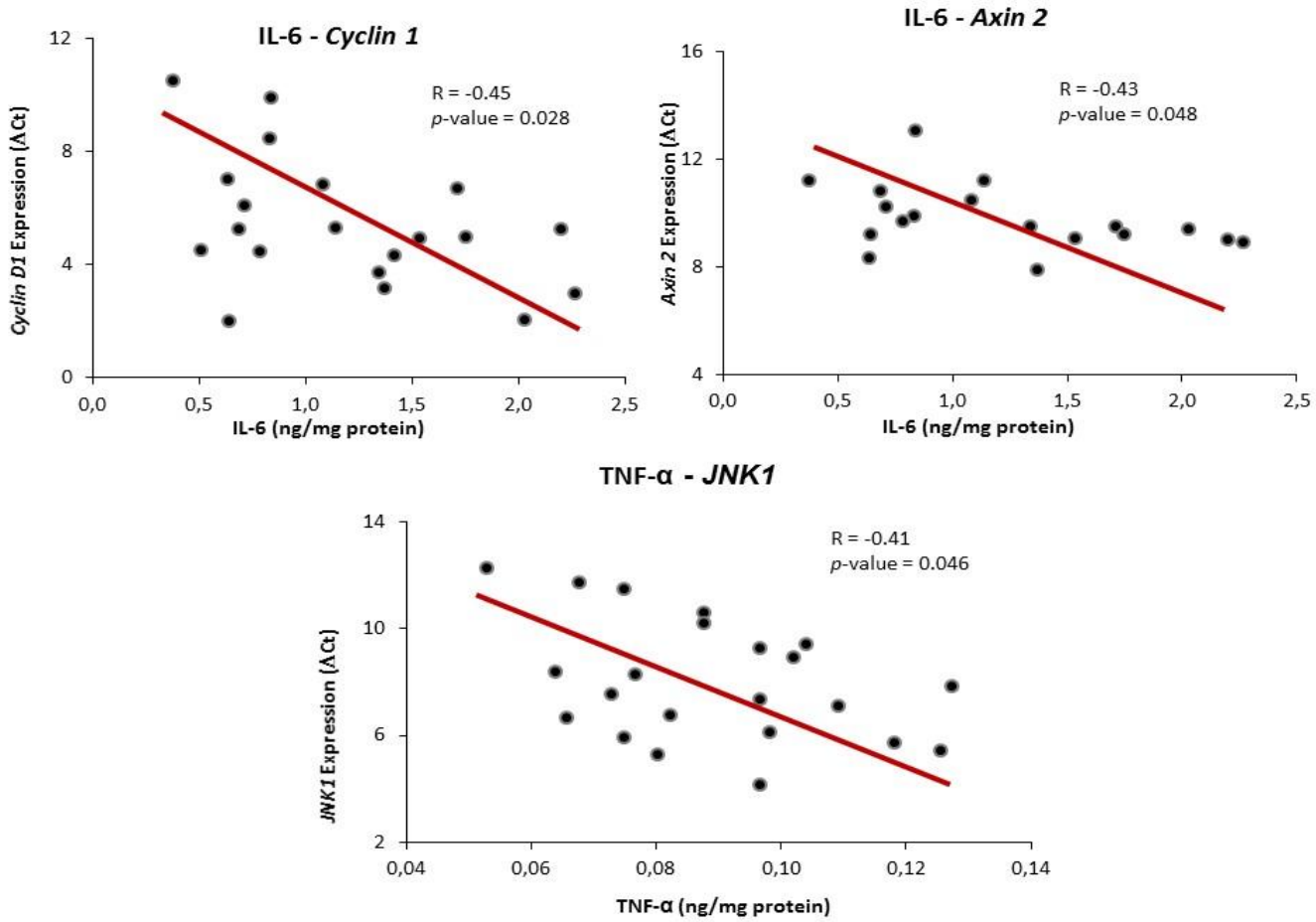

Figure 10: Protein levels of IL-6 are significantly correlated with *CyclinD1* and *Axin2* expression, while TNF-α correlates with *JNK1* expression.

