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Signals from adipose tissue in morbid obesity and effect on depot specific differences

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A thesis submitted to the University College London for the degree of MD (Research)

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DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University College London or any other University or Institute of learning. All the work presented is my own and any collaboration has been acknowledged.

ABSTRACT

Signals from adipose tissue, such as interleukin-6 (IL-6) and asymmetric dimethyl arginine (ADMA), an endogenous nitric oxide inhibitor, explain the link between obesity and metabolic diseases. Previously published human studies have used omental adipose tissue to study visceral depots, based on the assumption that all visceral adipose tissues are similar. This study, for the first time, assessed the release of five adipokines (adiponectin, leptin, IL-6, MCP-1 and RANTES) from the subcutaneous and two omental depots. Components of the cyclooxygenase (COX) and the nitric oxide (NO) pathways, that regulate cytokine release in other tissues, were also investigated for their putative role(s) in mediating adipokine release.

RANTES release was greatest from the gastric fat pad. However, significantly higher circulating RANTES levels suggest that adipose tissue is unlikely to be the main source of RANTES release.

Inhibition of the COX pathway, especially COX-2, reduced IL-6 release from subcutaneous adipose tissue. Prostacyclin synthase (PGI2S) activity was higher in the omental tissue and its protein expression was elevated in the stromavascular fraction from this depot. PGI2S activity appears to mainly reside in the non-adipocyte cells and is more coupled to IL-6 production in adipose tissue.

Serum insulin and CRP levels, and systolic blood pressure, directly associated with subcutaneous tissue ADMA content, while BMI correlated with omental ADMA release. ADMA release was higher from the omental depot. However, while DDAH2 expression was higher compared to DDAH1 in adipose tissue, there was no depot specific difference in the expression of either isoform.

In conclusion, this study showed adipose depot specific differences of RANTES release, a novel adipokine, from a hitherto poorly studied depot, the gastric fat pad. Characteristics of the omental adipose tissues differed depending on location and paracrine factors that may mediate the adipokine release. These regulatory pathways included components of the COX and NO pathways.

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This thesis is dedicated to my parents and my husband. To my husband who put up with my absences through this hectic period and always seemed to find the time to listen to my detailed description of the dilemmas! To my parents whose encouragement and upbeat attitude helped me get through the days when it felt that it was impossible to overcome the obstacles!

ABBREVIATIONS

ADMA	Asymmetric Dimethyl Arginine
AMP	Adenosine Mono Phosphate
ATP	Adenosine Tri Phosphate
BPD	Bilo-Pancreatic Diversion
BMI	Body Mass Index
cAMP	cyclic Adenosine Mono Phoshphate
COX	Cyclooxygenase
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) Ligand
DDAH	Dimethylarginine Dimethylaminohydrolase
DS	Duodenal Switch
eNOS	endothelial Nitric Oxide Synthase
G-CSF	Granulocyte Colony-Stimulating Factor
gm	Gram
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HDL	High Density Lipoprotein
HMW	High Molecular Weight
IDDM	Insulin Dependent Diabetes Mellitus
IL	Interleukin
IFN	Interferon
iNOS	inducible Nitric Oxide Synthase
IQR	Interquartile Range
JIB	Jejuno-Ileal Bypass
Kg	Kilogram
MCP-1	Monocyte Chemoattractant Protein-1
MIF	Macrophage migration Inhibitory Factor
MMW	Medium Molecular Weight
NEFA	Non Esterified Fatty Acid
ng	Nanogram
NGF	Nerve Growth Factor
NIDDM	Non Insulin Dependent Diabetes Mellitus

NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OD	Optical Density
PAI-1	Plasminogen Activator Inhibitor-1
PG	Prostaglandin
pg	Picogram
PGI_2S	Prostaglandin I_2 Synthase
PPAR	Proliferator Activated Receptor
PRMT	Protein Arginine Methyl Transferases
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RBP4	Retinol binding protein-4
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
RYGB	Roux en Y Gastric Bypass
SD	Standard Deviation
sIL-6R	Soluble Interleukin-6 Receptor
SDMA	Symmetric Dimethyl Arginine
LAGB	Laparoscopic Adjusted Gastric Banding
LDL	Low Density Lipoprotein
L-NMMA	N^G-mono-methyl-L-arginine
LPS	Lipo Polysaccharide
LMW	Low Molecular Weight
TNF	Tumour Necrosis Factor
TREM	Triggering Receptor Expressed on Myeloid cells
VBG	Vertical Banded Gastroplasty
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
WHR	Waist Hip Ratio

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CHAPTER 1: INTRODUCTION

1.1 OBESITY

1.1.1 Epidemiology

Obesity is the epidemic of the century and, contrary to some people's belief, it is not simply a cosmetic problem. The question of whether obesity should be called a 'disease' or, on the other hand, simply a 'condition' has gone on for more than 100 years and has significant connotations for the approach one takes in its treatment, to the regulation of medicines used for its treatment, and whether health care expenditures associated with obesity are reimbursed (Bray, 2004 (a)). During the early part of the 20th century the prevalence of obesity rose slowly, but around 1980 it began to rise more rapidly. Children are affected by obesity, with the prevalence rising from 5% in 1960 to 15% in 2000 (Ogden *et al.*, 2000). Obesity is the most prevalent nutritional disorder in industrialised countries (Kushner, 2002). Approximately 60% of overweight 5 to 17 year old children already have associated biochemical or clinical cardiovascular risk factors, such as hyperlipidemia, elevated blood pressure, or increased insulin levels, and 25% have 2 or more of these (Freedman *et al.*, 1999). It is associated with an increased risk of cardiovascular morbidity and mortality (Garrison *et al.*, 1996), type 2 diabetes mellitus (Ogden *et al.*, 2000), osteoarthritis and cancer. In a published report (Drenick *et al.*, 1980), mortality rates in patients who were morbidly obese is 12 times higher in men aged 25 to 34 years and 6 times higher in men aged 35 to 44 years compared to men with healthy weight of the same age. Obesity clearly increases health risk and the cost of health care (Klein *et al.*, 2004 (a)) and despite all this evidence; many clinicians consider obesity to be a self-inflicted condition of little medical significance. The future of these adults and children is complications of heart disease, renal failure, blindness and amputation disabling them during the next 20 years or so.

1.1.2 Causes of obesity

Obesity is not a single disorder but a heterogeneous group of conditions with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure (Kopelman, 2000).

1.1.2.1 Energy imbalance

An energy imbalance results in obesity. This state of imbalance can be described by the first law of thermodynamics, which says that the energy of a system is determined by the difference between the heat that enters that system and the heat that leaves. In the case of obesity, heat is from food energy, and the quantity of energy that is expended as chemical and physical energy comes from metabolism and work. In humans the unmetabolized foods are converted to fat and lead to obesity (Bray, 2004 (a)). The most variable component of energy expenditure is physical activity. Epidemiological data show that low levels of physical activity and watching more television predict higher body weight (Hancox *et al*., 2004). The defects in metabolic mechanisms that control energy expenditure have not been described in human obesity (Kopelman, 2000).

1.1.2.2 Genetics

Obesity might run in the families but the influence of the genotype on the etiology of obesity may be attenuated or exacerbated by non-genetic factors (Kopelman, 2000). Genetic factors can be divided into two groups, the rare genes that produce significant obesity and a group of more common genes that underlie susceptibility to obesity, the so-called "susceptibility" genes (Rankinen *et al.,* 2002). Single gene mutations, such as seen in humans with leptin and leptin receptor gene mutations, are rare and only constitute a minority of normal obesity (Zhang *et al.*, 1994). Importantly, genetics related to obesity are not responsible for the epidemics of obesity in recent times as the gene pool in communities has not changed significantly (Koplan & Dietz, 1999).

1.1.2.3 Environment

Environment plays an important role in the etiology of obesity. Availability of food, fast food or food with higher energy content, socializing with food and drink, increased numbers and marketing of snack foods, and, on the other hand, decreased energy expenditure, perhaps due to decrease in children's activity from watching TV, playing computer games or increasingly automated work places, are a few examples (Koplan & Dietz, 1999). Environmental factors have been shown to affect leptin sensitivity, as a high-fat diet leads to leptin resistance, although the basis for this is poorly understood (Friedman & Halaas, 1998).

1.1.2.4 Other causes

In most traditional societies, fatness has frequently been a symbol of motherhood in adult women (Brown & Bentley-Condit, 1998). In contrast, the cultural ideal of thinness is found in relatively few developed societies where motherhood is not the primary means of female status attainment (Anderson *et al.*, 1992). There is also evidence to suggest that under nutrition of the foetus during intrauterine development may determine the later onset of obesity, hypertension and type 2 diabetes independent of genetic inheritance (Barker, 1995). Viruses may also be a cause of obesity (Bray, 2004 (b)). The injection of several viruses into the central nervous system produces obesity in mice (Bray, 2004 (b)). A recent finding of antibodies to one of the adenoviruses at significant titres in obese human beings raises the possibility that viruses are involved in some cases (Dhurandhar *et al.*, 2000). In experimental animals, exposure in the neonatal period to toxins like monosodium glutamate, a common flavoring ingredient in food, also produces obesity. A similar effect of reduction in glucose can also produce obesity, suggesting that the brain of growing animals, and possibly those of human beings, may respond with damage to the metabolic sensors that regulate food needs (Bray & Champagne, 2005).

1.1.3 Indices and diagnosis of obesity

Body Mass Index (BMI): This is an index of obesity and acts as an indicator of heaviness and only indirectly of body fat (Garn *et al.*, 1986; Bouchard, 1992). It does not distinguish fat mass from fat free mass. Despite its limitations BMI is widely used clinically bacause of its simplicity to measure, low cost and reasonable correlation with body composition.

Waist Hip Ratio (WHR): The correlation between WHR and abdominal visceral fat is positive but the association is characterized by a wide scatter of score (Bouchard & Perusse, 1998) and it does not provide a precise estimate of abdominal visceral fat.

Skin fold thickness: This method is easy to use and it provides a precise assessment if performed at different sites and by the same person, but, the technique falls short in estimating the abdominal and intramuscular fat.

Bioimpedence: The principle of this technique is that lean mass conducts current better than fat mass because it is primarily an electrolyte solution. A weak current (impedence) is applied across extremities and the resistance is measured which in turn estimates the body fat. It is easy and practical to use. However it is no more accurate than anthropometric measurements (Kopelman, 2000) and results largely depend on the hydration status of the patient.

1.1.4 Types of obesity

The perception that not all obese individuals are alike and that it would be useful to distinguish several types of obesity is an ancient one (Bray, 1990). Initially it was observed that male pattern of fat distribution, android obesity, carried a greater health risk than the female profile (Vague, 1956). However it was not until 1980s that it was shown with human and animal studies that android obesity was a greater risk for cardiovascular disease and type 2 diabetes mellitus (Bjorntorp, 1985; Kissebah *et al.*, 1982). The Nurse Health Study found that the risk of type 2 diabetes mellitus increases with the degree and duration of overweight and with a more central distribution of body fat (Colditz *et al.*, 1995).

1.1.5 Consequences of obesity

Negative effects of excess weight on morbidity and mortality have been recognized for more than 2000 years. Hippocrates stated that "sudden death is more common in those who are naturally fat than in the lean". The pathophysiological effects of obesity may now be attributed to two factors: first, the increased mass of adipose tissue and, second, the secretion of pathogenic products from the enlarged fat and non-fat cells from these tissues. Sleep apnea, secondary to increase in para-pharyngeal fat deposits, osteoarthritis and social stigma are examples of the first category. The second category includes the metabolic effects brought on by distant effect of factors secreted from adipose tissue (Bray, 2004 (c)) namely type 2 diabetes mellitus, insulin resistance and the metabolic syndrome, hypertension, cardiovascular disease, some cancers, as well as, non alcoholic fatty liver and gallbladder disease.

1.1.5.1 Type 2 diabetes mellitus, insulin resistance and the metabolic syndrome

Weight gain appears to precede the onset of diabetes and increases its risk. Weight loss or moderate weight gain over years reduces the risk of developing type 2 diabetes.

The Swedish Obese Subject study reported 13 –16% of obese subjects presenting with diabetes at baseline (Sjostrom *et al.*, 1997) and in the Nurses Cohort Study, BMI was the dominant predictor of the risk of type 2 diabetes after adjustment for age (Colditz *et al.*, 1995). It is not surprising therefore that the worldwide epidemic of obesity has been accompanied by a surge in the incidence of type 2 diabetes (Engelgau *et al.*, 2004). Normally, control of blood glucose levels depends on the efficient action of insulin, which stimulates uptake of glucose from the blood into muscle and fat cells through GLUT4, an insulin-regulated glucose transporter. It also inhibits glucose production in liver, thereby maintaining normal glucose levels in the blood. In adipose tissue, glucose provides fuel for the synthesis of fat stores, which serve as the body's main energy reservoir (Muoio & Newgard, 2005). The insulin mechanism of glucose control fails in both obesity and type 2 diabetes. The onset of this 'insulin-resistant' condition is closely associated with weight gain (Engelgau *et al*., 2004), suggesting that increased adipose tissue generates signals that interferes with the action of insulin. On the other hand the expression of GLUT4 is greatly reduced in the adipocytes but not in the muscle cells of rodents and humans that are obese and have insulin resistance (Kahn, 1994). The changes in the body's insulin action occur through alterations in the sensitivity of muscle and liver cells to insulin. This implicates the presence of an adipose secreted substance that allows fat to communicate with peripheral tissues. However the responsible adipose-derived factor(s) has not been found (Muoio & Newgard, 2005). Recently a factor derived from fat cells, called retinol binding protein-4 (RBP4) has been shown to impair insulin sensitivity throughout the body. RBP4 joins a growing list of fat-derived peptides that modulate glucose homeostasis (Yang *et al*., 2005). Insulin insensitivity leads to compensatory hyperinsulinaemia which in turn, increases hepatic VLDL and triglyceride synthesis and secretion (Bray, 2004 (c)). It therefore has significant correlation with a dyslipoproteinaemic state (Sniderman & Cianflone, 1995). Expectantly, insulin resistance is the hallmark of the metabolic syndrome, which has been defined by the National Cholesterol Education Program Adult Treatment Panel III as having any three of the following five criteria: abdominal obesity, low HDL, high triglycerides, high fasting glucose and hypertension.

1.1.5.2 Cardiovascular disease and hypertension

Blood pressure often is increased in overweight individuals (Rocchini, 1998). In the Swedish Obese Subject Study, hypertension was present at baseline in 44-51% of the obese subjects. Also the Nurses Cohort Study found the risk of cardiovascular disease increased twofold in women with a BMI between 25 and 28.9, and 3.6-fold for a BMI >29, compared with women with a BMI of less than 21 (Willett *et al.*, 1995). On the other hand the association between obesity and the risk of death from cardiovascular disease was confirmed by a study of 8373 Finnish women (aged 30–59 yr) followed for 15 yr in the Finnish Heart Study. This study found that for each increase in body weight of approximately 1 kg, the risk of coronary mortality increased by 1–1.5% (Jousilahti *et al.,* 1996). Obesity is associated with an increase in total blood volume and cardiac output and a decrease in peripheral vascular resistance (Alpert *et al.*, 2001). The total blood volume is increased in proportion to body weight. This increase in blood volume contributes to an increase in the left ventricular preload and an increase in resting cardiac output (De Divitiis *et al.,* 1981). The increased demand for cardiac output is achieved by an increase in stroke volume while the heart rate remains comparatively unchanged. The obesity-related increase in stroke volume results from an increase in diastolic filling of the left ventricle (Licata *et al.,* 1991) and the left ventricular dilatation is accompanied by myocardial hypertrophy which over time can progress to heart failure (Kopelman, 2000). Left ventricular mass increases directly in proportion to BMI or the degree of overweight (Lauer *et al.*, 1991) and the severity of the defects in cardiac structure and function is associated with both the degree and duration of obesity (Alpert *et al.,* 1995). Weight loss, particularly in persons who are severely obese, can improve cardiac structure and function (Alaud-din *et al.*, 1990).

1.1.5.3 Other organs

An increased amount of fat in the chest wall and abdomen results in reduction in lung volume and decrease in its compliance. These changes are significantly exaggerated when an obese person lies flat. In some cases obstruction occurs in the larynx and is associated with loss of tone of the muscles controlling tongue movement (Kopelman, 2000). This leads to sleep apnea where sleep is disturbed with frequent awakening because of apnoeic episodes. The persistent hypoxia/hypercapnia, leads to pulmonary

hypertension secondary to the superimposed increased circulatory volume which in turn develops right sided cardiac failure. It can also lead to myocardial perfusion defects (Orea-Tejeda *et al*., 2003). There is a consensus that obesity is associated with an increased risk of cancer of the breast and endometrium in postmenopausal women, kidney, colon (strongest in men) and oesophagus (Calle & Thun, 2004). However, studies examining the influence of obesity and overweight on other malignancies i.e. prostate, liver, stomach, bladder, lymphoma and leukaemia, reveal inconclusive findings (Bianchini *et al*., 2002). Although the reasons are not clear the increased risk of endometrial and breast cancer in overweight women may be because of the increased production of oestrogens by adipose tissue (Schapira *et al*., 1994). Cholelithiasis is the primary hepatobiliary pathology associated with overweight (Ko & Lee, 2004). This is thought to be due to increased cholesterol turnover related to total body fat (Bray, 2003) which is in turn excreted in the bile. Non alcoholic fatty liver disease is also seen in obese individuals. This is a collection of liver abnormalities, including hepatomegaly, elevated liver enzymes, liver steatosis, steatohepatitis, fibrosis, and cirrhosis (Matteoni *et al*., 1999).

