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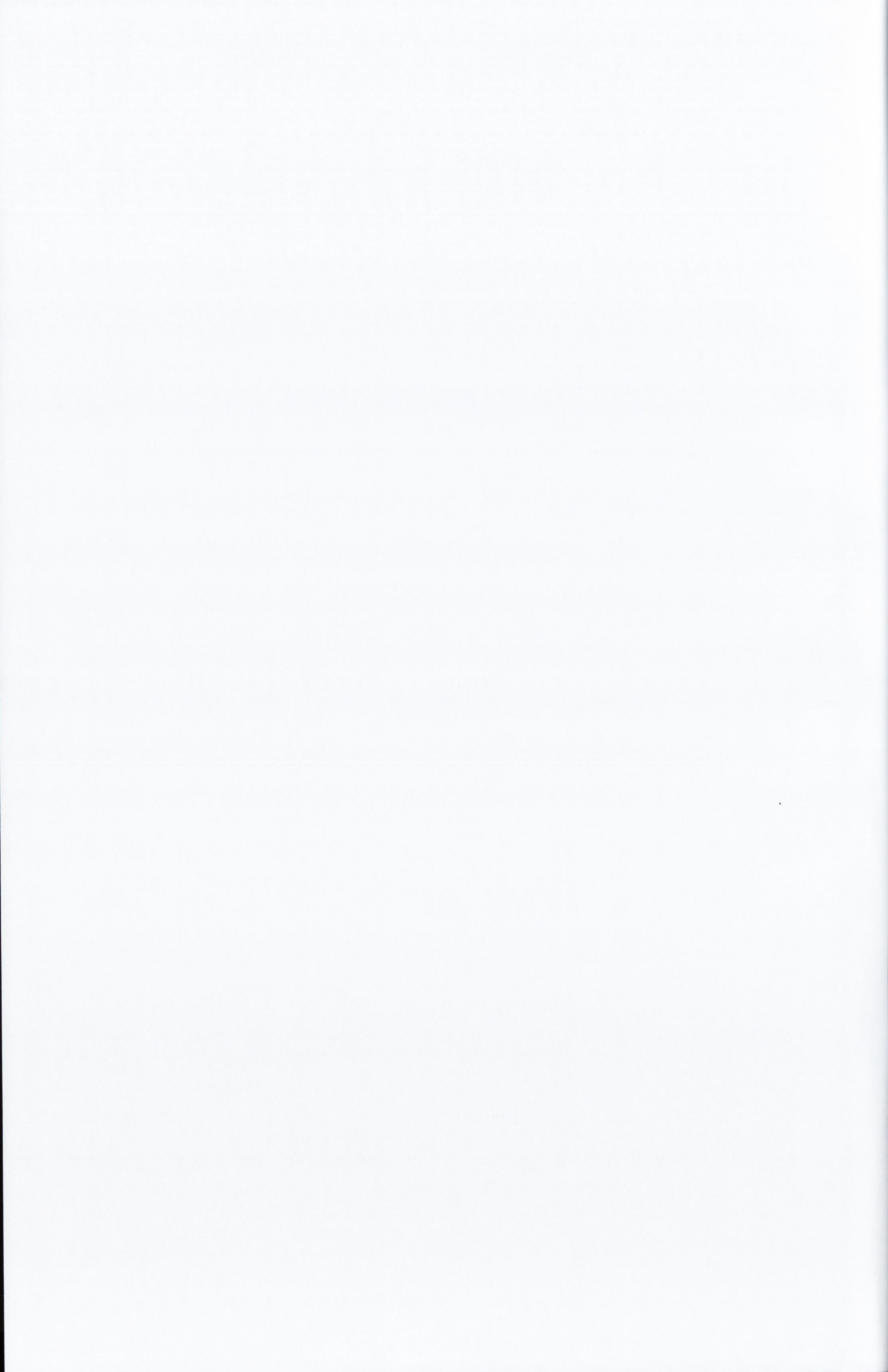

Investigation of the link between obesity and breast cancer - The role of adipose tissue in the development and propagation of malignancy

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A thesis submitted for the degree of Doctor in Medicine (MD)

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Declaration

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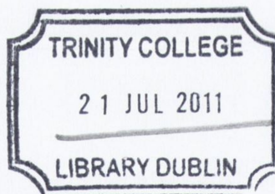
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Abstract

Introduction

Obesity and the metabolic syndrome are emerging risk factors for breast cancer. They are associated with increased incidence, an increased recurrence rate and a poorer prognosis. Despite this epidemiological evidence to suggest the associations between the two, very little data is forthcoming regarding the molecular mechanisms to link obesity with breast cancer. Adipose tissue is known to produce various chemokines, cytokines and peptides, collectively called 'adipokines'. The production of these adipokines is directly related to obesity status. In particular, leptin and adiponectin have stimulated great interest as their biological and pathophysiological properties are largely in opposition to one another. Furthermore it is postulated they function in an endocrine, paracrine or autocrine pathway to influence aspects of cancer progression. We hypothesise that adipokines are produced by mammary adipose tissue and that obesity status influences the production of these growth factors. We further propose that obesity status influences adipokine expression in human breast cancer tumour tissue.

Methods

A breast cancer biobank was established with specific concentration on gathering information on anthropometric and serological markers of obesity and the metabolic syndrome. Adipose conditioned media (ACM) was produced from harvested mammary adipose tissue from mastectomy samples. Using three breast cancer cell lines (MCF-7, MDA-MB-231 and T47-D), the influence of ACM from normal weight and metabolic syndrome patients on

various aspects of cancer progression was examined. Gene expression changes were investigated using cancer pathway profiler arrays to explain results observed using a co-culture model. Finally, the mRNA expression of adipokines and their ligand receptors was determined in mammary adipose and tumour tissue to examine the influence of obesity status, in particular metabolic syndrome status on gene expression.

Results

ACM from metabolic syndrome patients stimulated increased cell proliferation and invasion compared to normal weight cancer patients. Gene expression profiling revealed increased expression of genes involved in invasion/metastasis, cell signalling/transduction and angiogenesis. mRNA expression of leptin in mammary adipose tissue and leptin receptor in tumour tissue was increased in metabolic syndrome patients compared to normal weight women.

Conclusion

Our hypothesis sought to evaluate the effect of obesity status on mammary adipose tissue to establish a paracrine mechanism of action for adipokines in breast cancer. We concluded that mammary adipose tissue is under the influence of obesity status, principally by the presence of the metabolic syndrome. Furthermore, in tumour tissue, the metabolic syndrome induces increased expression of the leptin receptor. Further work is required to delineate the molecular mechanisms and alteration induced by obesity status. Understanding these mechanisms will allow for therapeutic interventions, either as preventative measures or as adjuvant treatments.

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This thesis would not have been possible without the help of numerous people and I am truly indebted to them for their advice, encouragement and guidance through the two years of this work.

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Presentations and Publications

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34th Sir Peter Freyer Surgical Symposium, Galway, 4th-5th Sept 2009

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Chapter 1:
General Introduction

1.1 Breast cancer epidemiology

Breast cancer is the commonest, non-dermatological malignancy diagnosed in women in Ireland. It accounts for 30% of all invasive cancers and 18% of all cancer deaths in women (National Cancer Registry, 2010) . On average 2,700 women are diagnosed with breast cancer each year. Approximately 1 in 12 women are at risk of developing the disease. Over the next 25 years (2010-2005), breast cancer is projected to increase by between 120% – 206% (National Cancer Registry, 2008).

The incidence of breast cancer in the western populations increases steadily with age until a plateau at approximately 50 years, after which incidence increases again at a more gradual rate. This pattern is explained by the existence of two distinct types of breast cancer, delineated by menopausal status. Other recognised factors influencing incidence include familial history of breast cancer (including genetic mutation, BRCA 1 and BRCA 2), particularly in first degree relatives. 90% of breast cancer cases have no family history. Nulliparous women and women whose first full-term pregnancy was after 35 years have a slightly increased risk compared to multiparous women. Late menarche and artificial menopause are associated with a lower incidence, whereas early menarche (<12 years) and late menopause (>50years) are associated with increased incidence. Endometrial cancer is also a risk factor for breast cancer as is previous cancer in the contralateral breast. More recent

developments have identified that dietary factors, western diet and obesity are contributory to increased incidence of breast cancer.

1.2 Obesity/ Metabolic syndrome epidemiology

Obesity is now a public health issue of global proportions. In Ireland, the obesity epidemic is gathering pace (Fig 1.1). Currently 33% of women are considered overweight (BMI with a further 26% classed as being obese (BMI >30) (Morgan K, 2008).

There are multiple methods of defining overweight or obesity status. Body fat distribution is a major factor in the definition of obesity and each specific depot has its own characteristics (Jensen, 2008). One may denote the adipose compartments of the body as lower body fat (adipose tissue caudad to the inguinal ligament and iliac crests and is composed of subcutaneous gluteal and upper thigh); upper body subcutaneous (superficial and deep abdominal) and intra-abdominal (visceral/omental). Upper abdominal obesity, incorporating the visceral adipose depot is measured most accurately (non-invasively) by waist circumference (WC). Body mass index (BMI) is a marker for general adiposity. Other potential anthropometric measurements that may be used include Visceral Fat Area and Waist Hip Ratio. In this body of work, BMI and waist circumference measurement are predominantly recorded and discussed. They are the predominant measurements used in obesity research and are also incorporated into the metabolic syndrome

definition (see below). Table 1.1 delineates obesity cut-off points for BMI and WC. BMI is calculated by the formula:

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{\text{Height (m)}^2}$$

Table 1.1 Definition of Obesity by BMI and Waist Circumference

Status	BMI	Waist Circum.
Normal	19.0 – 24.9	<80cm
Overweight	25.0 – 29.9	
Obese	≥30.0	≥80cm

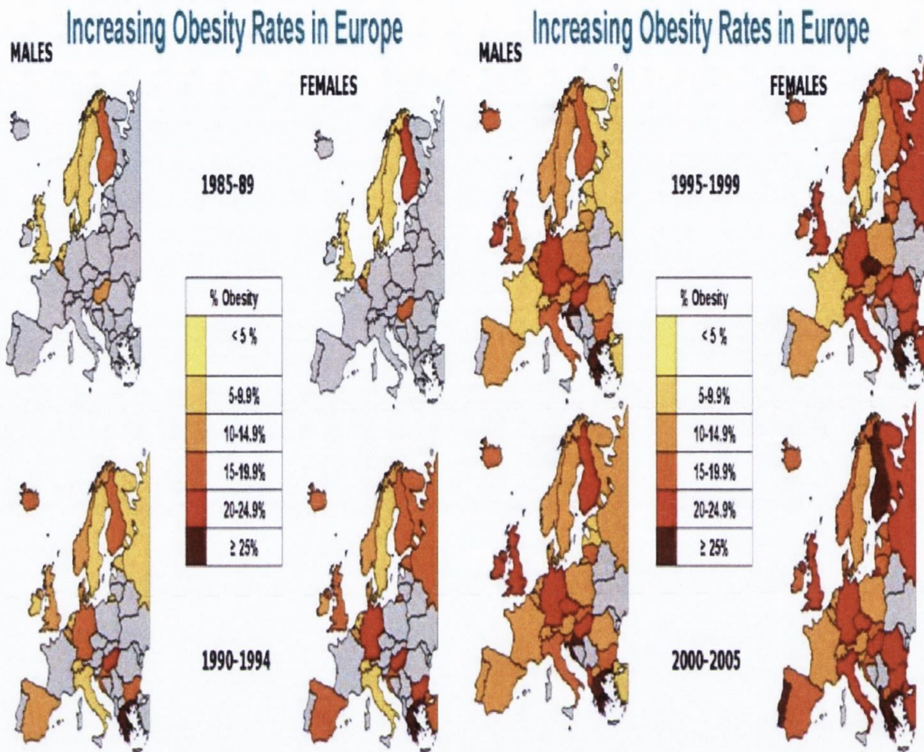


Fig. 1.1 **Increasing obesity rates in Europe from 1985-2005. Adapted from (Morgan et al, 2008)**

The sequelae of obesity are multitudinous, including, among them cardiovascular, peripheral vascular and renal disease. An important consequence of obesity is insulin resistance and diabetes. As obesity increases worldwide, a pattern of disease was identified using a clustering of closely

related cardiovascular risk factors. Originally denoted as the Syndrome X, it was diagnosed by the presence of risk factors including: Insulin resistance, hyperglycaemia, hypertension, low HDL– (high density lipoprotein) cholesterol and high LDL–(low density lipoprotein) cholesterol (Reaven, 1988). This constellation of risk factors was associated with much higher incidence of cardiovascular disease (Coronary artery disease, myocardial infarction) and type 2 diabetes mellitus (T2DM). The risk factors used to identify this syndrome have subsequently been refined and renamed as the *metabolic syndrome*.

Hyperinsulinaemia is a biomarker for insulin resistance and is a feature of the metabolic syndrome and precursor to T2DM. It can be calculated using the Homeostasis Model for assessment of Insulin Resistance (HOMA-IR) (Matthews *et al*, 1985) . Insulin concentrations are correlated to increasing waist circumference and BMI (Carnethon *et al*, 2002; Ross *et al*, 1996). The definition of metabolic syndrome used in this thesis is that published by the International Diabetes Federation (IDF) (Alberti *et al*, 2005) and is described in table 1.2. In Ireland, at present, the incidence of the metabolic syndrome in the general female population is 14% (Waterhouse *et al*, 2009).

Table 1.2 Metabolic Syndrome Definition (IDF 2005)

Central Obesity	
Waist Circumference	≥80 cm
Plus any two:	
1) Raised Triglycerides	> 1.7mmol/L
2) Reduced HDL-cholesterol	< 1.29 mmol/L
Or specific treatment for lipid abnormality	
3) Raised Blood pressure	Systolic ≥ 130mmHg
	Diastolic ≥ 85mmHg
Or specific treatment for previously diagnosed hypertension	
4) Raised fasting glucose	≥5.6 mmol/L
Or previously diagnosed T2DM	

1.3 Obesity in breast cancer: Epidemiological evidence

It has been long recognised that obesity is associated with multiple cancer types (Simopoulos, 1987). It is only recently that this association has been rigorously examined in epidemiological literature. The International Agency for Research into Cancer (IARC) concluded that excess body weight contributed to an increased risk of developing several cancer types (Bergstrom *et al*, 2001). In 2007, the World Cancer Research Fund reviewed a large body of literature and concluded that there was convincing evidence that body fatness is associated with an increased risk of oesophageal adenocarcinoma and

cancers of the pancreas, colorectum, endometrial, kidney and post menopausal breast cancer (WCRF, 2007).

Leading on from this data, it is now established that obesity is a strong risk factor for post-menopausal breast cancer. Using BMI as a marker, being overweight leads to a relative risk (RR) of 1.3 to the development of post-menopausal breast cancer, while being obese (BMI >30) leads to 1.5 RR. In other terms, being obese increases the risk of developing cancer by between 30-50% (Ballard-Barbash *et al*, 1996; Harvie *et al*, 2003; Trentham-Dietz *et al*, 1997). These associations between obesity and cancer become stronger with increasing age. La Vecchia reported that with BMI values above 28.4 there was a moderate association in women in the age groups 50–59 and 60–69 years, indicated by odds ratios of 1.30 and 1.24, this became noticeably stronger in those over 70 years of age, the odds ratio increasing to 2.14 (La Vecchia *et al*, 1997).

Intriguingly, this relationship does not hold for pre-menopausal breast cancer. An inverse relationship between BMI and cancer is demonstrated; the rationale being that excess adiposity induces anovulatory cycles and thus reduces the exposure to endogenous oestrogen (Zain *et al*, 2008). However, this must be tempered by the fact that adult weight gain between the ages of 20-50 is a strong risk factor for subsequent post-menopausal breast cancer (Howell *et al*, 2009; Santen *et al*, 2007). Obesity is also a poor prognostic indicator in breast cancer. Excess body weight is the

cause of approximately 20% of cancer deaths in women over the age of 50 years. A BMI >30 confers a relative risk of death from breast cancer of between 1.7 – 2.12 compared to normal weight women (Calle *et al*, 2003). Obese women are more likely to present with advanced breast cancer and ultimately have a worse outcome regardless of menopausal status (Harvie *et al*, 2003; Stephenson *et al*, 2003).

The metabolic syndrome is recognised as a high risk state for cancer in general (Cowey *et al*, 2006). The individual features of the metabolic syndrome have previously been studied with respect to breast cancer risk and direct associations have been suggested. Low HDL-cholesterol (Furberg *et al*, 2004), hyperglycaemia (Muti *et al*, 2002), high triglycerides (Potischman *et al*, 1991), hypertension (Soler *et al*, 1999), and hyperinsulinaemia (Hirose *et al*, 2003), have all been associated with increased breast cancer risk. A recent study, one of the first to study the metabolic syndrome as a complete entity, identified an increasing relative risk for the development of breast cancer in patients with increasing component of the MetS such that women with 3-5 risk factors had a RR of 2.48. Similar to obesity, MetS is associated with an aggressive phenotype at presentation (Healy *et al*, 2010) and ultimately has a worse prognosis.

Type 2 Diabetes Mellitus, the result of obesity and prolonged insulin resistance is recognised itself as an important risk factor for breast cancer (Xue *et al*, 2007). In a prospective cohort study, women with diabetes at

baseline were more likely to die from breast cancer compared to those without diabetes (HR = 1.27) (Coughlin *et al*, 2004). Similarly, Yanick *et al* demonstrated that breast cancer patients with diabetes were more likely to die prematurely from breast cancer compared to breast cancer patients without diabetes (RR 1.76) (Yancik *et al*, 2001).

Diabetes, obesity and breast cancer are all more prevalent in western society than in developing countries. More sedentary lifestyles, coupled with diets compose of increasing refined carbohydrates are thought to be responsible for these increases. As developing countries adopt western lifestyle and dietary characteristics, previously low rates of cancer incidence (breast etc) have begun to creep upward. The likelihood is that obesity, insulin resistance, the metabolic syndrome and diabetes are part of the same sequence of events that leads to the development and progression of breast cancer and cancer in general.

1.4 Current hypotheses in obesity related cancer

Several hypotheses have been proposed to explain the association between obesity and breast cancer. One hypothesis is that the biological/molecular cause of the association is elevated circulating oestrogens from peripheral aromatisation of androgens in adipose tissue in obese women compared to normal weight women. A second hypothesis is that obesity and its associated metabolic syndrome result in increased circulating levels of insulin

and insulin like growth factor (IGF) which are known mitogens. A final hypothesis is that adipose tissue is a source of growth factors (cytokines, chemokines), collectively termed adipokines, which are produced in a dysregulated fashion in obesity, to influence cancer development. Each of these hypotheses is discussed individually below.

1.4.1 Oestrogen, sex hormone biology and breast cancer

Oestrogen is presumed to be the major factor in obesity related breast cancer. Oestrogen biosynthesis is catalysed by the enzyme aromatase (aromatase cytochrome P450), a product of the *CYP19* gene. In obese women, the main sources of oestrogen synthesis are the buttocks, abdomen, thighs and adipose tissue of the breast. As adipose mass increases, aromatase levels in these tissues increase with a consequent elevation in oestradiol (Grodin *et al*, 1973). Sex hormone-binding globulin (SHBG) binds testosterone and oestradiol with high affinity. With increasing adipose mass, circulating SHBG decreases (McTiernan *et al*, 2003).Therefore the lower levels of SHBG in obesity allows an increase in free bioavailable oestrogen. Breast cancer risk has been demonstrated to be directly linked with sex-hormone concentrations, including oestrone, total and bioavailable oestradiol, while an inverse relationship with risk is seen with SHBG (Zeleniuch-Jacquotte *et al*, 2004).

In pre-menopausal women, aromatase is primarily expressed by the ovaries and circulating levels are under homeostatic regulation so that obesity and body fat mass do not influence oestrogen levels. However, it must be stressed that the study of oestrogens in pre-menopausal women is difficult due to the cyclical nature of sex hormone concentrations, especially during the early, and late, follicular phase (Rose *et al*, 2010) . No relationships have been demonstrated for oestrogen and breast cancer risk in pre-menopausal women in a prospective case control study nested in the large EPIC study (Kaaks *et al*, 2005).

The mechanism by which oestrogen stimulates cell proliferation is believed to be through activation of oestrogen receptor (ER) transcriptional activity and potentially through direct, intracellular signalling i.e. MAPK pathway. ER expression by tumour cells is a biomarker of the dependence of those cells on oestrogen for growth; its absence is a marker for poor prognosis. The production of a progesterone receptor (PR) depends upon normally functioning oestradiol - ER ligand interactions. By considering ER and PR together, a more accurate prediction of oestrogen dependence and therapeutic response to anti-oestrogenic therapy is improved. There is some debate that increased risk of breast cancer and a poor prognosis in obese populations is dependent on ER/ PR expression in tumours as suggested by a meta-analysis by Suzuki *et al*. The increased risk is solely related to tumours that are ER/PR +ve, with a 33% increased risk in postmenopausal women per 5 kg/m^2

increment in BMI. In pre-menopausal women, there is an associated 10% decrease in risk in this group per 5 kg/m^2 increment (Suzuki *et al*, 2009).

1.4.2 Insulin, Insulin like growth factor and breast cancer

Hyperinsulinaemia, a consequence of insulin resistance, is more common in obese women than normal weight women (Lazarus *et al*, 1998). Epidemiologic evidence from Gunter *et al*, demonstrated that insulin, in post-menopausal women, is an independent risk factor for the development of breast cancer in a large prospective study (Gunter *et al*, 2009). Goodwin *et al* demonstrated that a higher circulating plasma insulin level was associated with a greater risk of distant recurrence or death from disease (Goodwin *et al*, 2002). Furthermore, the insulin ligand receptor is over expressed in breast cancer compared to normal mammary epithelium. High expression of the insulin ligand receptor is associated with a worse 5-year survival in lymph-node negative breast cancer patients (Mathieu *et al*, 1997). Insulin is a recognised mitogen for normal mammary breast tissue and cancer cell lines (Chappell *et al*, 2001; Ish-Shalom *et al*, 1997). In animal models, the administration of exogenous insulin can promote breast tumour growth (Shafie *et al*, 1981; Shafie *et al*, 1981). These experimental data confirm that insulin is a potential mitogen in obesity related breast cancer.

Insulin like growth factor 1 (IGF-1) is a hormone related to insulin that has attracted interest as an endocrine risk factor for breast cancer. Both insulin and IGF-1 share similar sequence homology and downstream signalling pathways (Zapf *et al*, 1984). Most IGF in circulation is produced by the liver and is bound to IGF binding proteins (IGFBPs), with at least 75% bound to IGFBP-3. In obesity, IGFBP-1 in circulation is reduced so that approximately 1% of IGF-1 is unbound or free and this is considered the most biologically active form (Jones *et al*, 1995; Juul *et al*, 1997). IGF-1 promotes cellular proliferation and inhibits apoptosis in multiple tissues. Animal studies revealed that inhibition of the IGF-1 receptor or manipulation of IGF-1 concentrations in circulation could reduce tumour growth (Ish-Shalom *et al*, 1995). There is some evidence to support high circulating IGF-1 and breast cancer risk in women over 50 years (Baglietto *et al*, 2007; Rinaldi *et al*, 2006). However over expression of the IGF receptor in breast cancer has been associated with a favourable outcome, speculatively due to up regulation of receptors or better differentiation (Bonneterre *et al*, 1990; Peyrat *et al*, 1990; Toropainen *et al*, 1995).

1.4.3 Adipokines

White adipose tissue is the main source of energy storage in mammals and up until recently it was thought that this was the sole biological function of adipose tissue. Recent developments now indicate that adipose tissue is a highly dynamic organ with involvement in multiple physiological and metabolic processes beyond that of simple energy storage. This

observation was brought about by the recognition that adipose tissue is an endocrine organ secreting various proteins. This group of proteins synthesised exclusively or substantially by white adipose tissue is collectively termed adipokines, denoting their tissue of origin. Considerable interest is now being focused on the potential role of these adipokines in the development of cancers for which obesity is a risk factor.

1.4.3.1 Leptin

Leptin (greek, leptos, thin) (Ob) is a 16 kDa cytokine produced predominantly by adipose tissue and contributes to body weight homeostasis by regulating food intake and energy expenditure (Bray, 2002; Halaas *et al*, 1995; Zhang *et al*, 1994). Other physiological functions include bone formation, reproduction and angiogenesis (Saxena *et al*, 2007). Elevated circulating levels of leptin are found in obesity and are correlated with overall adipose tissue mass. Levels may also increase with overfeeding and decrease with starvation. The absence of leptin or a mutation in its receptor gene induces a massive hyperphagia and obesity in animal models (Friedman, 1999) and in humans (Farooqi *et al*, 1999). Both increased adipocyte number (hyperplasia) and size (hypertrophy) result in increased leptin levels (Considine *et al*, 1996). Leptin is also correlated with increasing insulin resistance and type 2 Diabetes Mellitus (T2DM) independent of BMI or body fat mass (Fischer *et al*, 2002). It is thus considered that leptin is a fundamental component of the metabolic syndrome. Indeed, concentrations of leptin increase in proportion to the number of individual components of the MetS (Sieminska *et al*, 2006).

The human leptin receptor (ObR) is a member of the class 1 cytokine receptor family. Six isoforms are known (ObRa-ObRf) but it is only the long isoforms that has an intracellular domain and thus capable of signalling (Fong *et al*, 1998). The leptin receptor engages both the signal transducers and activators of transcription 3 (STAT3) and the insulin receptor substrate phosphoinositide-3 kinase pathway (Buettner *et al*, 2006). STAT3 is essential for mediating food intake, liver glucose production and gonadotropin metabolism (Buettner *et al*, 2006). Leptin modulates the T-cell immune response, stimulates proliferation of T-helper cells, and increases pro-inflammatory cytokines by regulating immune cell response (Lord *et al*, 1998).

In breast cancer cell lines, exogenous leptin stimulated cellular proliferation of ER +ve cell lines in culture (Dieudonne *et al*, 2002; Garofalo *et al*, 2004; Hu *et al*, 2002). Vona-Davis *et al* would later describe proliferation in ER –ve cell lines including the MDA-MB-231 cell line at a concentration of 25-100 ng/ml, similar to that seen in ER +ve cell lines T 47-D and ZR-75-1. Leptin mediates multiple cellular processes including the ability to stimulate DNA synthesis and cell growth acting via multiple signalling cascades, such as the Janus-activated kinase 2/signal transducers and activators of transcription 3, extracellular signal-regulated kinase 1/2, protein kinase Ca, and Akt/GSK3 pathways (Dieudonne *et al*, 2002; Hu *et al*, 2002; Yin *et al*, 2004). Leptin induced cell cycle progression is accompanied by upregulation of cyclin-dependent kinase2 and cyclin D1 levels (Okumura *et al*, 2002) and

hyperphosphorylation/inactivation of the cell cycle inhibitor pRb (Garofalo *et al*, 2004). Leptin has been suggested to regulate angiogenesis by modulating VEGF activity (Gonzalez *et al*, 2006). It can increase the expression of VEGF and its receptor VEGFR-2 in a mouse model. Furthermore, the blockade of leptin receptor signalling can slow cancer growth with reduction in expression of VEGF, VEGFR-2 and cyclin D1 (Gonzalez *et al*, 2006). The functional effects of leptin in breast cancer are summarised in table 1.3.

1.4.3.2 Adiponectin

Adiponectin (AdipoQ) is a peptide from the collectin family and is exclusively secreted by white and brown adipose tissue (Hu *et al*, 1996; Nakano *et al*, 1996). It exists in plasma as a number of multimer complexes and combines via collagen domains to form three oligomeric forms: a low molecular weight trimer (LMW), a middle molecular weight hexamer (MMW), and high molecular weight (HMW) 12-18 mer adiponectin (Waki *et al*, 2003). It constitutes up to 0.1% of the total circulating plasma proteins (Arita *et al*, 1999). In contrast to leptin, adiponectin expression is decreased in obese humans, particularly visceral obesity, and an inverse relationship exists with insulin resistance (Arita *et al*, 1999). The metabolic syndrome is independently associated with hypoadiponectinaemia (Ryo *et al*, 2004). The principal physiological role of adiponectin is as an insulin sensitiser and is also involved in reduction of the inflammatory response.