Treatment	Cyclin D1	C-myc	Axin 2
Anti-TNF-α Antibody	0.36±0.12	0.76±0.30	2.01±1.08
p-Value	**0.031**	0.448	0.945
TNF-α Recombinant Protein	3.69±1.18	1.02±0.28	2.67±0.69
p-Value	**0.023**	0.836	**0.082**

Treatment	p53	Cox 2	JNK1	JUN
Anti-TNF-α Antibody	4.52±3.14	0.26±0.10	0.78±0.60	2.39±0.89
p-Value	0.225	**0.017**	0.323	0.703
TNF-α Recombinant Protein	0.51±0.08	2.46±1.96	2.00±0.25	7.38±4.12
p-Value	**0.014**	0.626	0.115	0.464

Table 1: Relative expression of Wnt targets after treatment with anti-TNF-α antibody (women with BMI ≥ 30) and TNF-α recombinant protein (women with BMI < 30).

CHAPTER 6

DISCUSSION

The expansion of adipose tissue observed in obesity is accompanied by an increase in the secretion of pro-inflammatory cytokines by the adipocytes, pre-adipocytes and macrophages that compose WAT, and those cytokines are commonly found elevated in the serum of obese individuals. In postmenopausal women specifically, Chedraui et al. (2012) showed that levels of IL-6 were elevated in women with the metabolic syndrome, a very common condition in obese individuals, and correlated with waist circumference. IL-6 and TNF-α also correlated with hypertension, another condition often found in obese individuals. Those cytokines may travel to other sites and affect distant tissues, including the breasts. Obesity has for example been associated with increased levels of TNF-α, IL-1β and IL-18 in the colon of obese mice compared to lean animals (Liu et al., 2012). Previous studies have shown elevated levels of macrophages, crown-like structures (CLS) and some of those inflammatory cytokines, especially IL-6 and TNF-α, in the mammary tissue of obese mice (Subbaramaiha et al., 2011; Santander et al.; 2015), but this relationship has not been investigated in humans. In this study, we measured the levels of proinflammatory cytokines in mammary tissue from postmenopausal women and examined their relationships with BMI.

Out of the six inflammatory cytokines tested, we demonstrated here that the levels of three major obesity-related pro-inflammatory cytokines, IL-6, IL-1β and TNF-α, are elevated in the breast tissue of obese women, which may create conditions favorable to the development of breast cancer. Indeed, cytokines seem to play important regulatory roles in tumor progression: some have been found in many cases

of breast cancers and are suspected to contribute to cancer development while others may have protective effects and are even used in therapeutics (Nicolini, Carpi and Rossi, 2006). Tissue levels of IL-1β and IL-6 are associated with tumor cell proliferation and invasion, while serum IL-1β and IL-8 are indicators of poor prognosis. The effects of IFN-γ seem less clear, and in some cases IFN-γ and IL-2 have even been used in therapy. Bozcuk et al. (2004) also found that elevated serum IL-6 was associated with lower progression free and overall survival in patients undergoing chemotherapy. Interestingly, they found that on the contrary elevated TNF-α (>6.20pg/mL) was associated with a 52% decreased risk of progression. However, other studies have shown that blockade of TNF-α inhibits cell proliferation and induces tumor cell apoptosis (Pileczki et al., 2012) and inhibits migration, invasion and metastasis (Hamaguchi et al., 2011). These apparently conflicting results can be explained by Balkwill's conclusions that TNF-α, when administered at supra-physiological levels, has powerful anti-cancer actions, but has tumor promoting effects when chronically produced in the tumor microenvironment (Balkwill, 2002). In the context of obesity, where TNF-α is chronically elevated, TNF-α should therefore be studied for its pro-carcinogenic potential. Indeed, as mentioned previously, higher numbers of CLS are found in the breasts of obese women with breast cancer than in the breasts of lean women with breast cancer (Morris et al., 2011), which suggests a higher pro-inflammatory cytokines secretion by macrophages. Blot et al. (2003) also showed that monocytes cultivated with breast cancer cells show a dramatic increase in TNF-α secretion and significantly reduced the pro-apoptotic effects of TNF-α, thus promoting cancer progression. Considering that obesity is well-established as a risk factor for breast cancer development (especially in post-menopausal women) and is associated with poor prognosis

regardless of menopausal status (Carmichael, 2006; Berdaz et al., 2004; Reeves et al., 2007), and that inflammation is now believed to play a role in this relationship (Santander et al., 2015), these findings may justify further research in this area and reveal new strategies for breast cancer prevention and treatment.