1.1.6 Treatments

Treatment of obesity is generally divided into medical and surgical options. Medical treatment usually consists of dietary and/or drug therapy in combination with behavior modification and exercise, while surgery is reserved for patients with clinically severe or morbidly obesity.

1.1.6.1 Behaviour and lifestyle

Behavioral treatments help obese individuals to develop adaptive thinking, eating, and exercise habits that enable them to decrease their weight and avoid regaining weight. People who combine caloric restriction and exercise with behavioral treatment may expect to lose about 5 to 10% of their body weight over a period of four to six months. Patients however are generally unwilling to continue behavioral treatment indefinitely (Wadden & Foster, 2000).

1.1.6.2 Diet

As energy imbalance is one of the main causes of obesity, the solution for obesity should be as simple as eating less and exercising more. Studies have shown that the

better the adherence to a diet, the greater the weight loss (Lyon *et al.,* 1995). Thus, it is adherence to diets, not diets themselves, that makes the difference (Dansinger *et al*., 2005). Although simple in theory, applying the ideas of energy balance and counting kilocalories to body weight control has proven unsuccessful.

1.1.6.3 Exercise

Exercise is commonly promoted as an important component of a weight loss regimen. However studies have shown that moderate exercise, per se, is ineffective as a means of achieving weight loss (Meredith *et al.*, 1989). Exercise however is an excellent way to promote preservation of lean body mass during dieting. It also appears to be more important in maintaining weight loss than in achieving it (Pavlou *et al.*, 1989).

1.1.6.4 Pharmacotherapy

There are five modes of action for drug therapy of obesity; 1) reducing food intake. 2) blocking fat absorption 3) increasing thermogenesis by uncoupling of fuel metabolism from the generation of ATP, thereby dissipating food energy as heat 4) regulating fat synthesis/lipolysis or adipose differentiation/apoptosis 5) modulating the central controller regulating body weight (Bray and Tartaglia, 2000). The two drugs currently available licensed for use in the United Kingdom are Sibutramine and Orlistat. Sibutramine is a β-phenethylamine that selectively inhibits the re-uptake of noradrenaline, serotonin and, to a lesser extent, dopamine and thus results in reduction of food intake. Studies have shown a dose-related reduction in body weight (Bray *et al.,* 1999), with weight loss of up to 9% below baseline, which can last up to 18 months with continued treatment. Orlistat is a hydrogenated derivative of a bacterial lipase inhibitor that blocks pancreatic lipase, thus decreasing triglyceride digestion (Guercolini, 1997). Two published clinical trials, each lasting two years, have shown that after one year the drug produces a weight loss of about 9–10% compared with a 4–6% weight loss in a placebo-treated group (Sjostrom *et al*., 1998; Davidson *et al.*, 1999). Drugs have a useful place as part of the treatment for some overweight patients. However studies indicate that regaining of weight is extremely likely when weight-loss medications are discontinued (National Task Force on the Prevention and Treatment of Obesity, 1993). The safety and efficacy of weight loss medications beyond two years of use have not been established (Yanovski & Yanovski, 2002) and therefore their long-term use must be risk assessed.

Metformin

Metformin is an insulin-sensitizing and antihyperglycemic agent used in the treatment of type 2 diabetes mellitus. The exact mechanism of action of Metformin is unknown, but one of its suggested actions is increased peripheral glucose disposal at lower insulin concentrations (Nosadini *et al.*, 1987; Prager *et al.*, 1988; Groop *et al.*, 1989). It has been shown that Metformin treatment of obese adults with NIDDM results in weight loss and improved glucose tolerance and lipid profiles (Hermann *et al.*, 1992; Bailey, 1992; Abbasi *et al.*, 1998). Furthermore, the use of Metformin in non-diabetic obese adults has been demonstrated to cause reduced food intake and weight loss with reduction in fasting plasma glucose, cholesterol, and insulin concentrations (Fontbonne *et al.*, 1996; Lee *et al.*, 1998). On the other hand, short-term Metformin treatment in women with polycystic ovary syndrome and insulin resistance has been shown to improve insulin sensitivity without a significant effect on body weight (Diamanti-Kandarakis *et al.*, 1998; Morin-Papunen *et al.*, 1998).

1.1.6.5 Surgical treatment

The epidemic of obesity has spawned a second epidemic; bariatric surgery. In recent years, there has been a dramatic increase in the number of bariatric surgical procedures performed worldwide (Santry *et al.*, 2005; Flum *et al.*, 2005). Bariatric surgery is not cosmetic surgery. It is major gastrointestinal surgery performed in extremely large patients whose obesity putting them at risk for complications and death, both from the medical problems associated with obesity and from the surgery itself. In a meta-analysis it was shown that surgery is more effective than non-surgical treatment for weight loss and control of some co-morbid conditions in patients with a BMI of 40 kg/m2 or greater (Maggard *et al.*, 2005). Surgical treatment of severe obesity also appears to be cost-effective by eliminating use of medications and absenteeism from work in patients who were previously morbidly obese (Martin *et al.*, 1995). The 1991 National Institutes of Health Consensus Development Panel published the recommended criteria for patient selection for surgical treatment shown below (Gastrointestinal surgery for severe obesity, 1992).

Criteria for Patient Selection

- Body weight 100% above ideal weight
- BMI ≥40 $kg.m^{-2}$
- BMI ≥35 $kg.m^{-2}$ with medical co-morbidities
- Failure of non-surgical attempts at weight reduction
- Absence of endocrine disorders that can cause morbid obesity
- Psychological stability
- Absence of alcohol and drug abuse
- Understanding of how surgery causes weight loss
- Realization that surgery itself does not guarantee good results
- Preoperative psychological evaluation for selected patients

1.1.6.5.1 Types of surgery

Bariatric surgery has continually evolved since its sporadic and tentative introduction in the 1950s. The first bariatric procedure, preceded by animal studies, subsequently presented to a recognized surgical society and published in a peer reviewed journal, was that of Kremen and associates in 1954, presenting a jejunoileal bypass (Kremen & Linner, 1954). Broadly speaking there are two types of surgery; malabsorptive and gastric restrictive surgery. The jejunoileal bypass (JIB) and its more recent modification, biliopancreatic diversion (BPD) and biliopancreatic diversion with duodenal switch (DS), are classified as primarily malabsorptive procedures, the gastroplasties (horizontal, vertical and vertical banded gastroplasty, VBG) are restrictive as is laparoscopic gastric banding. Roux-en-Y gastric bypass (RYGB) represents a combination of these two mechanisms (figure 1.1). Malabsorptive procedures produce the greatest degree of weight loss, but can be associated with serious and potentially life-threatening metabolic and nutritional complications. Restrictive procedures produce moderate degrees of weight loss and have the lowest incidence of metabolic and nutritional complications (Kral, 1992).

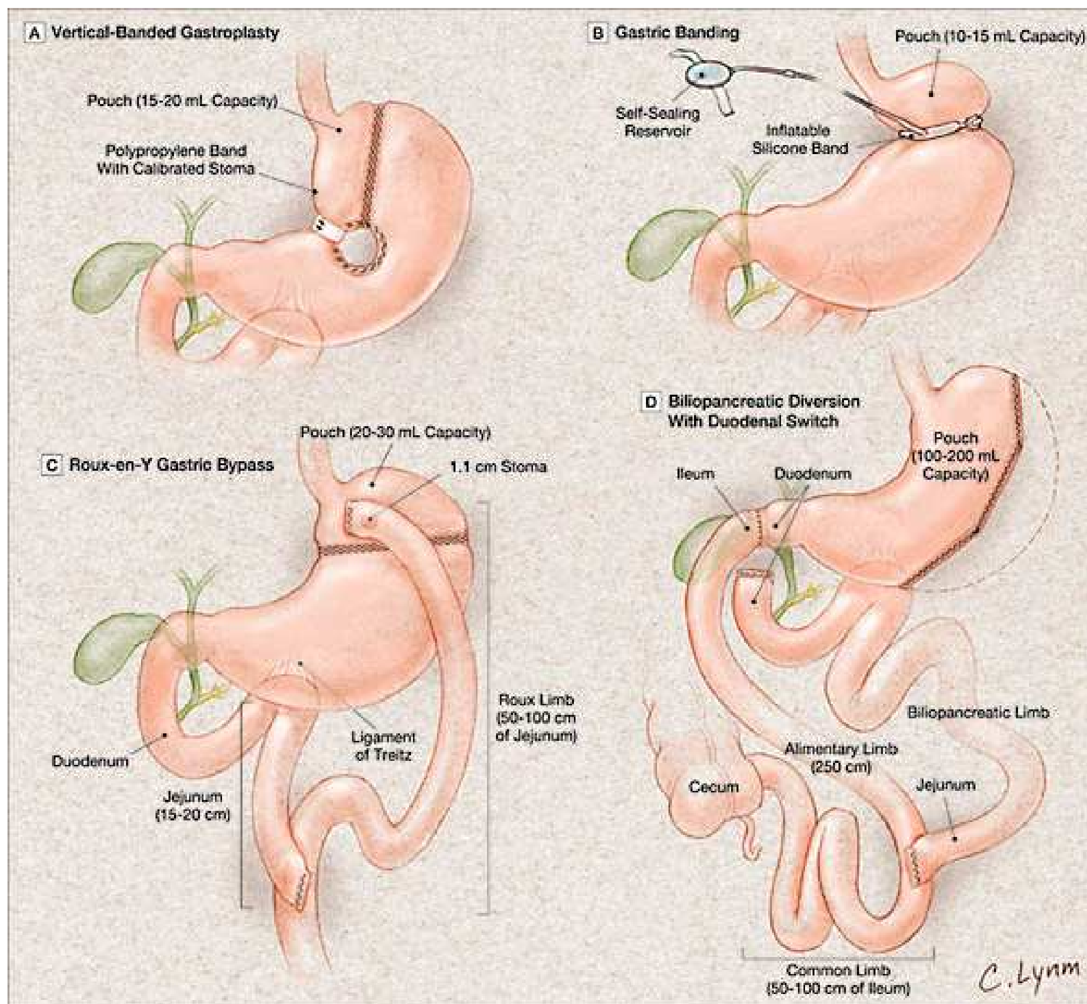


Figure 1.1: Types of bariatric surgery A: Vertical-banded gastroplasty. B: Gastric banding C: Roux-en-Y gastric bypass. D: Biliopancreatic diversion with duodenal switch (Brolin, 2002).

1.1.6.5.2 Benefits from surgical weight reduction

All bariatric procedures have been able to achieve loss of more than 50% of excess weight (O'Brien *et al.*, 2002; Buchwald *et al.*, 2004; Maggard *et al.*, 2005). Weight reduction surgery also has salutary effects on obesity-related hypertension and cardiovascular dysfunction. Weight loss resulting from bariatric surgery has been associated with significant improvement of left ventricular ejection fraction and lesser but measurable improvements in mean blood pressure, cardiac chamber size, and ventricular wall thickness (Alpert *et al.*, 1985). The Swedish Obese Subject study however, found no difference in blood pressure between surgically and medically treated patients with hypertension 8 years postoperatively (Torgerson & Sjostrom,

2001). In a four year follow up study of several hundred patients who underwent Laparoscopic Adjusted Gastric Banding (LAGB) elevated serum triglycerides had returned to normal and HDL-cholesterol levels had risen to normal values (Dixon & O'Brien, 2002 (b)). Insulin resistance was reduced and beta-cell function improved (Dixon & O'Brien, 2002 (a); Dixon *et al.*, 2003 (a)). Two-thirds of patients with type 2 diabetes return to having no clinical evidence of the disease, and have normal fasting blood glucose, serum insulin and HbA1c levels without therapy. There is also a marked improvement or resolution of obesity-hypoventilation syndrome and sleep apnea following surgically induced weight loss (Charuzi *et al.*, 1985; Sugerman *et al.*, 1988). Other reported improvements after surgical weight reduction have been in reflux oesophagitis (Dixon & O'Brien, 1999), depression (Dixon *et al.,* 2003 (b)), asthma (Dixon *et al.*, 1999), non alcoholic fatty liver disease (Dixon *et al.*, 2004) and quality of life (Dixon *et al.*, 2001). A randomised control study of 79 patients with mild to moderate obesity comparing surgery (LAGB) with medical treatment (O'Brien *et al.*, 2004) reported weight loss outcomes at 2 years of 71.5% excess body weight in the surgical group and 21.4% in the medical group. At 8 years follow-up, the Swedish Obesity Study reported the average weight loss of 20 kg among 251 surgically treated patients and this was maintained. A 10-year follow-up data on 1703 patients found those treated with surgery to have maintained their weight loss (16.1% decrease) contrary to ones on medical therapy (1.6% increase) (Sjostrom *et al.*, 2000; 2001).

1.2 ADIPOSE TISSUE

Obesity is defined by an excess of adipose tissue and in recent years this tissue has become a focus of intense study.

1.2.1 Depot variations in adipose tissue

Although the distribution of adipose tissue is significantly influenced by gender and obesity but it is unclear why some individuals gain relatively more upper body fat, and especially, visceral fat. Visceral fat mass is more closely correlated with obesity-associated pathology than overall adiposity (Peiris *et al.*, 1988; Stolk *et al.*, 2003; Dipietro *et al.*, 1999). Goodpaste *et al.* showed in a cross-sectional study of 3035 subjects that the distribution of body fat, measured with CT scan, was independently associated with the metabolic syndrome in older men and women, particularly among

those of normal body weight (Goodpaste *et al.*, 2005). Interestingly abdominal liposuction in a study, had not significantly improve obesity-associated metabolic abnormalities (Klein *et al.*, 2004 (b)). However in another study when obesity surgery was performed with omentectomy significant improvement was seen in the metabolic profiles of the patients (Thörne *et al.*, 2002). Cultured pre-adipocytes from omental fat, obtained from people with severe obesity, have a reduced capacity for differentiation (Tchkonia *et al.*, 2002) and proliferation (van Harmelen *et al.*, 2004) but are more susceptible to apoptosis (Niesler *et al.*, 1998; PaPineau *et al.*, 2003) than pre-adipocytes from subcutaneous abdominal fat. This implies a site-specific regulation of pre-adipocyte number. The release of adipokines also varies significantly between the depots and this suggests that the endocrine function of human adipose tissue as a metabolic organ is subject to regional variations. Adipose tissue is therefore a heterogenous metabolic organ (Arner, 1998) but the mechanism in which visceral obesity links to adverse outcomes is not clear. Montague & O'Rahilly have suggested the anatomical site and venous drainage of visceral adipose tissue and possible differences in the adipocytes' biology amongst few other possible explanations (Montague & O'Rahilly, 2000).

1.2.2 Functions of adipose tissue

1.2.2.1 Storage

Adipose tissue is the main site of energy storage in mammals and birds. Fat, in the form of triacylglycerols, is deposited in the adipocytes and in addition to being an efficient store of excess energy also provides thermal and mechanical insulation. Adipocytes have a central role in the deposition and release of fatty acids. (Mohamed-Ali *et al.* 1998; Fruhbeck *et al.* 2001; Trayhurn & Beattie, 2001). Adipocytes and the adipose tissue are able to dramatically change its size in accordance with changing metabolic needs. This ability gives the tissue an almost unlimited capacity for growth, making it perhaps the only tissue in the body with the ability to so drastically increase its size without an underlying transformed cellular phenotype.

1.2.2.2 Adipogenesis

Mature adipocytes differentiate from preadipocytes, which are present in the stromavascular fraction and can be recruited throughout adult life (Ailhaud *et al.*, 1992). This phenomenon seems to be reversible, and several reports describe the de-

differentiation of mature adipocytes into a preadipocyte state (Negrel *et al.*, 1985; Ron *et al.*, 1992). The fat cell number reflects the balance of preadipocyte proliferation, differentiation and apoptosis (Tchoukalova *et al.*, 2004). Adipogenesis results from the transcriptional activation and expression of adipocyte genes in fibroblast-like cells determined for preadipoctyes. Preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue the differentiation process, leading to the progressive acquisition of the morphological and biochemical characteristics of the mature adipocyte.

1.2.2.3 Lipolysis

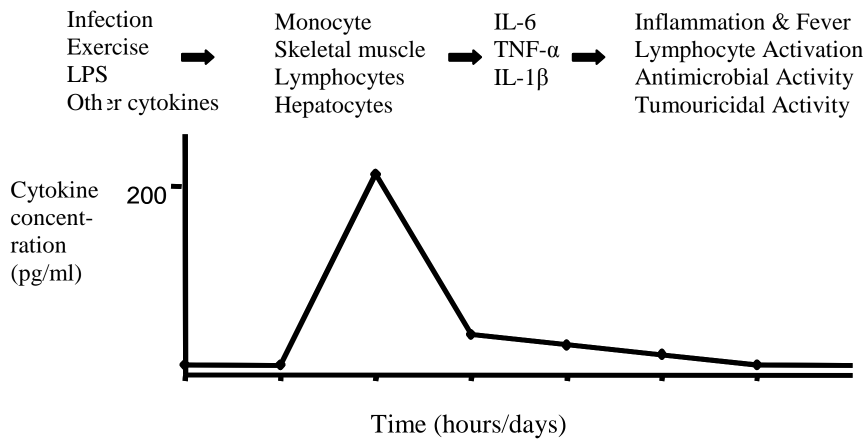
In times of fasting, tissues such as cardiac and skeletal muscle, and the liver, require substrate for energy production. Adipocytes are able to respond to these requirements by hydrolysing triglyceride into its component glycerol and fatty acids. Fatty acids are then free to diffuse out of the cell to be transported to where required. Stimulation of lipolysis is caused by many effectors, including catecholamines, prostaglandins and cytokines such as, TNFα, IL-1, IFNα, IFNβ and IFNγ. Conversely, glucose and insulin are able to inhibit lipolysis. In addition to the release of glycerol and fatty acids, as a result of metabolic activity, the adipose tissue has now been shown to be secretory organ.