There are two isoforms of the adiponectin receptor: AdipoR1 and AdipoR2. AdipoR1 is ubiquitously expressed, with abundant expression in skeletal muscle, while AdipoR2 is predominantly expressed in liver (Yamauchi *et al*, 2003). Adiponectin improves insulin resistance by increasing energy expenditure and fatty acid oxidation through activation of AMP-activated protein kinase (AMPK) and by increasing activity of PPARα target genes (Kadowaki *et al*, 2005). It is demonstrated in insulin resistant mouse models that the expression of adiponectin receptors are significantly decreased in skeletal muscle and adipose tissue, suggesting that not only does obesity decrease circulating levels of adiponectin but also influences expression of its receptors on target tissues. This potentiates the effect of insulin resistance leading to a vicious cycle, promoting hyperinsulinaemia (Tsuchida *et al*, 2004). In a human study a correlation was demonstrated between receptor gene expression and insulin sensitivity (Debard *et al*, 2004). Adiponectin also has vasculoprotective properties, mediated by an increase in endothelial nitric oxide production or by modulation of adhesion molecules (Kadowaki *et al*, 2005). Adiponectin via its receptors mediates several intracellular signalling pathways, including AMPKinase, JAK/STAT and MAP Kinase (p38 MAPK, ERK, JNK) (Luo *et al*, 2005; Miyazaki *et al*, 2005; Yamauchi *et al*, 2003). In the breast cancer cell line MCF-7, exogenous adiponectin, in multiple experiments demonstrated a reduction in cellular proliferation (25ng/ml) by decreasing c-myc and cyclin D1 cell, and also by promoting cell apoptosis (Dieudonne *et al*, 2006).

Table 1.3 Functional effects of adipokines in breast cancer cells

	Leptin	Adiponectin
Proliferation	↑	↓
Apoptosis	↓	↑
Cell cycle	↑	↓
Invasion	↑	↓
Angiogenesis	↑	↓

1.4.3.3 Other adipokines

An important development in the understanding of obesity is the concept that it (and diabetes) are characterised by a state of chronic low grade inflammation (Engstrom *et al*, 2003). Increased levels of pro-inflammatory cytokines and acute phase proteins are found in circulation in obesity. An increased number of macrophages resident in adipose tissue has been reported in human obesity and may contribute to the inflammatory process by secreting pro-inflammatory cytokines, or inducing their secretion from adipocytes (Di Gregorio *et al*, 2005). TNF was one of the first factors to be identified as being produced by adipocytes. Obesity induces increased secretion in mouse models (Hotamisligil *et al*, 1993). It is also recognised as playing a role in insulin resistance, through multiple pathways, including the inhibition of the insulin receptor signalling pathway (Hotamisligil, 2003). TNF-α acts both as an autocrine and a paracrine agent to influence numerous cellular processes,

including apoptosis, the production of numerous cytokines and adipokines (Coppack, 2001).

IL-6 is both expressed in, and secreted by, adipocytes and acts both locally (paracrine) and through circulation. Circulating plasma IL-6 and its expression in adipose tissue is higher in both obesity and insulin resistance (Vozarova *et al*, 2001; You *et al*, 2005). High serum IL-6 is associated with a poorer prognosis in breast cancer (Bachelot *et al*, 2003). It can promote cell migration via the activation of the MAPK pathway and is an effective anti-apoptotic agent, by inhibiting the proteases involved in apoptosis. TNF-α is a key regulator of IL-6 production (do Nascimento *et al*, 2004). Adipocytes secrete various chemoattractants that draw monocytes into adipose tissue. MCP-1, also known as chemokine (C-C) motif ligand 2 (CCL2), play an important role in the recruitment of macrophages. Obesity is associated with increased plasma levels of MCP-1, and over expression in adipose tissue (Sartipy *et al*, 2003). In breast cancer, high MCP-1 expression is associated with poor prognosis, recurrence and advanced disease (Lebrecht *et al*, 2004; Soria *et al*, 2008; Ueno *et al*, 2000). IL-8, a CXC chemokine has been shown to be released by isolated adipocytes and whole adipose tissue (Bruun *et al*, 2001). Besides its association with inflammatory processes it has been associated with the pathogenesis of atherosclerosis and coronary artery disease (Romuk *et al*, 2002). Plasma levels in diabetic patients are also higher compared to normal controls (Erbagci *et al*, 2001). In breast cancer, IL-8 is considered to be involved in cancer progression and metastasis. High

circulating IL-8 is associated with early dissemination of cancer and a poorer prognosis in breast cancer (Benoy *et al*, 2004). Vascular endothelial growth factor (VEGF) has a critical role in the angiogenic process, being a specific mitogen for vascular endothelial cells; angiogenesis is promoted by inducing matrix metalloproteinase (MMP) expression and stimulating vascular endothelial cell migration. Adipose tissue can produce VEGF, where it plays an important role in maintaining vascularity and in neovascularisation (an important process in the expansion of adipose tissue in obesity) (Thomas, 1996). The influence of obesity on these adipokines in breast cancer has not been established and requires investigation. Such data may suggest that obesity and its metabolic sequelae, as modifiable disease processes, could be manipulated to reduce breast cancer incidence and progression.

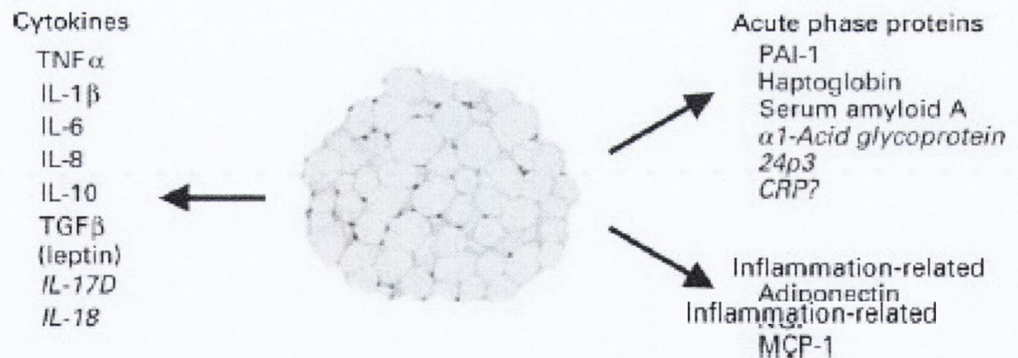


Fig 1.2 **Adipose tissue (Adipocytes and Stromal tissue) is a source of multiple cytokines, chemokines and peptides, collectively termed 'Adipokines' (Trayhurn *et al*, 2004).**

1.5 Pathways for adipokines in breast cancer

Obesity in cancer and the role of adipokines produces many interesting hypotheses and questions. Adipokines in circulation, produced by white adipose tissue (visceral, subcutaneous depots) suggest that the principle pathway by which they act is a classic endocrine pathway. In breast cancer, however, a second pathway potentially exists. As adipose tissue constitutes a large proportion of breast tissue, one must consider that a local or paracrine pathway for adipokines to work must exist. Finally, an autocrine, or autonomous pathway is hypothesised to be present. Figure 1.4 describes simply how these three pathways may interact.

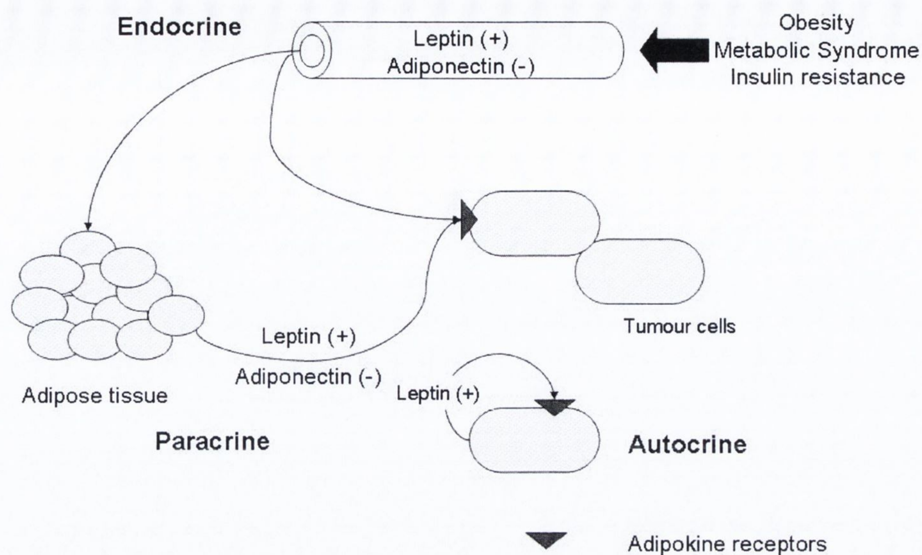


Fig. 1.3 **In obesity and breast cancer, adipokines circulate in the plasma to interact with cancerous breast epithelium. Endocrine-, paracrine-, and autocrine-mediated relationships exist between leptin and the cellular microenvironment to support the growth of tumour cells via leptin receptor activation. A paracrine relationship exists between adipokines and nearby mammary tumour cells to stimulate or inhibit growth. An autocrine relationship is established for leptin in breast cancer tissue but not for adiponectin.**

1.5.1 Endocrine mechanism

An exhaustive library of information demonstrates relationships between hormones and growth factors and breast cancer risk, clinico-pathological features and prognosis using circulating concentrations in plasma and serum. Principal among these being the evidence linking high oestrogen levels (total and free oestradiol and post menopausal breast cancer risk) (Toniolo *et al*, 1995) and similarly, high insulin levels with risk (Del Giudice *et al*, 1998; Lawlor *et al*, 2004) and prognosis (Goodwin *et al*, 2002).

Circulating leptin levels are speculated to be associated with increased cancer risk in several case-control studies. A number of negative or inconclusive studies have also been published (Table 1.4). Circulating leptin is also reported as a factor for a more aggressive tumour phenotype, being associated with increased tumour size (Chen *et al*, 2006), stage and grade (Goodwin *et al*, 2005). This study concluded however that circulating leptin was not a prognostic factor despite the link with aggressive tumour characteristics. The evidence for circulating leptin as risk factor for breast cancer is conflicting. Further, high powered studies are required to confirm this effect.

Adiponectin, in three published epidemiological studies have highlighted an inverse association between circulating adiponectin and breast cancer risk (Chen *et al*, 2006; Mantzoros *et al*, 2004; Miyoshi *et al*, 2003). Miyoshi *et al* found that lower adiponectin levels were associated with larger

tumour size and grade. Low levels of adiponectin were indicative of an aggressive phenotype, similar to what was observed with high circulating levels of leptin (Goodwin *et al*, 2005). Chen *et al* demonstrated that the adiponectin: leptin ratio was correlated strongly with tumour size, suggesting that the balance between these two adipokines, acting as circulating hormones, is important in an endocrine pathway in obesity related breast cancer.

1.5.2 Paracrine mechanism

The cellular and structural features of the mammary gland that favour stimulation of breast cancer cells by oestrogen in a paracrine manner must also apply to adipokines. Production of adipokines by the components of adipose tissue provides a microenvironment with opportunities for multiple and interactive paracrine activities between cancerous cells and mammary adipose tissue.

Table 1.4 Studies examining the role of circulating leptin in breast cancer risk

	Cases	Controls	Menopausal status	Results
Tessitore *et al* (Tessitore *et al*, 2000)	23	103	NA	Leptin higher in cases vs controls
Han et al (Han *et al*, 2005)	90	103	Leptin not related to menopausal status	Leptin higher in cancer vs benign/controls
Chen *et al* (Chen *et al*, 2006)	100	100	No significant effect between groups	Leptin higher in women with breast cancer
Petridou *et al* (Petridou *et al*, 2000)	75	75	Pre- and Postmenopausal	No association in post menopausal women Inverse relationship seen in premenopausal women
Ozet et *al* (Ozet *et al*, 2001)	58	25	Pre- and Postmenopausal	No association
Coskun *et al* (Coskun *et al*, 2003	85	25	NA	No association
Stattin *et al* (Stattin *et al*, 2004)	149	258	Postmenopausal	No association
Miyoshi *et al* (Miyoshi *et al*, 2006)	104	104	NA	No association

Table adapted from Vona-Davis and (Vona-Davis *et al*. 2007)

1.5.2.1 Anatomy and physiology of the breast

The adult female breast is composed of epithelial lactiferous ducts which terminate in secretory alveoli embedded within a framework of fibrous and adipose tissue (stroma) (Fig. 1.4). Normal growth and development of the breast is regulated by a complex interaction of hormones and growth factors. Some of these factors are secreted by breast epithelium and have autocrine function (cell-autonomous signals). Others are produced by stromal tissue and exercise paracrine control of epithelial development (heterotypic mechanisms). In cancer, these heterotypic mechanisms involve the secretion of soluble factors from tissues surrounding the tumour, extracellular matrix components and interactions between stromal and cancer cells to create a specific and local peritumoural microenvironment.

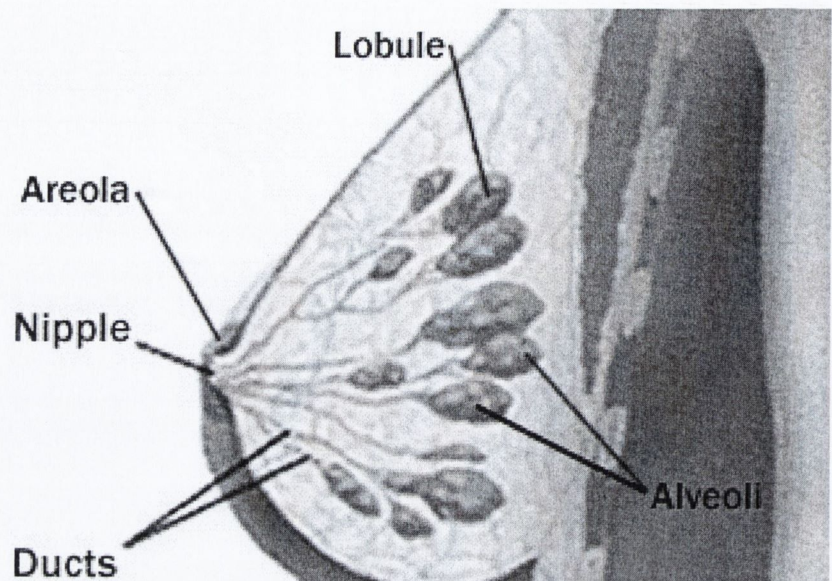


Fig. 1.4 **The structure of the female breast. The breast is composed of a number of structures including lobules (milk-producing glands), ducts (connecting the lobules to the nipple, adipose tissue, muscles (pectoralis major and minor) and the chest wall (rib cage).**

1.5.2.2 Mammary adipose issue as source of adipokines

Adipose tissue makes up the bulk of the breast, with epithelial cells comprising of only 10% of the total volume. It stands to reason that this adipose depot is a source of adipokines to influence cancer progression. Adipose tissue has been shown to influence breast cancer cell growth. Manabe *et al* demonstrated that secreted factors from adipocytes (rat) could promote cell proliferation of an ER +ve breast cancer cell line (MCF-7, T47-D) using

three dimensional collagen matrix co-culture systems (Manabe *et al*, 2003). *In vivo,* injection of a breast cancer cell line (SP1) with adipose tissue subcutaneously or intaperitoneally in mice induced mammary tumours and metastases. Omitting adipose tissue or avoiding a fat pad conversely did not produce tumour growth (Elliott *et al*, 1992). Iyengar *et al*, in an experiment treating the MCF-7 cell line with a conditioned media produced from murine adipocytes and fibroblasts, identified the upregulation of multiple genes involved in invasion, metastasis, and proliferation with a converse downregulation of genes involved in cell cycle checkpoint inhibition and tumour suppression using microarray technology (Iyengar *et al*, 2003). Using human mammary adipose tissue, from high risk premenopausal women, Celis *et al*, using a proteomic approach, identified many proteins secreted into an adipose conditioned media included signalling molecules, hormones, growth factors and cytokines including: Leptin, TNF-α, IL-6 and VEGF (Celis *et al*, 2005). This was the first study to demonstrate that mammary adipose tissue could be a source of factors in the microenvironment of a tumour that could potentially influence breast cancer progression.

1.5.2.3 Ligand-ligand binding receptors

The completion of a paracrine loop requires the presence of a specific ligand binding receptor on a target cell. Therefore adipokine receptors must be expressed on target tissues, in this case tumour cells. The leptin receptor isoforms (ObR long and short) are expressed in multiple breast cancer

cell lines indicating the potential for paracrine interactions between leptin, synthesised by adjacent adipocytes and the target breast cancer cells (Dieudonne *et al*, 2002; Hu *et al*, 2002; Laud *et al*, 2002). Similarly, the Adiponectin receptors 1 and 2 (AdiporR1 and AdipoR2) are present in the breast cancer cell lines MCF-7 and MDA-MB-231 (Dieudonne *et al*, 2006). Informative functional experimental evidence indicates the validity of this theory (section 1.4.3.1). Interaction between these principle adipokines and their receptors indicate that their expression, in an experimental setup, is mutually and inversely dependent. Jardé *et al* demonstrated that adiponectin decreased mRNA expression of leptin and the leptin receptor in the MCF-7 cell line and similarly, leptin decreased expression of adiponectin receptor 1 (Jarde *et al*, 2009).

1.5.3 Autocrine mechanism

Two studies have concluded that in primary tumours, the level of leptin expression positively correlates with ObR expression. Expression of leptin is found in normal mammary epithelium both leptin (Ob) and its receptor (ObR) are found to be over expressed in breast cancer tissue (Garofalo *et al*, 2006; Ishikawa *et al*, 2004). Garofalo *et al* examined adipokine expression in nodal metastasis and found that these metastases were detected in 34% of ObR positive cancers with strong immunoreactivity for leptin, but in none of the tumours that lacked either ObR expression or leptin over expression. The group

concluded that an autocrine loop may be operative and it may sustain the ability of breast cancer cells to metastasize.

An autocrine pathway for adiponectin has not been established. Takahata *et al* examined breast cancer cell lines and tissue for the presence of adiponectin and its ligand receptors AdipoR1 and AdipoR2. The receptors were identified but adiponectin itself was not thus confirming that an autocrine pathway is not plausible (Takahata *et al*, 2007).

1.6 Complex, interlinked relationships in breast cancer

Implicating a molecular basis for obesity and the metabolic syndrome for breast cancer remains thoroughly challenging. Considering the simplified schematic (Fig. 1.5), there is a huge potential for interaction (synergistic and antagonistic) at multiple levels between the multiple factors composing the three hypotheses (oestrogen, insulin and adipokines) proposed for obesity in breast cancer.

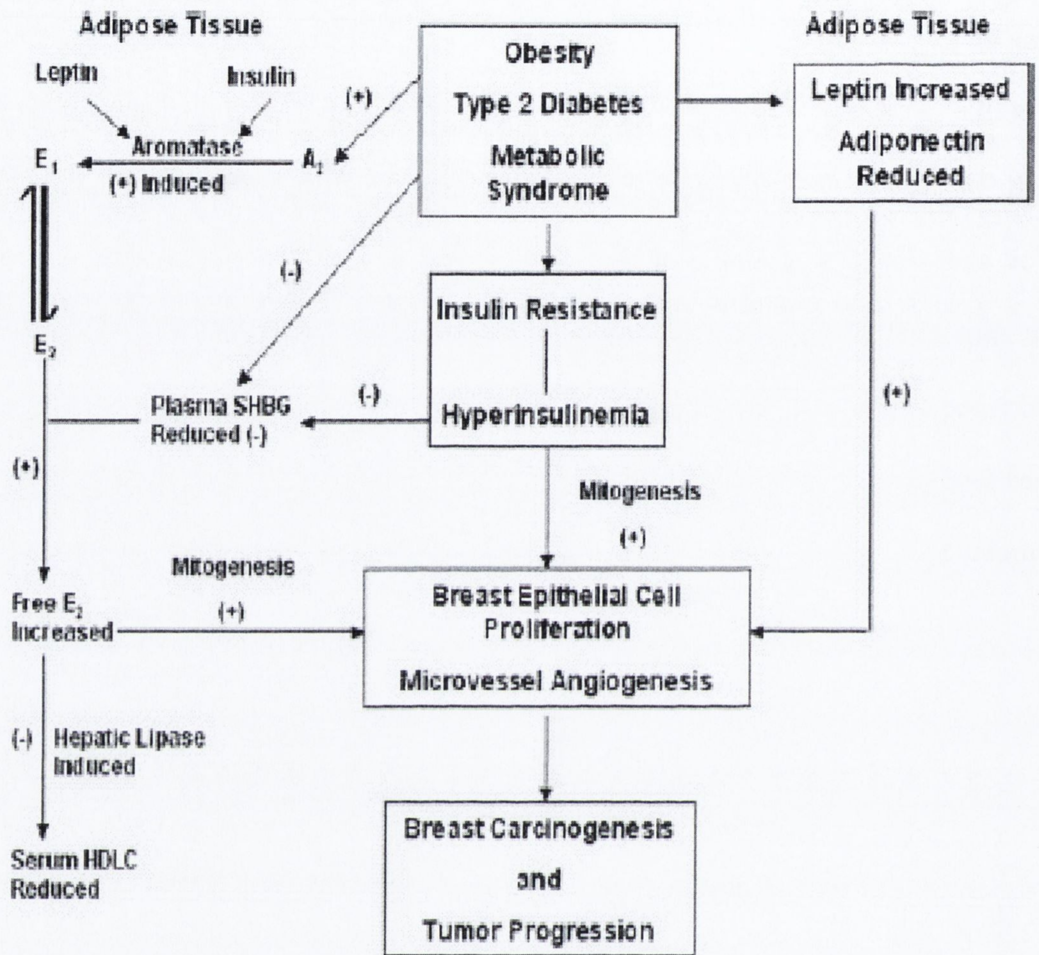


Fig. 1.5 **Intricate interactions between oestrogen, adipokines and insulin, modulated by obesity and insulin resistance demonstrate the complexity of obesity related influences on carcinogenesis.**

1.6.1 Oestrogen and Adipokines

Leptin is known to enhance the expression of aromatase and suggests that this adipokine may have influence on oestrogen synthesis (Catalano *et al*, 2003). Treatment with leptin upregulated signalling of oestradiol/ Oestrogen receptor of the breast cancer cell line MCF-7 treated with an aromatizable androgen, with a subsequent downregulation of the signals after treatment with the aromatase inhibitor letrozole. Further evidence suggests that leptin can cause direct activation of ER- in the absence of its usual ligand (Catalano *et al*, 2004). Similarly, oestrogen can stimulate leptin secretion in both *in vitro* and *in vivo* models. Another facet of this relationship is that leptin attenuates the effects of an anti-oestrogen (ICI 182,700) in experiments conducted by Garofalo *et al* (Garofalo *et al*, 2004). Therefore, high levels of leptin may potentially contribute to development of anti-oestrogen resistance in the adjuvant setting (Lorincz *et al*, 2006).

IL-6, along with TNF-α is capable of stimulating aromatase expression in adipose tissue (*in vitro* and *in vivo* experiments) and thus oestrogen biosynthesis, potentially contributing to cancer progression (Purohit *et al*, 1995).

1.6.2 Insulin, IGF and Adipokines

It is recognised that both leptin and IGF-1 are capable of regulating mammary tissue growth at multiple levels (Garofalo *et al*, 2006). Both hormones are secreted by abdominal adipocytes which results in an endocrine effect. A proposed mechanism sees mammary adipose tissue secreting these hormones locally, in a paracrine fashion to effect change in the breast. In addition, an autocrine pathway exists. IGF-1 and leptin are expressed by breast cancer cells and thus an autonomous signalling component is present. Pfeiler *et al* establishes that adiponectin receptors in breast cancer were not responsive to insulin resistance or hyperinsulinaemia (Pfeiler *et al*, 2009).

Ozbay *et al* highlighted that a unidirectional crosstalk could occur between IGF and ObR in breast cancer cell lines (Ozbay *et al*, 2008). In addition, Garofalo et al had demonstrated that IGF-1 could induce leptin transcripts in ER +ve MCF-7 cells (Garofalo *et al*, 2006). These in-depth studies indicate an intricate relationship between two major components of the hypothesis implicating obesity in breast cancer and require further study.

Strong associations between insulin, IGF-1 and sex hormone biology are present physiologically and pathologically (Hamelers *et al*, 2003)(see section 1.4.1). In cell based studies, insulin is also capable of increasing ER and PR levels in MCF-7 cells and with oestrogen, synergistically

promoting cell proliferation. It can also induce aromatase activity, thus producing an increase in mitogenic oestrogen (Plymate *et al*, 1988).

1.7 Avenues for primary prevention, recurrence reduction and prognosis in breast cancer

Obesity is a preventable, modifiable risk factor for breast cancer. Intervention for obesity and its metabolic syndrome should take the form of a combination of the following: diet, exercise and pharmacology.

1.7.1 Diet and exercise

Dietary patterns and moderate exercise have both been associated with improved survival in cancer (Holmes *et al*, 2005; Obermair *et al*, 1995; Rock *et al*, 2005). Incorporating moderate exercise and a high vegetable/fruit diet has been demonstrated to improve survival, independent of obesity status (Pierce *et al*, 2007). Weight loss, attributed to moderate exercise and a hypocalorific diet is associated with a decrease in serum leptin levels with a concomitant increase in adiponectin levels (Monzillo *et al*, 2003). Further research in this area is clearly required to establish if exercise and diet modification may be used as a method to prevent recurrence.

1.7.2 Pharmacology

Pharmacologic intervention is the main stay of treatment for diabetes when diet/exercise fails. Pharmaco-therapeutics is also most likely to be the best avenue for alteration or modification in obesity related cancer primarily due to limitations describes in section 1.7.2 and 1.7.3. To this end, recent evidence suggests that by treating individual components of the metabolic syndrome one may potentially reduce cancer recurrence.

1.7.3 Hyperglycaemia/Hyperinsulinaemia

Emerging evidence is now pointing towards insulin and insulin resistance as the impetus for the vast majority of changes seen in obesity related carcinogenesis (Goodwin *et al*, 2002). This area is also now the focus for therapeutic intervention in breast cancer with burgeoning interest, particularly in the biguanide derivative metformin, which is currently approved for treatment of non insulin dependent diabetes. An insulin sensitising agent, it has potent anti-hyperglycaemic properties, primarily inhibiting hepatic glucose production but may also sensitise peripheral tissues to insulin (Bailey *et al*, 1996). Another approved use for metformin is in the management of polycystic ovarian syndrome (PCOS, a disease characterised by chronic oligo-ovulation, excess androgen (decreased SHBG) and is often associated with similar metabolic abnormalities seen in metabolic syndrome (Nestler, 2008). In a large observational study (>10,000 diabetic patients) cancer related mortality was

significantly lower in those treated with metformin compared with other diabetic medications including sulphonylureas and insulin (Bowker *et al*, 2006). In a recent retrospective study it was found that metformin improved the pathological complete response (pCR) indicating response to treatment as compared to other diabetic medications (sulphonylureas, insulin) in diabetic women (Jiralerspong *et al*, 2009). In non-diabetic early breast cancer patients it can significantly lower insulin and LDL-cholesterol levels, as well as improving insulin resistance with minimal side-effects (Goodwin, 2008). How metformin works in cancer in still a matter of intense investigation, however preclinical studies indicate that the drug can inhibit cell proliferation by activation of AMPK (Phoenix *et al*, 2009). At present, at least two trials examining the influence of metformin in prognosis/survival and the effect of metformin on tumour biology are underway (Cazzaniga *et al*, 2009; Goodwin *et al*, 2009).

1.7.4 Hyperlipidaemia

Statins are thought to work by interrupting the biosynthetic pathway that produces melanovate. These affect multiple downstream pathways which are critical for cancer growth and progression. Thus statins may have potential as anti-cancer agents. Recently, it has been demonstrated that hypertriglyceridemia and low HDL-cholesterol, features of the metabolic syndrome, had the strongest association with breast cancer risk (Agnoli *et al*, 2010). Lipophilic statins have been shown to inhibit breast cancer cell growth,

particularly in ER-ve MDA MB-231 cell line. In animal models, these statins inhibited cancer growth, with greatest effect again seen in ER-ve tumours (Campbell *et al*, 2006; Koyuturk *et al*, 2007). In epidemiological studies, women taking lipophilic statins for greater than a year had a reduced frequency of ER-ve breast cancers (OR 0.63, p=0.02) (Kumar *et al*, 2008). In a newly reported human trial, reduced tumour proliferation and increased apoptotic activity in early high grade ER-ve tumours was observed following treatment with a statin (Garwood *et al*, 2010).