Many inflammatory pathways have been investigated for their roles in obesity-associated diseases. Regarding breast cancer, estrogen is often cited as one of the main contributors to the relationship between fat tissue accumulation and breast cancer risk. However, it does not seem to explain everything, especially when it comes to non-estrogen-responsive tumors. Wnt signaling has recently drawn attention since β-catenin accumulation has been found in a large percentage (~60%) of breast tumors and has been associated with poor prognosis (Howe & Brown, 2004; Lin et al., 2000). Also supporting the importance of Wnt signaling in breast cancer development, Mukherjee et al. (2011) showed that Wnt signaling was somehow altered in at least one Wnt-related gene in 99% (156/158) of the samples they analyzed. As mentioned previously, a few studies have shown that obesity might result in increased Wnt activity in the absence of antagonists (Gauger et al., 2014; Ouchi et al., 2010), which seem to be often downregulated in breast tumors (Suzuki et al., 2008; Mukherjee et al., 2011). Ouchi et al. (2010) also showed that obese individuals with CLS express lower levels of Sfrp5, suggesting that obesity might promote Wnt activity by down-regulating antagonists. Others have shown that cytokines can activate Wnt signaling: Gustafson and Smith (2006) showed that IL-6 and TNF-α both result in increased Wnt activity (high β-catenin, LRP6 and Dsh and low Axin) in preadipocytes, and Kaler, Augenlicht and Klampfer (2009a) showed that IL-1β produced by macrophages in colon cancer cells induced Wnt signaling, via the NF-κB/AKT pathway (Kaler et al., 2009b). In this study, after demonstrating that

high BMI is associated with an elevated inflammatory status manifested by increased levels of IL-1β, IL-6 and TNF-α in postmenopausl mammary tissue, we further investigated whether BMI is also associated with aberrant Wnt activity. We found a significant correlation between BMI and *CyclinD1* expression (p-value = 0.006). There was also a 13-fold increase in *CyclinD1* expression in obese women in comparison to women with BMI < 30. Our findings complete the study by Lin et al. (2000) who showed that *CyclinD1* expression paralleled β-catenin activity and was a predictor of poor patient survival, and the results of Tanić et al. (2013) who showed that CyclinD1 was overexpressed in 20% of breast cancer samples and was associated with the stage II of breast cancer patient, indicating its involvement in cancer progression. CyclinD1 could thus provide a potential explanation for the poor prognosis associated with obesity, regardless of menopausal status.

Axin2 expression was significantly increased (3-fold, p-value = 0.030) in the obese group in comparison to the leaner group. There also seems to be a positive correlation between BMI and *Axin2* expression, although the correlation is not significant due to an abnormally high data point (woman with BMI = 48). When this point is eliminated the relationship becomes significant (p-value = 0.028) and the correlation between BMI and *CyclinD1* also becomes even more significant (p-value = 0.002). Yook et al. (2006) showed that Axin2 plays a critical role in EMT and therefore promotes invasiveness via the zinc-finger transcription factor Snail1. Axin2 acts both as a tumor suppressor and as an oncogene (Fodde & Brabletz, 2007). It is part of the destruction complex and therefore participates in β-catenin degradation, and it controls the phosphorylation of Wnt targets (*c-Myc, Cyclins, Snail1*) by GSK3β. However, upon Wnt activation, Axin2 is upregulated and redirects GSK3β to the cytoplasm, leaving *Snail1* (and other Wnt targets) unphosphorylated and