1.2.2.4. Adipose tissue as a secretory organ

Production of signalling products places adipocytes among secretory cells. In 1985, Kelly suggested two pathways for the release of secretory products; regulated and constitutive pathways (Kelly, 1985). In the regulated pathway secretory products are stored in a secretory granule. These granules remain in the cytoplasm until stimuli e.g. neural or hormonal stimuli trigger the release of the products. The granule's half-life is hours to days and processing of precursors can occur in them. On the contrary in constitutive pathway, products are synthesised and packaged into membrane-limited vesicles in the Golgi apparatus and are rapidly released via exocytosis. There is no processing of the precursors, no storage and no regulation of their release. In some cells both pathways exist for the same product (Stachura & Frohman, 1975; Moore *et al*, 1983). The release of secretory products from adipose tissue seems to be predominantly via constitutive release (figure 1.2). In lean men body fat mass is 10-12% and in women 15-19%. This constitutive release of products is of paramount

importance in obese and morbidly obese subjects who have a body fat mass ranging between 40-65%. In these individuals, organs are surrounded by large amount of adipose tissue and are chronically exposed to their secretions over years.

(a) Acute cytokine release



(b) Chronic cytokine release

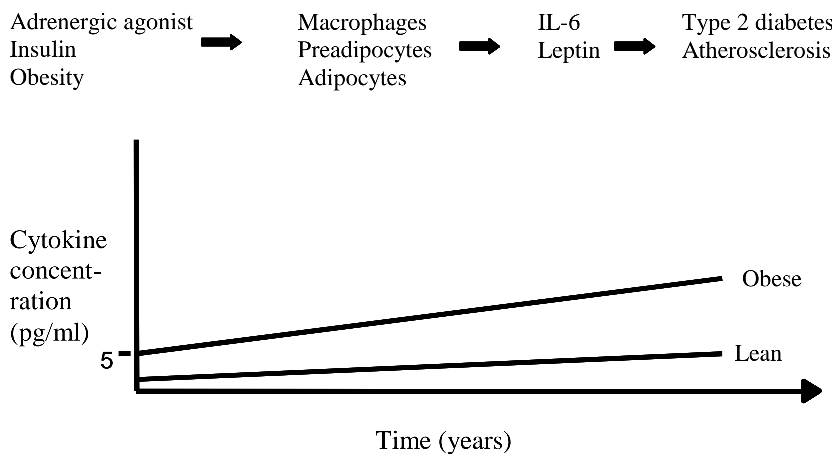


Figure 1.2: Acute and chronic cytokine release. (a) Stimuli such as infection or exposure to inflammatory stimuli such as lipopolysaccharide (LPS) and exercise results in an acute cytokine release and is mediated by immune and skeletal muscle cells, respectively. Cytokine levels rise sharply (50-100 fold) for a short period (hours). (b) Basal cytokine release increases with age and is higher in obese than lean individuals. The cytokine levels are much lower in obesity than those seen during infection and exercise however they are permanently elevated due to constitutive release from the excess white adipose tissue. (Mohamed-Ali *et al*., 2005)

1.2.2.5 Adipose tissue and adipokines

The apparent simplicity of adipose tissue, both histologically and metabolically, with triacylglycerols constituting more than 85% of the tissue weight and there being only a 'thin skim' of cytoplasm between the fat droplet and the plasma membrane, it is unsurprising that the tissue had been regarded as essentially limited in function to lipid synthesis and breakdown (Trayhurn, 2005). Quantitatively, the most important secretory product of adipocytes is fatty acids. The tissue also releases other lipid moieties, such as cholesterol, retinol, steroid hormones and prostaglandins (Trayhurn & Beattie, 2001). These substances are, however, not synthesized de novo within fat cells, although certain steroid transformations can take place. The significance of adipose tissue as an endocrine organ first surfaced in 1994 with the ground-breaking discovery of leptin (Zhang *et al.* 1994). Following research and analysis of expressed genes in adipose tissue has revealed that adipocytes produce and secrete a variety of bioactive substances, named adipokines.

The diversity of the adipokines, both in terms of protein structure and of function, is considerable. The group includes classical cytokines (e.g. TNF-α, IL-6), growth factors (e.g. transforming growth factor-β), proteins of the alternative complement system (e.g. adipsin, acylation-stimulating protein), proteins involved in vascular homeostasis (e.g. plasminogen activator inhibitor-1, tissue factor), the regulation of blood pressure (angiotensinogen), lipid metabolism (e.g. cholesteryl ester transfer protein, retinol-binding protein), glucose homeostasis (e.g. adiponectin) and angiogenesis (e.g. vascular endothelial growth factor), as well as acute-phase and stress responses (e.g. haptoglobin, metallothionein) (Trayhurn, 2005). Tissue culture studies have indicated, that adipocytes directly signal to other tissues such as skeletal muscle and the adrenal cortex (Dietze *et al.*, 2002; Ehrhart-Bornstein *et al.*, 2003). There is also, in particular, a distinct cross talk between adipocytes and the brain through leptin and the sympathetic nervous system (Rayner & Trayhurn, 2001).

1.2.3 Adipose tissue and inflammation

The issue of inflammation is one of the most important developing areas in obesity and the emergence of the concept that obesity, like diabetes, is characterized by chronic low-grade inflammation (Yudkin *et al.*, 1999; Bastard *et al.*, 2000; Das, 2001; Festa *et al.*, 2001; Engstro¨m *et al.*, 2003). An increasing number of adipokines are

directly linked to inflammation and the inflammatory response, mainly divided in to three categories as seen in figure 1.3.

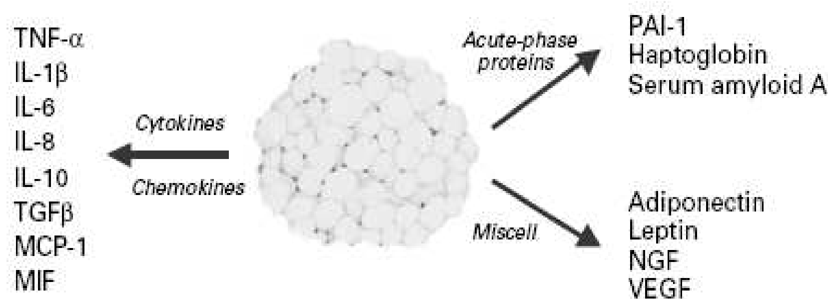


Figure 1.3: Adipokines related to inflammation. TGFβ: transforming growth factor b; MCP-1: Monocyte Chemoattractant Protein-1; MIF: Macrophage migration Inhibitory Factor; PAI-1: Plasminogen Activator Inhibitor-1; NGF: Nerve Growth Factor; VEGF: Vascular Endothelial Growth Factor; Miscell: miscellaneous (Trayhurn, 2005).

The basis for this view is that the circulating level of several markers of inflammation, such as IL-6, C-reactive protein and haptoglobin, are elevated in obesity and are reduced with weight loss (Yudkin *et al.*, 1999; Festa *et al.*, 2001; Chiellini *et al.*, 2004). Given that adipose tissue secretes a wide range of inflammation-related proteins, it is probable that it is the source of at least some of the increase in these factors in obese subjects. A key hypothesis is that adipose tissue-derived inflammatory factors may play a causal role in the development of the insulin-resistance and hence lead to metabolic syndrome (Hotamisligil, 2003; Yudkin, 2003). There is increasing support for such a concept and both the circulating levels and adipose tissue expression of several adipokines are increased in obese subjects, including leptin, plasminogen activator inhibitor-1, IL-6, TNF-α and haptoglobin (Considine *et al.*, 1996; Ostlund *et al.*, 1996; Mohamed-Ali *et al.*, 1997 (a); Alessi *et al.*, 2000; Bastard *et al.*, 2000; Chiellini *et al.*, 2004). In contrast, the production and circulating level of adiponectin, which has been reported to have an anti inflammatory effect (Yokota *et al.*, 2000), is reduced in obesity (Arita *et al.*, 1999; Hotta *et al.*, 2000). Obesity is also associated with increased macrophage infiltration of adipose tissue, and these macrophages may be an important component of the chronic inflammatory response playing a crucial role in the development of insulin resistance

(Neels & Olefsky, 2006). The reason for this chronic inflammatory response is not clear. One of the hypotheses is that the trigger for this inflammation is the hypoxia within clusters of adipocytes remote from the vascular supply in the expanding tissue (Trayhurn & Wood, 2004). Hypoxia leads to the expression of hypoxia-inducible factor-1a, which when combined with hypoxia-inducible factor-1b forms the transcription factor hypoxia-inducible factor-1. In turn the transcription of a number of genes, including those encoding the GLUT1 facilitative glucose transporter, glycolytic enzymes, such as lactate dehydrogenase, and inflammation-related proteins, has been shown in several tissues to be stimulated during hypoxia through the medium of hypoxia-inducible factor-1 (Wenger *et al.*, 2002).

1.3 SPECIFIC ADIPOSE TISSUE DERIVED FACTORS

1.3.1 RANTES

RANTES is made up of 68 amino acids and is a member of the chemotactic cytokine family. It recruits, activates and co-stimulates T cells and monocytes and so plays a role in immuno-regulatory and inflammatory processes (Luster, 1998; Gerhardt *et al.*, 2001). RANTES binds to specific receptors in the G-protein coupled receptor family, namely CCR1 and CCR5 (Appay & Rowland, 2001), however the mechanisms through which it activates leukocytes have yet to be elucidated. It appears that its function is dependant on its concentration. At low concentrations, RANTES acts in a monomeric, or dimeric, form directly on its specific receptor, whereas at high concentrations, it self-aggregates to form a multimer which interact with cell-surface glycosaminoglycans (Appay *et al.*, 1999). Its ability to self-aggregate is crucial in the activation and recruitment of leucocytes, as monomers have no effect, even though they are able to activate the G-protein coupled receptor. RANTES has therefore been implicated as a particular feature of inflammation, and its expression has been shown to increase in a number of imflammatory conditions such as allogenic transplant rejection, atherosclerosis, arthritis, atopic dermatitis, inflammatory airway disorders like asthma, delayed hypersensitivity reactions, glomerulonephritis, endometriosis, certain neurological disorders, such as Alzheimer's, and certain malignancies (Appay & Rowland, 2001). Following the discovery that RANTES was also secreted by adipose tissue (Xu *et al.*, 2003), its role in obesity and its co-morbidities has subsequently been the subject of intense research (Wu *et al.* 2007). Circulating RANTES levels have been shown to be higher in the pre-pubertal obese, compared to

the lean, individuals (Economou *et al.*, 2004) as well as in subjects suffering from cardiovascular disease (Parissis *et al.*, 2002). There was an association between the plasma RANTES levels and the cardiovascular risk factors, such as elevated serum triglycerides and a high waist-hip ratio (Koh *et al.*, 2008). It is known that CCR5 is expressed in human culture adipocytes and was present on the cell surface of adipocytes in subcutaneous tissue (Gerhardt *et al.*, 2001). Antagonism or knockout of the RANTES receptor (CCR5) has seemed to reduce the development of atherosclerosis in mice (Veillard *et al.*, 2004; Braunersreuther *et al.*, 2007). In contrast, up-regulation of RANTES and its receptors has been reported in adipose tissue in human obesity and has been associated with increased systemic inflammation (Huber *et al.*, 2008). Wu *et al.* found higher RANTES and CCR5 mRNA levels in obese compared to lean mice but this increase was more pronounced in males. RANTES expression in males was also higher in the obese mice but this difference was not apparent in females. In addition, authors demonstrated that visceral adipose tissue exhibits higher levels of RANTES and CCR5 mRNA than subcutaneous adipose of morbidly obese humans and that unlike IL-6 and MCP-1, RANTES is derived from both the stromavascular and adipocyte sections, but that this too was gender-specific and higher in male individuals. These findings have lead to the belief that RANTES may contribute to the inflammatory infiltration characterised in obesity.

1.3.2 Adiponectin

Adiponectin is made up of 30-kDa polypeptide and was identified by four different groups in mid 90s, using different methods. Adiponectin undergoes hydroxylation and glycosylation post translation. The isoforms then form trimers and polymers, which then form low molecular weight (LMW), middle molecular weight (MMW) and high molecular weight (HMW) compounds (Pajvani *et al.*, 2003; Waki *et al.*, 2003). These forms of adiponectin, contrary to the monomers, are found in the circulation (Chandran *et al.*, 2003). There are two adiponectin receptors, adipoR1 and adipoR2. AdipoR1 is expressed primarily in the muscle and adipoR2 in the liver (Yamauchi *et al.*, 2002). Therefore as well as circulating levels of adiponectin and its isoforms, its receptor subtypes also play a role in the effects exerted. Plasma adiponectin levels is higher in female suggesting a possible regulation by sexual hormones (Nishizawa *et al.*, 2002; Combs *et al.*, 2003; Xu *et al.*, 2005) and increase with age (Adamczak *et al.*, 2005). This adipokine circulates at high levels (3- 30 µg/ml) compared to other

adipokines, such as leptin with levels in the nanogram per milliliter range (Chandran *et al*., 2003; Fantuzzi, 2005). Adiponectin has a major role in energy homeostasis and inflammation (Lyon *et al*., 2003). It's known to have anti-inflammatory effects such as reducing the activity and production of TNF-α (Masaki *et al*., 2004), inhibiting production of IL-6 and induction of IL-10 and IL-1 receptor antagonists, which are anti-inflammatory cytokines (Kumada *et al*., 2004; Wolf *et al*., 2004; Wulster-Radcliffe *et al*., 2004).

Contrary to other adipokines, adiponectin's expression and circulatory levels are decreased as BMI increases. There was also a negative correlation between adiponectin levels and insulin resistance and type 2 diabetes with reduced plasma levels found in conditions associated with insulin resistance such as cardiovascular disease (Kumada *et al*., 2003; Pischon *et al*., 2004) and hypertension (Ouchi *et al*., 2003; Adamczak *et al*., 2003). In fact low adiponectin levels have been shown to be independently associated with metabolic syndrome (Matsushita *et al*., 2006). The exact mechanism of action and association with insulin sensitivity is not clear. However adiponectin has been shown, in the skeletal muscle, to increase the expression of Acyl-coenzyme A oxidase and hence the combustion of fatty acid, increase the uncoupling protein 2 expression which helps dissipate energy and increase CD36 that is involved in fatty acid transport (Yamauchi *et al*., 2001). All these help reduce tissue triglycerides and prevent insulin resistance (Shulman, 2000). It has also been shown to activate PPARα hence increase fatty-acid combustion and energy consumption and therefore reduce triglyceride in liver and muscle (Yamauchi *et al*., 2001) as well as activating AMP kinase and therefore stimulating phosphorylation of acetyl coenzyme-A carboxylase, fatty acid combustion and glucose uptake (Yamauchi *et al*., 2002). Adiponectin production was induced by insulin-sensitizing drugs, such as the PPARγ agonist, thiozolidinediones, as well as with weight loss (Yang *et al*., 2001; Weyer *et al*., 2001; Chandran *et al*., 2003; Diez & Iglesias, 2003). However the regulation of this adipokine and the molecular mechanisms of its action are still not clear.

1.3.3 MCP-1

MCP-1 or CCL2, like RANTES, is also a member of the chemotactic cytokine family. Infusion of MCP-1 into mice increased circulating monocytes, accumulation of monocytes in collateral arteries and neo-intimal formation (Takahashi *et al*., 2003;

van Royen *et al*., 2003). MCP-1 also plays an important role in the development of atherosclerosis as its expression is increased in atherosclerotic lesions (Yla-Herttuala *et al*., 1991; Takeya *et al*., 1993) and atheroma formation in hypercholesterolemic mice is reduced by inhibition of its expression or that of its receptor (Gu *et al*., 1998; Boring *et al*., 1998). In apolipoprotein E-knock out mice that are prone to severe atheroma formation this process was decreased when MCP-1 release was blocked through transfection of an N-terminal deletion mutant in MCP-1 gene (Inoue *et al*., 2002). MCP-1 was produced and released by the stromavacular fraction of adipose tissue, pre-adipocytes and isolated mature adipocytes (Gerhardt *et al*., 2001; Xu *et al*., 2003). The release of MCP-1 from pre-adipocytes was triggered by levels of TNF-α secreted by adipocytes in obesity (Xu *et al*., 2003). While it was not clear whether the MCP-1 from endothelial cells or that from adipocytes attracts the macrophages into adipose tissue, its expression precedes the appearance of macrophage markers (Xu *et al*., 2003). Circulating MCP-1 levels are elevated in genetic (ob/ob mice) and diet-induced obese mice and reduced after weight loss (Sartipy & Loskutoff, 2003; Takahashi *et al*., 2003). The mechanism of this process is not clear, however. It has been suggested that over-nutrition causes a metabolic overload with increased demands on the endoplasmic reticulum and on the mitochondria resulting in the release of pro-inflammatory mediators via excess production of reactive oxygen species (Wellen & Hotamiligil, 2005). However it has also been shown that treating adipocytes with MCP-1 causes a decrease in lipid accumulation as well as stimulation of leptin secretion by post-transcriptional mechanisms (Sartipy & Luskutoff, 2003). A study in obese children and adolescents found no significant correlation between circulating MCP-1 and BMI (Glowinska & Urban, 2003). More recently it is found that the MCP-1 levels, both circulating and mRNA content of subcutaneous adipose tissue, are elevated in human obesity and are greater in visceral compared to that in subcutaneous tissue (Christiansen *et al*., 2005; Bruun *et al*., 2003). MCP-1 is also elevated in patients with type 2 diabetes mellitus and is associated with an increase in cardiovascular disease (Nomura *et al*., 2000; Piemonti *et al*., 2003). In adipocyte cell lines MCP-1 was found to decrease insulin-stimulated glucose uptake and insulin-induced insulin receptor tyrosine phosphorylation suggesting an important role in insulin resistance of adipose tissue (Gerhardt *et al*., 2001; Sartipy & Loskutoff, 2003).