1.8 Specific Aims

There is clear epidemiological evidence that obesity and the metabolic syndrome are associated with breast cancer incidence, prognosis and recurrence. Much *in vitro* work has described potential obesity related mechanisms at play in various aspects of breast carcinogenesis. Translational evidence to marry the two is beginning to emerge. To this end, this body of work examines the role of obesity and the metabolic syndrome in influencing adipokine production and their associations with breast cancer using a translational, patient based approach. The specific aims of this thesis are:

1) Establishment of a bioresource for the study of obesity related breast cancer
2) Examination of the influence of obesity and the metabolic syndrome on mammary adipose tissue to establish a paracrine pathway for obesity related factors in breast cancer
3) Exploring the influence of obesity and the metabolic syndrome on the mRNA expression of adipokines in breast cancer.

Chapter 2:

Materials and Methods

2.1 Reagents

All laboratory chemicals and reagents were purchased from Sigma Chemical Company (MO, USA) unless otherwise stated, and prepared and stored according to manufacturer's instructions. Solid reagents were weighed using a Scout Pro electronic balance (Ohaus Corporation, NJ) or an Explorer Pro fine electronic balance (Ohaus Corporation, NJ), and made up using double distilled water. Solutions were autoclaved prior to use and stored at room temperature unless otherwise stated. Gilson pipettes were used to transfer liquid volumes up to 1 ml (Gilson S.A., France), electronic pipette aids (Drummond, PA, USA) and disposable Pasteur pipettes (Starstedt Ltd., Wexford, Ireland) were used for volumes greater than 1 ml and graduated cylinders were used for volumes in excess of 10 ml.

2.2 Bio-banking

2.2.1 Anthropometric and serological assessment of obesity and the metabolic syndrome

Weight was measured, using digital scales to 0.1kg. Height, measured barefoot using a portable stadiometer (Seca) to 0.1cm. Waist circumference (WC), to the nearest 0.5cm was measured at the midpoint between the lower border of the rib cage and the superior aspect of the iliac crest, with patient standing and after gentle expiration. Body composition was analysed using a Tanita BC 418 MA bioelectrical impedance analyser (Tanita UK Ltd, Middlesex, UK). This provides accurate information on lean/fat tissue mass in the trunk area, each limb as well as overall body composition.

Venous blood samples were obtained after an overnight fast. Fasting plasma glucose; fasting insulin levels; HbA1c level; fasting lipid profile (total-, HDL-cholesterol, and triglycerides) and sex hormone levels (oestradiol, progesterone, testosterone and sex hormone binding-globulin) were measured according to our institution's biochemistry department protocols. Insulin resistance was calculated using the HOMA method (Fasting Glucose x Fasting Insulin / 22.5) (Matthews *et al*, 1985).

2.2.2 Adipose conditioned media (ACM)

Mammary adipose tissue from the peritumoural (PT) area (<2cm from tumour site) and distal (D) area (>5cm from the tumour) were harvested from mastectomy samples immediately post resection (Fig 2.1). These adipose tissue samples were immediately transported in sterile transport buffer (glucose (0.1 %), gentamicin (0.05 mg/ml) in PBS) for processing in a Grade II laminar air flow cabinet. The adipose tissue was minced with a scissors and washed with sterile PBS. A small sample (approximately 500 mg) was flash frozen in liquid nitrogen and stored at – 80 °C for RNA extraction, while the remainder was cultured to produce adipose conditioned media (ACM). This was carried out as follows: in a tissue culture dish, 5 g minced adipose tissue was incubated in 10 ml serum free M199 medium (containing 0.05 mg/ml gentamicin) for 72 hours at 37 °C and 5 % CO_2 as previously described. ACM was then filtered (BD Biosciences, Bedford, MA, USA) to remove adipose tissue fragments and stored at – 80 °C.

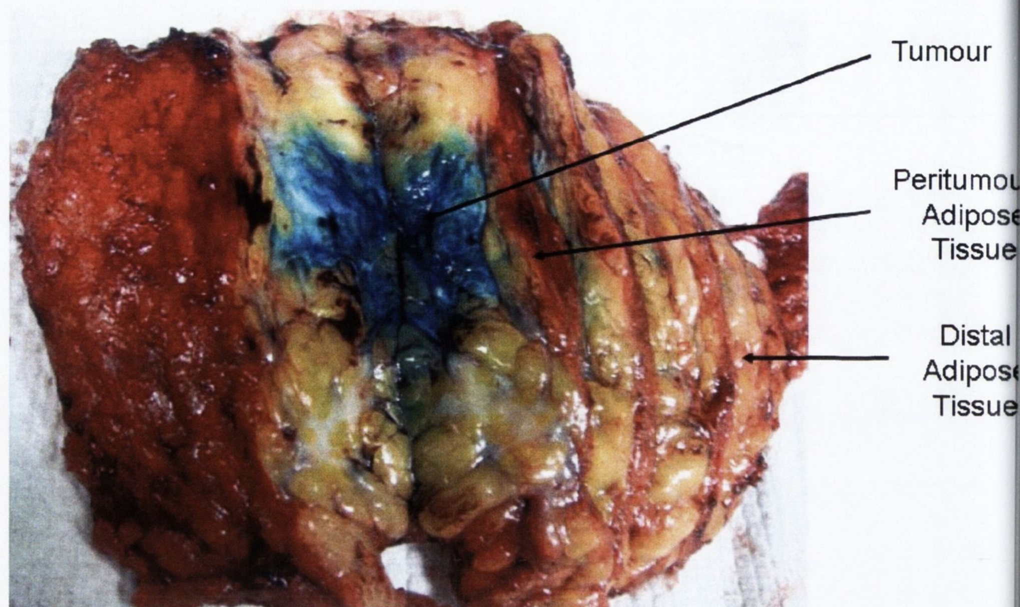


Fig 2.1 Mastectomy sample demonstrating sites for harvesting of peritumoural and distal mammary adipose tissue. Mastectomy samples are sliced into even sections from medial to lateral. The tumour site is identified by palpation and sections taken for bio-banking. Adipose tissue is then harvested with a fresh blade from both sites.

2.2.3 Tumour sampling

Tumour sampling for bio-banking was performed on all consenting patients undergoing wide local excision (WLE) or mastectomy for breast cancer. Exclusion criteria for bio-banking included: tumour sizes <1.5cm or cases where the supervising pathologist was concerned that tumour margins were likely to be compromised. After harvesting, tumour tissue is divided into 30-50mg pieces, placed into cryotubes and flash frozen in liquid nitrogen, and stored at -80 °C.

2.3 Cell Culture

2.3.1 Cell lines

Three human breast cancer cell lines were used throughout this project: MDA-MB 231 (Estrogen Receptor negative ER -ve), MCF-7 and T47D (Both Estrogen Receptor positive, ER +ve). All cell lines were purchased from European Collection of Cell Cultures (ECACC, Wiltshire, UK).

2.3.2 Cell line maintenance

The MDA-MB 231 cell line was cultured in Lebovitz-15 supplemented with 10% fetal calf serum (FCS), 5000 U/ml penicillin and 5000 ug/ml streptomycin, collectively entitled 'Pen-Strep' and L-Glutamine (Lonza, Verviers, Belgium). The MCF-7 cell line was cultured in EMEM supplemented 10% FCS, 5000 U/ml penicillin and 5000 ug/ml streptomycin, L Glutamine and non essential amino acids (NEAA). The T47D cell line was cultured in RPMI 1640, 10% FCS, 5000 U/ml penicillin and 5000 ug/ml streptomycin and L-Glutamine. MCF-7 and T47D cell lines were grown in T75cm^2 vented flasks (Nunc, Roskilde, Denmark), while MDA-MB 231 cell line was CO_2 independent and was maintained in non-vented T75cm^2 flasks. All cell lines were grown in a humidified incubator at 37°C with 5% CO_2. Cell lines were passaged weekly as described below (section 2.1.3) and fed by replacing culture media with fresh media.

2.3.3 Mycoplasma testing

Upon receipt of each new cell line, and every six months thereafter, cells were tested for mycoplasma infection using the MycoAlert™ mycoplasma detection system (Cambrex BioScience, Rockland, ME, USA). Mycoplasmal enzymes react with the MycoAlert™ substrate resulting in the conversion of ADP to ATP. The level of ATP in the sample before and after the addition of the substrate therefore indicates whether the sample is contaminated with mycoplasma. Cell lines were passaged twice in antibiotic free medium before a 1 ml sample of supernatant was taken. 100 μl of the supernatant sample was transferred to a luminescence compatible plate. 100 μl of MycoAlert™ reagent was added and incubated for 5 minutes before a one second (s) integrated reading ('Reading A') was taken on a luminometer (Wallac Victor 2 1420, PerkinElmer, Ballymount, Dublin). 100 μl of MycoAlert™ substrate was then added to the sample and incubated for 10 minutes before a second reading ('Reading B') was taken. A 'Reading B': 'Reading A' ratio <1 indicated that cells were free of mycoplasma. All cell lines consistently tested negative during the course of this work.

2.3.4 Passaging cell lines

Media was removed from the flasks containing the cell line. The flask was washed with 0.01M sterile phosphate buffered saline (PBS) (13.8mM NaCl, 2.7 mM KCL, pH 7.4), followed by the addition of 3-5mls of trypsin EDTA (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) (Lonza, Verviers, Belgium) and the flask was then placed in the incubator for 5minss at 37°C until the cells

lifted away from the flask surface. The trypsin was neutralised by adding 8-10mls of media containing fetal calf serum. The volume of media in the flask was then transferred to a 15ml sterile tube (Sarstedt, Wexford, Ireland) and centrifuged at 1300 x g for 3mins. All but 0.5 mls of the supernatant was discarded and the pellet was re-suspended in fresh media. An aliquot of cells was then transferred to a sterile T75cm^2 flask containing 15-20mls of fresh media. Cells were routinely passaged at a 1 in 5 to 1 in 10 dilution depending on cell confluency. All culturing experiments were carried out within 20 passages.

2.3.5 Counting cells

After cells were trypsinized and centrifuged. 1ml of media was added and the pellet thoroughly re-suspended, 20µl of cells was then added to 180µl of Trypan blue solution (Sigma-Aldrich Poole, UK). The cell/trypan blue solution, after appropriate mixing, was loaded onto each side of a bright line hemacytometer (Hausser Scientific, PA, USA) with a 22x40mm coverslip. Viable cells remain unstained due to their active exclusion of trypan blue, whereas dead cells stain blue due to membrane disruption. The number of viable cells was counted in each of the four corners of the grid. The number of cells/ml was then calculated using the following formula:

$$\frac{\text{Total number of cells counted} \times 10{,}000 \times 10 \text{ (dilution factor)}}{4} = \text{number of cells/ml}$$

2.4 BrdU Cell Proliferation Assay

Cells were seeded at a concentration of 5 x 10^3 /well in 96-well plates in the appropriate complete media and allowed to adhere overnight at 37°C. Following overnight incubation in serum depleted media (0.5% FCS); cells were treated for 24hrsrs with the appropriate stimulants. Cell proliferation was assessed using a BrdU cell proliferation assay (Roche Diagnostics, Sussex, UK). Following culture of cells in 96-well plates, 10μl of a 1/1000 dilution of BrdU labelling solution was added to each well (except appropriate controls) for 4h at 37°C During this labelling period, the pyrimidine analogue BrdU (5-bromo-2-deoxyyuridine) was incorporated in place of thymidine into the DNA of proliferating cells. The media was then removed and the cells fixed and denatured with 200 μl of a fixative solution for 30minss at room temperature (RT). 100 μl of anti-BrdU POD (mouse monoclonal antibody, peroxidise conjugated) working solution was then added to each well for 90 mins at RT. Cells were then washed with PBS and 100 μl of substrate solution was added for 5-10 mins (or until colour change was sufficient for photometric detection). 25 μl of 1mM H_2SO_4 was then added to stop the reaction. Absorbance was measured on a Versamax microplate reader with softmaxpro5 software (Molecular Devices, Silicon Valley, California, US) at 450nm with reference set to 690nm. Controls used in each experimental set up were as follows: Blank; Background.

2.5 Cell Invasion Assay

Tumour cell invasion was examined using a 96-well Cell Invasion Assay (Chemicon International, CA, USA). The cell invasion assay is performed in a 96-well invasion plate, in which each well contains an 8μm pore size polycarbonate membrane, coated with a thin layer of ECMatrix™ (extracellular matrix). The ECM layer occludes the membrane pore, blocking non-invasive cells from migrating through. Invasive cells do, however, migrate through the ECM layer and cling to bottom aspect of the membrane. Invaded cells on the bottom of the insert membrane are dissociated from it following incubation in Detachment Buffer, and subsequently lysed and detected with a fluorescent dye.

The assay was performed in a sterile cell culture hood where 100μL of pre-warmed serum-free media was added to the interior of the inserts (Fig. 2.2), and incubated at RT for 2hrs to rehydrate the ECM. The media was then carefully removed from the inserts (without disturbing the ECMatrix™ coated membrane). 150 μL of complete media was added to the wells of the feeder or lower chamber.

Breast cancer cell lines for this assay were grown in T75 flasks. Once a confluency of 70% was reached, the cells were serum starved overnight. Cells were then lifted using trypsin and counted. 7.5 x 10^5 cells in 100μl serum free media, was added per well to the invasion chamber (inserts containing ECM), which was then placed on the feeder tray. The plate was covered and incubated for 24hrsrs at 37°C. After this time period, the

cells/media were gently discarded from the top side of the insert by tapping. The invasion chamber was rinsed by placing the chamber plate into a new 96-well feeder tray containing 150µl of pre-warmed Cell Detachment solution, and incubated for 30mins at 37°C. Cells were dislodged from the underside by gentle tilting back and forth of the invasion chamber during this incubation. A Lysis Buffer/Fluorescent Dye solution was made up for all samples. The fluorescent CyQuant GR Dye was diluted 1:75 with 4X Lysis Buffer. 50 µl of this Buffer/Dye solution was added to each well of the feeder tray containing the Cell Detachment solution with cells that had invaded through the ECMatrix™-coated membrane. The tray was then incubated for 15minss at RT. 150µl of the mixture was then transferred to a new 96-well plate suitable for fluorescent measurement, and the fluorescent intensity was measured using a fluorescence plate reader with filters set at 480/520nm. The results of the assay were illustrated using a bar chart, with control cells (untreated) set to 100%. Samples without cells but which contained Cell Detachment buffer, Lysis Buffer, and CyQuant dye were used as a blank. The fluorescence values from these wells were subtracted from all the other values in order to interpret the data.

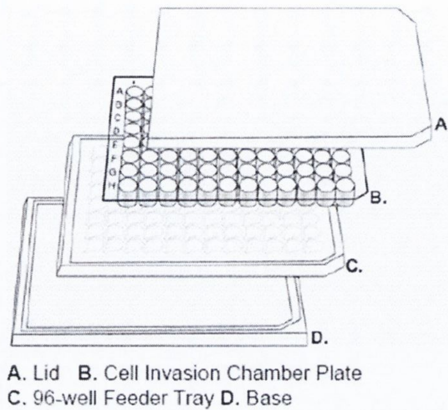


Fig. 2.2 Components of the 96-well Cell Invasion Assay plate

2.6 Cell Co-Culture

Co-culture experiments with mammary adipose tissue were conducted using Costar 12 well transwell plates, incorporating a 0.4 μm porous membrane (Corning Life Sciences, Amsterdam, Netherlands) (Fig 2.2). The plate is comprised of two separate chambers, a lower (feeding) layer and an upper (cell) layer separated by the membrane. The plates allow secreted factors from adipose tissue to diffuse into cell chamber above without allowing direct contact of the two cell types. Co-Culture experiments were conducted using the MCF-7 cell line.

Adipose tissue was harvested and processed as for ACM production. 1.0g of adipose tissue was placed into the lower wells and 2mls

M199 media was added and this was allowed to culture for 72hrs as per ACM protocol. MCF-7 cells after 24hrs incubation in serum depleted media (0.5% FCS) were seeded at a density of $5x10^4$ into the upper chamber at the 72hrs time point of ACM culture. This co-culture system was then incubated 37 ^oC for 24hrs. After this time period, RNA was extracted from the MCF-7 cells using the protocol outlined in 2.5.2.

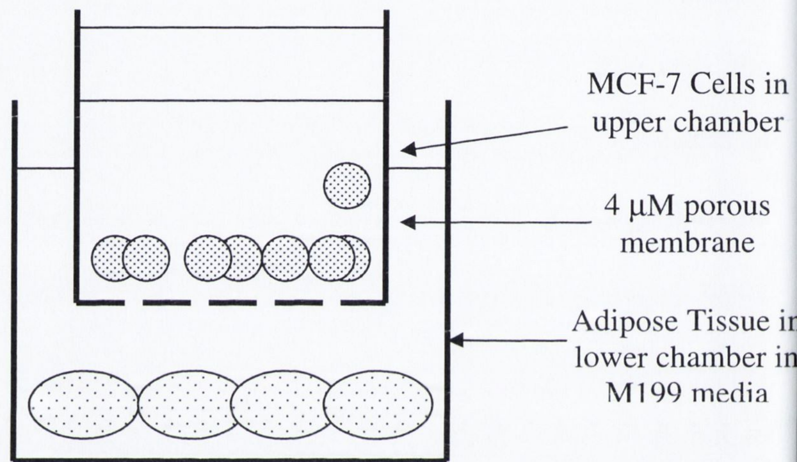


Fig 2.3 **Co- Culture System schematic**

2.7 Polymerase Chain Reaction experiments

2.7.1 Isolation of RNA from Tissue

RNA was isolated from human tissue (Mammary adipose tissue and tumour tissue) using a Qiagen RNeasy Lipid kit (Qiagen Inc., CA, USA). Tissue samples, in 2 mL cryotubes (Sarstedt, Wexford, Ireland) stored at -80 ^oC after harvest, were placed on ice and immediately 1mL of Qiazol Lysis reagent (Qiagen Inc., CA, USA), a mixture of guanidine thiocyanate and phenol in a monophasic solution was added. A 5mm stainless steel bead kit (Qiagen Inc., CA, USA) was then placed in each tube. 30mg of tumour tissue sample, or

100mg mammary adipose tissue, as per protocol, was used in RNA extractions. Samples were homogenised using a QIAtissuelyser (Qiagen Inc., CA, USA) at 25Hz for 5-10minss. The QIAtissuelyser racks were rotated to ensure even homogenisation and a further homogenisation period of 5-10mins at 25Hz was performed. Lysates were then transferred to new cryotubes and allowed to rest at RT for 5 mins. 100 μl of bromo-3-chloro-propane (BCP) was added and shook vigorously for 15 s. The samples were allowed rest for a further 2-3 mins. The samples were centrifuged for 15 mins at >8000 x g and at 4 °C. The aqueous, upper phase was transferred to a new tube and 600 μL of 70% ethanol and thoroughly mixed by vortexing. 700 μL of the sample was then transferred to an RNeasy mini spin column. The spin column was centrifuged at >8000 x g at RT for 15 s. The flow through was discarded from the lower part of the spin column and this step was repeated with the remainder of the sample. DNA clean up (as per 2.6.7.1) was performed at this stage if required. If not, 700 μL RW1 Buffer was added to the column, centrifuged at >8000 x g at RT for 15 s and the flow through discarded. 500 μL RPE Buffer was added and centrifuged at >8000 x g at RT, for 15 s and the flow through discarded. A further 500 μL RPE Buffer was added to the column and centrifuged at >8000 x g at RT for 2 mins, and the flow through discarded. The spin column was placed in a 1.5ml collection tube and 30 μL of RNase-free H_2O was added to the spin column. This was then centrifuged at>8000 x g at RT, for 1 mins. The resulting eluate contained the RNA sample and was stored at -80 °C until required.

2.7.2 Isolation of RNA from Cell Lines

Samples were collected in 1mL of Qiazol. A modified technique using a Qiagen RNeasy Lipid kit (Qiagen Inc., CA, USA) was then utilised. The tissue lysis step was omitted but all processes subsequently were the same as section 2.5.1.

2.7.3 RNA Quantification

RNA quantification was determined spectrophotometrically, using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). 1 μL of isolated RNA was loaded onto the Nanodrop. RNA was measured in ng/μL. 260:280 and 260:230 purity ratios were recorded. A 260:280 ratio of >1.8 was indicative of a relatively pure RNA yield, whereas a 260:230 ratio >1.7 demonstrated a sample free from phenol contamination.

2.7.4 RNA Integrity

RNA integrity was assessed using Agilent 2100 Bioanalyser, a 'lab-on-a-chip' micro-fluidics based platform. This technology uses pressure to apply an even and controlled distribution of the gel through a network of channels etched onto the surface of a small chip, to which the RNA samples are added. An electrophoretic trace of the RNA sample is produced, including any degradation products which may be present, in order to generate an RNA integrity number (RIN). Ladder and RNA samples were denatured at 72 ° C for 2 minutes and immediately placed on ice. The chip was prepared by evenly

distributing a gel-dye mix onto the surface of the chip using pressure, following which 1 μl of ladder and 1 μl of each sample were placed in the appropriate wells on the chip surface. The chip was vortexed at 2,400 rpm for 1 minute and read on the Agilent 2100 Bioanalyser within 5 minutes of preparation. A high RIN value indicates good RNA quality and suitability for use with microarray technology.

2.7.5 Human Cancer Pathway Finder RT^2 *Profiler*™ PCR Arrays

Changes in the expression profile of a panel of genes representative of the biological pathways involved in tumourigenesis in breast cancer (Cancer Pathway Finder) was examined using RT^2 *Profiler*™ PCR Array technology (Super Array Bioscience Corp., MD, USA). Each array is a 96-well plate containing primer sets for a thoroughly researched set of 84 relevant, pathway focused genes involved in 6 biological pathways of tumourigenesis: apoptosis, cell cycle, angiogenesis, invasion and metastasis, signal transduction and adhesion. 5 housekeeping genes normalise data, and 2 negative controls to determine potential contamination in the system.

2.7.5.1 RNA Clean-up

RNA samples prepared for examination on RT^2 *Profiler*™ PCR Arrays require a clean up step. RNA was prepared from cell lines as described in 2.6.2. A DNase digestion step was added in to ensure optimum RNA purity for the PCR arrays. This step was carried out using an RNase-free DNase set

(Qiagen Inc., CA USA). Briefly, after the remainder of the lysate-ethanol mixture has been centrifuged through the spin column for 15s at >8000 x g, 350 μL RW1 Buffer is added to the column and centrifuged for 15s at >8000 x g. 10 μL DNase stock 1 (Qiagen Inc., CA, USA) is added to RDD Buffer. This incubation mix is then added to the spin column and allowed to rest for 15 mins. 350 μL RW1 Buffer is added and the spin column was centrifuged for 15 s at >8000 x g. The protocol outlined in 2.6.2 was then continued.

2.7.5.2 cDNA synthesis for RT^2 *Profiler*™ PCR Arrays

A reverse transcription reaction was carried out using a First Strand cDNA Synthesis kit (SuperArray (SA) Biosciences Corporation, MD, USA) to generate cDNA from RNA samples. 250 ng of each RNA sample was combined with 2 μL 5X Genomic DNA elimination Buffer (SA Biosciences Corporation, MD, USA) and made up to a final volume of 10 μL with RNase free H_2O. This mixture was briefly mixed by pipetting before being incubated for 5 mins at 42 °C. It was then chilled on ice for 1 mins. 10 μL of this sample was then added to a reverse transcription cocktail made up as follows:

Table 2.3 **Reagents and volumes used in reverse-transcription reaction**

RT Cocktail	**Volume (μL)/Reaction**
5X RT Buffer	4
RNase free H_2O	3
RT Enzyme Mix 3	2
Primer & Ext Control Mix	1
Final Volume	**10**

Each sample was gently mixed and then incubated at 42 °C for 15 mins. The reaction was immediately stopped by heating the sample to 95°C for 5 mins. 91 μL dd H_2O was added to the sample and stored at -20 °C until further required.

2.7.5.3 Quantitative RT^2 *Profiler*™ PCR Array

Samples were prepared for loading onto the RT-PCR array according to the following tables:

Table 2.4 **Experimental cocktail Preparation**

2X SA qPCR Master Mix	1275 μL
Diluted cDNA synthesis reaction	102 μL
dd H_2O	1173 μL
Total Volume	**2550 μL**

25 μL of experimental mixture was carefully pipetted to each well of the 96-well PCR array. The array was tightly sealed with optical adhesive film (Applied Biosystems, Courtaboeuf, France) and then centrifuged briefly to remove any air bubbles. Real-time PCR was performed on an ABI Prism 7900 HT Fast Real-time PCR system (Applied Biosystems, Courtaboeuf, France) using the cycling conditions outlined in table 2.5. The threshold cycle (Ct) for each well was calculated using the instrument's software. Data analysis was carried out using a Microsoft Excel-based data analysis template, supplied by SA Biosciences (see template in Appendix 1). Analysis is based on the ΔΔCt method with raw data being normalised to house-keeping genes on the array plate. Fold-changes in gene expression observed in the array analysis were validated using a quantitative real-time PCR approach, as in the protocol outlined in 2.6.8.

Table 2.5 Thermal cycling conditions for real time PCR reactions

No. Cycles	Duration	Temperature
1	10 mins	95°C
40	15 s	95°C
	1 min	60°C

2.7.6 cDNA synthesis for Quantitative real-time PCR (Human Study)

1μg mRNA from samples was added to 1μL of random primers (Promega, WI, USA) and made up to a volume of 12μL with RNase-free H_2O. This was incubated for 10 mins at 70°C and placed on ice for 1 min. 8 μL of a reverse transcription master mix (0.5 μL dH_2O, 0.5 μL RNase inhibitor, 0.5 μL dNTPs, 0.5 μL SuperScript, 4 μL 5X RT Buffer, 2 μL 0.1mM dTT) was then added to the reverse transcriptase mixture and placed in a thermal cycler for 30 mins at 37°C.

2.7.6.1 Gene Expression protocol

Taqman fluorogenic gene expression probe sets (Applied Biosystems, Courtaboeuf, France) were used for all gene expression experiments. All probe sets used were FAM labelled (table 2.6). Quantitative normalisation of cDNA in each sample was performed using the ribosomal RNA subunit 18s as an endogenous control.

Quantitative real time PCR was performed using an ABI Prism 7900 HT Fast Real-time PCR system (Applied Biosystems, Courtaboeuf, France). The final reaction mixture (20 μL volume) consisted of 2 μL cDNA, 1 μL probe, 10 μL Taqman Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France) and 7 μL dH_2O. Each sample was assayed in triplicate. Thermal cycling conditions were as follows: Step 1 – 2mins at 50°C, Step 2 –

10 mins at 95°C, Step 3 – 15 sec at 95°C and 1 mins at 60°C repeated for 40 cycles.

Relative quantification of gene expression was examined using the comparative threshold (CT) method (AppliedBiosystems, 1997). A well described technique (Revillion *et al*, 2006) where the CT variable is defined as the cycle number which the fluorescent signal emitted by the cleavage of the dual labelled probe is first detected. The method requires the use of a calibrator sample, by which unknown samples can be quantified. The MCF-7 cell line was chosen for this purpose as it expresses all 5 targets in question (Dieudonne *et al*, 2006; Garofalo *et al*, 2004) and its target expression value was set to 1. Relative target expression was then given by the formula: 2 (-ΔΔCT), where ΔΔCT = ΔCT patient sample - ΔCT Calibrator sample; with ΔCT = CT target – CT 18s.

Table 2.6: Taqman Primer probe set nomenclature

Abbreviation	Assay ID	Amplicon length
Ob	Hs 00174877_m1	74
AdipoQ	Hs00605917_m1	71
ObR	Hs00174492_m1	78
AdipoR1	Hs00360422_m1	94
AdipoR2	Hs 01047563_m1	148
CDK4	Hs 00897129_m1	72
TIMP-3	Hs 00165949_m1	59
VEGFa	Hs 00900055_m1	59
MTA2	Hs 03987601_m1	59
NME1	Hs 00264824_m1	71
18s	Hs 99999901_s1	187

2.8 Millipore Milliplex MAP Technology

Milliplex MAP assays (Millipore, Watford, UK) are based on Luminex xMap technology, allowing multiplex detection of proteins from a single biological sample. The technology is based on the coating of microspheres or beads with capture antibodies of interest, to which the sample is then added. The used of multiple conjugated beads allows multiple readouts from the same sample, thus conserving valuable biological specimens. Adipose conditioned media (ACM) samples were outsourced to Millipore for testing on the Human Cytokine and Human Adipokine panels to screen ACM for the presence of cytokines. Adipokines of interest identified from Milliplex Map results were further quantified using ELISA (described in section 2.8).