transcriptionally active. Upregulation of Axin2 in women with high BMIs could thus promote cancer invasion and aggressiveness, and also contribute to the relationship between obesity and poor prognosis for breast cancer patients. We also found that *JNK1* (*MAPK8*) was upregulated 7-fold in the obese group (borderline significant: p-value = 0.058). *JNK1* is traditionally considered a target of the non-canonical Wnt pathway, but recent studies suggest that the canonical and non-canonical Wnt pathways may cooperate and contribute to carcinogenesis (Saadeddin et al., 2009). JNK seems to be involved in both cell proliferation and cell apoptosis. Some studies report increased tumor formation upon JNK inhibition (Cellurale et al., 2012) while others showed that JNK inhibition in breast cancer cells resulted in apoptosis and growth inhibition and therefore could be used to decrease proliferation (Mingo-Sion et al., 2004). JNK thus seems to participate in normal breast development, but its dysregulation may have consequences on tumorigenesis. Interestingly, JNK is involved in immunity and is activated by many stressors including the inflammatory cytokines TNF-α and IL-1, and inhibits cell growth in inflammatory conditions (Mingo-Sion et al., 2004). However, Ouchi et al. (2010) showed that blockade of Wnt5a-JNK signaling reduced IL-6 and TNF-α secretion by adipocytes and macrophages, and concluded that JNK activation significantly contributes to inflammation. Our results are consistent with their finding that Wnt5a and Wnt5a:sFRP5 ratio was increased in obesity, and that the presence of CLS was associated with decreased sFRP5 antagonist, and resulted in increased activation of JNK1. JNK1 has been found to have opposing functions and its role in tumorigenesis is not fully understood yet, but because it is influenced by inflammatory mediators and that those are abnormally regulated in obesity, further investigation on the mechanisms involved would be beneficial. Obesity promotes chronic low-grade

inflammation rather than the acute inflammatory response seen in normal immune responses and may therefore impair normal JNK regulation.

One surprising result was that the tumor suppressor *p53* was close to being significantly up-regulated 8-fold ($p = 0.065$) in obese women. *P53* is regulated by many pathways and therefore this increase in its expression may not be due to Wnt signaling exclusively. Also, it is important to note that *p53* is often altered in breast cancer, and that *p53* signaling may be significantly impaired in obesity. Indeed, Ford et al. (2013) showed that obesity promoted mammary tumor progression in mice regardless of the level of expression of *p53*, and Ayyanan et al. (2005) found that *p53* functions in breast tumors are impaired upon Wnt1 activation. Therefore, elevated *p53* expression may not be as beneficial as expected upon development of breast tumors in obese individuals.

In addition to the correlation between BMI and Wnt activity, we attempted to establish a causal relationship between the presence of inflammatory cytokines and Wnt activation. We first looked at the correlation between each elevated inflammatory cytokine and each elevated Wnt target, and found that IL-6 correlates with *CyclinD1* and *Axin2* expressions, and that TNF-α correlates with *JNK1*. This may suggest that inflammatory cytokines at least partially explain the effects of obesity on Wnt activity. TNF-α is one of the main obesity-related inflammatory cytokines, and has been shown to activate the Wnt pathway in adipocytes (Gustafson & Smith, 2006; Isakson et al., 2009), in the colon of obese mice (Liu et al., 2012), and in gastric tumors (Oguma et al., 2008). Oguma et al. also showed that TNF-α inhibition reduced Wnt activity while TNF-α protein stimulated it. We thus suspected that TNF-α blockade would reduce Wnt activity while addition of TNF-α recombinant protein would increase it. We did find that *CyclinD1* expression reflected TNF-α treatment: it