1.3.4 IL-6

IL-6 is a 21 to 28 kDa protein, depending on the cellular source and preparation (Hirano *et al.*, 1986). IL-6 plays a central role in diverse host defense mechanisms such as the immune response, haematopoiesis, and acute-phase reactions (van Snick, 1990) and along with other cytokines it represents an important frontline component of the body's defence against infection or tissue damage (Nicola, 1997). Site of production of IL-6 has been documented in a seemingly endless variety of cells namely fibroblasts (van Damme *et al.*,1987), endothelial cells (Corbel & Melchers, 1984), keratinocytes (Baumann *et al.*, 1984), peripheral blood mononuclear cells (van Damme *et al.* , 1988), monocytes/macrophages (van Snick *et al.*, 1986), T-cell lines (Hirano *et al.*, 1985), mast cells (Plaut *et al.*, 1989; Hfiltner *et al.*, 1989), a variety of tumor cell lines (Hirano *et al.*, 1986), and more recently, adipose tissue and adipocytes (Mohamed-Ali *et al.*, 1997 (a); Kern *et al.*, 1995). In the steady state, IL-6 was usually not produced constitutively by normal cells, but its expression was readily induced by viral infections (Cayphas *et al.*, 1987; Sehgal *et al.*, 1988; Frei *et al.*, 1989; Nakajima *et al.*, 1989), lipopolysaccharide Lipase (LPL) (Nordan & Potter, 1986), IL-1 (Shalaby *et al.*, 1989), tumor necrosis factor-α , platelet-derived growth factor (Kohase *et al.*, 1987), IL-3 and GM-CS. However not all cells respond similarly to all these factors. Chronic IL-6 release has been shown to inhibit monomeric LPL, an endogenous inhibitor of active (dimeric) LPL (Wallberg-Jonsson *et al.*, 1996) and to stimulate lipolysis (Hauner *et al.*, 1995; Path *et al.*, 2001). This results in further production of free fatty acids and allows macrophages to take up triglycerides and become atherogenic foam cells, thus promoting atherosclerosis. Bruun *et al.* showed that sustained IL-6 release can inhibit adipose tissue expression of adiponectin (Bruun *et al.*, 2003; Clarke & Mohamed-Ali *et al.*, 2004). Furthermore, IL-6 can stimulate fibroblasts to differentiate into adipocytes (Kajkenova *et al.*, 1997). The IL-6 expressed and released by adipose tissue (Kern *et al.*, 1995; Mohamed-Ali *et al.*, 1997 (a)) can influence endothelial function (Bhagat & Balance, 1997) and induce endothelial expression of chemokines and adhesion molecules (Romano *et al.*, 1997) which are central in the early stages of the atherogenetic process (Jang *et al.*, 1994). It has been shown that the production of IL-6, as well as its systemic concentrations, increase with adiposity (Mohamed-Ali *et al.*, 1997 (a)) and also decrease with weight loss (Dandona *et al.*, 1998; Bastard *et al.*, 2000). There was also a difference in

amount of production from different adipose tissue depots, with *in vivo* and *in vitro* studies suggesting a greater contribution from visceral than subcutaneous fat (Fried *et al*., 1998, Fontana *et al.*, 2007). Insulin resistance in obesity may be directly caused by increased IL-6 as this cytokine has been shown to impair insulin signalling *in vitro* (Hotamisligil *et al.*, 1996). Subsequently, during sustained adiposity, insulin resistance becomes a maladaptive consequence of obesity

1.3.5 Leptin

Leptin has revolutionized the study of adipose tissue. Discovery of leptin, largely produced by adipocytes, reclassified the adipose tissue as an endocrine organ. Leptin is a 16 kDa peptide which has a cytokine-like structure (Zhang *et al*., 1994; Gaucher *et al*., 2003). Its receptor is a member of the gp130 class I cytokine receptor family (Baumann *et al*., 1996). Consequently, leptin was occasionally grouped with the more traditional cytokines, such as IL-6 and TNF-α. Leptin is encoded by the *ob* gene and is expressed predominantly in adipose tissue, in correlation with the amount of fat present in adipocytes, with the larger, more lipid engorged cells secreting more leptin (Friedman & Halass, 1998). It has been shown to have a role in a range of processes including the regulation of appetite and energy expenditure, glucose homeostasis, bone formation, regulation of puberty and reproduction, immunity and inflammation (Rajala & Scherer, 2003). It was present in the circulation and cerebrospinal fluid. It crosses the blood-brain barrier and binds to receptors in the hypothalamus, where it stimulates anorexigenic peptides, and inhibits orexigenic peptides, thus regulating food intake (Ronti *et al*., 2006). Leptin also acts on skeletal muscle, pancreas, liver and adipose tissue (Rajala & Scherer, 2003). Mice with leptin (*ob/ob)* or leptin receptor (*db/db)* gene mutations were massively obese, infertile and insulin resistant (Zhang *et al*., 1994; Fantuzzi, 2005). Similarly, in very rare cases of human leptin deficiency due to chromosomal mutations, individuals were morbidly obese, hyperphagic and did not undergo normal sexual maturation (Clement *et al*., 1998; O'Rahilly *et al*., 2003). In both cases, leptin administration led to dramatic weight loss, improved insulin sensitivity and resolution of hypogonadism. Leptin treatment was, however, less effective in the majority of obese patients because human obesity, in the main, is associated with leptin resistance (Bakker *et al*., 2004). It is unclear as to the cause of this resistance and its molecular basis, but, it has been suggested that it could be the result of impaired leptin transport through the blood brain barrier and/or signalling

defects of the leptin receptor in the hypothalamus (Farooqi *et al.*, 2002). Leptin is involved in regulating many inflammatory and immune processes (Fantuzzi, 2005). Plasma leptin levels are increased in diet induced obesity (Li *et al.*, 1997) and correlate with BMI (Maffei *et al.*, 1995) and percentage body fat (Considine *et al.*, 1996) as well increasing with age. Leptin was expressed in significantly larger amounts in the subcutaneous tissue compared to visceral depots (Lefebvre *et al.*, 1998). It also resulted in inhibition of the action of insulin and lipogenesis, and stimulation of lipolysis (Wang *et al.*, 1999; Aprath-Husmann *et al.*, 2001). The effect of leptin on insulin is not clear, however. Some studies have shown a correlation between leptin and insulin resistance (Zimmet *et al*, 1996; Girard, 1997), some have found have found no relation (Mohamed-Ali *et al.*, 1997 (b)) and others have shown leptin to be an anti-diabetic agent (Sivitz *et al.*, 1997).

1.4 REGULATION OF ADIPOSE TISSUE DERIVED FACTORS

The molecular pathways behind the constitutive release of these adipokines and their regulation are poorly understood. These signals produced by adipose tissue potentially mediate adverse cardiovascular effects, modulate prostaglandins/Interleukins release, insulin resistance and endothelial NO bioavailability (Clarke & Mohamed Ali, 2004). Many adipokines are cytokines with 'immunological' origin; and cytokines in immune cells are often regulated by the COX or NOS pathways. Therefore these two pathways are reviewed in greater detail in the following sections.

1.4.1 Prostaglandins and COX pathway

Prostaglandins are formed from arachidonic acid. Arachidonic acid is released from membrane phospholipids in response to various physiological and pathological stimuli by the action of phospholipaseA_2 which is followed by the actions of COX and the respective synthases (Narumiya *et al.*, 1999) (figure 1.3). COX-1 and COX-2 are its two closely related forms. COX-1 is predominantly constitutively released and is expressed throughout the body and provides certain homeostatic functions, such as maintaining normal gastric mucosa, influencing renal blood flow, and aiding in blood clotting by abetting platelet aggregation (Turini & DuBois, 2002). In contrast, COX-2 is the inducible form (Hla & Neilson, 1992) and is expressed in response to inflammatory and other physiologic stimuli and growth factors and is involved in the production of those prostaglandins that mediate pain and support the inflammatory

process. The two isoforms are different in their amino acid sequence of the arachidonic acid binding site and this difference allows for their selective inhibition (Marnett & Kalgutkar, 1999). Release of prostanglandins from rat adipose tissue was first noted in 1968 (Shaw & Ramwell, 1968). IL-6 is also produced by adipose tissue and especially in obesity a significant portion of systemic IL-6 may be adipose tissue derived (Mohamed-Ali *et al.*, 1997 (a)). It may be that constitutive IL-6 release from the expanded fat mass leads to its chronic elevation in this condition. It has been shown that the treatment of epithelial cells with prostaglandinE_2 (PGE_2) caused a dose-dependent increase in IL-6 release (Tavakoli *et al.*, 2001) and that both PGE_2 and carbacyclin, an prostacyclin analogue, increased IL-6 release from human placenta (Turner *et al.*, 2004) . In our group (unpublished results) IL-6 release in primary human adipose tissue cell cultures was increased after treatment with PGE_2, PGI_2, PGD_2 and especially with carbacyclin, an IP receptor agonist. Understanding the molecular mechanisms regulating adipose IL-6 secretion could lead to dietary and pharmacological strategies to modulate this cytokine in obesity associated pathologies.

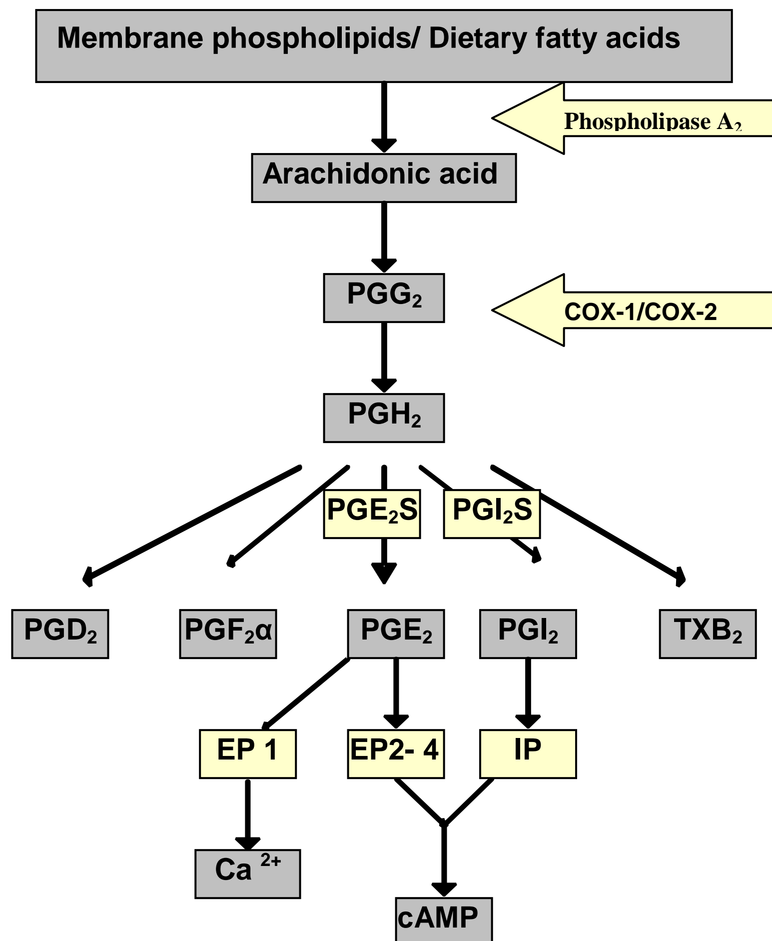


Figure 1.4: The COX pathway: IL-6 release occurs through changes in the COX/Prostaglandin axis. Various synthases convert PGH_2 into the corresponding prostaglandins. PGE_2 signals via EP receptors and PGI_2 via IP receptors. IL-6 secretion is regulated via receptors that are mediated by elevation in cAMP (EP2, 4 and IP). PGI_2S: Prostacyclin synthase. PGE_2S: ProstaglandinE_2 Synthase.

1.4.2 NOS Pathway

1.4.2.1 NO

NO is synthesized from L-arginine by NOS. It has a cruicial role in endothelial function and relaxation. Endothelium-derived NO regulates arterial tone through a dilator action on vascular smooth muscle cells. Studies demonstrating increased blood pressure in animals lacking endothelial NOS provide evidence for a role of NO in the regulation of arterial pressure (Huang *et al*., 1995). Endothelial dysfunction understandably is an early abnormality in the pathogenesis of atherosclerosis. Alterations of the NOS pathway and impairment of NO-dependent vasodilation has been linked to several well-known atherogenic risk factors, including hypercholesterolemia, hypertension, type 2 diabetes, smoking, and ageing (Boger *et al*., 1996). Reduced NO and therefore endothelial dysfunction can arise because of low NO bioavailability, enhanced NO degradation triggered by high oxidative stress, low availability of the substrate of NO synthase, L-arginine, or because of an increase in the endogenous NO inhibitors such as asymmetric dimethylarginine (ADMA). Recently, increased focus has been on ADMA and there are reports that the plasma concentrations of ADMA are elevated in human disease states.

1.4.2.2 Asymmetrical Dimethyl Arginine

This amino acid is structurally similar to L-arginine with which it competes as substrate for NOS (Kielstein & Zoccali, 2005). The arginine analogues identified to date include N^G-mono-methyl-L-arginine (L-NMMA), N^G, N^G-di-methyl-L-arginine (asymmetric dimethyl-arginine; ADMA) and NG,N′G-dimethyl-L-arginine (symmetric di-methyl-arginine; SDMA). The asymmetrically (L-NMMA and ADMA), but not the symmetrically methylated arginine residues (SDMA), are competitive inhibitors of the NOS enzymes (Leiper, 2005). In pre-contracted endothelium-intact rat aortic rings, ADMA has been shown to cause dose dependent increases in tone. ADMA was able to raise blood pressure in guinea-pigs and reduce resting forearm blood flow. Thus, it was postulated that ADMA, which was identified as circulating in human plasma at a concentration ten times greater than that of naturally occurring L-NMMA, might act as an important endogenous regulator of the L-arginine/NO pathway and/or arginine-handling enzymes *in vivo* (Vallance *et al*., 1992). Protein methylation is a post-translational modification (Clarke, 1993; Aletta *et al*., 1998) and

is catalysed by a family of enzymes termed protein-arginine methyltransferases (PRMTs). The genes encoding PRMTs have only been cloned in the last decade and their cellular functions are only beginning to be understood. McDermott observed that contrary to earlier belief (McDermott, 1976), ADMA was not fully excreted via urine but was hydrolysed by an enzyme named N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH) (Ogawa *et al*, 1989). DDAH hydrolyse ADMA and L-NMMA, not SDMA, to give citrulline and dimethylamine or monomethylamine, respectively.

ADMA is raised in a variety of conditions associated with high cardiovascular risk, such as in familial hypercholesterolemia and essential hypertension, and was particularly evident in patients with end-stage renal disease (Vallance *et al*., 1992; Zoccali *et al*., 2001). Overall, ADMA is emerging as a cardiovascular risk marker of primary importance (Zoccali, 2006) and as a potential target for interventions aimed at reducing atherosclerotic complications. Only recently studies have looked at ADMA in obesity. Eid *et al.* has shown a close relationship between BMI and plasma levels of ADMA and the L-arg/ADMA ratio, independent of other metabolic risk factors (Eid *et al*., 2004). Overweight and obese subjects had significantly elevated ADMA levels and lower L-arg/ADMA ratio compared to lean or normal individuals.

1.4.2.2.1 ADMA, Insulin and endothelial dysfunction

Obesity, hypertension, type 2 diabetes and dyslipidaemia are all commonly associated with reduced sensitivity to the metabolic actions of insulin (Pinkney *et al*., 1997; Reaven, 1988). Although it is frequently assumed that the increased insulin secretion is a result of insulin resistance, there appears to be some evidence that hyperinsulinaemia may precede the development of insulin resistance, which can play a role in the pathogenesis of obesity (Penicaud *et al*., 1989; Le Stunff & Bougneres, 1994; Rizza *et al*., 1985; Sigal *et al*., 1997). An association has been seen between insulin resistance and raised serum ADMA and treatment with an insulin-sensitizing drug, Rosiglitazone, enhanced insulin sensitivity and reduced plasma ADMA concentrations (Stuhlinger *et al*., 2002). It is now established that activation of the PPARγ, by thiazolidinediones, induces differentiation of preadipocytes into mature fat cells leading to increased NEFA uptake and decreased plasma concentrations. This was often accompanied by a reduction in plasma glucose concentrations and oxidative

stress. The mechanism for thiazolidinedione's ADMA lowering effect (Stuhlinger *et al*., 2002), is not yet known. It could be mediated through a reduction in oxidative stress, and/or increased DDAH activity and hence increased ADMA degradation (Chan & Chan, 2002). Reduced plasma ADMA levels have also been seen with Metformin treatment in poorly controlled type 2 diabetic patients (Asagami *et al*., 2002). Weight loss studies in insulin resistant obese individuals have shown plasma ADMA concentrations decreased in conjunction with improvement in insulin sensitivity after weight loss. However this was not evident in insulin sensitive obese subjects (Mclaughlin *et al*., 2006). This and other published literature suggest that insulin resistance is the cause of vascular dysfunction (Ferrannini *et al*., 1987; Reaven, 1988; Russell *et al*., 1998; Pinkney *et al*., 1997;Hsueh *et al*., 1997; Jin & Bohlen, 1997). This hypothesis remains unproven. A study on diet-induced obese rats found markedly impaired arterial relaxation, mainly due to endothelial dysfunction and these defects occurred in the absence of significant insulin resistance suggesting endothelial dysfunction precedes insulin resistance (Naderali *et al*., 2001).