2.9 Enzyme Linked Immuno-Sorbent Assays

Enzyme Linked Immuno-Sorbent Assay (ELISA) kits (Human DuoSet® ELISA kits, R&D Systems Europe, Abingdon, UK) were used to determine the levels of various adipokines (leptin, adiponectin, IL-6, IL-8, MCP-1, and VEGF) in ACM and serum isolated from blood samples taken from breast cancer patients. The ELISA technique is based on the antibody sandwich principle. First, 50µl of the capture antibody specific to the analyte of interest is bound to a microtiter 96-well plate (Nunc, Roskilde, Denmark) to create the solid phase and is allowed to bind to the well for 24hrs at 4°C. Unbound antibody is removed by washing the plate three times using wash buffer (0.05% Tween® 20 in PBS) and a blocking reagent (Reagent diluent 1%

BSA (Bovine serum albumins) in PBS is added for 2hrs. Following a wash, 50 μl of samples, standards, and controls are then incubated with the solid phase antibody for 24hrs at 4°C, which captures the analyte. After washing away unbound analyte, 50 μl of conjugated detection antibody (*e.g.* biotin conjugated) is added and incubated for 2h at 4°C. This detection antibody binds to a different epitope of the molecule being measured, completing the sandwich. Following a wash to remove unbound detection antibody, 50 μl of the detection reagent, streptavidin-HRP, is added and the plate incubated fro 30mins at RT. The plate is washed and 50μl substrate solution, TMB (Tetramethylbenzidine, R&D Systems Europe, Abingdon, UK), is added and colour develops in proportion to the amount of bound analyte. Colour development is stopped using 25μL of 1M H_2SO_4 solution and the intensity of the colour is measured. The optical density (O.D.) of the yellow colour is read at A450 with wavelength correction set to 570nm on a Versamax microplate reader (Molecular Devices, Silicon Valley, California, US) with softmaxpro5 software. Concentrations of the analytes in question are then calculated using the optical density of the samples in relation to a standard curve using known concentrations of the recombinant protein in question (representative curve shown in appendix 2). Table 2.7 describes the various dilutions for capture and detection antibodies as well as the top standards for each ELISA kit.

Table 2.7 Concentrations for ELISA capture and detection antibodies, and top standards

	Capture Antibody	Detection Antibody	Top standard
Leptin	4 µg/ml	12.5 ng/ml	2000 pg/ml
Adiponectin	2 µg/ml	2 µg/ml	4000 pg/ml
IL-6	2 µg/ml	200 ng/ml	600 pg/ml
IL-8	4 µg/ml	20 ng/ml	2000 pg/ml
MCP-1	1 µg/ml	100 ng/ml	1000 pg/ml
VEGF	1µg/ml	100 ng/ml	2000 pg/ml

2.10 Statistical Analysis

Data is expressed as mean ± SEM or median ± IQR (Interquartile range) as stated in figures, and as appropriate. For continuous variables, distribution normality was determined using the Kolmogarov-Smirnov test. If the distribution was Gaussian, then unpaired Students *t*-tests for the comparison of means of two groups was employed. For statistical analysis involving 3 or more groups, the analysis of variance (ANOVA) test was used. Post-hoc analysis was carried out to determine significance between individual groups. The Bonferroni multiple comparisons test was used for this purpose. In cases were Gaussian distribution was not evident, the Mann-Whitney and Kruskall-Wallis tests were used to examine data. Similarly, post-hoc analysis was performed using Dunn's multiple comparison tests. For categorical data, the Fischer's exact or Pearson's Chi Square (χ^2) test was employed.

Chapter 3:

Establishment of a Bioresource

3.1 Introduction

Biobanking is the organised collection of biological samples and its associated data (Cambon-Thomsen, 2004). Traditionally, biobanks have been set up and organised by individual principal investigators to meet specific research objectives. With the emergence of advance genomic technology, particularly tissue-based RNA and DNA microarray platform, the understanding and treatment of cancer is rapidly changing. By far the most common, disease-orientated biobanks may contain many different types of tissue ranging from tumour tissue, normal tissue, blood and other components which are collected from the patients in the framework of medical diagnosis and treatment. The specific strengths of this type of biobank include the ability to compare different stages or types of disease at molecular and genetic levels (Asslaber *et al*, 2007).

The overall value of bioresource collection in medical research is dependent on the quality and detail of clinical data included with donated samples. The St. James's breast cancer biobank collects in-depth anthropomorphic and serological assessment of obesity and metabolic syndrome of the patients included. Liaising with the dedicated breast cancer data manager ensures comprehensive and validated data on all aspects of the patients contained within the resource. This specific aims of this chapter were:

1) Describe and denote the clinical (including anthropometric and serological measurements of obesity) characteristics and pathological features of the cohort included in the St. James's breast cancer biobank.

2) Describe the biobanking process from consent through sample procurement to eventual storage

3) Examine the integrity of tissue and extracted RNA stored in the biobank as a quality control exercise to confirm suitable material for various studies

4) Screen and quantify potential adipokines in adipose conditioned media to assess suitability for use as representative model of obesity in breast cancer

3.2 The biobanking process

3.2.1 Recruitment

A disease-orientated breast cancer and mammary adipose tissue bank was established in St. James's Hospital in July 2008. Informed, written consent prior to surgery was the initial step that began the process of biobanking and was obtained in accordance with the Declaration of Helsinki. Institutional ethical review board approval was granted for the recruitment and sample collection for research purposes. The consent forms were in triplicate, a copy kept in the patient file as a permanent record, a second copy was transported with the specimen and kept on record in the biobank facility and third copy was given to the patient with contact details if the patient wished to rescind consent in the future. Potential patients for recruitment were identified by review at the breast cancer multidisciplinary team meeting (MDT) held

once weekly, at which all diagnoses of breast cancer over the preceding weeks were discussed. A dedicated biobank technician is informed of all cases and coordinates with the surgeons, research staff and pathologists in the biobank process.

For each entry into the bioresource, a full anthropometric screen for obesity parameters including weight, height, BMI, and waist circumference was recorded by the biobank coordinator. Percentage (%) fat mass was recorded using a Tanita Body Impedance Analyser (BIA). Coupled to this was a complete serological assessment of metabolic parameters as described in section 2.11 according to the IDF definition for metabolic syndrome in section 1.2.

3.2.2 Patient population

To date, 120 patients have been consented for sample collection into the biobank over the 2 year period July 2008-June 2010 (table 3.1). Of these, 106 cases have had a full metabolic screen performed. Matched tumour tissue was available in 85 cases. Tissue sampling did not proceed due to small size and potential compromise of margins at pathological examination in 35 cases. There were 14 patients that did not undergo metabolic assessment; reasons included poor mobility, contraindication to use of Tanita body analyser due to presence of a pacemaker and failure/refusal at phlebotomy). Median age of women consented to the biobank was 55.0 years (range 23.0-94 years). Post-

menopausal women accounted for 60% (n=72) of women recruited. As stated above, the unique aspect regarding this cancer biobank is the presence of anthropomorphic and serological data providing insight into the metabolic status of cancer patients. Of the 106 women who had a metabolic assessment, 39 (36.8%) were classed as normal weigh, 40 (37.7%) as being obese and a further 27 (25.5%) determined to have the metabolic syndrome. The significant differences in anthropomorphic measurements (weight, BMI, waist circumference and % fat mass) and metabolic parameters (glucose, insulin, HOMA-IR, triglycerides, HDL-cholesterol and SHBG) indicate that patients with the metabolic syndrome are anthropometrically and biochemically different to normal weight and obese patients. Thus within the St. James's Breast Biobank there exists three separate sub-groups of patients to evaluate the influence of obesity and the metabolic syndrome in breast cancer.

Table 3.1 Demographics of patients consented to the St. James's Breast Biobank 2008-2010

	n	%
Median age	55.0 (23-94)	
Menopausal status		
Premenopausal	48	40
Postmenopausal	72	60
Tumour type		
Ductal	84	70
Lobular	17	14.2
Other	19	15.8
Tumour size (cm)		
<2	40	33.3
2-4.9	62	51.7
≥ 5	18	15
Nodal status		
0	59	49.2
1-3	41	34.2
>3	20	16.6
Grade		
1	16	13.3
2	51	42.5
3	53	44.2
ER status		
Positive	89	74.2
Negative	31	25.8
PR status		
Positive	70	58.3
Negative	50	41.7
Her2 status		
Positive	23	19.2
Negative	97	80.8

Table 3.2 **Anthropomorphic and serological markers of obesity and the metabolic syndrome in patients incorporated into the St. James's Breast Biobank**

	Normal Weight n=39		*Obese only n=40*		*Metabolic Syndrome n=27*		
	Median	*95% CI*	*Median*	*95% CI*	*Median*	*95% CI*	*P*
Anthropometry							
Weight (kg)	56.2	54.3-60.3	75.7	68.1-78.9	85.1	74.1-96.0	<0.001[b,c]
BMI (kg/m^2)	23.0	22.5-24.4	28.5	27.5-30.7	34.0	30.3-39.7	<0.001[b,c]
Waist circum (cm)	76.0	70.0-76.0	92.0	90.0-98.0	108.0	100-120	<0.001[a,b,c]
% fat mass	26.90	25.9-28.5	38.35	36.6-41.8	42.4	39.1-45.3	<0.001[a,b,c]
Homeostasis							
Glucose (mmol/L)	5.0	4.9-5.2	5.0	4.6-5.2	5.8	5.3-6.4	0.001[a,b]
Insulin (mU/L)	5.75	4.4-7.0	7.5	5.3-9.1	15.6	11.6-23.0	0.005[a,b]
HOMA-IR	1.44	1.24-2.10	1.75	1.16-3.28	3.99	2.40-6.55	0.001[a,b]
Lipid profile							
Total Cholesterol	5.1	4.3-5.4	5.1	4.4-5.5	5.3	4.6-5.6	0.882
Triglycerides	1.0	0.8-1.1	1.3	1.0-1.6	1.9	1.8-2.5	0.001[b]
LDL	2.6	2.3-3.3	2.8	2.1-3.2	3.0	2.1-3.2	0.645
HDL	1.67	1.5-1.8	1.60	1.4-1.8	1.19	1.1-1.4	<0.001[b,c]
Sex Hormones							
Oestradiol							
Premenopausal	389	315-980	352	142-433	209	200-238	0.279
Postmenopausal	108	73-115	127	91.0-191	100	84-148	0.190
Testosterone							
Premenopausal	1.1	0.8-1.6	1.1	0.6-1.4	0.8	0.6-0.1.2	0.356
Postmenopausal	0.9	0.6-1.0	0.7	0.3-1.5	0.8	0.5-1.1	0.743
Progesterone							
Premenopausal	2.3	1.3-35.0	1.6	0.9-7.1	1.3	0.9-12.4	0.381
Postmenopausal	1.1	0.9-1.5	0.5	0.4-1.1	0.7	0.5-1.2	0.047[c]
SHBG							
Premenopausal	68.0	45.0-75.0	49.0	37.0-73.0	28.0	24.0-68	0.084
Postmenopausal	52.0	45.0-72.0	68.0	34.0-93.0	32.5	27.0-45.0	0.005[a,,b]

a Metabolic syndrome vs Obese only
b Metabolic syndrome vs Normal Weight
c Obese only vs Normal Weight

3.2.3 Quality assurance in biological material procurement

Of vital importance in any biobanking process is ensuring that the quality and integrity of tissue procurement, and its subsequent processing, is of a sufficient satisfactory standard. The lack of reproducibility of gene signatures in cancer tissues raises concerns as to discrepancy in quality of tissue used for such studies (Acharya *et al*, 2008; Brenton *et al*, 2005). To minimise such variation, a number of quality assurance steps have been incorporated into the biobanking process in our institution.

The transfer of the biological sample is required to be rapid so as to ensure ex-vivo time is kept to a minimum. The sample is transferred fresh, immediately post resection in pre-labelled containers. A time indication is given to the biobank coordinator for excision so that the coordinator can be present in theatre at time of final excision ain order that the specimen can be transported with little delay. At this point, the biobank technician is also informed so that preparations for biobanking are completed prior to arrival of the sample. Upon transfer to the pathology department's laboratory, the sample is catalogued, given a specific biobank reference number and prepared for analysis. A senior pathologist then orientates measures and describes the specimen. In the case of a wide local excision (WLE), the exterior margins are marked with tissue dyes, or with mastectomies, the posterior surface is marked. Incisions are then made into the specimen at 1cm intervals, examining the tumour and its relations to margins. An area of tumour is then excised (0.8 x

0.8 x 0.3-0.5 cm) with a scalpel. If there is any doubt of margin compromise by the senior pathologist, the sample will be deemed not suitable for biobanking. This is an essential component to the biobank as patient care must not be affected by the actions involved in the process of sample acquisition. Where possible, a sample of normal tissue and adipose tissue is also excised for biobanking purposes. The same cataloguing process is performed for these tissues. The excised sample is then divided into 30-50 mg aliquots and snap frozen in liquid nitrogen until transfer to a dedicated -80°C freezer.

3.2.4 Frozen section analysis of tumour and normal tissues

A major issue for biobanking and subsequent utilisation of such material is the heterogeneity of the tumour and normal tissues. In order to ensure that material submitted to the biobank was indeed tumour or normal tissue, 36 randomly selected aliquots of tumour and normal tissue were subjected to frozen section analysis. A full face section of the aliquot was taken and examined. The percentage of tumour tissue or normal tissue was recorded, other features present in the tissue were recorded, these included DCIS, LCIS, necrosis, or in normal tissue, the presence of glands, lobules, stroma, or adipose tissue. Interestingly, these data demonstrate that there is marked variability of tumour present in individual aliquots. Furthermore, it demonstrates the difficulty in acquiring definite normal tissue for analysis.

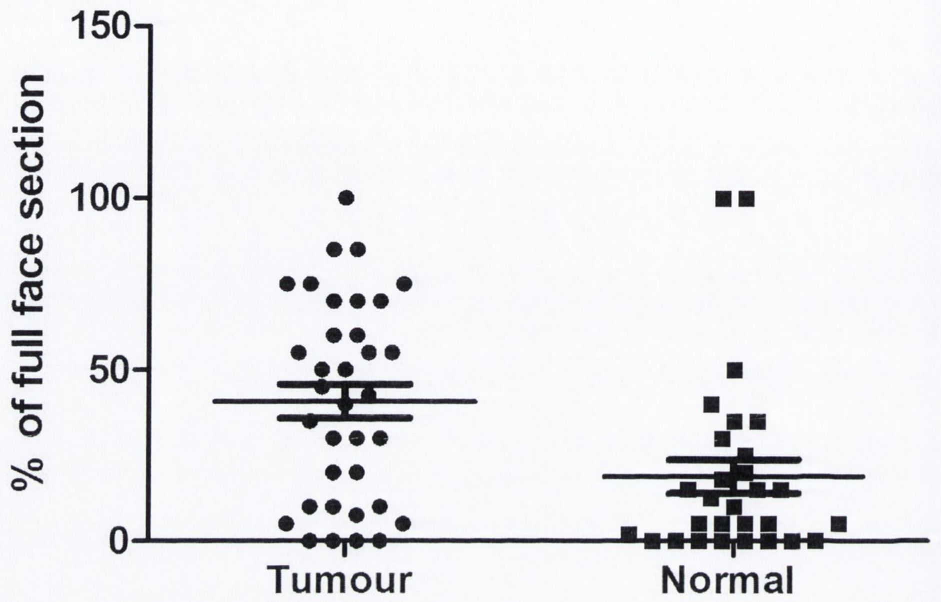


Fig 3.2 **Percentage tissue present in full face frozen section analysis of tumour and normal tissue aliquots (n=36), indicating the variability and heterogeneity present in individual aliquots.**

3.2.5 Effect of storage conditions on RNA extraction from intact tissue

Another aspect to the determination of optimal storage state of biological material is the effect of storage at -80°C on intact tissue. 10 matched tumour samples had RNA extracted at 0 and 6 months to determine if quality and purity of the tumour samples were degraded by storage at -80°C after being snap frozen post harvest after tumour resection. Purity, as measured by the A260/280 remained stable in all RNA extracted from tumour tissue in this experiment (2.064 ±0.018 vs 2.068 ±0.014, p=0.863). No differences were seen with regards to yield of RNA extracted (490.7 ±76.2 ng/ml vs 539.6 ±86.2, p=0.676) however from figure 3.3 B it is apparent that the yield from tissue samples is highly variable and is likely to be related to the size of the tissue aliquot and heterogeneity of the sample at time of processing rather than a storage related phenomenon.

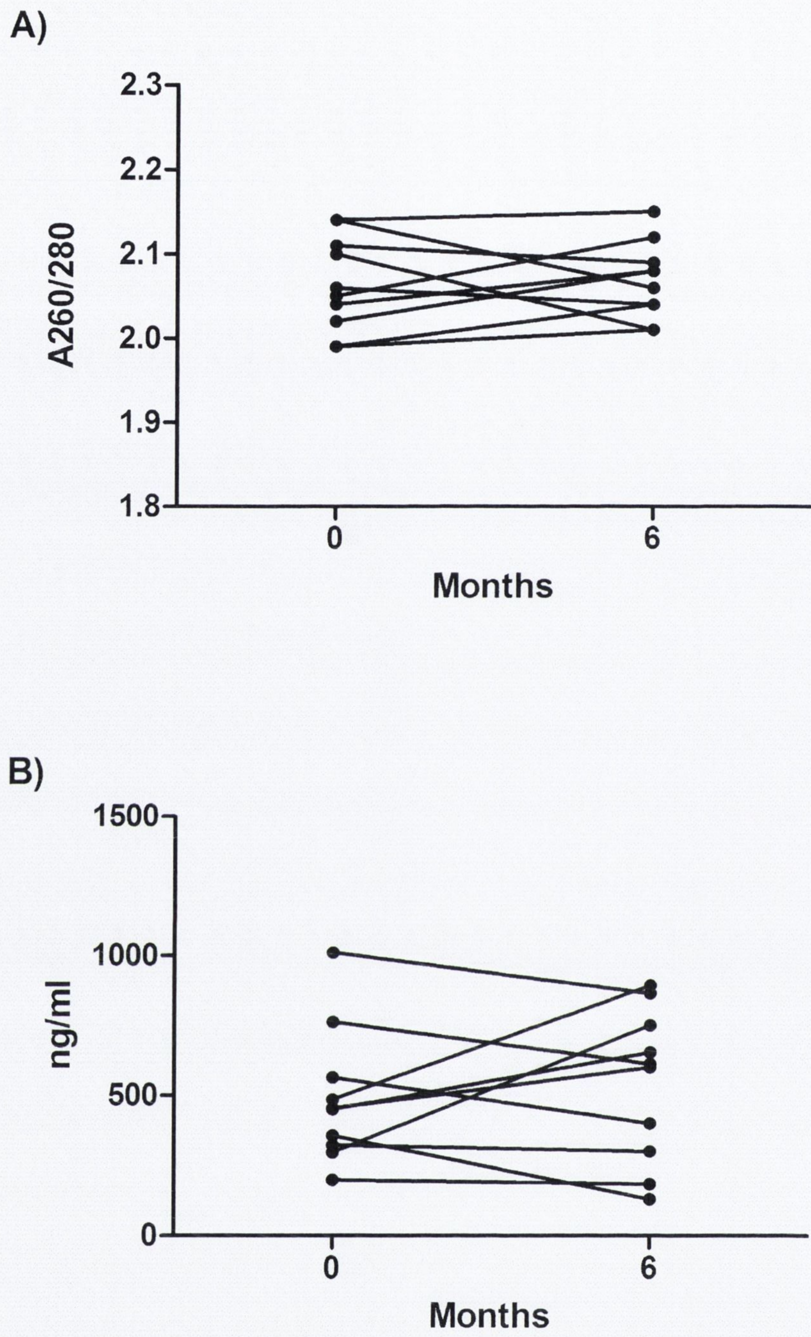


Fig 3.4 **Effects of storage at -80°C on RNA extracted from intact biological specimens as measured by purity (A260/280 ratio) (A), and yield (ng/ml) (B), in breast tumour samples at 0 and at 6 month interval (n=10)**

3.2.6 Effect of storage at -80°C on extracted RNA

RNA yield and quality was examined at 3, 6 and 12 month intervals to assess potential degradation of extracted RNA in storage at -80°C. The RNA extraction process is described in section 2.7.1. Briefly, RNA extraction is performed using Qiagen RNeasy Lipid kits and is based on trizol method of RNA extraction using spin column technology. Yield and quality (purity) was measured using the Nanodrop spectrophotometer. The A260/280 ratio was used as a measure of the purity of the RNA extracted. A value of greater than 1.8 is considered pure and suitable for expression profiling. 15 RNA samples were examined at the various time points to determine stability or degradation.

Storage at -80°C did not cause major degradation of RNA purity as denoted by the A260/280 ratio at 3 months (2.014 ±0.012 vs 2.092 ±0.015, p=0.527), 6 months (2.054 ±0.036 vs 2.084 ±0.016, p=0.469) and 12 months (2.076 ±0.030 vs 2.092 0.024, p=0.669) (Fig 3.4 A). Similarly, yield remained unaffected at the time intervals described: 3 months (1158 ±498.3 ng/ml vs 1206 ±538.3ng/ml, p=0.949), 6 months (1106 ±575.0 ng/ml vs 1342 ±620 ng/ml, p=0.788) and 12 months (426.4 ±73.7 ng/ml vs 545.3 ±92.3 ng/ml, p=0.343) (Fig 3.4 B).

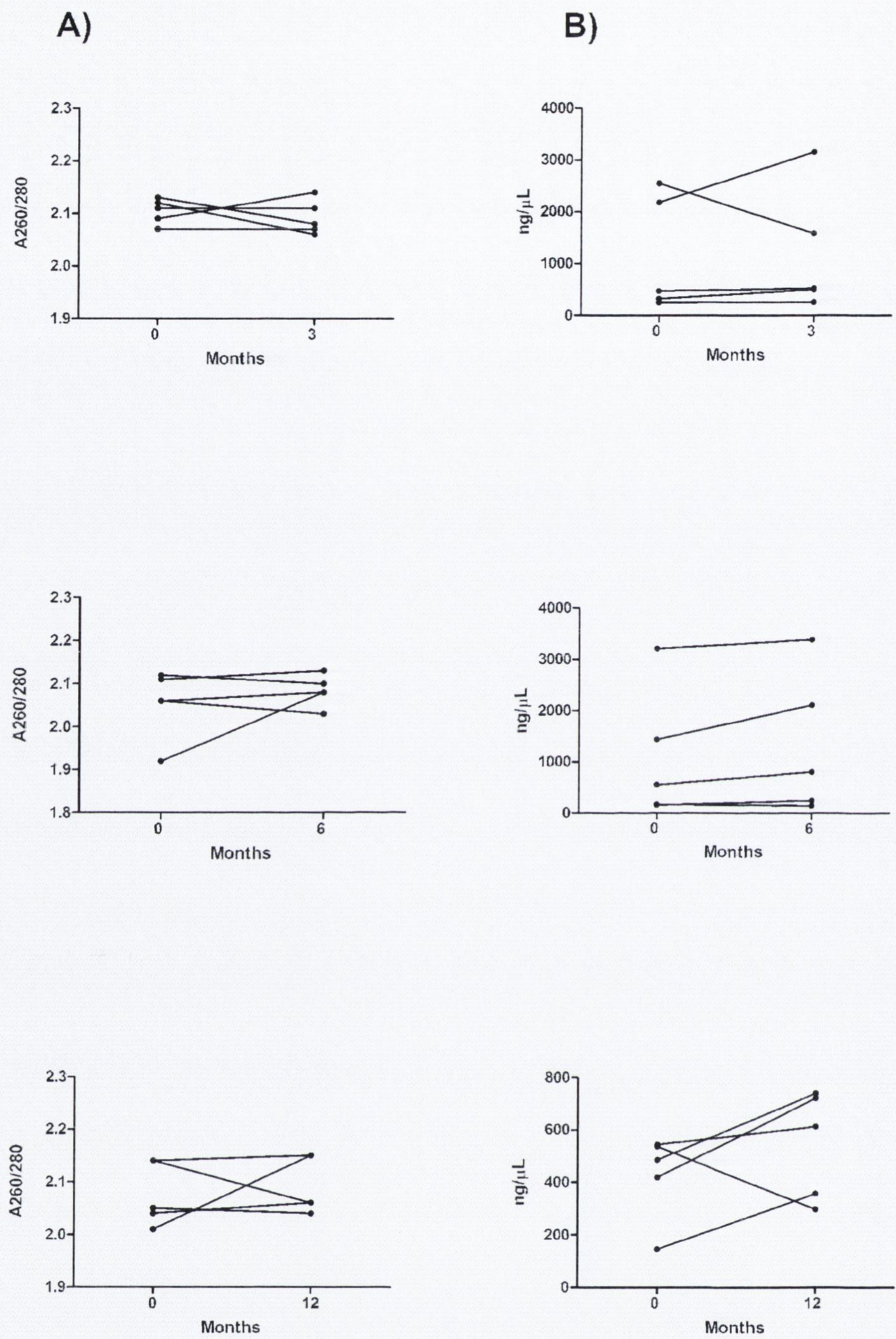


Fig 3.4 **The effect of storage at -80°C on extracted RNA as measured by purity (A260/280 ratio) (A), and yield (ng/ml) (B), in breast tumour samples at 3, 6 and 12 month intervals (n=5 for each time point)**

3.3 Adipose Conditioned Media (ACM) adipokine screening

An additional component to this biobank was the inclusion of a conditioned media, produced form mammary adipose tissue, and collectively termed adipose conditioned media (ACM). This adipose conditioned media (production described in 2.2.2) was designed to be used as model to represent the environment of the breast that envelops tumour and thus to identify obesity related factors that could potentially influence breast cancer. Multiplex cytometric bead array assay technology using the Luminex platform was initially chosen to screen ACM to determine the presence of various adipokines and other cytokines. The technology is based on the coating of microspheres or beads with capture antibodies of interest, to which the sample is then added. The used of multiple conjugated beads allows multiple readouts from the same sample, thus conserving valuable biological specimens.

Multiple adipokines and cytokines were present in the adipose conditioned media. Table 3.3 describes the adipokines present and their respective concentrations. It was found that there was considerable variability in concentrations between the peritumoural and distant samples. This was an unsuspected and surprising result and could suggest that tumour proximity influenced cytokine and adipokine production. The Milliplex arrays were deemed to be too costly to run on a larger cohort of ACM samples procured in the biobank. Therefore, to proceed, it was decided that further quantification of

adipokines was subsequently to be performed using ELISA kits (as described in 4.2.5).

Table 3.3 **Quantification of adipokines and cytokines in adipose conditioned media (n=15) using Milliplex assay arrays.**

	Peritumoural	Distant	P value
Leptin	31066.2 ±3963.8	50070.9 ±8877.2	0.055
Adiponectin	343235.3 ±12301.5	382088.2 ±12068.5	0.028
HGF	5634.7 ±563.9	6472.7 ±436.0	0.244
PAI-1	8732.7 ±1241.8	12841.2 ±1691.4	0.055
IL-1β	1.53 ±0.51	0.98 ±0.19	0.299
IL-6	6241.7 ±195.5	8160 ±274.3	<0.0001
IL-8	5063.6 ±687.7	8417.0 ±3687.7	0.178
MCP-1	4696.5 ±38.9	4995.3 ±74.5	0.0007
NGF	56.43 ±10.13	60.08 ±8.35	0.782
TNF-α	5.15 ±1.01	4.80 ±0.52	0.764
Resistin	1382.6 ±173.3	1946.5 ±263.7	0.079

All data represented as pg/ml

3.4 Discussion

The development of biobanks is critical for basic scientific research and subsequent translation to progress medical understanding of disease. To fully achieve the promise of translational science, molecular characterisation of disease states with reference to demographics, anthropometrics and ultimately long term follow up, including recurrence and survival, post therapeutic intervention is necessary. The place of the biobank is essential in order to attain this goal. The establishment of a national breast biobank network is also required. Common standard operating procedures for the harvesting and storage of biological material as well as the necessary informatics back up will enable the capacity for high throughput of translational research with improved diagnostics, treatment, prognostic indicators and ultimately screening and prevention.