was increased in samples treated with protein, and reduced in samples treated with neutralizing antibody. This is consistent with the study by Cawthron et al. (2007) who found, while investigating the effects of TNF-α on adipogenesis, that TNF-α treatment resulted in increased *CyclinD1* expression, and seems of major interest since *CyclinD1* is a well-established oncogene in many cancers, including breast cancer (Arnold & Papanikolaou, 2005), and has been found elevated in a significant portions of human breast tumors (Tanić et al., 2013). *Axin2* also seems to be upregulated after treatment with TNF-α recombinant protein ($p = 0.082$), but it was not significantly affected by treatment with anti-TNF-α antibody in our study. Ayyanan et al. (2005) found *Axin2* to be consistently upregulated in human breast carcinomas, which further supports our hypothesis that inflammatory cytokines result in Wnt activation and thus promote breast tumor development. We also found that TNF-α blockade significantly reduced *Cox2* expression. Cox2 has been shown to be involved in the development of several types of tumors, including breast tumors, and is associated with poor prognosis and its expression increases with breast tumor progression (Cho et al., 2005). Our data thus suggest that blockade of inflammatory cytokines may be an effective way to reduce breast cancer risk, possibly by decreasing Wnt activity. Finally, we did find that addition of TNF-α recombinant protein reduced *p53* expression. As mentioned previously, *p53* is often impaired in breast cancer (Ford et al., 2013; Ayyanan et al., 2005), and although we found that it may be upregulated in obese women who are disease free, these results suggest that the elevated levels of TNF-α observed in the breasts of women with high BMI may impair its tumor suppressor functions and thus allow breast cancer development. Together, these results reveal that TNF-α may be an important mediator between obesity and Wnt activation. Although further investigation is required, TNF-α seems

to be an important modulator of Wnt signaling and may be considered as a target for therapeutics in the treatment of breast cancer.

Our results thus do indicate the presence of a pro-inflammatory environment in the breasts of obese women, and seem to support our hypothesis that inflammation causes Wnt activity to increase with BMI. However, a few limitations must be taken into consideration when interpreting our results. Our sample size is relatively small and we do lack information regarding the donors – we were provided age and BMI only – so we may not be accounting for confounders including body fat distribution, breast size, parity, actual menopausal status, breast feeding, use of hormone therapy, etc. These factors have all been listed as potential risk factors, and therefore further studies that control for these would help strengthen our findings. However, while many of these are suspected to increase breast cancer risk via the actions of sex hormones, we propose a mechanism that is estrogen-independent, and could therefore explain the link between obesity and breast cancer risk for both estrogen-responsive tumors after menopause and triple negative breast cancer before menopause, and between obesity and poor prognosis regardless of menopause status. Most women studied for the correlations between BMI and cytokines levels and BMI and Wnt activity were disease free, so further research to clearly establish the link between Wnt signaling and breast cancer development needs to be done. Previous studies have shown increased Wnt activity in breast cancer, and others have shown increased presence of macrophages, markers of inflammation, near breast tumors so we suspect a link between the two conditions, but further research is necessary and a follow-up to investigate whether women with increased BMI and increased Wnt activity develop breast tumors more frequently than leaner women would be ideal. On the contrary, although all our samples are normal mammary tissues, some of the women from

whom samples were obtained for ex-vivo culture with TNF-α recombinant protein or anti-TNF-α antibody had breast tumors, which may influence our results. Finally, as mentioned above, many, if not all, of the Wnt targets are also influenced by other pathways. Our study does not allow us to discriminate between them and therefore Wnt signaling may not be the only one at play and the observed aberrant expression of the Wnt pathway downstream genes may not exclusively be the consequence of obesity-induced inflammation. Nevertheless, our results provide promising evidence of a link between obesity-induced inflammation and the activation of Wnt signaling, which may influence the development of breast cancer.