1.5 AIMS OF THE STUDY

The aim(s) of this study were to investigate the signals from adipose tissue in morbid obesity and explore some of the molecular mechanisms regulating adipokine secretion. Specific aims were to investigate:

1) in a cross-sectional study of morbidly obese individuals undergoing surgery, the:
    a) *in vitro* release of RANTES, MCP-1, adiponectin, IL-6 and leptin,
    b) site specific differences of adipokine secretion, including comparison of two visceral depots; omental fat and the gastric fat pad
2) components of the COX pathway and its role in IL-6 release from adipose tissue
3) components of the NOS pathway and its association with indices of insulin sensitivity

CHAPTER 2: MATERIAL AND METHODS

2.1 RECRUITMENT

Patients undergoing bariatric, reflux operations or cholecystectomy were recruited from Mr Majid Hashemi's surgical outpatient clinic or identified from his operating lists at the Whittington NHS Hospital Trust**.** The inclusion and exclusion criteria were as shown below:

Subject Inclusion Criteria

- Adults aged >= 21 years
- Obese subjects with BMI >28 $kg.m^{-2}$

Subject Exclusion Criteria

- Current treatment with aspirin, NSAID's, steroids, warfarin, ACE inhibitors, statins or other agents known to affect prostaglandin or cytokine synthesis
- Diabetes mellitus
- Coronary artery disease
- Connective tissue disease or other inflammatory conditions likely to affect cytokine levels
- Malignancy or other terminal illness
- Severe uncontrolled hypertension
- Immunocompromised subjects
- Substance abuse or other causes or poor compliance

All patients undergoing bariatric surgery were on liver shrinkage diet for 2 weeks prior to surgery and were advised to drink 2 liters of fluid daily. Patients were allowed to have: cheese, egg, mayonnaise, butter, meat, nuts, seed, salad, vegetables, herbs, stock cubes, lemon juice, artificial sweetners, and fizzy drinks. They were not allowed to have: alcohol, biscuits, chocolates, cakes, cereals, bread, pasta, rice, potato, sugar, pastry, pies, fruit juice, ice cream and tinned soup. The following foods were allowed in limited amounts: 1/3 pint of milk/yoghurt, three portions of fruit; 1 slice of melon, medium bowl of strawberries, orange, apple, small banana, pear, peach, 100 g of tinned fruit in natural juice and 1 portion of vegetables (2 tablespoons); sweetcorn, peas, green beans, kidney beans, baked beans.

The patients recruited from clinic had the study explained and an invitation letter and information sheet provided to them. Patients not seen in clinic, but planned to have one of the above procedures, were sent an invitation letter and information sheet by

post. All patients were contacted after 48 hrs, in order to answer their queries and obtain their decision with regards to joining this study. The study had been approved by the Moorfields and Whittington Ethical Committee.

Schematic of study design	
Screening	Identification of suitable patients from the clinic and theatre list
Clinic/Telephone	Explanation of study, answer questions and issue information sheet.
Operation day	Consent was obtained. Medical history questionnaire was completed Anthropometric measurements were obtained Fasted blood were taken Intra-operatively, subcutaneous, omental and gastric fat pad biopsies were taken.

Table 2.1: Schematic of study design. The recruitment, consent, measurements and samples taken from patients

2.1.1 Medical history questionnaire

Preoperatively a questionnaire was completed, detailing past medical history, drug history, social history and family history of heart disease or diabetes history, whether they had taken or were prescribed any medication recently.

2.1.2 Anthropometric measures

Random blood pressure and heart rate were measured by electronic blood pressure monitoring devices. Weight (in kg), height (in m), waist circumference (in cm) and hip circumference (in cm) were measured and the waist/hip ratio was calculated. Waist and hip measurements were made by plastic measuring tape. BMI was calculated by weight in kilograms divided by the square of height in metres. Body composition was measured by electrical bioimpedence analyzer (Biostat, Douglas, U.K.). This devise measured the impedance or resistance to the flow of a safe and low-level electric current through the body fluids and estimated fat and lean mass and body water as a percent of total body mass.

2.1.3 Fasting blood samples

Fasting blood samples were used for measurement of serum lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyvcerides), plasma glucose, plasma insulin and adipokines. HOMA-R index was calculated by the following formula: Glucose (mmol/l) x Insulin (MU/l) / 22.5

2.1.4 Fat biopsy

A small sample (2-4gms) of adipose tissue was obtained from the subcutaneous tissue of anterior abdominal wall and from two visceral fat samples: Omental and Gastric fat pad (figure 2.1), during the primary surgical procedure under general anesthetic. In some patients the gastric fat pad was not obtainable.

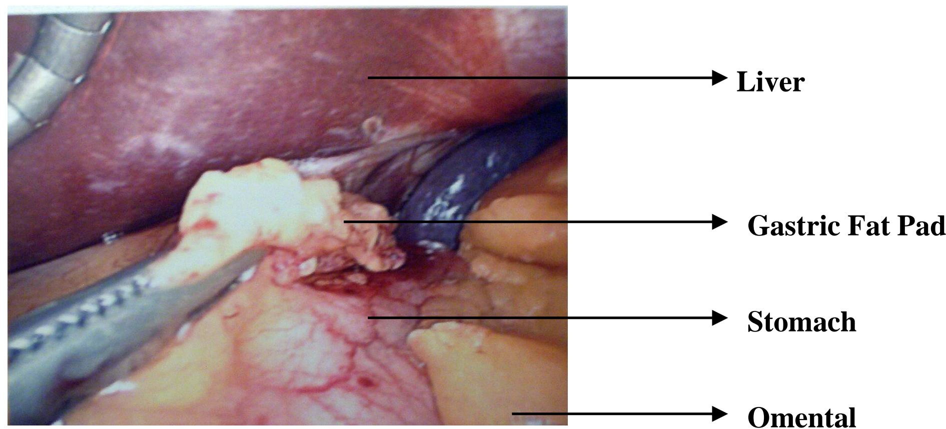


Figure 2.1: Gastric Fat Pad (GFP). This fat depot is in proximity of the stomach and macroscopically looks different from omental fat.

2.2 RNA

2.2.1 RNA extraction

Fat tissue obtained from the biopsies was stored in RNA*Later* (Ambion, USA) and kept at -20°C until RNA extraction and analysis. 0.2 gram of fat was ground in a pestle and mortar while being kept frozen with liquid N_2. The fat powder was placed in a 1.5 ml eppendorf tube and RNA extracted with the RNeasy kit (Qiagen, USA) as per manufacturer's instructions. First step of this process was the addition of 1ml of QIAzol lysis buffer to the ground tissue and the mixture was left sitting for 5 minutes at room temperature. Then 200µl chloroform was added, vigorously mixed by vortexing, left standing for 3 minutes at room temperature and spun at 13000 rpm, for 15 minutes, at 4°C. The clear top layer was then separated and equal volume of 70%

ethanol was added. The mixture was vortexed and pippetted in the RNeasy Mini Spin Column provided. The column was spun at 13000 rpm, for 15 seconds, at room temperature. The flow through was discarded and the buffers provided were next added to the column as per protocol: first with 700 µl of RW1 buffer, followed twice by 500 µl of RPE buffers. Each time the columns were spun at 13,000 rpm for 15 seconds at room temperature and the flow through discarded. Then 50µl of nuclease free water was added to the column with a fresh collection tube. After a 2-minute spin, at 13000 rpm, at room temperature, RNA was collected and stored at -80°. For RNA extraction from stromavascular fraction or adipocytes, 10µl of nuclease free water was used to achieve higher RNA concentrations.

2.2.2 cDNA synthesis

The concentration and purity of isolated RNA was assessed by measuring the optical density at 260nm (OD_{260}) and 280 nm (OD_{280}). The Ultrospec 3000 analyser, an UV/Visible spectrophotometer (Amersham pharmacia biotech, Uppsala, Sweden) was calibrated with nuclease free water. 5 µl of RNA sample was diluted in 55 µl of nuclease free water. The diluted sample was transferred into a crystal cuvette and placed in the analyser, which calculated the OD_{260}/OD_{280} ratio and the RNA concentration of the sample in µg/ml. The purity of RNA preparation was assessed by the $OD_{260}/OD_{280\ and}$ ratios of 1.4 to 1.8 were considered acceptable. Samples were done in batches of ten, before the analyser was re-calibrated.

cDNA was then synthesised from the RNA by using Taqman Reverse Transcription Reagents (Applied Biosciences, USA). Volume of RNA needed for obtaining 0.5 µg and the total reaction volume was calculated. Usual reaction volume was 25µl. Master mix was made up with dNTP, RNAse inhibitor, and random oligonucleotide primers, provided by the kit. Mixture was added up to the required volume with nuclease free water. Reverse transcriptase buffer was added during the last step. The mixture was heated at the following steps: 48 ºC for 30 seconds, 42 ºC for 2 minutes and 72 ºC for 15 minutes. Samples were held at 4ºC, the cDNA stored at - 20° if necessary prior to PCR analysis.

2.2.3 Real-time rt-PCR analysis

Specific primer sequences for Taq-man® rt-PCR analysis were purchased from Qiagen, USA. Primers were diluted to 10 µM concentration. The volume of primer used was equivalent to 20 ng of template in each tube. For each primer, a mixture containing forward primer, reverse primer and nuclease free water was added to make up a total reaction volume of 20 µl. Twenty µl of SYBR Green Master Mix (Applied Biosciences, USA) was added to each tube. Samples were run in triplicate with 10 µl total volume per well in 96-well rt-PCR plates. For each rt-PCR reaction, ß-Actin was used as the housekeeper gene.

2.2.4 ct calculations

Data were analysed according to Livak and Schmittgen for the relative quantification of real time rt-PCR data (Livak & Schmittgen, 2001). This method known as $2^{-\Delta\Delta ct}$, calculates the relative changes in gene expression between two or more samples, in which one sample is nominated as baseline (or calibrator) and all remaining samples are expressed relative to this control.

2.3 PROTEIN

2.3.1 Protein extraction and Western Blot

Fat tissue obtained from biopsy was placed in RNA*Later* and stored at -20°until analysis. The tissue (0.2 gram) was ground, while being kept frozen with liquid N_2 and lysed in protein lysis buffer (1% Triton X-100, Tris-HCL, PH 7.6, 150 mM Nacl) supplemented with protease inhibitors at 100 µM (Boehringer Mannhein, Sussex, U.K.). The homogenate was centrifuged at 13,000 rpm at 4ºC for 15 minutes and the supernatant was recovered. The protein content was estimated with a kit from BioRad, U.K., and 25 µg of total protein was separated for by SDS-PAGE electrophoresis with a 12.5% resolving gel and 5% stacking gel. Gels were blotted onto PVDF membranes and blocked with 5% non-fat dried milk and probed with polyclonal antibodies against human PGIS and α-tubulin. Polyclonal antibodies to PGI_2S were purchased from Cayman Chemical (Ann Arbor, USA) and those to α-tubulin, used as control, were obtained from Sigma (Poole, U.K.). Secondary antibodies conjugated to horseradish peroxidise were from Amersham Bioscience. Antigen- antibody complexes were detected by chemiluminescence with an ECL kit (Amersham

Biosciences, U.K.) and blots exposed to high performance chemiluminescence film (Hyperfilm ECL, Amersham Biosciences).

2.3.2 Cytokine Protein Array

The assay was carried out as per manufacturer's instructions (Proteome Profiler Array, Human Cytokine Array Panel A, R & D Systems, U.K.). Capture antibodies specific to thirty six cytokines/chemokines (mentioned below) were spotted in duplicate on nitrocellulose membranes. Each membrane was incubated for 1 hour at room temperature with blocking buffer (provided by the kit, # 895022); 350 µl of organ culture supernatant were diluted in assay buffer (provided by the kit, # 895876) to a final volume of 1.5 ml and incubated with buffer containing biotinylated detection antibody for 1 hour at room temperature. The sample/antibody mixture was then incubated with the membrane on a rocking platform at 4 ^{o}C overnight. Following a wash to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescent detection reagents (substrate provided by the kit) were added sequentially. Membranes were exposed to X-ray film (Amersham Hyperfilm ECL, GE Healthcare, U.K.) for up to 3 min. Films were scanned and quantified as pixel density using Adobe Photoshop. Signals from the negative control spots (background value) were subtracted from each spot. Positive control spots were assigned as 100% and other spots expressed relative to this. Spots with densities less than 10% of the positive controls were considered negative.

The 36 cytokines were as follows:

C5a, GCSF, CXCL1, CD54, IL-1RA, IL-6, IL-8, IL-13, IL-16, MCP-1, MIF, PAI-1, IP-10, RANTES, CD154, GM-CSF, CCL1, IFNγ, IL-1 α, IL-β, IL-2, IL-4, IL-5, IL-10, IL-12 p70, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL-11, CCL-3, CCL-4, CXCL-12, TNF-α, soluble TREM-1.

2.4 STROMAVASCULAR FRACTION EXTRACTION

Fat tissue was cleaned from vessels, weighed and cut into small pieces with fine scissors, digested with 0.2% collagenase in a shaking water bath at 37°C, 100 rpm for 1 hr. The mixture was homogenized and the stromal cells were separated from mature adipocytes by adding 2 ml of Di-isononyl phathalate and centrifuging at 2060 rcf, for 15 minutes, at 4°C. The adipocytes were separated, frozen in dry ice and stored at -80°C. The supernatant was removed and 1ml of serum free medium (Cell-gro,

Hyclone, USA) was added to the pellet (stromavascular fraction) and centrifuged at 2060 rcf, for 2 minutes, at 4°C. This process was repeated three times. The pellet was re-suspended in Cell-gro for 1 hr. The volume of Cell-gro used for re-suspension depended on the size of the pellet. The samples were then frozen in dry ice and stored at -80°C.

2.5 ORGAN CULTURE BATH

2.5.1 Adipose tissue organ cultures

The adipose organ cultures were set up with 0.2 gm of adipose tissue in 1 ml of serum-free media (Cell-gro, Hyclone, USA), incubated for 24 hours at 37°C/5% CO2. The explant supernatants were then stored at -80° and used to estimate *in vitro* release of adipokines by ELISA.

2.5.2 Incubation with COX inhibitors

Adipose tissue from subcutaneous and omental depots were divided into seven 0.1gm samples. Each tissue was incubated over 5 hrs at 37°C/5% CO_2 with two concentrations of non-selective COX inhibitors (Aspirin 0.2 mM and 5.0 mM), two COX-1 selective inhibitors (NS-398 0.01 μM and 1.0 μM) and two selective COX-2 inhibitors (Sc-560 0.01 μM and 1.0 μM) as well as a control sample incubated with only Cell-gro. Aspirin (acetylsalicylic acid) was obtained from Sigma (Poole, U.K.). NS-398 and SC-560 were purchased from Alexis Corporation (Nottingham, U.K.). Supernatant explants were then stored at -80°C. The cell culture supernatants were assayed for prostaglandins: PGE_2 and 6-keto $PGF_1\alpha$ (a stable metabolite of PGI_2).
The prostaglandin concentrations were determined using Prostaglandin E_2 (PGE_2) Biotrak Enzyme-immunoassay system and 6-keto-Prostaglandin $F_1\alpha$ (6-keto $PGF_1\alpha$) Enzyme-immunoassay Biotrak system (Amersham Biosciences, Little Chalfont, U.K.).

2.6 ELISA

2.6.1 Glucose, lipids, C-reactive protein, prostaglandins,

Plasma glucose concentration was assayed with glucose oxidase reagent (Beckman, CA, USA).
Serum triglycerides and total cholesterol were assayed with commercial reagents (total-cholesterol: Boehringer-Mannhein, Sussex, U.K. and triglycerides: Roche Diagnostics, Herts, U.K.). HDL-cholesterol was measured by the same method after the low density lipoproteins were quantitatively precipitated out by the addition of phosphotungstic acid in the presence of magnesium ions. LDL-cholesterol was calculated using the Friedwald formula (Friedewald *et al*., 1972).
C-Reactive Protein (CRP) was measured by a latex-enhanced immuno-turbidimetric (agglutination) procedure. The turbidity of the anti-CRP antibody-latex was measured,

which related to the concentration of CRP. The turbidity was measured photometrically at 340nm. 3 ul of serum samples was used. CRP was measured on the Roche/Hitachi Modular PP analyser (leased 2002) using a Roche Tina-Quant CRP (Latex) turbidemetric kit (CRPLX code 3002039). Assay time was 10 minutes, at room temperature.

Glucose, lipids and CRP assays were performed by Dr David Wickens at Chemical Pathology, Whittington Hospital (London, U.K.).

2.6.2 Cytokines

Plasma and explant supernatant levels of IL-6, RANTES, MCP-1, leptin and adiponectin were measured by 2-site ELISAs (R & D Systems, Oxon, U.K.). Insulin ELISA kit was from Mercodia (Uppsala, Sweden). Human plasma IL-6 concentrations were assayed with the high sensitivity ELISA with a limit of detection of 0.09pg/ml. All other ELISAs for the measurements of adipokine levels in culture supernatants or plasma were normal sensitivity with inter and intra-assay CVs less than 10% (R & D Systems, Oxon, U.K.).

The following dilutions were necessary for assaying plasma and explant adipokine levels:

	Serum	**Explant**
IL-6	Neat	1/200
MCP-1	1/5	1/50
Adiponectin	1/100	1/50
RANTES	Neat	Neat
Leptin	1/100	1/20
Insulin	Neat	-

Table 2.2: Dilution of sample prior to assay by ELISA

2.7 ADIPONECTIN (MULTIMERIC) EXTRACTION

Serum Multimeric Adiponectin was assayed with “Alpco diagnostic” ELISA kit (Salem, NH). With this assay, total adiponectin (total-Ad), high-molecular (HMW-Ad), middle-molecular (MMW-Ad), high-medium-molecular (HMWMMW-Ad) and low-molecular weight adiponectin (LMW-Ad) fractions could be assessed. Samples were pre-treated with protease for measurement of a specific adiponectin multimere and levels were determined either directly or indirectly. Total-Ad, HMW-Ad and

HMWMMW-Ad fractions were assayed directly. LMW-Ad values were obtained by subtracting the combined concentration of MMW-Ad and HMW-Ad from the total concentration. MMW-Ad values were obtained by subtracting HMW-Ad from the concentration of HMWMMW-Ad.