In the development of this biobank, it was important to recognise variables in the pre- and post-acquisition periods that may alter the integrity and quality of tissue harvested. These include: 1) Time to harvest: The transfer and harvesting of tissue is required to be as swift as possible to ensure integrity of the sample Type of surgical procedure, ischaemia time, and surgical trauma all contribute to changes in gene transcription that may lead to variation in scientific data accrued (Foster *et al*, 2006; Werner *et al*, 2000). By monitoring the surgical theatre system (Sapphire Systems, Newgate Technology Limited, UK), having transfer and storage equipment prepared and maintaining regular

communication with the surgeon and theatre staff ensured that time to harvest was as brief as possible. 2) Heterogeneity of tissue types present in tumour pose a significant problem to planned gene expression/microarray studies. By thoroughly examining the tissue extracted, one can ensure the tissue put forward for analysis is indeed homogenous tumour, thus providing for accurate and true results. The difficulties observed in accruing normal breast tissue including the paucity of homogenous normal tissue, or contamination with adipose tissue or stroma further highlight the importance of quality tissue. As such, it was decided that normal breast tissue was not to be studied further until an accurate, consistent method of acquisition is identified. 3) Methods of storage: Long term storage of tissue either in the intact form (fresh frozen) or as RNA/DNA is another step which requires optimisation and careful monitoring. Our brief experiments indicate that storage of tumour tissue, either intact or in the form of extracted RNA at -80°C is a viable practice. However continued quality control is imperative to ensure the reliability of the bioresource going forward.

The body of work in this thesis was focused on the influence of obesity on breast cancer, and in particular the paracrine effect of mammary adipose tissue on breast cancer. To this end, conditioned media from mammary adipose tissue harvested from mastectomies was manufactured. The results from the Milliplex assay screen of a subset of ACM samples were encouraging as they identified and quantified a number of cytokines and adipokines. The technology was found to be difficult to use and ultimately too costly to be used

regularly. However, as a screening tool, it confirmed the presence of adipokines in ACM and therefore it could be used as a representative model of the mammary fat pad in the investigation of the paracrine effect of obesity and adipokines in breast cancer. Further work to consolidate the strength of this bioresource will include the addition of tissue microarrays (TMA) of all consented patients to the biobank. TMAs provide a powerful tool for helping in the validation of gene expression and large scale genomic studies (Dhir, 2008; Signoretti *et al*, 2008). Tissue microarrays will aid in further tumour profiling, screening of gene expression studies, and in the identification of prognostic or diagnostic markers, collectively termed 'biomarkers'. Ultimately it is hoped that each patients stored in the biobank will have serum, tumour and normal tissue (in the form of intact frozen tissue, and extracted RNA and DNA), and a paraffin embedded tissue sample on the tissue microarray.

Chapter 4:

Adipose Conditioned Media as a functional model of Obesity in Breast Cancer

4.1 Introduction

Adipose tissue, including the mammary fat pad, up until recently had been thought of as being an energy depository for excess dietary calorie intake and a hormonally inert tissue. Now, it is considered a functionally active endocrine organ producing steroid and peptide hormones, including adipokines (Kershaw *et al*, 2004). Adipose tissue, in general, is made up of two parts: Adipocytes and a stromal-vascular fraction (SVF) component (Fibroblasts, Macrophages, Stem cells). It is from these two components that the vast majority of adipokines are produced. It is also known that adipokine production from adipose depots (subcutaneous and visceral) is influenced by obesity as described in 1.4.4, such that with increasing obesity circulating levels of leptin are increased and adiponectin levels, conversely, are decreased. In studies examining visceral/ omental (VAT) and subcutaneous (SAT) adipose tissue, it has been demonstrated that at the mRNA and protein level, adipokine production is obesity status dependent. Skurk *et al* also highlighted that this adipokine expression also depended on adipocyte size (Skurk *et al*, 2007). As discussed in 1.5.2.1, mammary adipose tissue (MAT) is a potential source of these adipokines. Of particular interest is the potential for these adipokines acting in a paracrine or a local manner to influence cancer progression.

Cancer cell – stromal interactions play crucial roles in growth and invasion of in cancers of many organs (Heber *et al*, 1996). Fibroblasts are well recognised for their involvement in promoting breast cancer invasiveness (Ruohola *et al*, 2001). Despite the breast being composed of most adipose

tissue, the role of adipocytes in breast cancer progression was, up until recently unexplored. Experiments using cultured adipocytes (murine) demonstrated that factors secreted into a culture media could promote cell proliferation, invasion and migration in breast cancer cell lines (Iyengar *et al*, 2003; Kim *et al*, 2008). *In vivo*, the subcutaneous or intraperitoneal injection of mice with the breast cancer cell line SP1 together with adipose tissue promoted tumour growth and metastases; no tumours were detected however with injection of SP1 cells in sites distant from a fat pad (Elliott *et al*, 1992). The mechanisms by which adipose tissue promoted these events *in vitro* and *in vivo* remained unclear but it was hypothesised that mammary adipose tissue could influence aspects of carcinogenesis by the release of adipokines and other factors into the milieu surrounding cancer cells.

The influence of obesity and the metabolic syndrome on this adipose depot and its subsequent effect on cancer has heretofore been unexamined. Therefore the aims of this chapter were:

1) To examine if mammary adipose tissue (MAT) could influence various aspects of cancer progression (particularly proliferation, invasion).

2) Quantify adipokine production in ACM and determine the influence of obesity and metabolic syndrome (MetS) status on the production of adipokines from MAT.

3) Examine how MAT may influence cancer related gene expression in a co-culture system.

4.2 Results

4.2.1 Breast cancer cell lines are responsive to adipokines

In order to examine the potential role of adipokines in breast cancer, the effect of recombinant leptin and adiponectin on proliferation in the breast cancer cell lines MCF-7 and MDA-MB-231 was examined using BrdU cell proliferation assays. Dose response curves were carried out using increasing concentrations of leptin (0.5ng/ml, 5ng/ml, 50ng/ml, 500ng/ml) and adiponectin (5ng/ml, 50ng/ml, 500ng/ml, 5000ng/ml) at 24 and 48hrs.

Examining leptin dose response curves (Fig. 4.1), in the MCF-7 cell line, there is demonstrable increase in cell proliferation in response to increasing concentrations of leptin with a significant increase in cell proliferation observed following treatment with 500ng/ml leptin at 24hrs relative to an untreated control (128.77 ±5.86 % vs 100%, p=0.039). At 48hrs, at the same concentration, cell proliferation was increased but not significant (142.71 ±29.72 % vs 100%, p=0.287). In the MDA MB 231 cell line does not mirror this relationship. A non significant increase in proliferation is witnessed at 5ng/ml (147.8 ±21.82%, p=0.156) and 50 ng/ml (157.1 ±26.56, p=0.164) at 24hrs. At the 48hr timepoint, MDA MB-231 cells do not appear to proliferate in response to leptin.

Examining adiponectin dose response curves (Fig. 4.2), in the MCF-7 cell line, a decreasing cell proliferation curve is observed with increasing concentrations of adiponectin at both 24hrs and 48hrs. At 48hrs and

at a concentration of 5000ng/ml, there is significant decrease in cell proliferation (90.2 ±0.83% vs 100%, p=0.007). At the coinciding concentration at 24hrs cell there is a small, non significant decrease (97.21 ± 11.72, p=0.818). In the MDA MB-231 cell line, a similar decreasing proliferation curve is seen at the 48hrs timepoint. At 5000ng/ml, there is a non significant decrease in cell proliferation (79.46 ±19.05%, p=0.382).

These experiments demonstrate that adipokines may influence cell proliferation in breast cancer. This suggests that in *vivo*, breast cancer may be responsive to these adipokines. Furthermore, the observed responsiveness to increasing and decreasing concentrations of leptin and adiponectin respectively may implicate obesity as a factor in increased tumour proliferation, given that, in obesity, leptin concentrations are increased and adiponectin levels are conversely decreased.

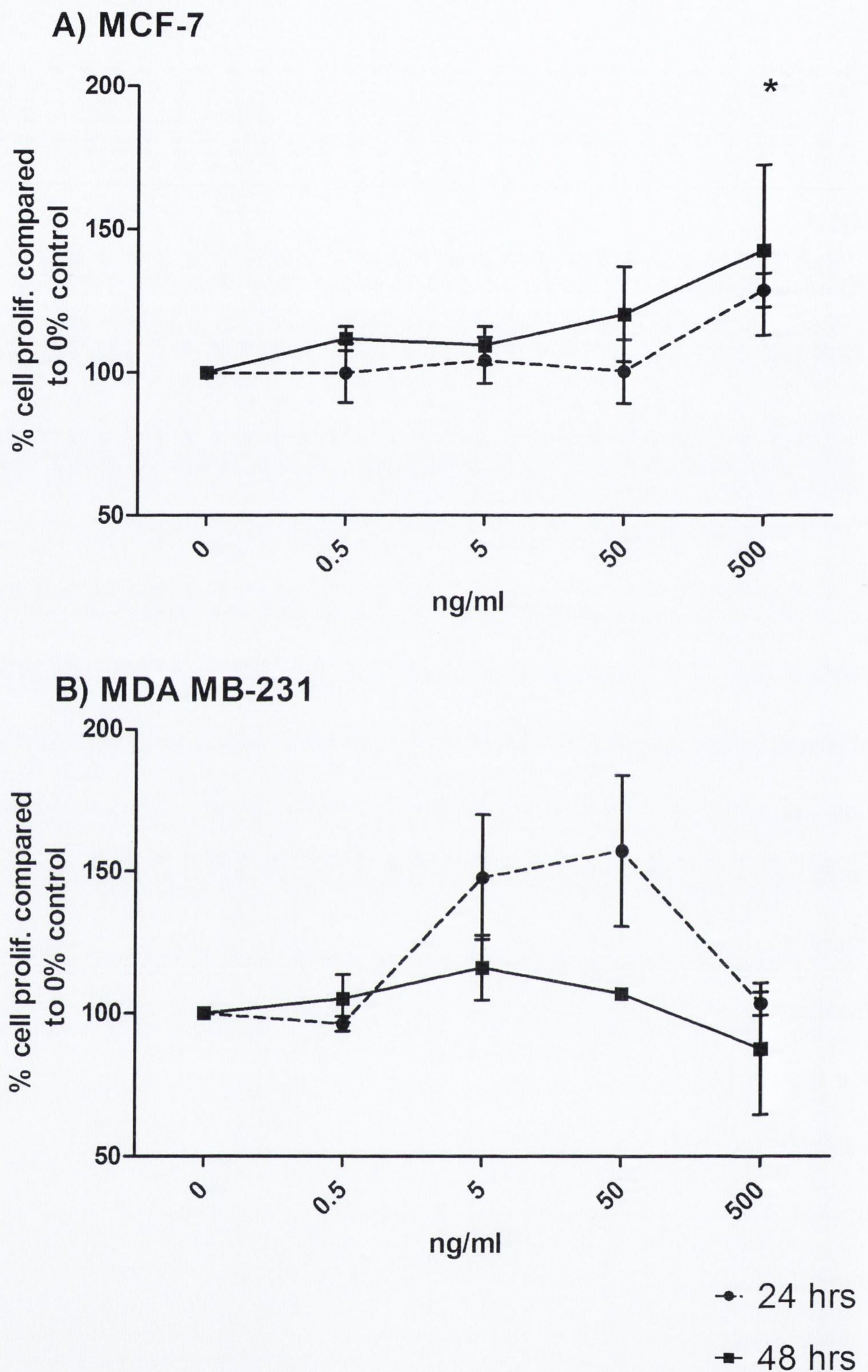


Fig. 4.1 **The effect of recombinant leptin on MCF-7 (A) and MDA MB-231 (B) cell proliferation in a dose response experiment at 24hrs and 48hrs. Data is expressed as mean ± SEM, with control cells taken as 100%. Statistical analysis was carried out by ANOVA with post test analysis by Tukey-Kramer multiple comparisons test. Results shown are the mean of 3 independent experiments. * $p < 0.05$ 24hrs**

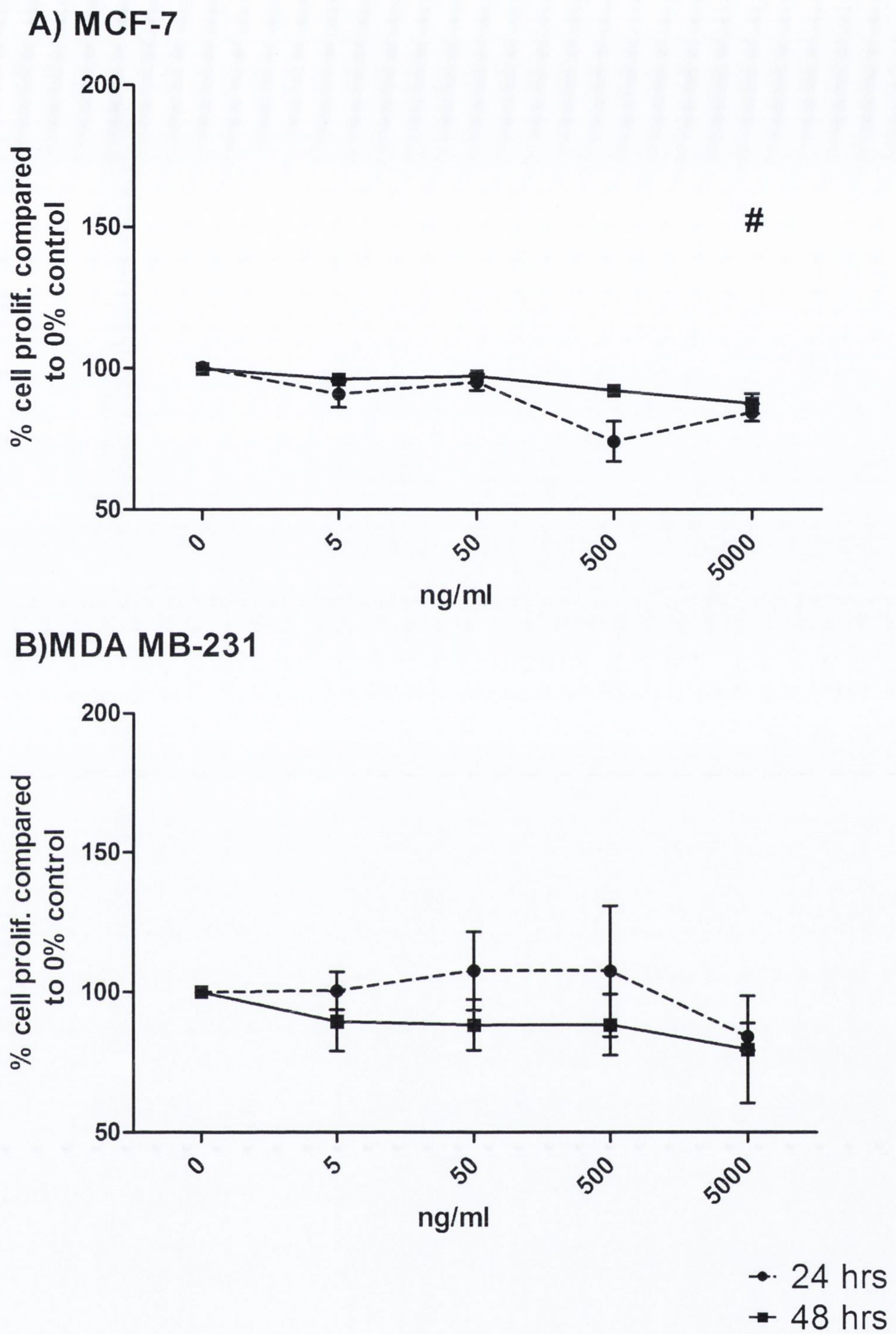


Fig. 4.2 **The effect of recombinant adiponectin on MCF-7 (A) and MDA MB-231 cell proliferation in a dose response experiment at 24hrs and 48hrs. Data is expressed as mean ± SEM, with control cells taken as 100%. Statistical analysis was carried out by ANOVA with post test analysis by Tukey-Kramer multiple comparisons test. Results shown are the mean of 3 independent experiments. # p <0.05 48hrs**

4.2.2 ACM stimulates cell proliferation

Following on from the discovery that adipose conditioned media contained multiple growth factors (cytokines, adipokines), we wished to examine the functional properties of ACM to determine if it could influence various aspects of cancer progression. Initially, as a proof of concept, two breast cancer cell lines (MCF-7, MDA-MB-231) were treated with ACM (produced from postmenopausal women, n=10) for 24hrs and 48hrs.

ACM demonstrated the ability to stimulate proliferation of breast cancer cell lines. In Peritumoural (PT) and Distal (D) ACM, proliferation was similar in both depots in the MCF-7 and MDA-MB-231 cell lines at both 24 and 48hrs (table 4.1).

Table 4.1 ACM cell proliferation by site of production at 48hrs

	PT	D	P value
24hrs			
MCF-7	163.62 ±15.09	158.99 ±16.13	0.742
MDA-MB 231	96.16 ±5.53	83.74 ±7.00	0.064
48hrs			
MCF-7	152.27 ±9.36	152.56 ±10.52	0.983
MDA-MB-231	110.53 ±5.66	113.48 ±2.28	0.631

Examining this effect further, it was demonstrated that in the ER +ve cell line MCF-7 at 48hrs, ACM from patients with the MetS stimulated significantly greater proliferation compared to NW patients. This effect was seen in both ACM depots (PT and D) (PT: 203.6 ±24.23 vs 136.8 ± 11.58 %, p

= 0.022; D: 198.4 ± 17.01 vs 137.6 ± 9.02 %, p = 0.006, Fig.. 4.3 A). The ER –ve MDA-MB-231 cell line did not demonstrate this effect (PT: 115.45 ± 9.47 vs 108.05 ± 7.30 %, p = 0.944; D: 112.85 ± 15.07 vs 114.9 ± 26.7 %, p = 0.376, Fig. 4.3 B). These data imply that ACM from patients with MetS contains factors that are secreted in differing concentrations to influence the increased proliferation observed and that it is the entire gland that is altered, ruling out a local tumour adipose tissue interaction that may influence secretion.

Also, as the proliferation effect was seen in the ER +ve MCF-7 cell line, a second ER +ve cell line, T47-D, was cultured with a combination of peritumoural and distant ACM from 15 MetS and 15 normal weight patients (due to low supply). It was observed that MetS group had greater cell proliferation but this did not reach significance (116.6 ± 11.16 vs 95.85 ± 12.47 %, p = 0.252) (Fig. 4.3 C). These data may indicate that in an *in vitro* model, ACM induced cell proliferation is oestrogen dependent.

Cell proliferation assays using ACM from premenopausal women demonstrated a significant increase in proliferation in both peritumoural and distal ACM depots (Fig 4.4). The diagnosis of MetS in pre-menopausal women was infrequent so obesity, as defined by WC > 80cm was used to delineate two groups. There were no differences noted between normal weight and obese premenopausal women in either peritumoural or distal ACM cell proliferation experiments. (PT: 226.14 ± 37.91 vs 187.53 ± 19.16%, p=0.333; D: 216.33 ± 29.1 vs 225.53 ± 32.21%, p=0.856).

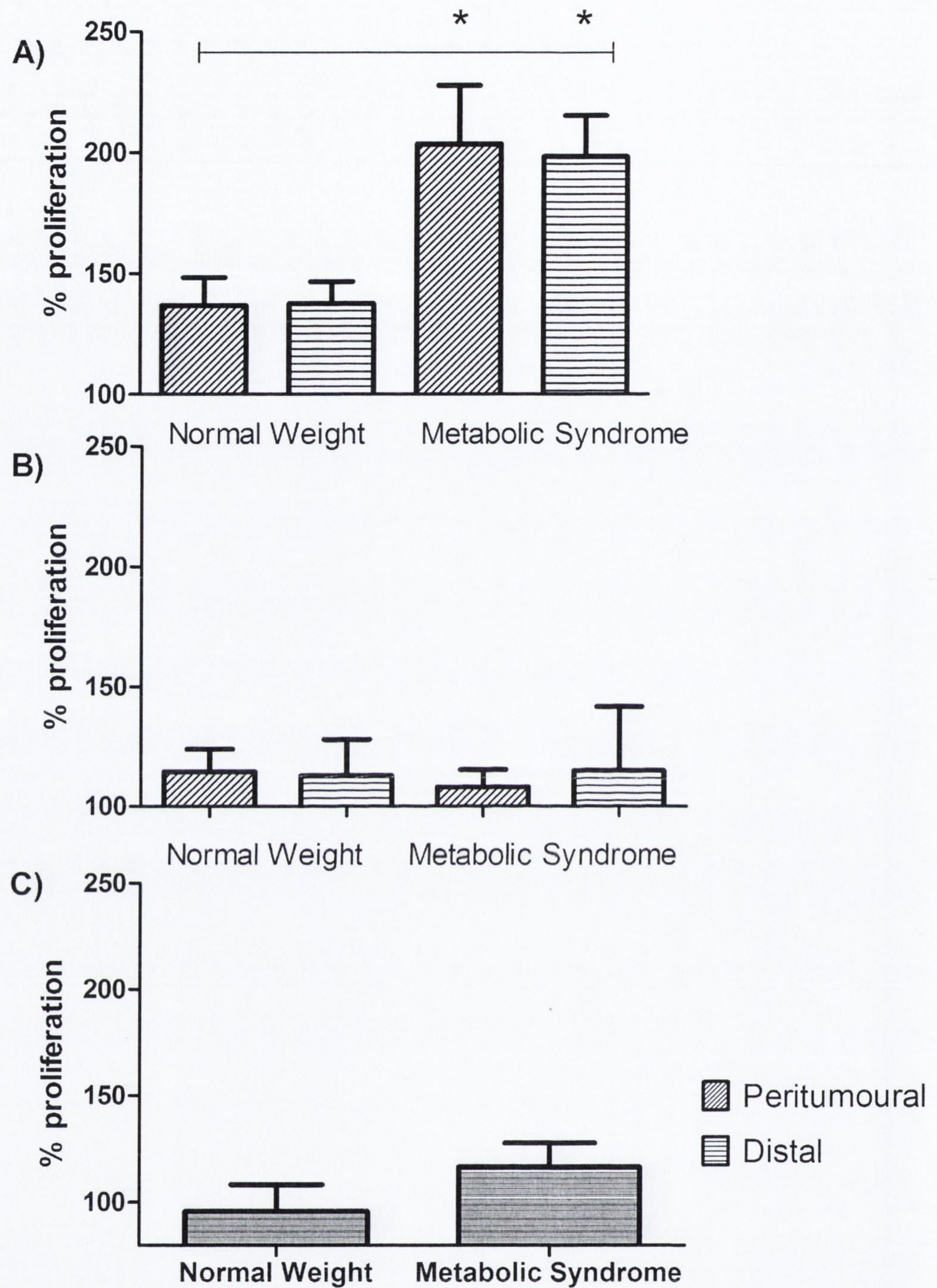


Fig. 4.3 **ACM cell proliferation by BrdU. MCF-7 (A) and MDA-MB-231 (B) were cultured in peritumoural and distant ACM for 48hrs. T 47-D (C) cultured in distant ACM only. All data expressed relative to control (0% M199 media) set at 100% proliferation. Normal weight ACM n=15, Metabolic syndrome n=15). Each sample repeated in triplicate. Data are expressed as mean ± SEM. Statistical analysis performed by unpaired student t-test.**
*** p<0.05**

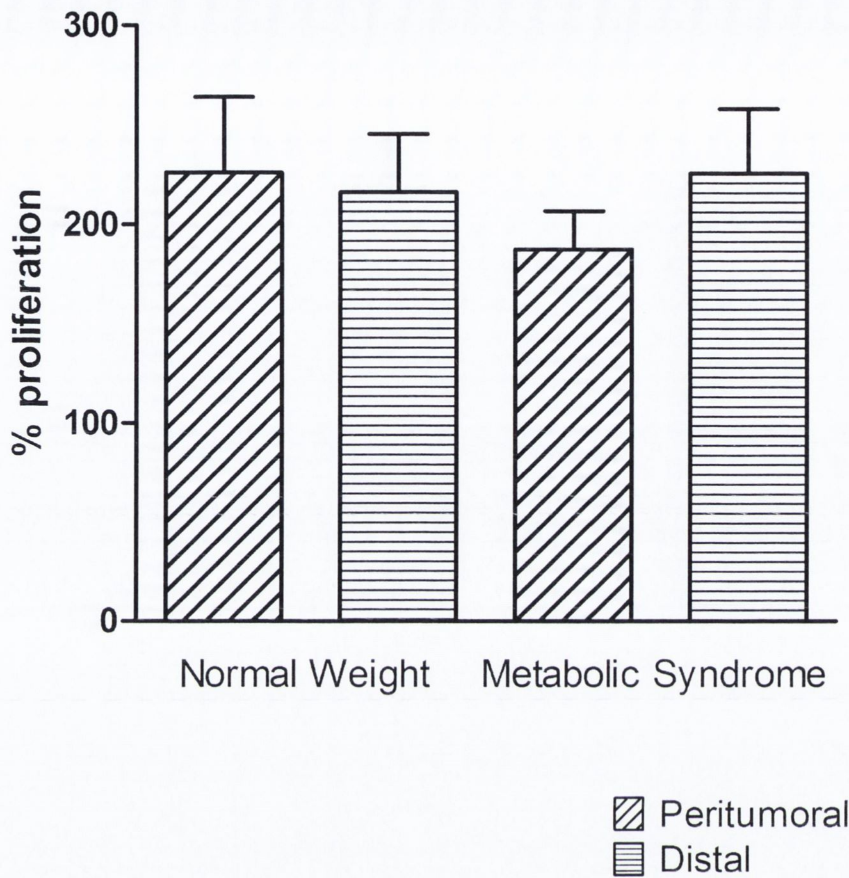


Fig 4.4 **Pre-menopausal ACM cell proliferation by BrdU. MCF-7 cells were cultured in peritumoural and distal ACM for 48hrs. All data expressed relative to control (0% M199 media) set at 100% proliferation. Normal weight ACM n=6, Obese ACM n=8). Each sample repeated in triplicate. Data are expressed as mean ± SEM. Statistical analysis performed by unpaired student t-test.**

4.2.3 Effects of ACM on expression of genes implicated in pathways involved in cancer: qRT-profiler PCR array

In order to elucidate potential molecular mechanisms involved in the observed finding in 4.2.2 suggesting that ACM from patients with MetS promoted significantly greater proliferation compare to ACM from normal weight cancer patients, we examined changes in breast cancer gene expression profiles of cellular pathways involved in carcinogenesis using Human Cancer Pathway Finder quantitative reverse-transcription PCR arrays. The arrays are loaded with 88 genes implicated in several classic hallmarks of carcinogenesis (Invasion/metastasis, angiogenesis, transduction/transcription, adhesion, apoptosis and cell cycle). A co-culture system using whole adipose tissue and the MCF-7 cell line as outlined in 2.4 was used for this experiment. RNA was isolated from the cell lines after 24hrs incubation with mammary adipose tissue pieces from 3 postmenopausal women with MetS. For each patient sample, a control (cells treated with M199 media without supplementation) was set up. Purity of RNA was determined spectrophotometrically using the Nanodrop. Following examination of RNA purity, 250ng of each sample was reverse transcribed to cDNA as per manufacturer's instructions. Synthesised cDNA samples were then loaded onto the qRT-PCR array plates for analysis of cancer pathway gene expression profiles (see appendix 2 for a list of genes examined on the Human Cancer Pathway Finder array).

The arrays provided an insight into how mammary adipose tissue as a paracrine mediator of obesity may affect cancer progression (Fig. 4.5 A).