CHAPTER 7

SUMMARY & CONCLUSIONS

Overall, our study provided evidence of a link between obesity-induced inflammation and Wnt signaling activation, which may contribute to breast cancer development. We were the first laboratory, to our knowledge, to investigate the relationship between BMI, inflammatory cytokines and the Wnt pathway in human mammary tissue. Although not quite sufficient to determine the exact role of obesity-driven Wnt signaling in the breast and its implications on breast cancer development, our results do show that an inflammatory environment is found in the breasts of obese women and is associated with increased Wnt activity, which may drive the development of breast cancer. Since Wnt activity is upregulated in the majority of breast cancer cases (~60%), these finding are very relevant to the research on the link between obesity and cancer. TNF-α, one of the most important inflammatory cytokines associated with obesity, may play a role in this relationship. Our results demonstrate that its blockade reduces the expression of several Wnt targets, while addition of TNF-α recombinant proteins had the opposite effect. We thus outlined a potential new mechanism linking obesity-associated inflammation and particularly the critical TNF-α, Wnt activity, and possibly breast cancer development and progression, which will contribute to our understanding of the relationship between obesity and breast cancer. Other inflammatory cytokines are found at high levels in obese individuals, including IL-6 and IL-1β, and therefore investigating their roles as well would be beneficial and may reveal complementary effects on Wnt signaling. Evaluating Wnt activity at different stages of breast cancer development would further contribute to our understanding of the role of Wnt in breast cancer.

APPENDIX

SAMPLES AND PROCEDURES

A. Samples

Sample	BMI	Age
72	25,8	49
74	25,9	56
80	22	52
81	21	55
83	25	56
91	32	56
92	34	52
110	32	59
134	35	56
140	37	53
142	26	65
143	33	66
147	23	42
163	28	62
165	37	65
166	48	59

Sample	BMI	Age
180	33	51
190	41	54
192	41	55
194	23	43
211	27,8	57
213	39,7	46
214	39	59
222	42	31
225	32,6	35
226	31	61
233	40	59
241	28	51
243	23	56
249	39	62
250	32	56

Table 2: Sample characteristics (age and BMI) for cytokines profile and Wnt activity (Batch 1)

Sample	BMI	Age	Treatment	Surgery
148	30,4	48	antibody	single
269-R	24,7	53	protein	rm
269-L	24,7	53	protein	rm
286	33,9	50	antibody	single
290-NAB	23,8	50	protein	bilateral
290-AB	23,8	50	protein	bilateral
307	50,1	67	antibody	single
312-NAB	33,6	52	antibody	bilateral
312-AB	33,6	52	antibody	bilateral
318	25	53	protein	single
336	26,6	59,5	protein	single

Legend:

rm = reduction mammoplasty

single = mastectomy of the affected side

bilateral = mastectomy of both the affected (AB) and non-affected (NAB) sides

Table 3: Sample characteristics (age, BMI, surgery and treatment) for effects of TNF-α on Wnt activity (Batch 2).

B. Protein preparation

Prepare *Tris Lysis buffer (1X, Incomplete):* 150 mM NaCl; 20 mM Tris, pH 7.5; 1 mM EDTA; 1 mM EGTA; 1% Trition-X-100

Store at 2-8°C

Prepare *Complete Lysis Buffer:* 10 mL 1X Tris Lysis Buffer; 100µL Phosphatase inhibitor I; 100µL Phosphatase inhibitor II; 100µL Protease inhibitor solution

Preparation of tissue samples

- Prepare 10~20 mg of tissue per 400 µl Complete Lysis Buffer and put the tissue in 2ml microcentrifuge tubes with 400 µl pre-chilled Complete Lysis Buffer.
- Homogenize (30,000 rpm) using a power homogenizer.
- Incubate on ice for 30 min.
- Centrifuge (~12,000 g) for 30 min at 4°C.
- Remove and discard the fatty layer, transfer the clear supernatant into a new tube.
- Determine the protein concentration using Pierce™ BCA protein Assay kit and a microplate Reader (SpectraMax M2, by Molecular Devices)
- Divide into aliquots and store at -80°C.

C. Cytokine profiling (MSD protocol)

Materials:

- Proinflammatory Panel 1 (human) Kits (Proinflammatory Panel 1 (human) Calibrator Blend; Diluent 2; Diluent 3; Read Buffer T (4X); SULFO-TAG Detection Antibodies (IFN-γ, IL-1β, IL-2, IL-6, IL-8, TNF-α).
- Phophate-buffered saline (PBS) plus 0.05% Tween-20

- Deionized water

Turn on MSD instrument

Prepare calibrators according to the company's protocol from the lyophilized calibrator

Prepare samples in 96-well polypropylene plate (so multichannel pipette can be used)