2.8 PROSTAGLANDIN EXTRACTION FROM ADIPOSE TISSUE

Protein was extracted from adipose tissue as described above (section 2.3.1). 100µl of protein was diluted with 200µl of methanol and made up to 1ml with distilled H_2O. The sample was acidified to pH 3.5 with 1 N HCL. Sep-Pak® Vac 3cc (500mg) C18 cartridges (Waters, Elstree, U.K.) were preconditioned by washing the column with 2ml methanol followed by 2ml H_2O. The sample was applied to the column, washed with 5ml H_2O, followed by 5 ml of Hexane (Fisher scientific, leister, U.K.) and eluted with 2ml of methyl formate. Methyl formate was evaporated in speedvac at 4°C, over 2 hrs, with vacuum. The residue was reconstituted with 0.5ml of Cell-gro (Hyclone, USA). Prostaglandins were assayed as specified above (section 2.6.1)

2.9 ADMA EXTRACTION

ADMA extraction with High-Performance Liquid Chromotograpgy (HPLC) was adapted from Vallance *et al.* (Vallance *et al*., 1992). The elusion buffer was made with 10% ammonia (NH_3), 40% ddH_2O and 50% HPLC methanol.

Internal Standard:

To use L-NMMA as internal standard, 40.3µl of 100mM stock of L-NMMA stock (Calbiochem, Damstadt, Germany) was added to 59.7µl of ddH_2O to obtain a concentration of 10,000 ng/µl. This was diluted with ddH2O to100 ng/µl and then to 10 ng/µl L-NMMA stock.

To use homoarginine as internal standard, 1ml of ddH_2O was added to 1 mg of Homoarginine (MW 224.7, Sigma, U.K.) to obtain a stock solution of 1mg/ml. This was then diluted with ddH_2O to obtain a 0.1 mg/ml stock.

Standards for the HPLC machine

36.3µl of 100mM ADMA or SDMA (Calbiochem, Damstadt, Germany) was added to 63.7µl of ddH_2O to obtain a 10 µg/µl concentration stock solution, which was diluted to achieve 100, 10 and 1 ng/µl concentrations. Standards of ADMA and SDMA calibrated to give 40, 20, 10, 5 and 1 ng on the column. To obtain 40 ng on column,

40µl of 10 ng/µl of ADMA and 40µl of 10 ng/µl SDMA were added, for 20 ng on column, 20µl of 10 ng/µl of ADMA and 20µl of 10 ng/µl SDMA, for 10 ng on column, 10µl of 10 ng/µl of ADMA and 10µl of 10 ng/µl SDMA, for 5 ng on column, 50µl of 1 ng/µl of ADMA and 50µl of 1 ng/µl SDMA and for 1 ng on column, 10µl of 1 ng/µl of ADMA and 10µl of 1 ng/µl SDMA were added. All solutions were made up to 100µl by adding the appropriate amount of ddH_2O. L-NMMA standard was made by adding 20µl (the volume used as internal standard) of 10 ng/µl L-NMMA with 80µl of ddH2O to add to 100µl.
Homoarginine standard was made with 5µl (the volume used as internal standard) of 0.1mg/ml of homoarginine, with 95µl ddH2O.

HPLC running buffers
Three buffers were used: Buffer A was made with 935 mls of ddH_2O, 50 mls of 1M potassium phosphate stock (MW 136, PH 6.9, Sigma, U.K.) and 15 mls of 1.5% acetronitrile for HPLC (VWR, Leister, U.K.). Buffer B was made with 45% acetronitrile, 45% methanol for HPLC (VWR, Leister, U.K.) and 10% ddH_2O. Buffer C was 5% methanol.

2.9.1 ADMA extraction from plasma
Plasma was centrifuged at 2000rpm, for 10 minutes, at 4ºC. A volume of 200 µl of plasma was removed. 20µl of internal standard (10ng/µl) L-NMMA and 780µl of PBS was added to the plasma to obtain a total volume of 1ml. Samples were loaded into a labelled solid phase extraction (SPE) column (Waters, Oasis®, U.K.). Vacuum pump was turned on (Fisher Scientific, Leistershire, U.K.) and the flow maintained at 1ml/min. The flow through was discarded and 1 ml of HCL (100mM) was added to the column. Vacuum pump was once again turned on and a flow of 1ml/min was obtained. The flow through was discarded and 1ml of methanol (CH_3OH) was added and flow through discarded as above. Next 1ml of elution buffer was added to the column and flow through was collected in labelled 1.5 ml Eppendorf tubes. The sample was placed on a heating block (80 ºC) and the solvent was evaporated to dryness under constant flow of nitrogen. The dried extract was redissolved in 100µl of ddH_2O and mixed well by vortexing. The sample was centrifuged for 2 minutes at 13000 rpm at 4ºC and 80µl was transferred into a labelled flat bottom insert (Metlab, U.K) within a screw-top glass vial. The vial was placed and sample injected into a

HPLC column (Agilent, Cheshire, U.K.), consisting of a gradient pump, an autosampler and a fluorescence detector and the absorbence was monitored.

2.9.2 ADMA extraction from adipose tissue

Adipose tissue (0.1 g) was ground with a pestle and mortar with liquid nitrogen. It was then lysed in protein lysis buffer (1% Triton X-100, Tris-HCL, PH 7.6, 150 mM Nacl) supplemented with protease inhibitors at 100 µM (Complete, Boehringer Mannhein, U.K.). The Homogenate was centrifuged at 13,000 rpm at 4 ºC, for 15 minutes and the supranatant were recovered. 100µl of lysate was mixed with 880µl of PBS and 20µl of internal standard to make a total of 1ml. The next steps for ADMA extraction were as done in plasma samples explained above in 2.9.1.

2.9.3 ADMA extraction from cell culture

Cell culture samples were centrifuged for 10 min at 2000 rpm at 4ºC. 200µl of the cell culture media was removed, 5µl of internal standard (0.1 mg/ml of homoarginine) added and the total volume made up to 1ml with 795µl of PBS. The next steps are as in described in 2.9.1.

2.10 STATISTICS

Data were analysed using SPSS version 14 for Windows software (Statistical Package for the Social Sciences, SPSS UK Ltd, Chertsey, U.K.). Data in the text are expressed as mean (SD) or median (IQR) and in figures as median (IQR) unless otherwise stated. Comparisons were carried out by *Student's* t-test or Wilcoxon for non parametric data. Correlations were determined by Spearman's Rho. Significance was defined as $p<0.05$.

CHAPTER 3: RESULTS

3.1 PATIENT RECRUITMENT

A total of 31 patients were recruited (25 female/ 6 male). Of the 25 females only 1 subject was lean (Caucasian), 5 were Afro-Caribbean, 2 were Caucasian with type 2 diabetes mellitus and 1 was non- diabetic Caucasian on Metformin. Of the 6 males, 3 were lean (1Afro-Caribbean and 2 Caucasian) and 1 Caucasian with type 2 diabetes mellitus. The studies outlined in the following sections used these patients to investigate various parameters including depot specific release and the effect of systemic insulinaemia. However, the number of patients in each of the studies and the characteristics of these sub-sets are set out separately.

3.2 CROSS SECTIONAL STUDY OF ADIPOKINES

In this section the circulating and depot specific release of five adipokines, namely MCP-1, RANTES, adiponectin, IL-6 and leptin, were assessed in a cross-sectional study of morbidly obese patients undergoing surgery. The last two adipokines have been extensively investigated in the past and were partly used as reference molecules here (Madani *et al*., 2009 (b), in press). However, none of these adipokines have been previously assessed in two different abdominal depots, namely omental and gastric fat pad.

3.2.1 Circulating adipokines

Circulating levels of the adipokines were measured. Male, Afro-Caribbean, patients who had type 2 diabetes mellitus or were on Metformin and lean patients were excluded from the analysis in order to remove the effect of known disease, sex and race bias. From three patients no gastric fat pad biopsy was obtained and they were also excluded. Therefore out of the possible 31 patients, 14 Caucasian females were analysed in this section. Anthropometric and metabolic characteristics of this sub-set of patients are shown in table 3.1.

Patient's Parameter	Value
Female / male	14 / 0
Age (years)	44 (6.5)
BMI (kg/m^2)	46.4 (6.5)
Body Fat (%)	53.4 (7.8)
Lean Mass (%)	47.3 (7.6)
Diastolic blood pressure (mmHg)	84.6 (8.6)
Total Cholesterol (mmol/l)	4.6 (0.9)
HDL cholesterol (mmol/l)	1.2 (0.3)
LDL cholesterol (mmol/l)	3 (0.9)
Triglyceride (mmol/l)	1 (0.3)
Glucose (mmol/l)	5.2 (0.5)
Insulin (MU/l)	7.8 (6.3 – 17.0)
Adiponectin (µg/ml)	4.9 (2.1– 11.0)
Leptin (ng/ml)	28.7 (19.1 –38.6)
RANTES (ng/ml)	57.0 (35.1 – 67.0)
MCP-1 (pg/ml)	220.0 (170.0 – 240.0)
IL-6 (pg/ml)	2.0 (1.0-3.0)
CRP (mg/l)	7.1 (4.9 – 11.2)

Table 3.1: Anthropometric and metabolic characteristics of patients. Data are shown as mean (SD) or median (IQR).

Interestingly all these morbidly obese female individuals, had normal glucose and lipid profiles. However in almost two thirds of the patients the normal glucose control was achieved by high insulin levels. The systolic blood pressure correlated positively with triglycerides ($r = 0.65$, $p = 0.02$) and % body fat ($r = 0.61$, $p = 0.03$) and negatively with % lean mass ($r = -0.61$, $p = 0.03$). Diastolic blood pressure, similarly,

directly related to % body fat (r = 0.55, p = 0.05) and indirectly with % lean mass (r = -0.55, p = 0.05) as well as with total cholesterol (r = -0.62, p = 0.03). Blood pressure was not associated with glucose, insulin or BMI. Measured bioimpedence was indirectly related to the systolic (r = -0.55, p = 0.05) and diastolic blood pressure (r = -0.88, p = 0.000) but directly related to total cholesterol (r = 0.54, p = 0.05) and LDL (r = 0.55, p = 0.041).

In this morbidly obese group, plasma adipokines did not correlate with age, indices of obesity, glucose or insulin sensitivity. Total adiponectin was negatively associated with both systolic blood pressure (r = -0.62, p = 0.024) and diastolic blood pressure (r = - 0.52, p = 0.07). Serum RANTES inversely correlated with HDL-cholesterol (r = -0.62, p = 0.02), serum IL-6 (r = -0.68, p = 0.008) and serum leptin levels (r = -0.67, p = 0.01). CRP did not correlate with indices of obesity, glucose, insulin or any of the circulating adipokines.

3.2.2 Depot specific release; Subcutaneous, Omental and Gastric Fat Pad

Protein array was used as the first tool to analysize the presence of 36 cytokines, including RANTES and MCP-1 (figure 3.1).

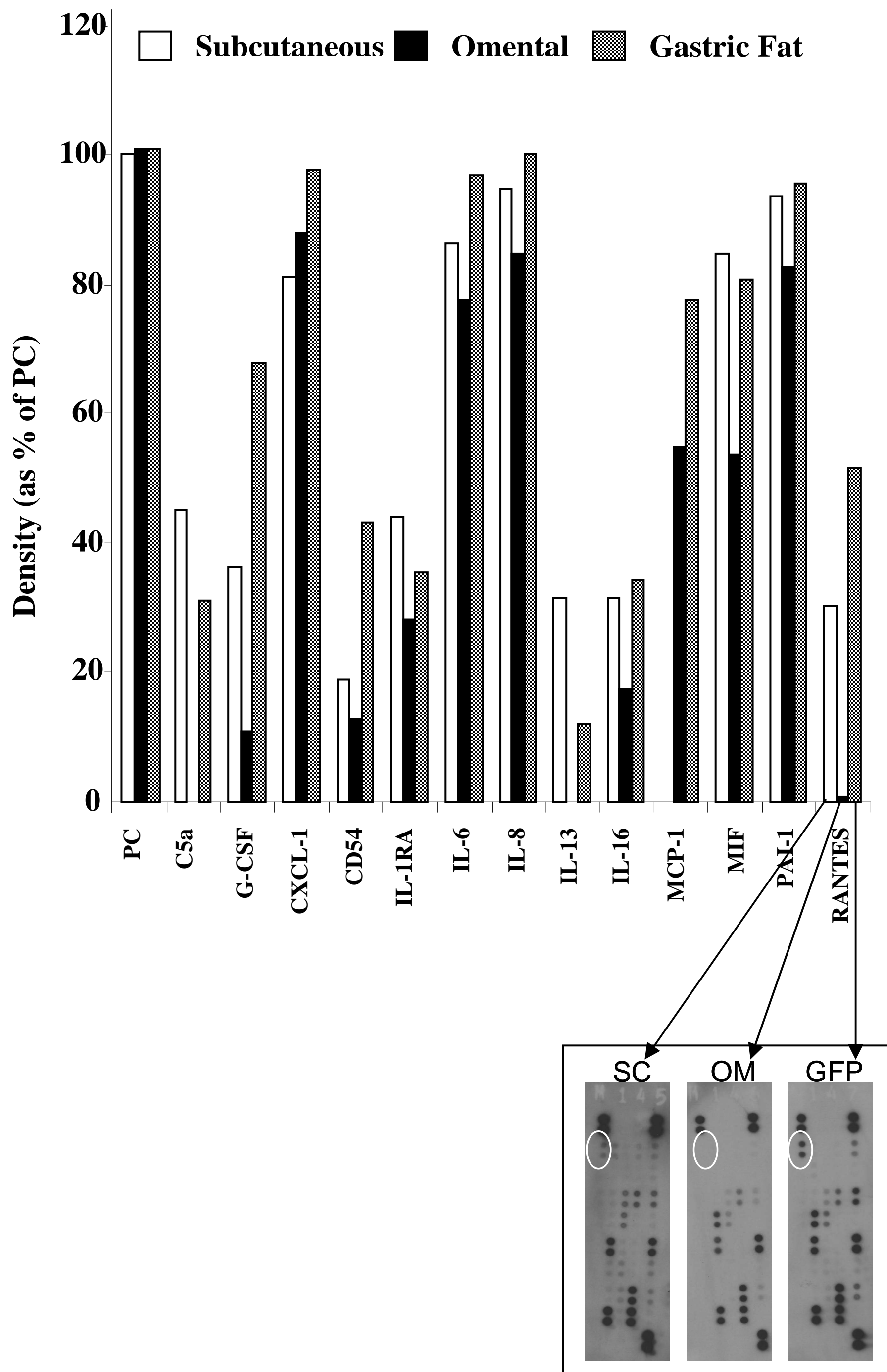


Figure 3.1: Protein array of cytokines from subcutaneous, omental and gastric fat pad depots. Thirty six cytokines were captured. Out of the possible 36 cytokines, 13 cytokine were present in the human adipose tissue with densities at least >10% of positive control (PC). G-CSF, CXCL-1, IL-6, IL-8, MCP-1, MIF, PAI-1 and RANTES had densities ≥50% of the positive control in at least one of the adipose tissue depots studied. G-CSF: Granulocyte Colony-Stimulating Factor, CXCL-1: Chemokine (C-X-C motif) Ligand-1, IL-6: Interleukin-6, IL-8: Interleukin-8, MCP-1: Monocyte Chemoattractant Protein, MIF: Macrophage migration Inhibitory Factor, PAI-1: Plasminogen Activator Inhibitor-1, RANTES: Regulated upon Activation, Normal T cell Expressed and Secreted.

Out of these 36 cytokines, 13 were present in the human adipose tissue with densities at least >10% of positive control and G-CSF, CXCL-1, IL-6, IL-8, MCP-1, MIF, PAI-1 and RANTES had densities ≥50% of the positive control in at least one of the adipose tissue depots studied. G-CSF, CD54 and RANTES showed greater expression in the gastric fat pad compared to either the subcutaneous or the omental depots (fig 3.1). To validate the protein array data using more quantitative techniques, the release of some of the adipokines were analysed by ELISA. In contrast to IL-6 and MCP-1, RANTES was released in small amounts from the adipose tissue (table 3.2) and its systemic levels were relatively high suggesting that adipose tissue is not the main source of the release of RANTES. Therefore, although it is clear that RANTES is secreted from adipose tissue but it is not certain whether these levels are high enough to act as a local chemoattractant.

	Sub-cutaneous	**Omental**	**Gastric Fat Pad**	**Plasma**
RANTES (ng)	0.03 (0.01 – 0.07)	0.04 (0.02 – 0.09)	0.1 (0.06 - 0.21)	57.0 (35.1 – 67.0)
MCP-1 (ng)	6.1 (2.6 – 9.5)	6.2 (2.5 – 13.2)	6.6 (3.9 – 8.6)	0.22 (0.17 – 0.24)
Adiponectin (ng)	21.2 (11.0 – 26.8)	20.3 (10.2 – 28.7)	16.7 (7.3 – 22.1)	4929.1 (2055.8– 10981.2)
IL-6 (ng)	2.3 (1.0 – 9.9)	2.4 (0.5 – 21.5)	6.2 (1.0 - 11.7)	0.002 (0.001-0.003)
Leptin (ng)	1.2 (0.2 - 2.9)	0.4 (0.1 - 0.6)	0.2 (0.09 - 0.9)	28.7 (19.1 –38.6)

Table 3.2: *In vitro* production rate of adipokines. Adipokine production is calculated as ng per gm of adipose tissue per hour in organ culture baths and ng per ml in circulating levels. Data shown in Median +/- IOR (n = 14).