The genes that were altered were predominantly in invasion/metastasis (33%) (Upregulated: *NME1, MTA2, TIMP-3*) and transduction/transcription pathways (34%) (Upregulated: *ERBB2, PIK31A, AKT1*; Downregulated: *FOS, NFKB1A*). Other pathways that were altered included: Angiogenesis (Upregulated: *IFN1A, TGFBR1*; Downregulated: *VEGFa*) and Cell cycle (Upregulated: *CDK4;* Downregulated *E2F1*)

Based on the results of the qRT-PCR arrays, a number of genes interest were selected for validation. Sample size was increased to 5 obese women and the co-culture experiment was repeated. Changes in gene expression were validated using qPCR (Fig. 4.5 B). Five genes were selected for validation: *NME1, MTA2, CDK4, VEGFa and TIMP-3*. These genes were expressed >2fold in the original qRT-PCR arrays and thus selected. *NME1, MTA2* and *CDK4* were significantly upregulated compared to the control. *VEGFa*, which was down-regulated in the Human Cancer Pathway Finder Array data, was found to be upregulated in the validation set. *TIMP3* was not significantly up-regulated in the validation set as compared to a control. These results identify a number of genes, whose expression patterns are influenced by adipose tissue and its secreted factors. They suggest that factors secreted by mammary adipose tissue, acting via a paracrine mechanism, may influence aspects of carcinogenesis, particularly invasion/metastasis. Further investigation is required to determine if obesity status plays a role.

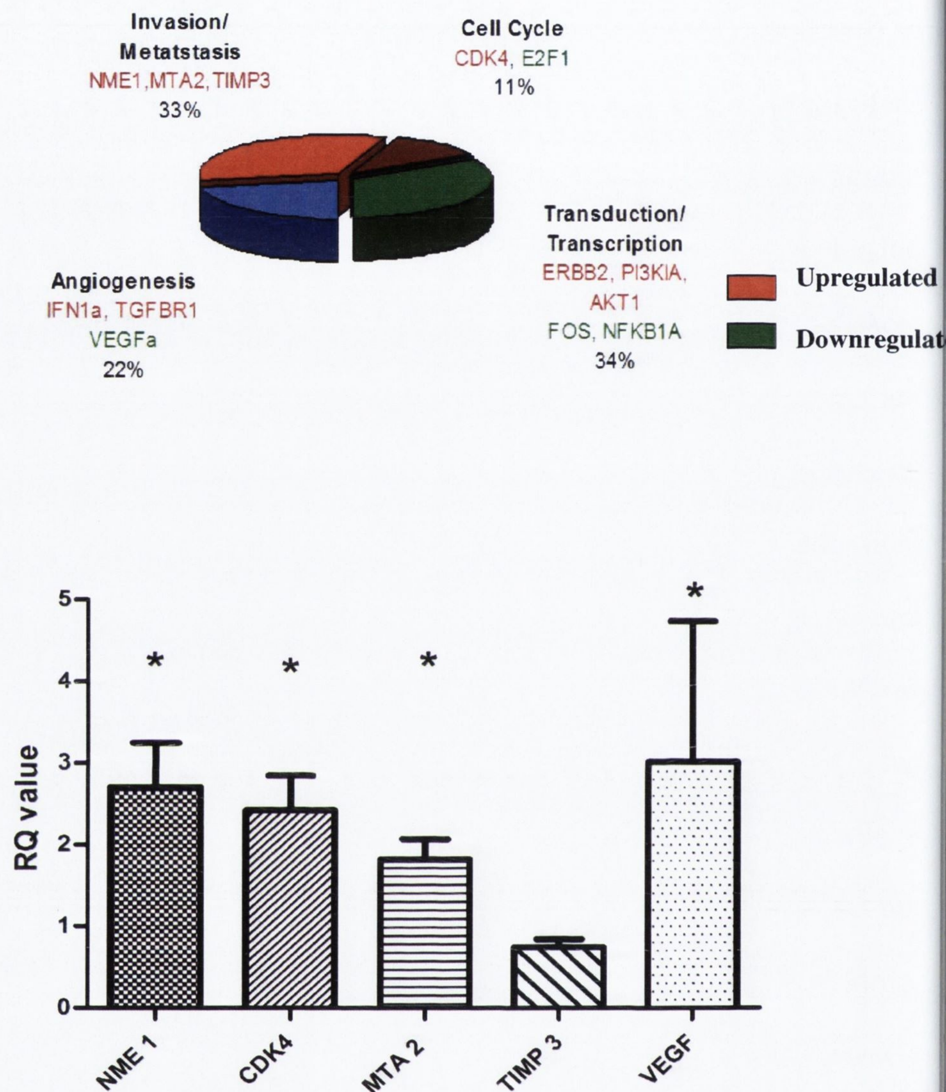


Fig. 4.5 **A) Changes in Human Cancer Pathway Finder qRT-PCR Array following 24h co-culture with mammary adipose tissue. Fold changes in gene expression >2, calculated using the CT method are demonstrated.**

B) Validation using qPCR primer probe sets for NME1, MTA2, CDK4, VEGFa, and TIMP3 using the co-culture model on obese cancer patients (n=5).
Control (cells treated with M199 media without supplementation) is set to an RQ (Relative Quantification) value of 1 for each gene. Data is expressed as mean ± SEM. Statistical analysis performed by unpaired student t-test. *p<0.05

4.2.4 Effect of ACM on tumour cell invasion

It is recognised that obese women, particularly those with MetS are likely to present with advanced or metastatic disease. Invasion through the extracellular membrane is an important step in tumour metastasis. From 2.2.3, we determined that adipose tissue promoted numerous genes involved in invasion and metastasis. The effects of ACM on cell invasion in a functional model, and the influence of MetS status on this process was examined by Cell Invasion Assays.

ACM from MetS patients significantly increased invasion of the MCF-7 cell line compared to ACM normal weight patients (153.4 ±6.027 vs 126.3 ± 6.03 % RFU, $p=0.006$, Fig. 4.6 A). Cell invasion was increased in MetS ACM in the T 47-D (125.2 ± 19.52 vs 110.2 ±10.78 % RFU, $p=0.512$, Fig. 4.6 B), while in the MDA-MB-231 cell line, cell invasion was decreased compared to the control (87.28 ± 4.80 vs 83.26 ± 6.02 % RFU, $p=0.607$, Fig. 4.6 C).

This indicates mammary adipose tissue and its secreted factors in ACM can influence cell invasion. The metabolic syndrome stimulated increased invasion, indicating a potential paracrine role for adipose tissue and by extension obesity/Mets in breast cancer to promote cancer progression. It also validates the gene expression data that highlight the role of invasion/metastasis pathways in a co-culture model.

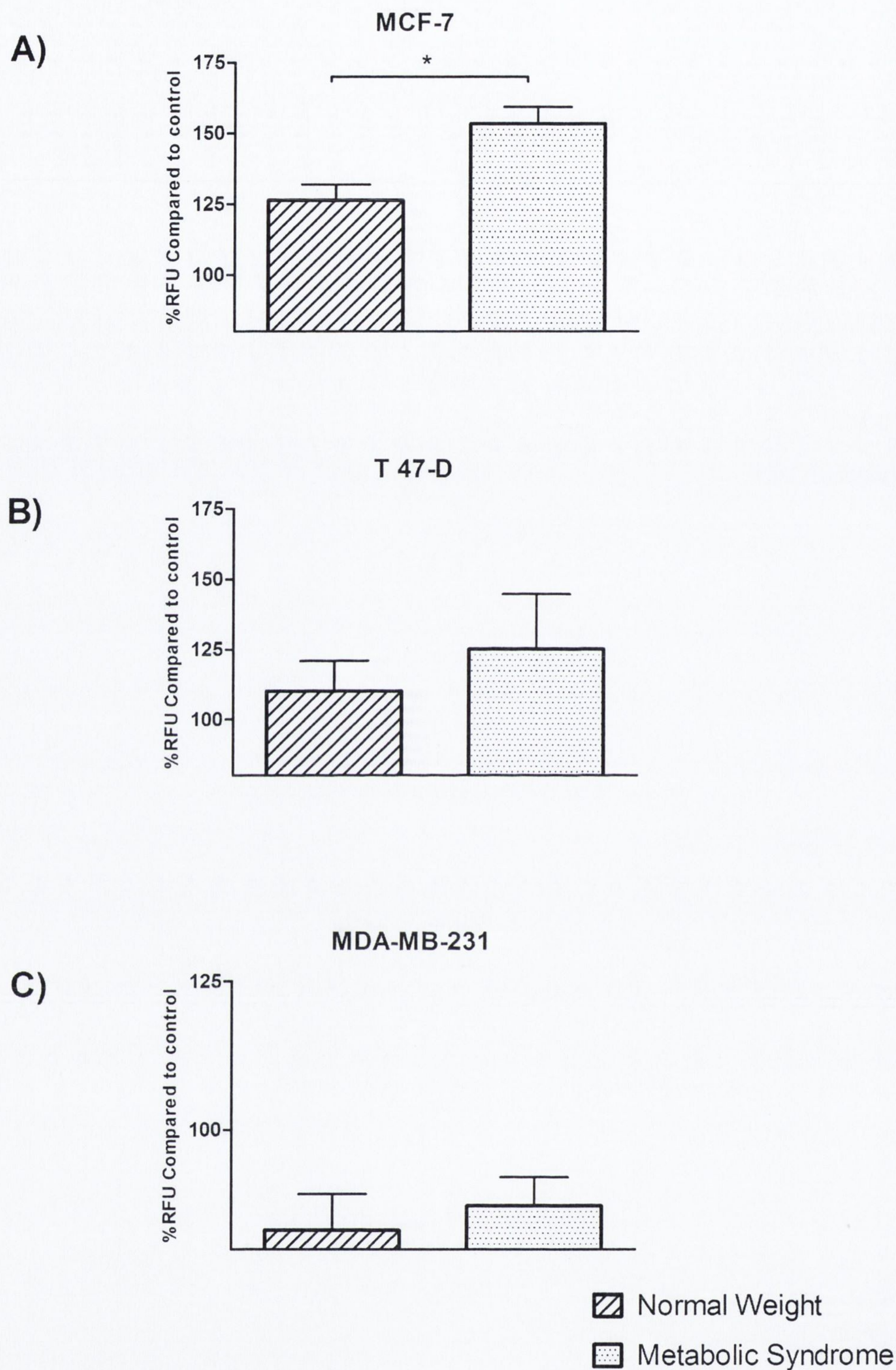


Fig 4.6 **The effect of ACM on breast cancer cell line invasion. Cell invasion was examined following a 24hr incubation of ACM (Normal weight n=12, MetS n=12) with MCF-7 (A), T 47-D (B), and MDA-MB-231 (C) cell lines. Each ACM sample was loaded in triplicate onto 96-well invasion plates. Data is represented as a percentage of a 0% control, set to 100% relative fluorescent units (RFU) and expressed as mean ± SEM. Statistical analysis performed by unpaired student t-tests. *p <0.05**

4.2.5 Adipokine expression in ACM

Adipose conditioned media was initially screened (section 3.2.4) to examine what potential adipokines were present. Following on from this, using Human ELISA DuoSet kits, a number of adipokines of interest were examined to quantify secreted levels and determine if obesity status influenced expression. The ELISAs chosen were leptin, adiponectin, IL-6, IL-8, MCP-1 and VEGF. ACM was examined in postmenopausal women in 3 groups for this study: Normal weight (n=15), Obese (n=6) and MetS (n=15).

Adipokines from both depots (peritumoral and distal) expressed similar concentrations in postmenopausal women. Individual adipokines and significance values are provided in table 4.2. Metabolic syndrome status appeared to influence the expression of leptin and adiponectin in both peritumoural and distal ACM (Fig 4.7), with increasing levels of leptin and conversely, decreasing levels adiponectin as expected. An increase in MCP-1 levels was also seen in MetS patients. ACM IL-6 and IL-8 did not appear to be influenced by MetS status in his study.

The oestrogen concentration in a small cohort of ACM was examined by EIA to determine levels secreted by adipose tissue into the conditioned media. It was found that between the 3 groups (Normal weight, Obese, and MetS) that there was no significant difference in oestradiol concentrations (30.82 ±4.59, 32.82 ± 4.59, 35.36 ±9.5 *fg*/ml, p = 0.823) (Fig. 4.7 C)

Table 4.2 Adipokine levels in ACM (Peritumoral and Distal)

	Peritumoural ACM				Distal ACM			
	Normal Weight	**Obese**	**MetS**	**P**	**Normal Weight**	**Obese**	**MetS**	**P**
Leptin	66.15 ±19.57	77.78 ±29.23	113.95 ±42.02	0.568	50.12 ±17.47	77.79 ±29.23	85.47 ±14.88	0.807
Adiponectin	220.8 ±45.07	219.04±59.29	174.06 ±19.33	0.123	229.9 ±0.79	217.34 ±49.29	170.97 ±24.01	0.438
IL-6	38.47 ±10.38	40.72 ±5.88	44.72 ±6.88	0.881	37.50 ±16.04	37.51 ±16.13	29.78 ±13.44	0.784
IL-8	34.65 ±7.27	40.94 ±6.76	43.94 ±5.24	0.967	39.83 ±1.08	40.12 ±6.91	27.69 ±2.20	0.378
MCP-1	75.47 ±17.61	70.03 ±13.51	89.99 ±11.07	0.277	76.42 ±17.61	70.03 ±13.51	101.57 ±11.07	0.471

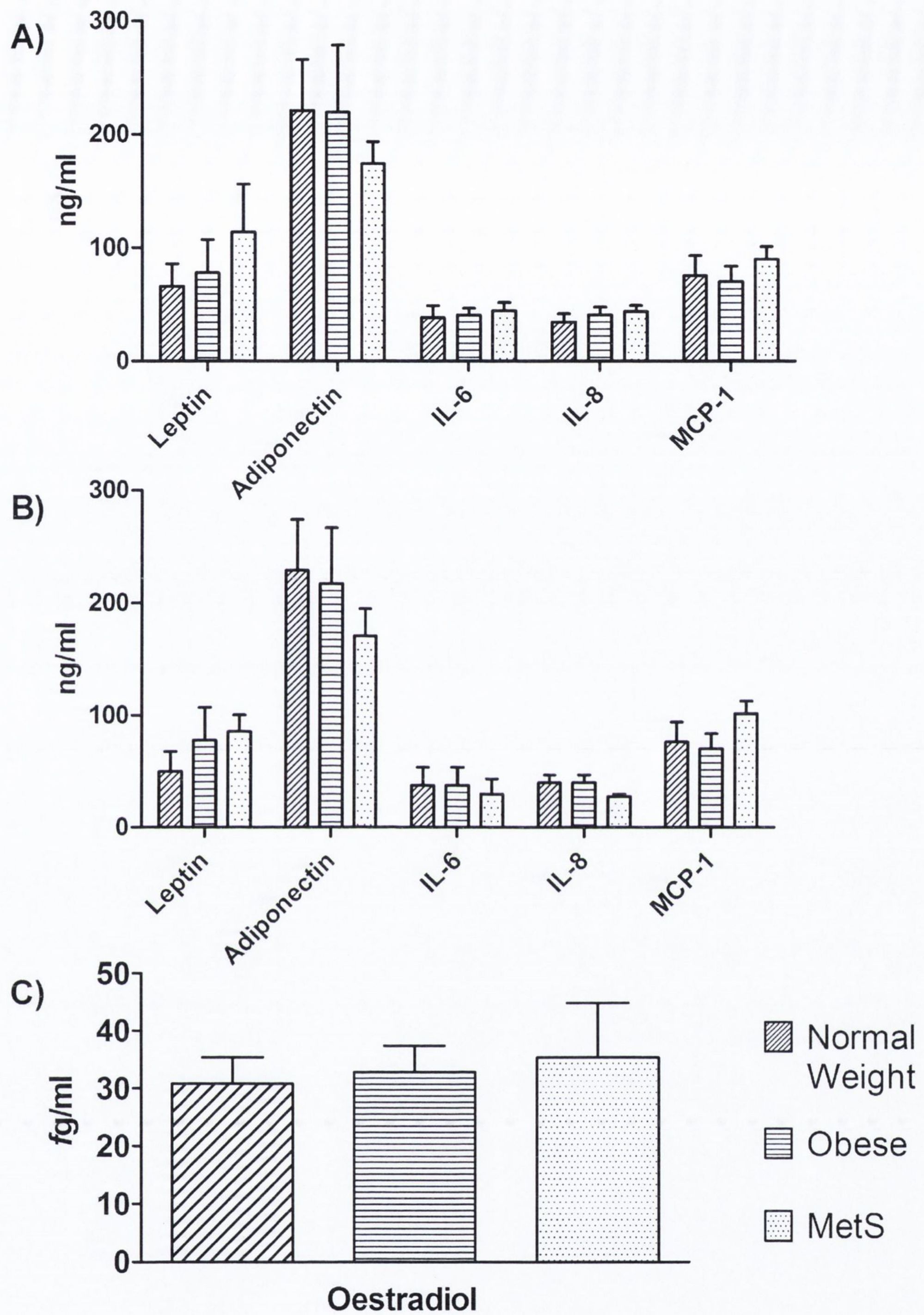


Fig. 4.7 **Adipokine quantification in ACM by ELISA. Adipokine concentrations were assessed using Human ELISA DuoSets in Peritumoural (A), and Distal (B) ACM from normal weight (n=15), Obese (n=6) and MetS (n=15). Each ACM sample was assayed in triplicate against a standard curve for the adipokine in question. Oestradiol in the ACM (C) was examined using an EIA kit in Peritumoral ACM only. Data is represented as mean ± SEM. Statistical analysis was performed by ANOVA.**

4.2.6 The influence of obesity status on identified genes: ACM experiment

A further experiment was conducted to determine whether obesity status could influence the expression of the 5 genes identified in 4.2.4. The MCF-7 cell line was treated with ACM from normal weight (n=10) and MetS (n=10) patients and incubated for 24h. RNA was extracted, cDNA made and expression of the 5 genes, identified in 4.2.5 was examined (Fig. 4.8 A). As an additional component to this experiment gene expression of the adipokines leptin and adiponectin, and their ligand receptors were examined to determine if their expression was altered in this experiment in response to ACM from MetS and normal weight patients (Fig. 4.8 B). Individual RQ values for each set of genes in this experiment are described in Table 4.3.

Although no significant differences are noted, it is interesting to note that there is increased expression in the metabolic syndrome group for 4 of the 5 genes (*NME1, MTA2, CDK4, VEGF-a*), perhaps indicating that in a functional model, the invasion/metastasis pathway is stimulated to a greater degree in MetS patients.

Adipokine gene expression data demonstrated similar trends to that observed above, with the expression of leptin *(Ob)* and leptin receptor *(ObR)* increased and adiponectin *(AdipoQ)* decreased in the metabolic syndrome group compared to the normal weight group. These experiments should be repeated in a larger cohort to ascertain significance but the data suggests that

secreted factors in ACM are different in the two groups and subsequently have influence on gene expression of adipokines.

Table 4.3 Relative Quantification (RQ) values for ACM co-culture experiment

Gene	N. Weight	MetS	P value
NME1	4.50 (±0.62)	4.82 (±1.51)	0.850
MTA2	1.62 (±0.26)	2.02 (±0.57)	0.880
CDK4	0.73 (±0.09)	2.01 (±0.57)	0.099
TIMP-3	1.41 (0.32)	1.32 (±0.22)	0.824
VEGFa	3.31 (±0.74)	4.53 (±0.91)	0.312
Ob	3.74 (±0.66)	6.81 (±1.97)	0.261
ObR	0.44 (±0.06)	0.68 (±0.13)	0.104
AdipoQ	20.05 (±7.56)	10.7 (±4.73)	0.310
AdipoR1	1.50 (±0.25)	1.63 (±0.39)	0.788
AdipoR2	1.30 (±0.20)	1.44 (±0.36)	0.734

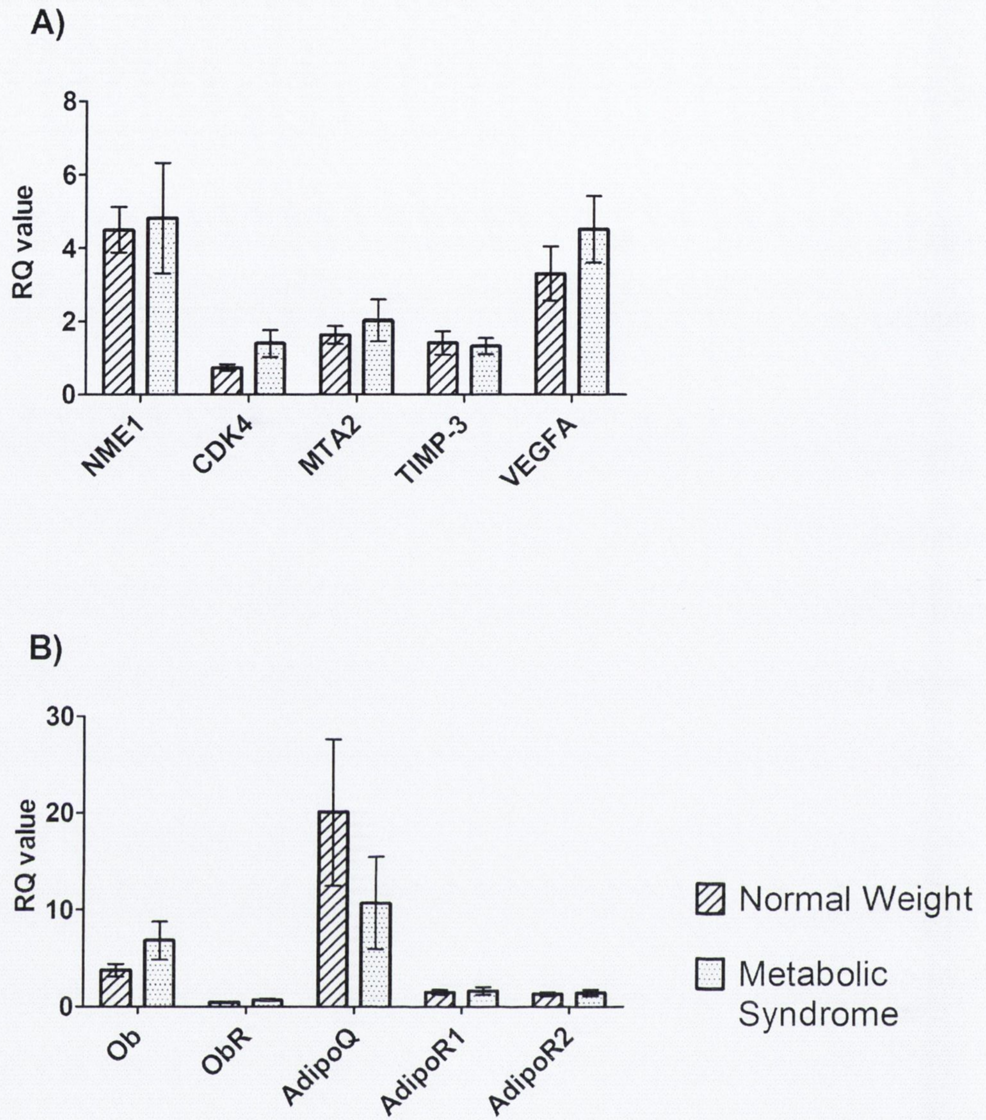


Fig. 4.8 A) mRNA expression of validated genes in a co-culture experiment treating MCF-7 cells with ACM from Normal Weight and Metabolic Syndrome patients (n=10 per group)

B) mRNA expression of adipokines, leptin (Ob) and adiponectin (AdipoQ), and their ligand receptors (ObR, AdipoR1 and AdipoR2) in the same experiment.

Control (cells treated with M199 media without supplementation) is set to an RQ (Relative Quantification) value of 1 for each gene. Each sample is repeated in triplicate. Data is expressed as mean ± SEM. Statistical analysis performed by, unpaired student t-test.

4.2.7 Examination of MetS influence on gene expression in a cohort of tumour

From the previous experimental data, MetS may potentially play a role in influencing the expression of a number of genes involved in angiogenesis and invasion/metastasis in breast cancer. To further elucidate the potential of MetS to influence gene expression, we examined whether the genes, VEGFa, CDK4 and MTA2 in human breast tumour tissue were responsive to MetS status. This experiment was performed using RNA extracted from tumour tissue in 20 normal weight and 20 MetS postmenopausal women using an RQ-PCR approach.

CDK4 gene expression was non-significantly higher in tumours from patients with MetS compared to normal weight patients (21.10 ±11.12 vs. 11.95 ± 4.935, p = 0.884). A similar non- significant increase was observed for MTA2 between the two groups (40.35 ±21.25 vs. 18.09, p = 0.815). VEGF-a gene expression was found to be lower in the MetS group (1071 ± 650.1 vs 1255 ± 599.1, p = 0.773) (Fig 4.9).

The mRNA expression of these genes in breast tumour tissue is high but variable. These data suggest that the genes examined are not influenced by MetS status in human breast cancer tissue. Other molecular pathways ought to be examined in an effort to explain the molecular mechanisms by which obesity influences cancer incidence and prognosis.

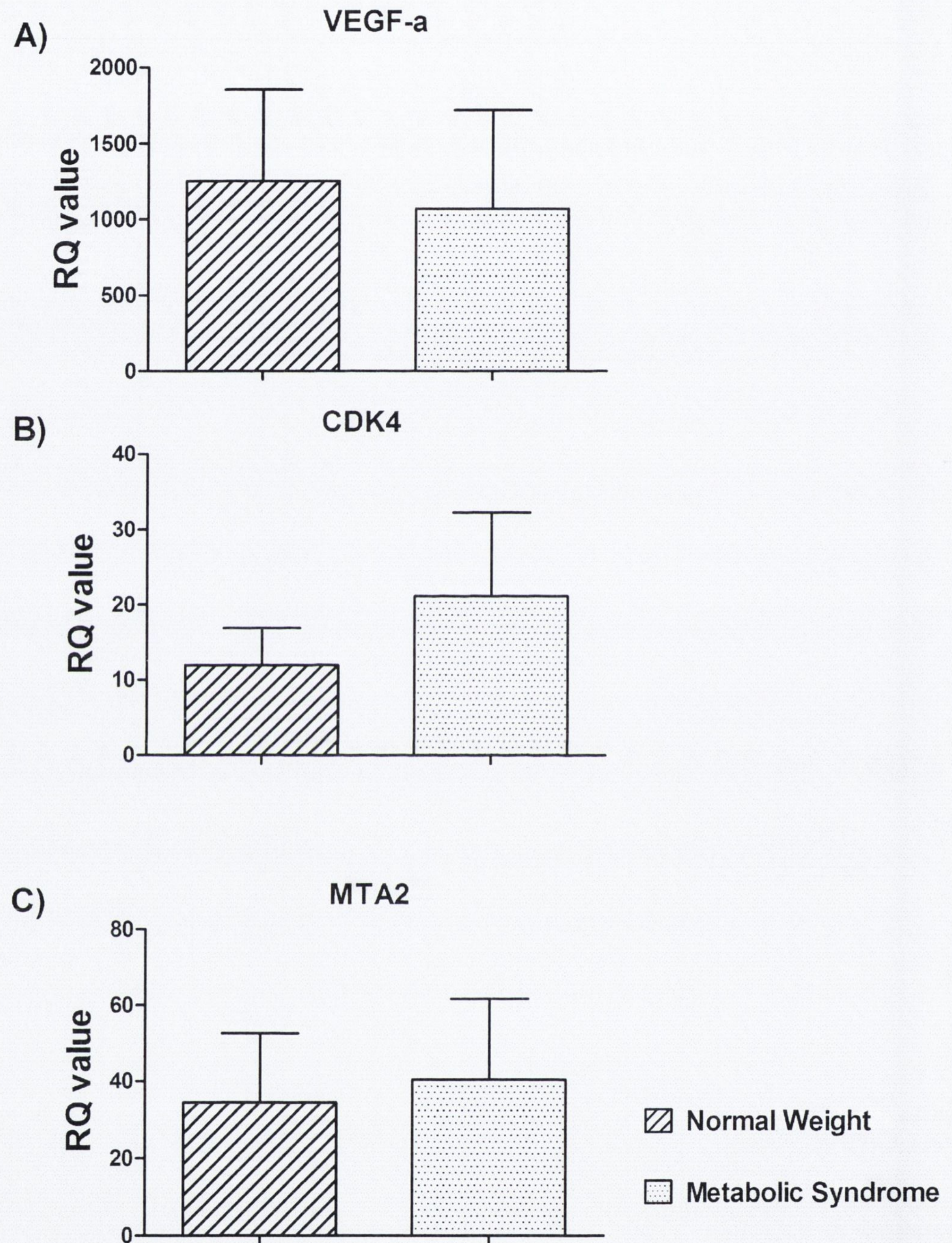


Fig. 4.9 **Examination of the influence of the metabolic syndrome on tumour expression of VEGF-a (A), CDK4 (B), and MTA2 (C). Gene expression was studied on human tumour tissue from 20 normal weight and 20 MetS postmenopausal patients using RQ-PCR. Data is expressed as mean ± SEM. Statistical analysis was carried out using the Mann-Whitney Test.**

4.3 Discussion

Hanahan and Weinberg, in a seminal review, proposed that tumourigenesis was a multistep process with all cancer genotypes a manifestation of altered cell physiology that collectively dictated malignant growth. These altered physiological processes were: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Part of this proposal also described the contributions of ancillary cells present in the milieu of the tumour. In essence, cancer development is dependent upon changes in the heterotypic interactions between tumour cells and their normal neighbouring tissues (Hanahan *et al*, 2000). In breast cancer, adipose tissue, and by extension adipokines, appear to fit this description of neighbouring tissues interacting with tumour cells to aid progression. Furthermore, the recognition that obesity is a modulator of adipokine expression reinforces the hypothesis that mammary adipose tissue, influenced by obesity and may be involved in cancer development and progression. In this chapter, the potential role for mammary adipose tissue to influence cancer progression and the influence of the metabolic syndrome was examined.