- For duplicates, place 60µL of sample in well
- Add 60µL of Diluent 2 (2-fold dilution)
- Mix well (centrifuge) for a few seconds

Prepare antibody solution: In one 3-mL tube, add 60µL of each detection antibody, and add Diluent 3 to bring total volume to 3000µL (vol. Diluent 3 = 3000 – 60*(# of Ab))

Prepare the Wash Buffer by combining 15mL of buffer and 285mL of deionized water

Prepare Read Buffer T (2X) by combining 10mL of Read Buffer T (4X) and 10mL of deionized water

Protocol:

- Add 50µL of sample (or calibrator) per well using a multichannel pipette
- Seal plate with adhesive tape
- Incubate at room temperature for 2 hours with shaking (350rpm on plate shaker)
- Wash plate 3 times with 150µL/well of wash buffer
- Add 25µL of detection antibody solution to each well
- Seal plate with adhesive tape

- Incubate at room temperature for 2 hours with shaking (350rpm on plate shaker)
- Wash plate 3 times with 150µL/well of wash buffer
- Add 150µL/well of 2X Read Buffer T, using reverse pipetting
- Read plate using MSD instrument

D. Real-time PCR

a) Isolate RNA using TRIzol®

- Homogenize samples (~10mg) in 1mL TRIzol® Reagent in 3-mL centrifuge tubes using a power homogenizer.
- Incubate for 5min at room temperature
- Add 0.2mL of chloroform, cap the tube and shake vigorously for 15sec.
- Incubate for 2-3min at room temperature and centrifuge at 12,000 x g for 15min at 4°C.
- Remove the aqueous (top) phase and place in new 1.5-mL centrifuge tube
- Add 0.5mL of 100% isopropanol
- Incubate at room temperature for 10min, and centrifuge at 12,000 x g for 10min at 4°C.
- Remove the supernatant from the tube.
- Add 1mL of 75% ethanol, vortex briefly, and centrifuge at 7500 x g for 5min at 4°C.
- Discard the wash and air dry the RNA pellet for 5-10min.
- Resuspend the pellet in 50µL RNase-free water
- Incubate in a heat block at 55-60°C for 10-15min.

- Proceed to cDNA synthesis or store at -70°C.

b) Perform cDNA synthesis using QuantiTect® Reverse Transcription (Qiagen®)

- Add 2µL of gDNA Wipeout Buffer, 7x; 75ng of RNA; and enough RNase-free water to bring the volume to 14µL
- Incubate for 2min at 42°C and place on ice.
- Add 1µL of Quantiscript Reverse Transcriptase; 4µL of Quantiscript RT Buffer, 5x; and 1µL of RT Primer Mix.
- Incubate for 15min at 42°C, then incubate for 3min at 95°C.
- Proceed to real-time PCR or store at -20°C.

c) Perform real-time PCR

- Prepare the primer mixture: mix 5µL of the forward primer, 5µL of the reverse primer, and 90µL of DNase, RNase free distilled water. Primers used are listed in Table 4 below.
- In each well (384- or 96-well plate, or 8-tube strip), add 2µL of DNase/RNase free distilled water and 5µL of Power SYBR® Master Mix (Life Technologies).
- Add 1µL of primer mixture
- Add 2µL of cDNA sample.
- Cap/seal and centrifuge to eliminate bubbles.
- Run RT-PCR to measure ΔC_T using the standard mode.

Wnt Targets	Forward	Reverse
MYCBP (Myc)	ATGGCCCATTACAAAGCCG	TTTCTGGAGTAGCAGCTCCTAA
CCND1 (CyclinD1)	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
AXIN2	CAACACCAGGCGGAACGAA	GCCCAATAAGGAGTGTAAGGACT
TP53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
PTGS2 (COX2)	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
MAPK8 (JNK)	TGTGTGGAATCAAGCACCTTC	AGGCGTCATCATAAAACTCGTTC
JUN	TCCAAGTGCCGAAAAAGGAAG	CGAGTTCTGAGCTTTCAAGGT
B-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

Table 4: Wnt targets and β-actin (housekeeping gene) primers.

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