RANTES was released in significantly higher amounts, per gm of tissue, from the gastric fat pad compared to the omental (p = 0.03) or subcutaneous (p = 0.01) depots (figure 3.2). Subcutaneous leptin release was significantly higher compared to omental depot (p = 0.05), but there was no difference between either of these depots and the gastric fat pad release. There was a trend towards higher subcutaneous adiponectin release compared to that from the gastric fat pad (p = 0.08) but did not

reach significance. Neither MCP-1, nor IL-6 release were significantly different between the depots. Comparisons were made using Wilcoxon rank test.

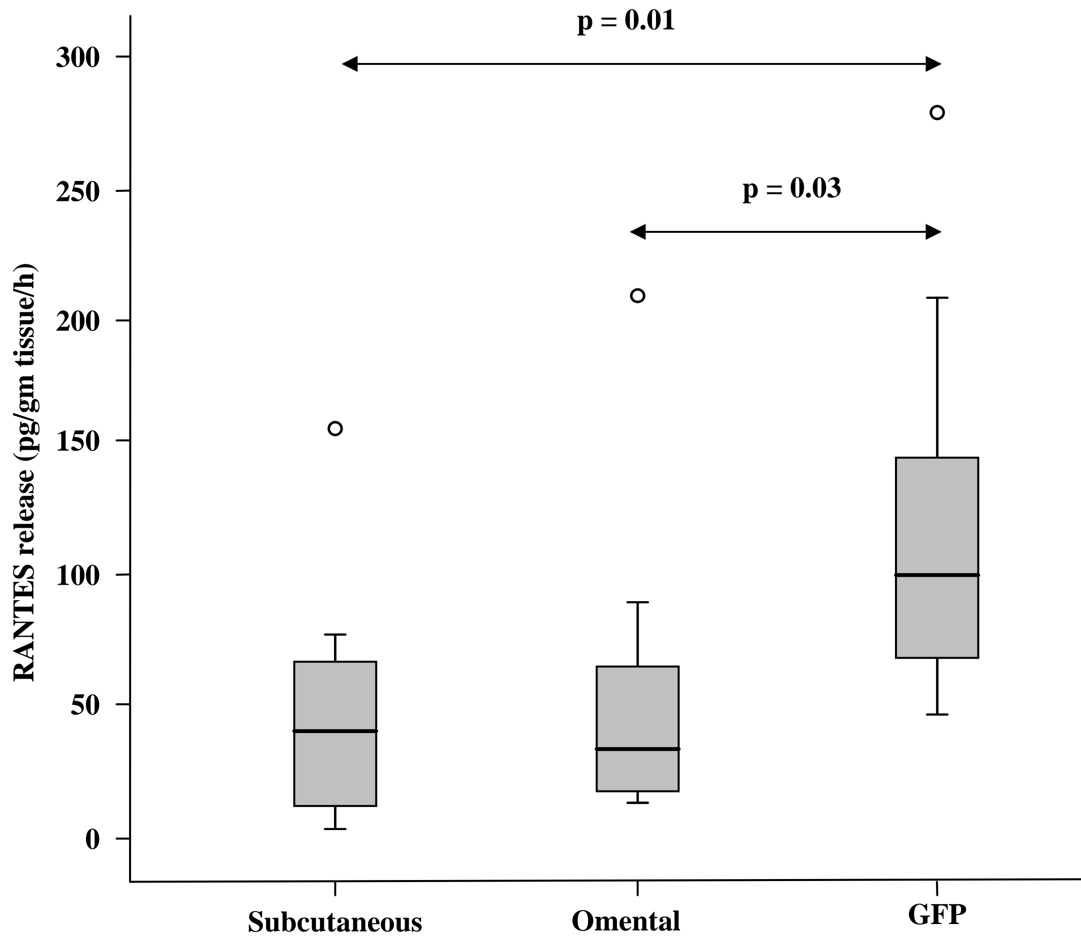


Figure 3.2: Depot specific RANTES release. RANTES was released in significantly higher amount from the Gastric Fat Pad (GFP) compared to subcutaneous (p = 0.01) or omental (p = 0.03) depots. Comparisons were made by Wilcoxon rank test.

3.2.3 Chemokine interactions

Subcutaneous RANTES release correlated significantly with that from omental tissue ($r = 0.54$, $p = 0.05$). The release of RANTES from both these depots correlated significantly with omental MCP-1 (for subcutaneous RANTES $r = 0.66$, $p = 0.02$; for omental RANTES $r = 0.80$, $p = 0.001$) and omental IL-6 (for subcutaneous RANTES $r = 0.62$, $p = 0.02$; for omental RANTES $r = 0.55$, $p = 0.04$). There were no significant correlations between the release of RANTES from the gastric fat pad and the other adipokines. Furthermore, subcutaneous MCP-1 release correlated with gastric fat pad MCP-1 ($r=0.74$, $p=0.01$) and omental MCP-1 with omental IL-6 ($r = 0.65$, $p = 0.02$). There were no associations between the release of any of the adipokines from the organ bath and serum CRP.

In the next two sections, the study focused on two pathways that may mediate adipokine secretion and function, namely the COX and NOS pathways. In section 3.2, the *in vitro* inhibition of the components of the COX pathway was investigated; namely the activity of PGE_2S and PGI_2S and the secretion of the related prostaglandins. In section 3.3 the generation of ADMA and the expression DDAH, as components of the NOS pathway, as well as the release of adiponectin and the effect of insulin, were assessed.

3.3 IL-6 AND COX PATHWAY

In this section the effect of *in vitro* inhibition of the COX pathway, with non-selective and selective COX inhibitors, on the release of IL-6 from different depots was assessed in a subgroup of patients. Activities of PGI_2S and PGE_2S were measured as well as the protein expression in both subcutaneous and omental depots. The expression was then compared between the adipocytes and the stoma vascular fraction of the adipose tissue.

In this group of morbidly obese female (patient characteristics shown in table 3.1) no significant difference was seen between the subcutaneous and omental IL-6 release (figure 3.3).

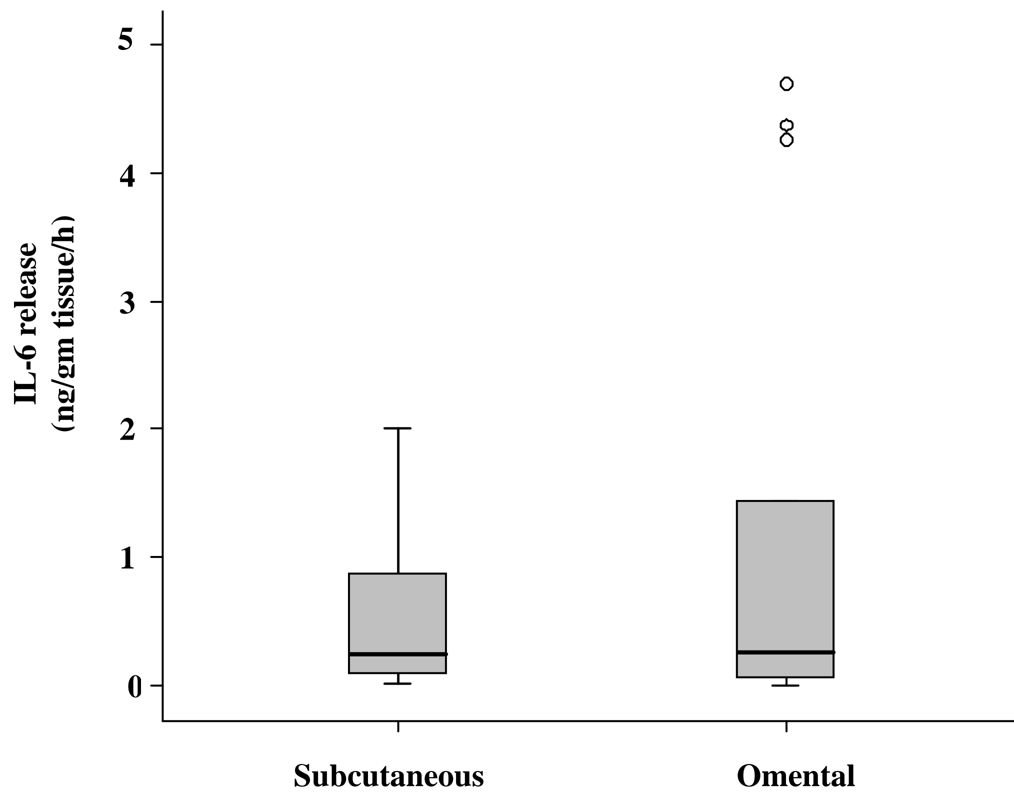


Figure 3.3: Depot specific differences in IL-6 release in human white adipose tissue. No significant difference between the two depots (n=14). Graph shown as box plot and data as median ± IQR. Comparisons were made by Wilcoxon Rank test.

The effect of Aspirin at low dose (0.2 mM) and high dose (5 mM) as well as NS-398 (COX-2 selective inhibitor) and SC-560 (COX-1 selective inhibitor) at low dose (0.01μM) and high dose (1μM) on IL-6 release in human organ culture was assessed in a subgroup of patients. IL-6 release from the subcutaneous depot was significantly reduced by low dose aspirin but not by high-dose aspirin (n=5, p = 0.043, figure 3.4). This effect was not seen in the omental depot. Subcutaneous IL-6 release was reduced by low (p = 0.08) and higher dose (p = 0.07) NS-398, but, they did not reach significance. No effect was seen in the omental depot. SC-560, contrary to that in mouse adipose tissue had no significant effect on IL-6 release from subcutaneous or omental depots at either dose (data not shown).

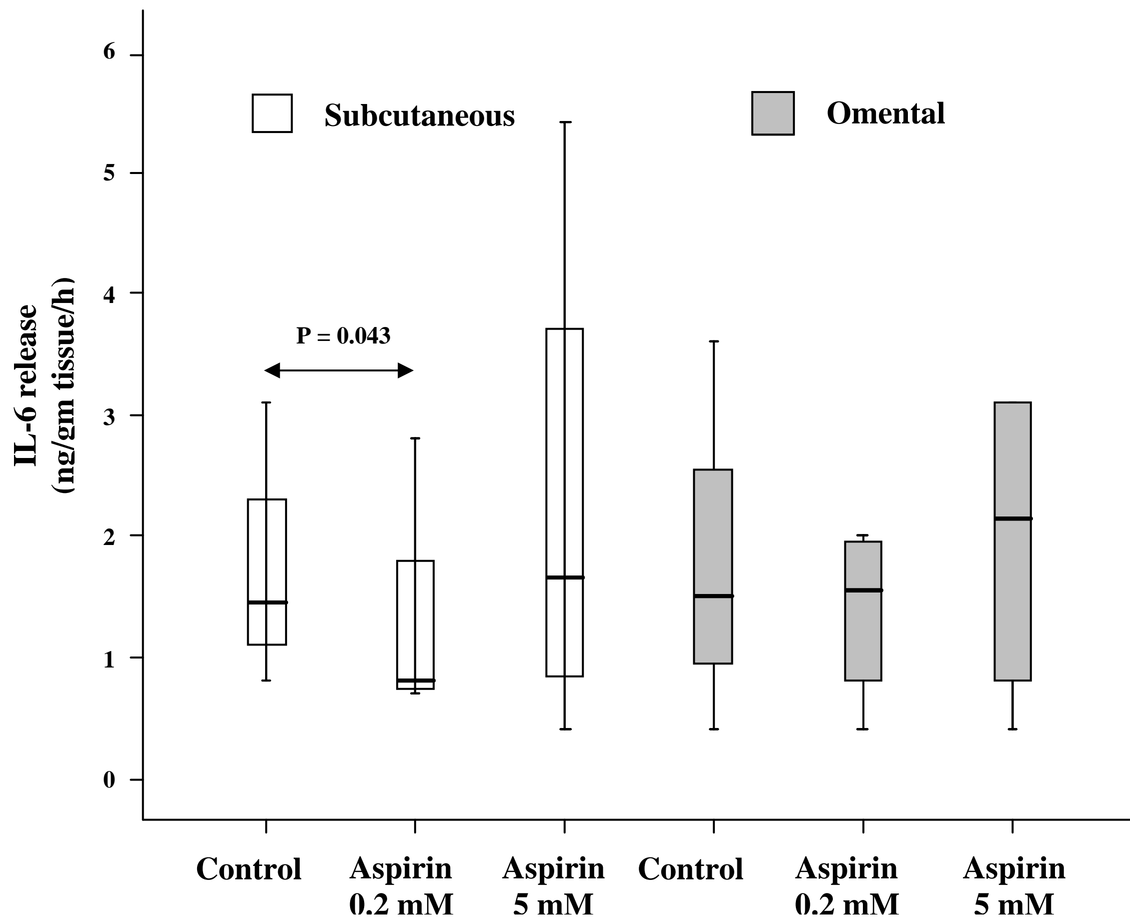


Figure 3.4: The effect of *in vitro* COX inhibition on IL-6 release from human subcutaneous and omental white adipose tissue. Aspirin, a non-selective COX inhibitor, significantly reduced subcutaneous IL-6 release at the low dose only (p = 0.04). This was not seen in the omental depot. Graph shown as box plot and data as median ± IQR (n = 5). Comparisons were made by Wilcoxon Rank test.

Activities of PGI_2S and PGE_2S were measured as 6-keto $PGF_1\alpha$ (stable prostacyclin metabolite) and PGE_2 release respectively and expressed in ng of the prostaglandin released per gm of adipose tissue per hour. There was no significant correlation between IL-6 release and PGE_2S or PGI_2S activity in either subcutaneous (PGE_2S: $r = -0.205$, $p = 0.74$; PGI_2S: $r = 0.316$, $p = 0.541$) or omental (PGE_2S: $r = 0.316$, $p = 0.684$; PGI_2S $r = 0.632$, $p = 0.368$) depot. However there was significant direct correlation between subcutaneous PGI_2S and omental PGE_2S activity ($r = 0.949$, $p = 0.014$). PGI_2S activity was found to be higher in the omental depots (figure 3.5) but Aspirin, COX-1 or COX-2 had no effect on PGI_2S or PGE_2S activity. All correlations were performed by Spearman's Rho.

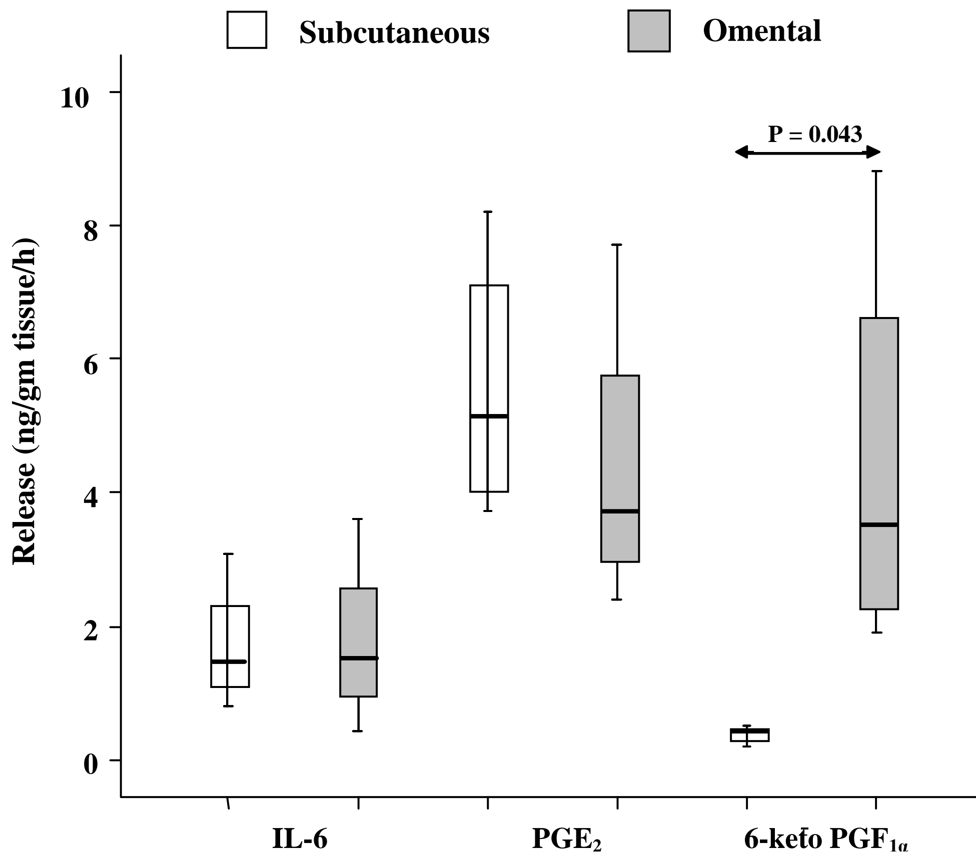


Figure 3.5: Comparison of subcutaneous and omental PGE_2S and PGI_2S activity. 6-keto $PGF_1\alpha$ (PGI_2S activity) is lower in subcutaneous compared to omental depots ($p = 0.043$). No difference is seen in the IL-6 or PGE_2 release between depots. Graph shown as box plot and data as median ± IQR ($n = 5$). Comparisons were made by Wilcoxon.

In a subgroup of patients, the stromavascular fraction was separated from adipose tissue and PGI_2S protein expression was investigated by Western blot analysis. There was higher PGI_2S expression in the stromavascular fraction of the omental depots compared to the subcutaneous stromavascular fraction (figure 3.6).