In this study, the mRNA expression of the leptin and adiponectin receptors was established. By using recombinant forms of the adipokines it was determined, in dose response curve experiments, that these cell lines were responsive to adipokines, such that increasing concentrations of leptin increased proliferation while adiponectin had the reverse effect, inhibiting

proliferation. These adipokines are not found in isolation *in vivo*. These cell proliferation experiments ought to be performed again, with the two adipokines combined in varying concentrations to determine if these adipokines in concentrations mimicking differing degrees of obesity can influence cell proliferation.

These cell proliferation experiments were repeated using adipose conditioned media (ACM) to establish whether this source of growth factors influenced growth. In the MCF-7 cell line, ACM stimulated growth at 24hrs and 48hrs, while the MDA-MB-231 cell line stimulated growth at 48hrs only. This indicated that ACM was a valid model to study the role of mammary adipose tissue in cancer progression. In the ER +ve MCF-7 cell line, a significant increase in proliferation was observed in cells treated with ACM from patients with MetS compared to normal weight patients. A second ER +ve cell line, T47-D, demonstrated a similar, but non-significant increase in proliferation in cell treated with ACM from MetS patients. These data would suggest that in an experimental model examining the role of adipose tissue and adipokines in breast cancer, mammary adipose tissue has the potential to stimulate cell proliferation and also that obesity status, particularly the presence of the metabolic syndrome stimulates this proliferation to a greater degree.

It must also be noted in this experimental model that the oestrogen receptor appears to be a necessary component to stimulate cell proliferation. These data corroborate what has been shown previously in *in vitro* work

examining adipokines in breast cancer. Indeed ER +ve cell lines have been used extensively and almost exclusively, in this work. For example, leptin was found to promote proliferation in MCF-7 and T47-D cell lines (Dieudonne *et al*, 2002; Hu *et al*, 2002). Furthermore, experimental work by Manabe *et al*, demonstrated the potential role of murine adipose tissue to influence proliferation was performed in the MCF-7 cell line (Manabe *et al*, 2003).

Determination of adipokine concentrations in ACM revealed some interesting results. Although no individual data set attained significance, one may conclude, particularly in the case of adiponectin and leptin that the secretion of these adipokines, as in other fat depots, is responsive to obesity and the MetS. Leptin was found in a higher concentration in the MetS group compared to normal weight patients, similarly adiponectin trended towards decreasing amounts in conditioned media. These data further advance the hypothesis that adipokines, secreted by mammary adipose tissue are likely to act in a paracrine manner to influence breast cancer. In a relatively similar experimental set up, Berstein *et al* examined the expression of adipokines in mammary adipose tissue. This group focused primarily on subdividing their cohorts by menopausal status and by the presence of hyperglycaemia. The mean BMI in this study was 28.1 ± 0.53. No differences were noted with regards adipokine expression and hyperglycaemia in this study. Neither was any difference noted with regards menopausal status. Interestingly, leptin levels were relatively similar between the two studies (mean leptin concentration 27.04 ± 7.37 ng/ml in Berstein et al vs 91.39 ng/ml in this body of work) providing evidence that both studies are likely to be valid in the

analysis of adipokines secreted by mammary adipose tissue (Berstein *et al*, 2007). MCP-1 also demonstrated an increase in MetS patients compared to normal weight cancer patients. The other adipokines examined, IL-6 and IL-8 did not appear to demonstrate a relationship with obesity. Also, as was noted above, the two ACM depots (Peritumoural and Distal) were similar in their levels of adipokine expression. This would imply view that obesity influences the whole mammary fat pad with respect to adipokine expression.

Oestrogen levels were examined in ACM due to the findings describing the increased cell proliferation in MetS patients in the ER +ve cell lines MCF-7 and T 47-D. ACM Oestrogen levels were similar between normal weight, obese and MetS patients implying that in mammary adipose tissue, obesity does not influence oestrogen secretion. This suggests that potential paracrine obesity related mechanisms in breast cancer require oestradiol as a co-factor to stimulate cell proliferation. This is supported by data from Catalano *et al* that suggests that leptin can stimulate the oestrogen receptor in the absence of its usual ligand (Catalano *et al*, 2004). Bidirectional cross talk between the leptin and oestrogen is also evident potentially indicating a synergism that may stimulate increased cell proliferation in obese patients (Fusco *et al*, 2010).

In this study, co-culture experiments with the MCF-7 cell line and mammary adipose tissue indicated that various components of cancer progression were altered using RT^2 *Profiler*™ cancer profiler array technology. These included invasion/metastasis, cell cycle/transduction and angiogenesis.

From these pathways, a number of genes were selected to be validated and examined further.

CDK4 (Cyclin-dependent Kinase 4) is a cyclin D1-associated kinase required for mammary neoplasia. Cyclin D1 is a key component of the cell cycle machinery and its levels are controlled by extracellular mitogenic stimulation and are regarded, along with the other D cyclins, as links between the cell microenvironment and the cell cycle machinery (Sherr *et al*, 1999). CDK4, in our studies, was found to have higher expression in MCF-7 cells treated with ACM from patients with MetS. In examining human tumour tissue, expression of CDK4 was also expressed to a higher degree in MetS patients. These data suggest that components of the cell cycle machinery may be stimulated to a greater degree in MetS patients by obesity related growth factors and mitogens. Experimental evidence has demonstrated that leptin can upregulate multiple cell cycle component gene expression in the MCF-7 cell line (Perera *et al*, 2008). In colon cancer, the obese state is thought to increase tumour cell proliferation by activating the PI3K/Akt pathway which in turn can promote activation of components of the cell cycle, including CDK4, to stimulate proliferation (Huang *et al*, 2009; Miliani de Marval *et al*, 2004; Sears *et al*, 2000). The influence of obesity status on cell cycle in breast cancer has not previously been undertaken and it may shed further light on this novel finding.

Vascular endothelial growth factor (VEGF-a) is an important component of the angiogenic process. It can induce matrix metalloproteinase

(MMP) expression to promote angiogenesis and also stimulate vascular endothelial cell migration. Serum VEGF is positively correlated with BMI and visceral fat area (Miyazawa-Hoshimoto *et al*, 2003). It is recognised that certain adipokines, including leptin can induce VEGF gene expression in breast cancer cells (Gonzalez *et al*, 2006). VEGF mRNA expression in adipocytes is potently induced by insulin whereas leptin does not have an effect in this cell type (Mick *et al*, 2002). In this chapter, VEGF was initially found to downregulated in the profiler data. This was a result we had not expected and was investigated further within the larger validation cohort which conversely demonstrated an increase in VEGF gene expression. VEGF mRNA expression was subsequently found to be non-significantly higher in MetS patients compared to normal weight patients in the ACM study, indicating that secreted factors in ACM could stimulate increased VEGF expression in MetS patients. VEGF expression has not previously been demonstrated to be expressed differentially with regards obesity status in any cancer type. Tumour VEGF expression did not appear to be MetS status dependent in our human tissue study.

The Metastasis Associated protein (MTA) family and MTA2 in particular, are involved in nucleosome remodelling and possess some histone deacetylase (HDAC) activity. The function of MTA2 is thought to be as a suppressor of p53 dependent activity (Luo *et al*, 2000). MTA2 may also be a co-repressor of ERα and modulate its activity by the associated HDAC activity (Mazumdar *et al*, 2001). In breast cancer, MTA1 and MTA3 overexpression was associated with hormone independent tumour growth and the development

of increased invasive properties (Fujita *et al*, 2004; Mazumdar *et al*, 2001). Cui *et al* demonstrated that MTA2 may also be involved in this process of oestrogen independent growth (Cui *et al*, 2006). No formal links with obesity or adipokines have been established for MTA2 in breast cancer but these initial data should be explored in the future.

From data derived from the cancer pathway finder array, it was evident that proliferative and invasive pathways were stimulated in cells treated with ACM from patients with MetS. A functional assessment of these properties was assessed using cell invasion assays. It was confirmed that MetS ACM stimulated cell invasion to a greater degree in MCF-7 cells, and to a lesser extent in the other ER+ve cell line T47-D, compared to ACM form normal weight patients. Obesity is associated with an increased metastatic burden in breast cancer. *In vitro* work has demonstrated that leptin can promote MCF-7 cell invasiveness by increasing MMP-2 activity (McMurtry *et al*, 2009). Saxena *et al* observed that leptin an IGF-1, in combination, could promote breast cancer invasion in MCF-7 cells (Saxena *et al*, 2008). Adiponectin, on the other hand, may inhibit cancer invasiveness by stimulating expression of the tumour suppressor LKB1 (Taliaferro-Smith *et al*, 2009). In obese and metabolic syndrome patients, the unique high leptin and low adiponectin environment may thus promote breast cancer invasiveness as observed in the ACM studies demonstrated here.

A major limiting factor in studies using ACM was the procurement of adipose tissue to produce the conditioned media. At each step in the process

of obtaining mammary adipose tissue lay particular issues and hurdles to overcome. As a limited resource, it was difficult to obtain large numbers in each group to expand and analyse results in depth. It would have also been favourable to perform sub group analysis, examining differences between pre- and postmenopausal women, ER +ve vs ER –ve groups etc. As such, the results and conclusions we draw must be tempered by the low numbers involved.

In conclusion, the presence of metabolic syndrome in breast cancer patients, influences adipokine expression in mammary adipose tissue. A conditioned media produced from this adipose depot in post-menopausal women, stimulates cell proliferation and invasion to a greater degree compared to normal weight women. A non-significant increase in leptin and MCP-1, and a converse decrease in adiponectin concentration in ACM from patients with MetS were observed. Co-culture of MCF-7 cells with adipose tissue revealed multiple gene changes in proliferation and invasion pathways which were investigated. Further experiments with ACM revealed non-significant increases in genes associated with invasion and metastasis (NME1, MTA2, CDK4, VEGF-a) in MetS patients. Tumour expression of CDK4 and MTA2 was non-significantly higher in MetS patients. These data provide novel evidence to suggest that adipokines produced in mammary adipose tissue are obesity status dependent and is similar to what is observed in other adipose depots. As such, this differential expression may explain some of the epidemiological evidence that breast cancer patients with obesity or metabolic syndrome have increased rates of metastasis and poorer overall survival. Further work in this area to

examine, in depth, the differences between normal weight and obese/metabolic syndrome patients may provide information for novel treatment targets for breast cancer. Ultimately, primary prevention and recurrence of breast cancer through diet, exercise, and pharmacological means may be achievable by modulation of adipokine expression.

Chapter 5:

An investigation of adipokine expression in Breast Cancer

5.1 Introduction

The epidemiological association between obesity and breast cancer is well established, with consistent and significant relationships with incidence and mortality, particularly, but not exclusively in postmenopausal women (Daling *et al*, 2001; Dawood *et al*, 2008; Harvie *et al*, 2003). The metabolic syndrome (MetS), a consequence of obesity and characterised by insulin resistance, is also associated with increased incidence (Agnoli *et al*, 2010), a more aggressive tumour phenotype (Healy *et al*, 2010), and a poorer prognosis in breast cancer (Pasanisi *et al*, 2006). With up to 20% of cancer deaths in women in the U.S. attributed to obesity, and with the obesity pandemic showing no sign of abating, it is likely that incidence and mortality rates will continue to rise (Calle *et al*, 2003).

The molecular basis by which obesity and the metabolic syndrome (MetS) influence cancer remains largely unknown. It is likely a multifactorial aetiology exists, primarily based around a chronic inflammatory state with high circulating levels of tumour and obesity related markers (IL-6, TNF-α), hyperinsulinaemia, bio-available insulin-like growth factor-1 (IGF-1) and oestrogen. Adipokines, secreted by adipose tissue are biologically active factors whose production is directly influenced by obesity (Fischer-Posovszky *et al*, 2007). Circulating levels of the adipokine leptin are positively correlated with obesity status, whereas lower adiponectin levels are present with increasing obesity (Arita *et al*, 1999; Maffei *et al*, 1995). Their biological actions are largely opposing, such that leptin is pro-mitogenic and pro-angiogenic whereas adiponectin has pro-apoptotic and anti-inflammatory

properties (Barb *et al*, 2007; Dieudonne *et al*, 2006; Dieudonne *et al*, 2002). It is these curious and intriguing properties which have stimulated much interest and debate on the role of adipokines in cancer. It is postulated that adipokines act by three interlinked pathways in breast cancer (Vona-Davis *et al*, 2007): 1) *Endocrine pathway:* acting as circulating hormones, adipokines are thought to influence cancer growth and development. Leptin is reportedly higher in cancer patients compared to controls (Chen *et al*, 2006; Tessitore *et al*, 2000), although negative or inconclusive reports also abound (Coskun *et al*, 2003; Woo *et al*, 2006). Conversely, circulating adiponectin, levels are reported to be lower in breast cancer cases (Mantzoros *et al*, 2004; Miyoshi *et al*, 2003). 2) *Paracrine pathway:* Adipose tissue (adipocytes and stromal component) can influence various aspects of cancer progression (Iyengar *et al*, 2003; Manabe *et al*, 2003). The breast parenchyma consists of up to 90% adipose tissue and as both leptin and adiponectin are intrinsically expressed, it stands to reason that these adipokines, produced from within the breast may form part of a paracrine loop (Korner *et al*, 2005; Simons *et al*, 2005). The completion of a paracrine loop requires the expression of ligand receptors on the target cell. 3) *Autocrine pathway:* It is known that leptin (Ob) and its ligand receptor (ObR) are expressed in breast tumour tissue (Jarde *et al*, 2008; Miyoshi *et al*, 2006; Revillion *et al*, 2006) and it is suggested that tumours may be able to promote proliferation/invasion via the leptin pathway autonomously. This mechanism does not appear to hold true for the adiponectin pathway, because while the adiponectin ligand receptors AdipoR1 and AdipoR2 are expressed by tumour tissue, adiponectin is not, and thus an autocrine pathway is not plausible (Takahata *et al*, 2007).

While it is established that leptin and adiponectin and their associated ligand receptors are expressed at the mRNA level in human breast tumour tissue, no study has examined both adipokines simultaneously. Very little data are available on the influence of obesity status on adipokine expression in mammary adipose tissue or tumour tissue, an important feature in a potential paracrine adipokine pathway in breast cancer. The present study, hence, seeks to determine the influence, if any, of obesity and the metabolic syndrome on adipokine expression in breast cancer tissue.

5.2 Results

5.2.1 Patient Cohort

A cohort of 77 women was divided into three groups depending on their obesity/metabolic syndrome status: Group 1 – Normal weight n=25; Group 2 – Obese only, n=27; Group 3 – Obese with Metabolic Syndrome (MetS), n=25. Median age of the cohort was 55.5 (range 31-85). 46 were postmenopausal (59.7%), defined by absence of menses for 1 year. 6 premenopausal women (19.3%) were diagnosed with MetS as compared to 19 women (46%) in the postmenopausal group. There were 47 node positive cases (61%) and 52 were considered ER+ve (67%). There were no associations noted between clinico-pathological features and obesity status.

Significant differences were noted between the MetS patients and the other patient groups with respect to all anthropomorphic markers (Weight, BMI, WC and % Fat mass) (Table 5.1). Similar differences were identified for serological markers of the metabolic syndrome (Glucose, $p<0.001$;

Triglycerides, p=0.001; and HDL-cholesterol, p<0.001). Plasma insulin levels and the HOMA-IR, a marker for insulin resistance, were also noted to be significantly higher in the MetS group (p=0.005, p=0.001 respectively).

Table 5.1 Anthropomorphic/Serological measures of obesity and Metabolic Syndrome

	Normal Weight n=25		Obese only n=27		Metabolic Syndrome n=25		
	Median	*95% CI*	*Median*	*95% CI*	*Median*	*95% CI*	*P*
Anthropometry							
Weight (kg)	57.6	54.3-61.7	71.8	65.0-75.0	75.9	66.4-87.2	<0.001[b,]
BMI (kg/m^2)	23.6	23.0-25.4	28.1	27.0-30.7	31.0	26.9-36.8	<0.001[b,]
Waist circum (cm)	76.0	64.0-78.0	96.0	90.0-101.0	106.0	100-120	<0.001[a,]
% fat mass	27.0	26.4-31.5	38.0	35.4-39.2	42.4	39.5-45.3	<0.001[a,]
Homeostasis							
Glucose (mmol/L)	5.1	4.8-5.4	5.0	4.6-5.2	5.8	5.3-6.4	0.001[a,b]
Insulin (mU/L)	6.7	5.6-9.2	7.7	5.2-16.4	13.3	8.2-22.0	0.005[a,b]
HOMA-IR	1.44	1.24-2.10	1.75	1.16-3.28	3.99	2.40-6.55	0.001[a,b]
Lipid profile							
Total Cholesterol	4.9	4.3-5.7	5.1	4.1-5.6	5.3	4.6-5.6	0.930
Triglycerides	1.0	0.9-1.1	1.2	1.0-1.8	1.8	1.4-2.9	0.001[b]
LDL	2.6	2.3-3.3	2.6	2.1-3.4	3.1	2.7-3.3	0.6287
HDL	1.7	1.6-1.8	1.5	1.3-1.7	1.26	1.1-1.4	<0.001[b]
Sex Hormones							
Oestradiol							
Premenopausal	727	358-1072	298	104-433	220	220-238	0.030[c]
Postmenopausal	96	73-143	121	91.0-191	100	92-148	0.5603
Testosterone							
Premenopausal	1.6	1.0-2.0	0.7	0.6-2.3	0.6	0.6-0.72	0.022
Postmenopausal	0.7	0.5-1.1	0.6	0.3-0.8	0.8	0.5-1.5	0.420
Progesterone							
Premenopausal	35.0	1.4-84	1.3	0.7-12.5	6.7	0.9-12.4	0.053
Postmenopausal	1.1	0.8-1.6	0.5	0.4-1.1	0.8	0.5-0.9	0.078
SHBG							
Premenopausal	68.0	39.0-97.0	55.5	34.0-104.0	66.5	43.4-68	0.614
Postmenopausal	48.5	41.0-68.0	72.0	66.0-93.0	31.0	27.0-58.0	0.003[a,,b]
Adipokines							
Leptin (ng/ml)	14.3	4.5-23.1	33.4	18.9-44.1	70.3	31.9-104.9	<0.001[b]
Adiponectin (mcg/ml)	736.8	714.0-809.5	725.0	659.1-767.2	752.2	672.4-897.6	0.458

a Metabolic syndrome vs Obese only
b Metabolic syndrome vs Normal Weight
c Obese only vs Normal Weight

5.2.2 Adipose tissue mRNA expression of adipokine receptors

The mRNA expression of the adipokines Leptin *(Ob)* and Adiponectin *(AdipoQ)* were examined in mammary adipose tissue samples from 50 women belonging to the breast cancer cohort indicated in table 5.1. *Ob* was expressed to a greater degree in patients who had the MetS as compared to the obese only or normal weight groups (p<0.001). This relationship was not demonstrated for *AdipoQ* (p=0.715).

5.2.3 Tumour tissue mRNA receptor expression of adipokine receptors

MetS patients had significantly higher *ObR* expression compared to the obese only (p<0.005) and normal weight patient groups (p<0.001). However, *AdipoR1* (0.221) and *AdipoR2* (p=0.259) expression did not demonstrate this relationship (Figure 5.2). No differences were demonstrable between pre- and post-menopausal groups (*ObR* p=0.208, *AdipoR1* p=0.602, *AdipoR2* p=0.987), nor between ER +ve and ER –ve groups (*ObR* p=0.713, *AdipoR1* p=0.630, *AdipoR2* p=0.991). *ObR* expression tended towards higher expression in lobular than in other tumour types (P=0.074). A trend towards increased expression in nodal burden status (0, 1-3, >3) was noted but was not significant (0 nodes: 17.0; 1-3 nodes: 29.3; >3 nodes: 22.7 p=0.258). *AdipoR1* or *AdipoR2* expression did not correlate with any clinico-pathological features (Table 5.2).

Table 5.2 **Expression of adipokines and adipokine receptors in response to clinico-pathological features**

	Leptin R (ObR)			*Adiponectin R1 (AdipoR1)*			*Adiponectin R2 (AdipoR2)*		
	Median	95% CI	*P*	Median	95% CI	*P*	Median	95% CI	*P*
Menopausal Status*									
Premenopausal	20.5	15.5-37.3		0.14	0.13-0.39		0.55	0.31-1.1	
Postmenopausal	20.6	9.0-29.5	0.208	0.20	0.11-0.41	0.602	0.54	0.32-0.89	0.990
Tumour Type†									
Ductal	17.1	9.1-22.6		0.18	0.11-0.29		0.55	0.35-0.92	
Lobular	34.6	17.7-48.0		0.14	0.04-0.17		0.34	0.34-0.76	
Other	27.0	15.9-82.2	0.074	0.23	0.05-1.17	0.763	0.33	0.26-7.6	0.856
Tumour size (cm)†									
<2	22.6	15.9-49.4		0.23	0.03-0.33		0.51	0.07-0.92	
2-5	17.3	7.9-38.5		0.18	0.13-0.35		0.70	0.24-1.19	
>5	20.6	9.0-19.8	0.617	0.18	0.11-0.38	0.748	0.66	0.34-1.12	0.549
Nodal Status†									
0	17.0	9.1-13.6		0.16	0.07-0.30		0.36	0.26-1.26	
1-3	29.3	15.5-39.8		0.30	0.14-0.89		0.81	0.261.57	
>3	22.7	7.9-33.8	0.281	0.17	0.09-0.33	0.307	0.64	0.03-1.00	0.578
Grade†									
1	20.5	15.9-60.9		0.23	0.12-0.26		0.38	0.31-3.90	
2	30.9	12.0-38.9		0.25	0.12-0.44		0.77	0.33-1.67	
3	17.0	8.7-23.6	0.280	0.17	0.11-0.33	0.838	0.53	0.34-0.93	0.853
ER Status*									
Positive	20.5	15.5-29.3		0.17	0.12-0.30		0.51	0.33-1.00	
Negative	23.1	8.7-28.5	0.713	0.24	0.09-0.38	0.630	0.66	0.19-1.00	0.991
PR Status*									
Positive	20.6	15.5-36.7		0.18	0.11-0.44		0.55	0.33-1.26	
Negative	24.9	7.7-29.8	0.505	0.22	0.12-0.32	0.615	0.51	0.33-0.77	0.540
Her2 Status*									
Positive	24.2	2.7-38.5		0.38	0.17-0.78		0.92	0.35-1.97	
Negative	20.5	15.5-29.3	0.758	0.18	0.12-0.26	0.075	0.51	0.33-0.77	0.241

*mRNA data expressed as arbitrary units, * Mann-Whitney-U test, † Kruskal-Wallis Test*

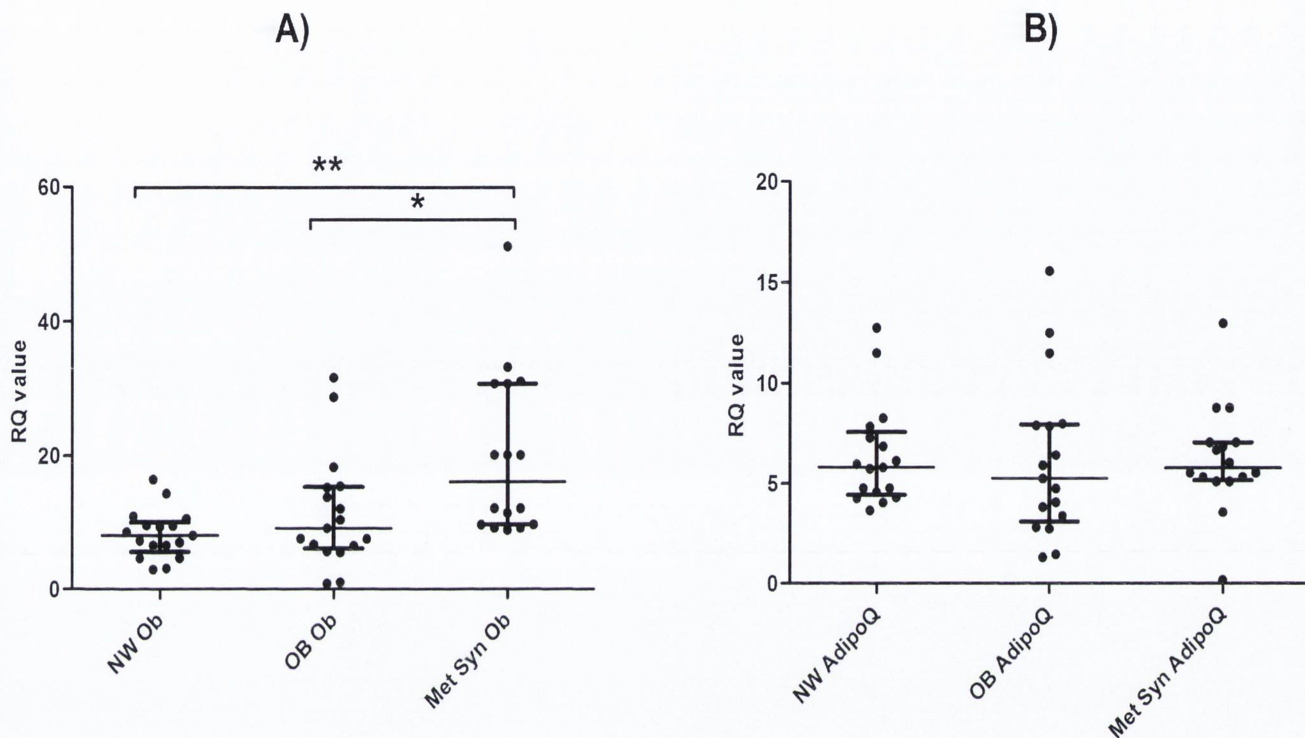


Figure 5.1 mRNA expression of adipokines in mammary adipose tissue

Adipokine expression of A) Leptin (Ob) and B) Adiponectin (AdipoQ) in mammary adipose tissue. NW (Normal weight), OB (Obese only) and Met Syn (Metabolic Syndrome). N=50: Data expressed as median + IQR (Interquartile range); Statistical analysis was carried out using the Kruskall Wallis test, with post hoc analysis using the Dunn's Multiple Comparisons test (* $p<0.005$,** $p<0.001$

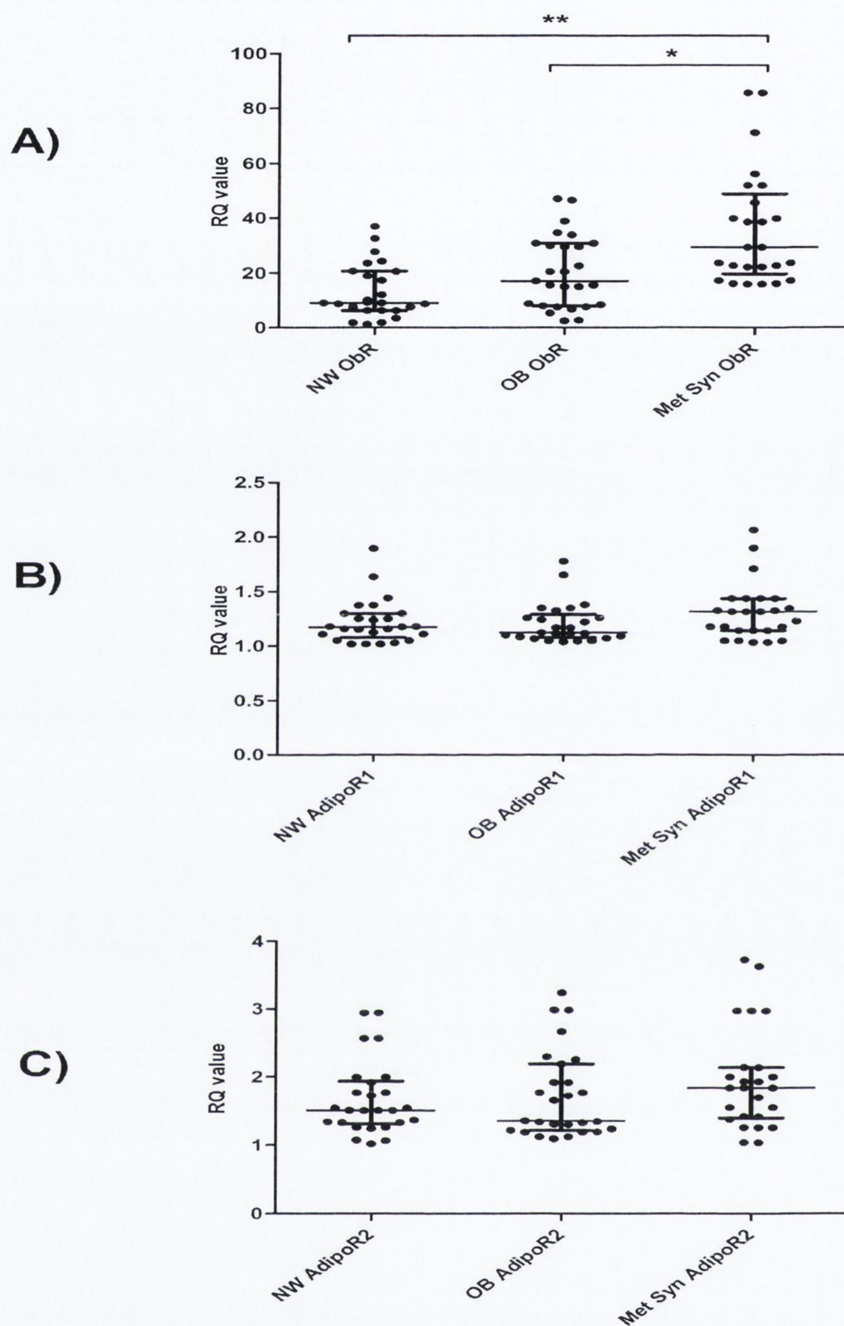


Figure 5.2 mRNA expression of Adipokine receptors in tumour tissue

Adipokine receptor expression of A) Leptin (*ObR*), B) Adiponectin (*AdipoR1/AdipoR2*) in tumour tissue. NW (Normal weight), OB (Obese only) and Met Syn (Metabolic Syndrome). N=77: Data expressed as median + IQR (Interquartile range); Statistical analysis was carried out using the Kruskall Wallis test, with post hoc analysis using the Dunn's Multiple Comparisons test (* $p<0.005$,** $p<0.001$)

5.2.4 Correlations with adipokine mRNA expression and individual features of the metabolic syndrome

Significant associations were evident between *Ob* and *ObR* expression and several components of the metabolic syndrome, including insulin (*Ob* *r* 0.45, p=0.006; *ObR* *r* 0.34, p=0.018), HOMA-IR (*Ob* *r* 0.44, p=0.001; *ObR* *r* 0.3, p=0.025) and triglycerides (*Ob* *r* 0.29, p=0.032; *ObR* *r* 0.45, p=0.002) (Table 5.3). Serum leptin levels reflected the differential findings for *Ob* / *ObR* mRNA expression in mammary adipose and tumour tissue respectively with correlations evident for serological features of obesity and MetS (Insulin *r* 0.4, p=0.012; HOMA-IR *r* 0.42, p=0.007; triglycerides *r* 0.62, p<0.0001). Additionally, serum leptin reflected obesity status with positive correlations for BMI (*r* 0.69, p <0.0001), WC (*r* 0.59, p<0.0001) and % Fat Mass (*r* 0.65, p<0.0001).

Table 5.2: Correlations for mRNA expression of mammary adipose leptin, tumour leptin receptor and circulating serum leptin with individual features of obesity and the metabolic syndrome

	Ob (MAT)		*ObR*		*Serum Leptin*	
	r	*p*	*r*	*p*	*r*	*p*
Insulin	0.45	0.006	0.34	0.018	0.40	0.012
HOMA	0.44	0.001	0.33	0.025	0.42	0.007
Glucose	0.13	0.572	-0.23	0.073	0.40	0.009
Triglycerides	0.29	0.032	0.45	0.002	0.62	<0.0001
HDL	-0.33	0.021	-0.38	0.002	-0.50	0.001
Waist circum	0.10	0.987	0.13	0.361	0.59	<0.0001
BMI	0.02	0.937	0.16	0.196	0.69	<0.0001
% Fat Mass	0.05	0.872	0.01	0.937	0.65	0.0001
Ob (MAT)	–	–	0.30	0.08	0.65	0.0001
ObR	0.30	0.08	–	–	0.39	0.021
Serum leptin	0.65	0.0001	0.39	0.021	–	–

r = Spearman Correlation coefficient

5.3 Discussion

Recent data indicate that adipose tissue derived adipokines in serum are associated with obesity and may be involved in development and/or progression of breast cancer. Consequently, our aims were to a) examine with respect to obesity status, relative mRNA expression of adipokine receptors *ObR*, *AdipoR1* and *AdipoR2* in breast tumour tissue, b) relative expression of adipokine mRNA expression (*Ob* and *AdipoQ*) in MAT, and c) to establish what individual features of obesity and the associated metabolic syndrome influenced expression.

In this study, obese patients with the MetS had significantly greater *Ob* expression in MAT coinciding with a greater *ObR* expression in matched tumour tissue compared to normal weight cancer patients. First, these data provide evidence that mammary adipose leptin (*Ob*) mRNA expression is influenced by obesity status and this potentially points to a paracrine role for leptin secretion in the local tumour milieu (Schaffler *et al*, 2007). Secondly, increased tumour *ObR* expression demonstrates the direct effect of obesity status on tumour tissue.

We were unable to correlate clinico-pathological features with *Ob/ObR* expression, possibly due to our inability to sample small tumours for inclusion in the biobank (potential to interrupt margins for staging purposes) and because 67% of the patient cohort had node positive breast cancer. These factors are likely to disrupt any connections between histological characteristics and *ObR* expression. Previously, Fiori *et al* indicated that *ObR*

expression was greater in larger tumours (Fiorio *et al*, 2008) and Garofalo had demonstrated that *ObR* expression was associated with nodal metastasis (Garofalo *et al*, 2006), thus it is likely that leptin/leptin receptor expression is associated with increased tumour aggressiveness. From the literature it is apparent that *ObR* expression in breast tumour tissue is linked with survival. Revillion *et al* indicated, that a high *ObR-L: ObR-S* (ObR isoforms) ratio indicated tumour aggressiveness with associated reduction in relapse free survival in a 322 patient cohort (Revillion *et al*, 2006). Meanwhile, Miyoshi *et al* reported, that a poorer prognosis was evident in a subgroup of patients with high *ObR* levels and high serum leptin levels (Miyoshi *et al*, 2006). These studies did not examine obesity status in depth, therefore, is it reasonable to assume that high *ObR*/ high serum leptin is an indicator of obesity status. If so, the apparent inherent link between obesity and adipokine/ adipokine receptor expression, demonstrated in our study, is potentially an indicator of poor prognosis.

We demonstrated that several features of the metabolic syndrome (a consequence of increasing obesity) correlate with increased *Ob / ObR* expression in MAT and tumour tissue respectively. The mechanisms responsible for increased adipokine expression in MAT and adipokine receptor expression in tumour tissue remains unclear. *In vitro*, obesity related stimuli, including insulin, hypoxia, and IGF result in an upregulation of expression of the Ob/ObR system in ER+ve and ER –ve breast cancer cell lines (Garofalo *et al*, 2006). Further examination by Bartella *et al* elucidated the mechanism for insulin dependent transcription of leptin in the ER –ve cell line MDA-MB-231 and concluded that hyperinsulinaemia could induce cancer progression through

leptin dependent mechanisms (Bartella *et al*, 2008). Our study demonstrated significant, positive correlations for insulin and HOMA-IR (insulin resistance) with MAT *Ob* and tumour *ObR* expression. These data possibly define a direct, 'dose response' type relationship between obesity/MetS and the *Ob / ObR* pathway in breast cancer. Similarly, serum leptin levels positively correlated with all features of obesity and MetS, and also correlated with the expression of *Ob* in MAT and *ObR* in tumour tissue. This may indicate that the leptin pathway is upregulated, in its entirety (endocrine and paracrine), in breast cancer.

A positive correlation with plasma triglycerides and inverse relationship with HDL-cholesterol was intriguing. Recently, it has been demonstrated that these features of the metabolic syndrome had the strongest association with breast cancer risk (Agnoli *et al*, 2010). Statins, in a newly reported human trial, reduced tumour proliferation and increased apoptotic activity in early high grade ER-ve tumours (Garwood *et al*, 2010). Since the main effect of leptin is cellular proliferation, one could assume that statin activity may dampen the expression of the *Ob / ObR* pathway in breast cancer. Further research needs to be performed to elucidate this mechanism.

No evidence was observed regarding the influence of obesity status on *AdipoQ* expression in MAT or of the ligand receptors *AdipoR1* or *AdipoR2* in tumour tissue. Serum adiponectin levels were not influenced by obesity status in this study, however it is well described that adiponectin levels are lower in obese groups and in breast cancer patients (Miyoshi *et al*, 2003; Tworoger *et al*, 2007). Recent studies have failed to show associations between

AdipoR1/ AdipoR2 expression and clinico-pathological characteristics or prognosis in breast cancer (Korner *et al*, 2007; Takahata *et al*, 2007). Our data corroborate evidence proposed by Pfeiler *et al* who reported that *AdipoR1 / AdipoR2* expression in breast tumour tissue is neither responsive to serum insulin nor insulin resistance (Pfeiler *et al*, 2009). These data are in variance to what we had expected, and to established observations for the down regulation of expression of *AdipoQ, AdipoR1* and *AdipoR2* in obese mouse adipose tissue (Tsuchida *et al*, 2004) and human skeletal muscle in response to insulin resistance (Civitarese *et al*, 2004; Debard *et al*, 2004) Overall, our study indicates that in breast cancer tissue, the adiponectin receptors appear unresponsive to obesity related stimuli, including the metabolic syndrome and hyperinsulinaemia.

In conclusion, we demonstrate that mRNA expression of leptin in mammary adipose tissue and the ligand receptor leptin expression in tumour tissue is obesity status dependent with the strongest expression observed in patients with the metabolic syndrome. Individual features of the metabolic syndrome were found to correlate with *Ob / ObR* expression. We believe these novel data add weight to the amassing literature implicating the role of obesity in breast cancer and also lend credence to recent calls to investigate the potential to treat the metabolic syndrome in order to reduce breast cancer incidence and recurrence.

Chapter 6:

Concluding Discussion

6.1 Discussion

Obesity, nutritional status, diet and physical activity are now recognised as new risk factors for the development of several cancer types, including breast cancer. Breast cancer incidence worldwide is increasing unabated, however reasons for this remain undetermined. The concomitant emergence of the obesity pandemic has led some investigators to associate the rise in obesity with increased cancer rates. Epidemiology has now successfully demonstrated the association of obesity status with incidence (Harvie *et al*, 2003) and poor survival (Calle *et al*, 2003) in breast cancer. The metabolic syndrome, a consequence of obesity and defined by the conglomeration of anthropomorphic and serological markers of obesity, is also recognised as a risk factor for breast cancer. The metabolic syndrome attributes breast cancer with an aggressive phenotype (Healy *et al*, 2010) and ultimately a poorer outcome.

While epidemiology has demonstrated associations between obesity and breast cancer, molecular mechanisms for these links have trailed behind. The emergence of adipokines produced by adipose tissue and their close relationships with body habitus naturally instigated investigations into the part they may play in breast cancer. *In vitro* work demonstrated that the principal adipokines leptin and adiponectin are in direct biological opposition in breast cancer, with influence over proliferation, invasion, angiogenesis and apoptosis. It was hypothesised that these adipokines could act through endocrine (circulating), paracrine (local) and autocrine (intra-tumoural) mechanisms to influence carcinogenesis (Vona-Davis *et al*, 2007).

Furthermore, human studies highlighted potential associations of serum leptin and adiponectin with breast cancer incidence, and in particular, gene expression of tumour leptin and leptin receptor with prognosis (Revillion *et al*, 2006).

A paracrine model for adipokines in breast cancer was an intriguing aspect to the obesity question and formed a considerable part of this body of work. It had been hypothesised that mammary adipose tissue was a source of adipokines that could act locally to influence breast cancer (Schaffler *et al*, 2007). We had further hypothesised that adipokine production from this source was influenced by obesity status and that it could influence various aspects of cancer progression. Adipose conditioned media, produced from mammary adipose tissue of women undergoing mastectomy was found to increase cell proliferation of three breast cancer cell lines: ER +ve MCF-7, ER +ve T47-D and the ER –ve MDA-MB-231. ACM from patients with the metabolic syndrome stimulated the greatest degree of proliferation in MCF-7 cells. A similar result was demonstrated with cell invasion in the MCF-7 cell line. Profiling of the conditioned media indicated the presence of a number of adipokines which were subsequently quantified in larger numbers using ELISA. It was found that leptin levels were higher and adiponectin levels lower in the ACM produced from metabolic syndrome patients compared with that from normal weight patients. These novel data indicate that mammary adipose tissue and the adipokines produced by this fat pad are obesity status dependent. Thus in a functional paracrine model of obesity in breast cancer, metabolic syndrome patients stimulate greater proliferation and invasion, suggesting a potential reasons to explain the relationship between obesity and

breast cancer and why obese patients, in particular metabolic patients, present with more aggressive disease and have a poorer prognosis.

Reasons for the observed increased proliferation and invasion in cells treated with ACM from metabolic syndrome patients were investigated using cancer pathway profiler arrays. A number of genes were identified and validated, the majority of which are associated with cell cycle, invasion and metastasis: NME1, MTA2, VEGF-A, TIMP-3 and CDK4. Experiments were repeated in a larger cohort to determine if the expression of these selected genes were altered between normal weight and metabolic syndrome patients. No significant differences were identified, however, gene expression NME1, MTA2, VEGF-A and CDK4 were all increased in metabolic syndrome patients suggesting that there may be a general upregulation of cancer pathways in these patients, further implicating obesity and metabolic syndrome in tumour aggressiveness. This area of work requires further investigation to determine the overall significance of a paracrine mechanism of action in breast cancer in relation to obesity. The use of more in-depth microarray technology, in particular the powerful Affymetrix system, would be suitable to examine a huge number of gene changes in multiple pathways involved in carcinogenesis to provide a much greater strength of understanding of the role that obesity plays in breast cancer.

A final aspect of this thesis was to examine the influence of obesity status on tumour expression of the adipokines and their ligand receptors. Revillion *et al* and Miyoshi *et al* *(Miyoshi et al, 2006)* had both described the significance of over expression of leptin and its receptor in a poorer survival in

breast cancer patients. The question of the influence of obesity remained an unanswered issue in these seminal articles. We hypothesised that expression of these adipokines and their receptors were altered by the presence of obesity status. We demonstrated that the leptin receptor (ObR) in breast tumour tissue was expressed to a greater degree in metabolic syndrome patients compared to normal weight cancer patients. Interestingly, no such relationship was identified for the adiponectin receptors (AdipoR1 and AdipoR2). Gene expression of the adipokines was also investigated in mammary adipose tissue. Leptin (Ob) was again found to be expressed to a greater degree in metabolic syndrome patients, while adiponectin (AdipoQ) was not altered by obesity status. Firstly, from this data, it is evident that obesity and in particular the metabolic syndrome alters expression of the leptin pathway in both mammary adipose tissue and associated tumour tissue. This suggests that the leptin pathway is responsive to obesity related stimuli and further suggests a role for this obesity in breast cancer. Secondly, clinico-pathological features in this relatively small study did not relate to increased expression, however, with increasing biological specimens recruited to the biobank it would be an interesting and worthwhile exercise to revisit this data once a suitable time period has elapsed to determine the significance of increased expression of leptin receptor in cancer tissue and correlate with survival.

In conclusion, this thesis has described the influence of obesity in breast cancer in both a functional model and the direct effect in tumour tissue. Ultimately, obesity and the metabolic syndrome are preventable risk factors for breast cancer and focus should be applied by all parties (Government, Medical associations) to improve the level of education concerning obesity. Of great

interest is the number of prospective studies now emerging to examine strategies to prevent or reduce the risk of obesity related breast cancer. Trials including the use of metformin (Goodwin *et al*, 2009) to improve insulin resistance, a key component of obesity and the metabolic syndrome, in particular will be followed with keen attention to ascertain its potential to improve prognostic factors.

6.2 Future work

Future directions for this body of work would include the expansion of Adipose Conditioned Media experiments to examine other areas of carcinogenesis including apoptosis. Incorporating precancerous (DCIS), high risk patients (BRCA) or non cancer patients (undergoing reduction mastoplasty) into the ACM experiments would provide insight into the influence of obesity in these groups; particularly it would allow us to determine if mammary adipose tissue from non-cancer patients is similar to cancer patients. Finally, one could then establish if ACM is a true model of obesity. As described above, using the Affymetrix platform above would provide us with a much greater understanding of the complexity of gene changes at play in obesity related breast cancer.

Finally, the emergence of metformin as a potential therapeutic agent provides for exciting new ideas. One could repeat the ACM studies using metformin in the MetS group to determine if it modified response. Furthermore, a small window trial, similar to that previously published for statins in a neoadjuvant group by Garwood *et al* (Garwood et al, 2010), could

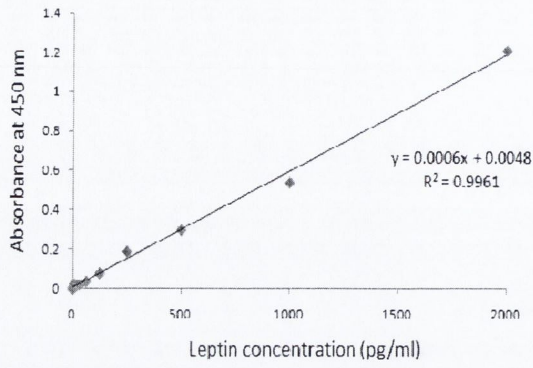
be repeated using metformin. Examining alteration in tumour clinico-pathological features as well as adipokine expression and other markers of obesity may suggest if metformin is indeed a potential therapeutic agent.

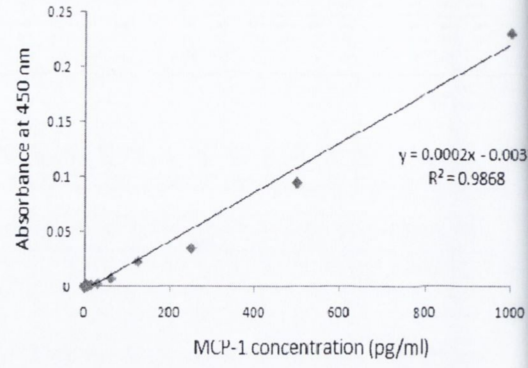
Appendix 1: Human Cancer Pathway Finder Array Data

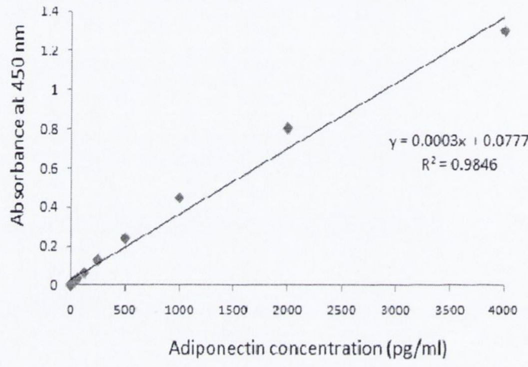
List of genes on Human Cancer Pathway qRT-PCR Profiler Array

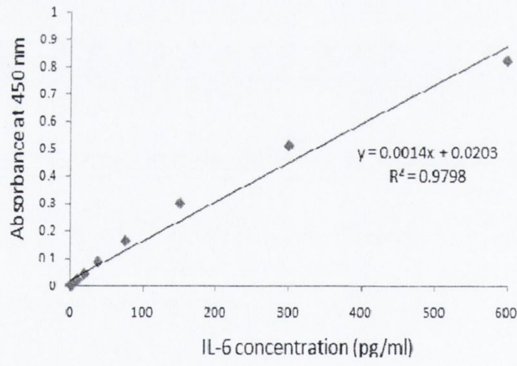
Position	UniGene	RefSeq	Symbol	Description	Gene Name
A01	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	AKT/PKB
A02	Hs.369675	NM_001146	ANGPT1	Angiopoietin 1	AGP1/AGPT
A03	Hs.553484	NM_001147	ANGPT2	Angiopoietin 2	AGPT2/ANG2
A04	Hs.552567	NM_001160	APAF1	Apoptotic peptidase activating factor 1	CED4
A05	Hs.367437	NM_000051	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	AT1/ATA
A06	Hs.370254	NM_004322	BAD	BCL2-antagonist of cell death	BBC2/BCL2L6
A07	Hs.159428	NM_004324	BAX	BCL2-associated X protein	Bax zeta
A08	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2
A09	Hs.516966	NM_138578	BCL2L1	BCL2-like 1	BCL-XL/S
A10	Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	BRCAI/BRCC1
A11	Hs.591630	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase	CAP4/FLICE
A12	Hs.244723	NM_001238	CCNE1	Cyclin E1	CCNE
B01	Hs.437705	NM_001789	CDC25A	Cell division cycle 25 homolog A (S. pombe)	CDC25A2
B02	Hs.19192	NM_001798	CDK2	Cyclin-dependent kinase 2	p33(CDK2)
B03	Hs.95577	NM_000075	CDK4	Cyclin-dependent kinase 4	CMM3/PSK-J3
B04	Hs.370771	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CAP20/CDKN1
B05	Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	ARF/CDK4I
B06	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	CASH/CASP8AP1
B07	Hs.291363	NM_007194	CHEK2	CHK2 checkpoint homolog (S. pombe)	CDS1/CHK2
B08	Hs.517356	NM_030582	COL18A1	Collagen, type XVIII, alpha 1	KNO
B09	Hs.96055	NM_005225	E2F1	E2F transcription factor 1	E2F-1/RBBP3
B10	Hs.446352	NM_004448	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	HER-2/HER-2
B11	Hs.652288	NM_005239	ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	c-Ets2
B12	Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	ALPS1A/APO-1
C01	Hs.533683	NM_000141	FGFR2	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	BEK/BFR-1
C02	Hs.25647	NM_005252	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	c-fos
C03	Hs.90708	NM_006144	GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	CTLA3/HFSP
C04	Hs.90753	NM_006410	HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	CC3/TIP30
C05	Hs.37026	NM_024013	IFNA1	Interferon, alpha 1	IFL/IFN
C06	Hs.93177	NM_002176	IFNB1	interferon, beta 1, fibroblast	IFB/IFF
C07	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)	IGFI
C08	Hs.624	NM_000584	IL8	Interleukin 8	3-10C/AMCF-I
C09	Hs.652204	NM_181501	ITGA1	Integrin, alpha 1	CD49a/VLA1
C10	Hs.482077	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR/CD49B
C11	Hs.265829	NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C/GAP-B3
C12	Hs.440955	NM_000885	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	CD49D/IA4
D01	Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	CD51/MSK8
D02	Hs.643813	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	CD29/FNRB
D03	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	CD61/GP3A
D04	Hs.536663	NM_002213	ITGB5	Integrin, beta 5	FLJ26658
D05	Hs.525704	NM_002228	JUN	Jun oncogene	AP1/c-Jun
D06	Hs.145442	NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1	MAPKK1/MEK1
D07	Hs.599039	NM_006500	MCAM	Melanoma cell adhesion molecule	CD146/MUC18
D08	Hs.567303	NM_002392	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	HDMX/hdm2
D09	Hs.132966	NM_000245	MET	Met proto-oncogene (hepatocyte growth factor receptor)	HGFR/RCCP2
D10	Hs.83169	NM_002421	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	CLG/CLGN
D11	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4/CLG4A
D12	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B/GELB
E01	Hs.525629	NM_004689	MTA1	Metastasis associated 1	Mta-1
E02	Hs.173043	NM_004739	MTA2	Metastasis associated 1 family, member 2	DKFZp686F2281/MTA1L1
E03	Hs.336994	NM_014751	MTSS1	Metastasis suppressor 1	MIM/MIMA
E04	Hs.202453	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	c-Myc
E05	Hs.431926	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	DKFZp686C01211/EBP-1
E06	Hs.81328	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,	IKBA/MAD-3
E07	Hs.118638	NM_000269	NME1	Non-metastatic cells 1, protein (NM23A) expressed in	AWD/GAAD
E08	Hs.9235	NM_005009	NME4	Non-metastatic cells 4, protein expressed in	NM23H4/nm23-H4
E09	Hs.645488	NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF-A/PDGF1
E10	Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGF2/SIS
E11	Hs.132225	NM_181504	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	GRB1/p85-ALPHA
E12	Hs.77274	NM_002658	PLAU	Plasminogen activator, urokinase	ATF/UPA
F01	Hs.466871	NM_002659	PLAUR	Plasminogen activator, urokinase receptor	CD87/UPAR
F02	Hs.409965	NM_002687	PNN	Pinin, desmosome associated protein	DRS/SDK3
F03	Hs.159130	NM_002880	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	CRAF/Raf-1
F04	Hs.408528	NM_000321	RB1	Retinoblastoma 1 (including osteosarcoma)	OSRC/RB
F05	Hs.81256	NM_002961	S100A4	S100 calcium binding protein A4	18A2/42A
F06	Hs.55279	NM_002639	SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	PI5/maspin
F07	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	PAI/PAI-1
F08	Hs.349470	NM_003087	SNCG	Synuclein, gamma (breast cancer-specific protein 1)	BCSG1/SR
F09	Hs.371720	NM_003177	SYK	Spleen tyrosine kinase	Syk
F10	Hs.89640	NM_000459	TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	CD202B/TIE-2
F11	Hs.492203	NM_198253	TERT	Telomerase reverse transcriptase	EST2/TCS1
F12	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	CED/DPD1
G01	Hs.494622	NM_004612	TGFBR1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	AAT5/ACVRLK4
G02	Hs.164226	NM_003246	THBS1	Thrombospondin 1	THBS/TSP
G03	Hs.522632	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1	CLGI/EPA
G04	Hs.644633	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	HSMRK222/K222
G05	Hs.241570	NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)	DIF/TNF-alpha
G06	Hs.521456	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	CD262/DR5
G07	Hs.279594	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	CD120a/FPF
G08	Hs.462529	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	APO-3/DDR3
G09	Hs.408312	NM_000546	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	LFS1/TRP53
G10	Hs.66744	NM_000474	TWIST1	Twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) (Drosophila)	ACS3/BPES2
G11	Hs.563491	NM_017549	EPDR1	Ependymin related protein 1 (zebrafish)	EPDR/MERP-1
G12	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	VEGF/VEGF-A
H01	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	B2M
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	HGPRT/HPRT
H03	Hs.546356	NM_012423	RPL13A	Ribosomal protein L13a	RPL13A
H04	Hs.544577	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
H05	Hs.520640	NM_001101	ACTB	Actin, beta	PS1TP5BP1
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC

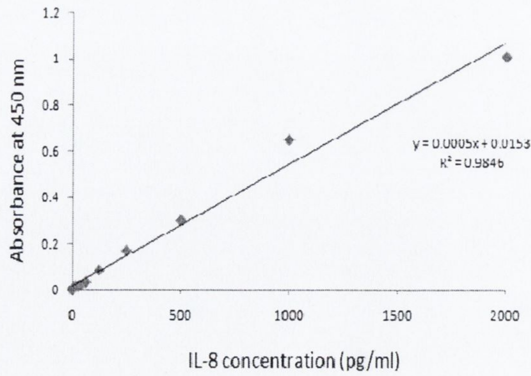
Appendix 2: ELISA Standard Curves











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