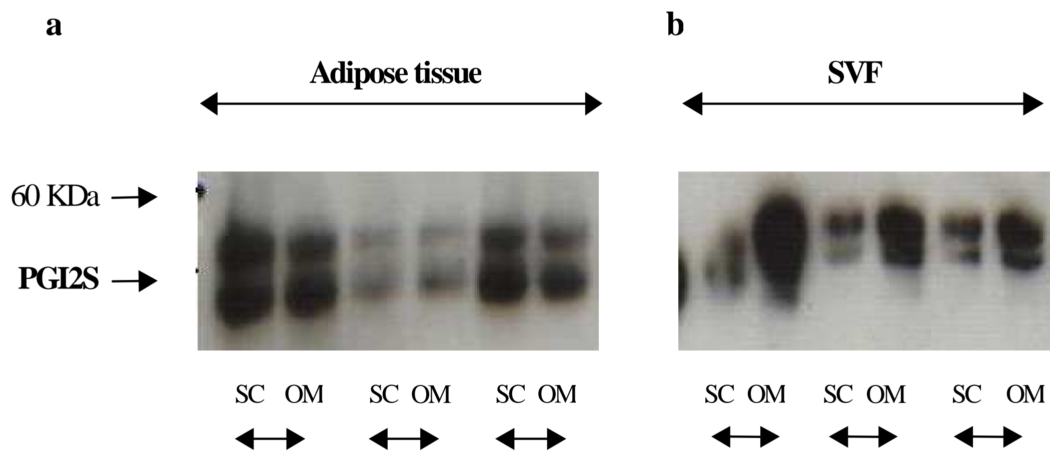


Figure 3.6: Western blot analysis for PGI_2 synthase in: (a) human adipose tissue (b) the enriched the stromavascular fraction (SVF). The depot specific difference in PGI_2S expression is apparent in the SVF but not in the intact adipose tissue. Conditions: Primary antibody anti-human PGI_2 synthase; 1:1000 dilution overnight at 4^oC. Secondary ant-rabbit IgG-horseradish peroxidase conjugated antibody at 1:3000 dilution for 2 h at room temperature. Film exposed for 10 minutes. Molecular weight of PGI_2S is 57 KDa.

3.4 ADMA, DDAH, ADIPONECTIN AND THE EFFECT OF INSULIN

Although all patients in this study group were normoglycaemic, this was achieved by high insulin levels in some patients. Therefore the aim of this study was to investigate the effect of insulin by assessing the differences in the expression of DDAH, ADMA and release of adiponectin between the normoinsulinaemic and hyperinsulinaemic groups, as well as comparing the subcutaneous and omental depots.

In this section of the possible 31 patients 17 were analysed. In order to exclude the effect of sex and race; 5 Afro-Caribbean female, 6 male, 2 patient with type 2 diabetes and 1 obese patient on Metformin, were excluded. Details of the patients are in table 3.3.

Patient's Parameter	Value
Female / Male	17 / 0
Age (years)	43.44 (7.87)
BMI (kg/m^2)	43.83 (10.14)
Body fat (%)	52.57 (7.05)
Lean mass (%)	48.3 (7.7)
Insulin (MU/l)	7.75 (7.13 – 15.03)
Total Cholestrol (mmol/l)	4.55 (3.85-4.88)
HDL cholesterol (mmol/l)	1.2 (1.0 – 1.4)
LDL cholesterol (mmol/l)	2.95 (2.3 – 3.28)
TG cholesterol (mmol/l)	1.1 (1.0 – 1.2)
CRP (mg/l)	7.1 (4.9 – 11.2)

Table 3.3: Anthropometric and metabolic characteristics of patients. Data are shown in mean +/- SD or median +/-IQR.

Systolic blood pressure positively correlated with BMI ($p = 0.05$, $r = 0.48$) and glucose ($r = 0.47$, $p = 0.05$). Diastolic blood pressure, although not statistically significant, was closely related to BMI ($r = 0.43$, $p = 0.08$). Bioimpedence was associated with total cholesterol ($r = 0.5$, $p = 0.04$), LDL ($r = 0.53$, $p = 0.03$) and percentage lean mass ($r = 0.5$, $p = 0.04$) but negatively associated with BMI ($r = -0.71$,

p = 0.001), percentage body fat (r = -0.5, p =0.04) and diastolic blood pressure (r = -0.6, p = 0.02). Systemic CRP levels did not correlate with insulin, glucose, blood pressure or any indices of obesity.

3.4.1 ADMA

Plasma ADMA and SDMA were analysed and ADMA/SDMA ratio was calculated. Serum ADMA correlated with serum SDMA (r = 0.79, p= 0.000). Circulating ADMA correlated negatively with triglyceride (r = -0.53, p= 0.03) and there was no association with BMI or glucose. However there was a trend towards positive correlation between circulating ADMA and insulin (r = 0.45, p = 0.061). ADMA/SDMA ratio was directly associated with systolic blood pressure (r = 0.53, p = 0.03) and although closely related, it did not reach statistical significance with the diastolic blood pressure (r = 0.46, p = 0.07).

ADMA extracted from subcutaneous and omental explants were successful in 13 patient. Explant ADMA release was significantly higher from the omental depot compared to the subcutaneous (figure 3.7). Explant SDMA extraction was completed in 9 patients. Tissue ADMA and SDMA was only successfully extracted from adipose tissue of six patients and no significant differences were found between depots. Insulin also positively correlated with subcutaneous tissue ADMA (r = 0.83, p = 0.04) and subcutaneous tissue SDMA (r = 0.70, p = 0.03) and nearly associated with omental tissue ADMA (r = 0.7, p = 0.08) and omental tissue SDMA (r = 0.7, p = 0.08). However insulin negatively correlated with subcutaneous explant SDMA (r = -0.89, p = 0.02). BMI was positively (r = 0.57, p = 0.45) and percentage lean mass was negatively (r = -0.56, p = 0.05) associated with omental explant ADMA. Systolic blood pressure indirectly related to omental tissue SDMA (r = -0.83, p = 0.02) and there was a close indirect, but not statistically significant, association with subcutaneous tissue ADMA (r = -0.78, p = 0.07). HDL correlated negatively with subcutaneous explant ADMA (r = -0.56, p = 0.05) and positively with subcutaneous explant SDMA (r = 0.85, p = 0.04) depots. Triglyceride negatively correlated with omental tissue SDMA (r = -0.74, p = 0.06). Subcutaneous tissue SDMA correlated negatively with cholesterol (r = 0.78, p = 0.04) and LDL (r = 0.78, p = 0.04). CRP was strongly related to subcutaneous tissue ADMA (r = 0.9, p = 0.03). All correlations were by Spearman's Rho.

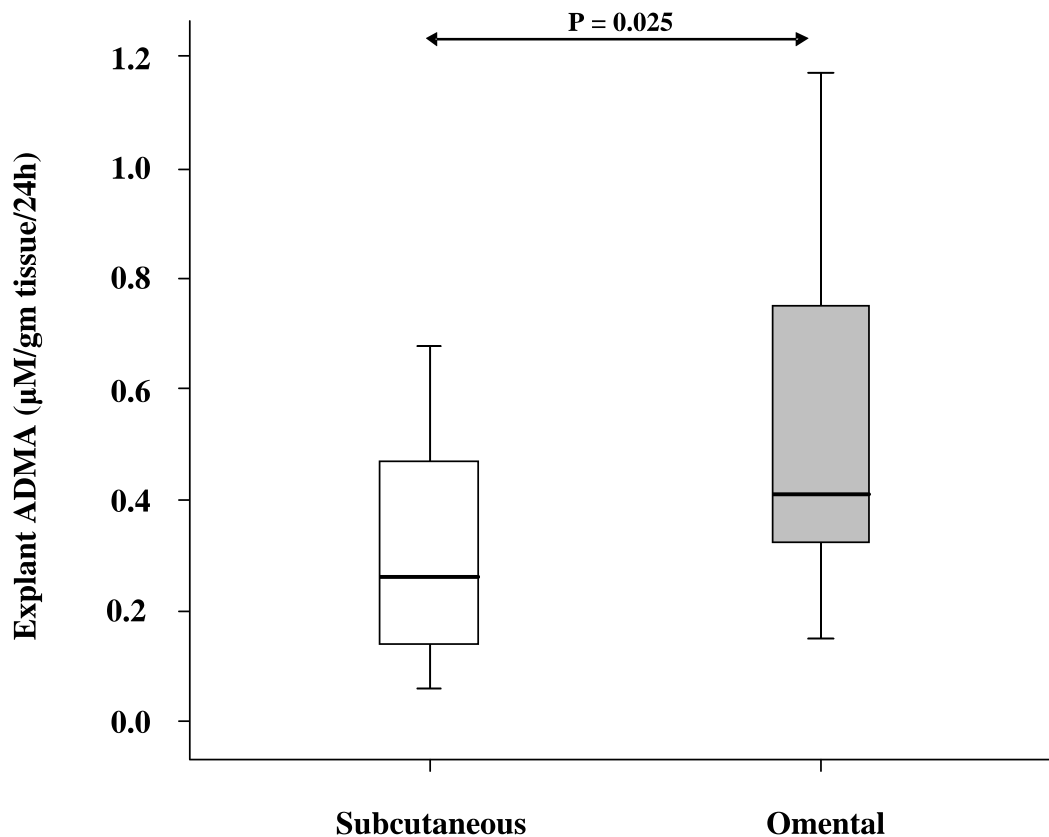


Figure 3.7: ADMA extraction from explants. Omental ADMA levels were significantly higher than those from the subcutaneous depot (p = 0.025). Explants were set up as 0.2gm of adipose tissue in 1 ml of organ culture for 24 hrs. Graph shown as box plot and data as median ± IQR (n = 13, 4 missing data). Comparisons were made by Wilcoxon Rank test.

3.4.2 DDAH expression

DDAH1 and DDAH2 primers were tested with dilution series (figure 3.8). GAPDH or/and β-actin were used as control.

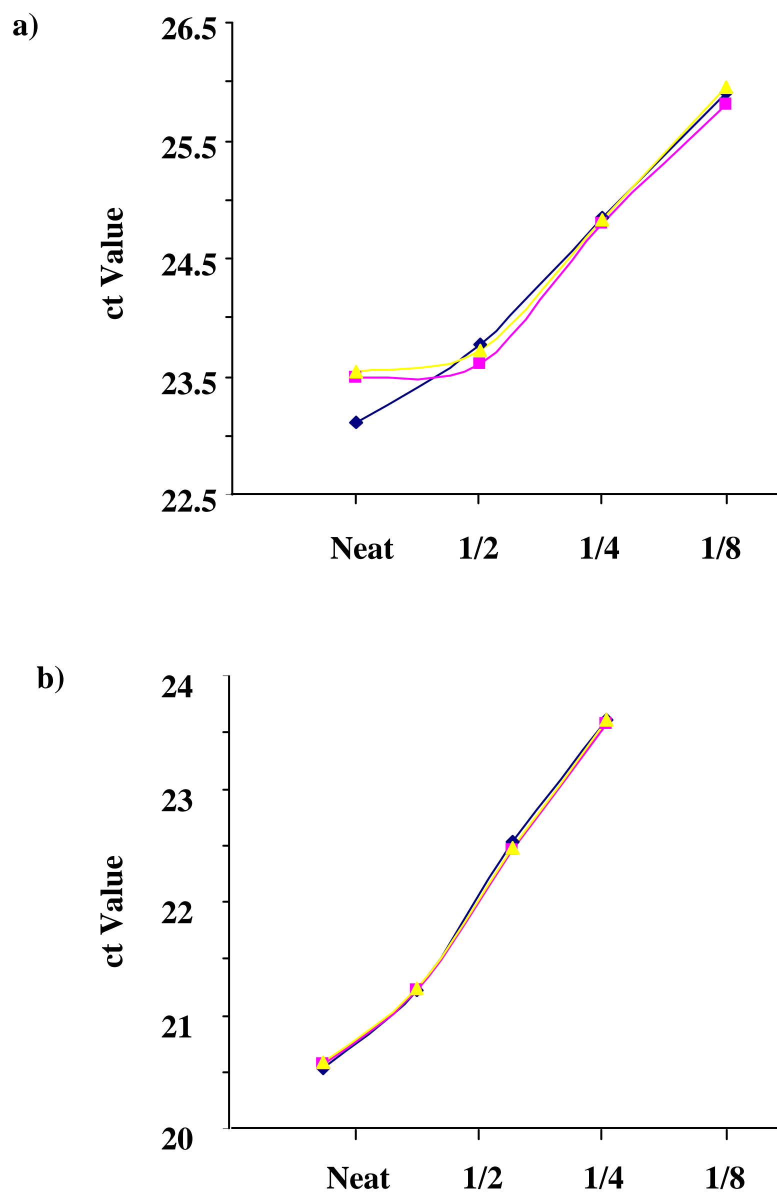


Figure 3.8: Dilution series. a) Dilution series of DDAH2 b) Dilution series of β-actin. Dilutions used were: neat, one in two, one in four and one in eight. Samples were done in triplicate. Each colour represents one series of dilution.

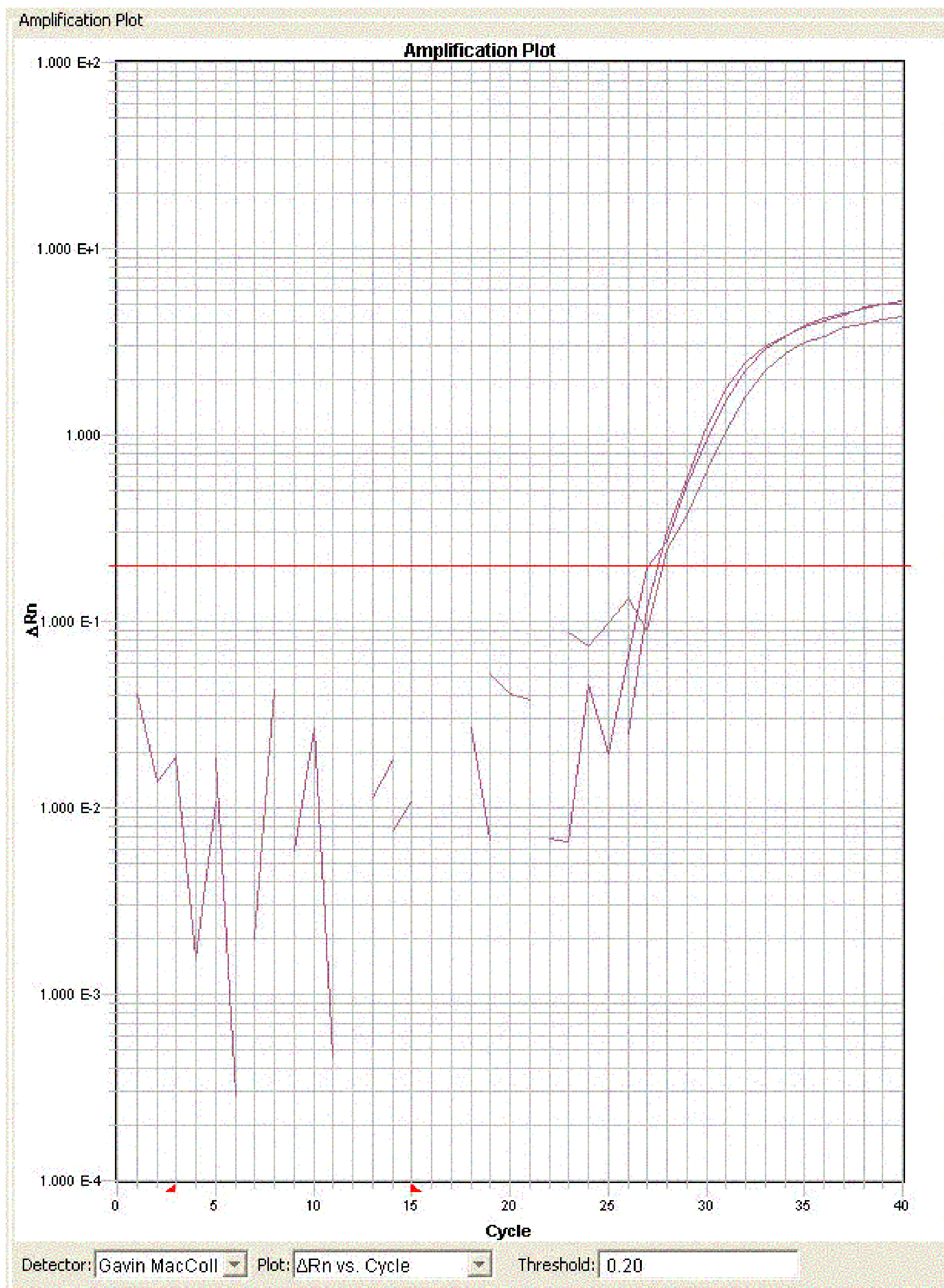


Figure 3.9: Amplification plot of DDAH1. Taq-man® rt-PCR amplification plot of DDAH1. Samples were tested in triplicates. Amplification plot shows a close overlap of the ct values of the triplicates.

Omental DDAH1 expression was directly related to percentage lean mass ($r = 0.54$, $p = 0.04$). There was no correlation between DDAH1 or DDAH2 expression and BMI, blood pressure, serum glucose, insulin, lipids or CRP.

Not surprisingly there was a direct correlation between subcutaneous and omental; Δct DDAH1 ($r = 0.69$, $p = 0.004$) and this nearly reached significance with subcutaneous Δct DDAH2 ($r = 0.48$, $p = 0.06$). There was no difference in expression of DDAH1 or DDAH2 between subcutaneous and omental depots (figure 3.10). $\Delta\Delta$ct values were used as explained in section 2.2.4.

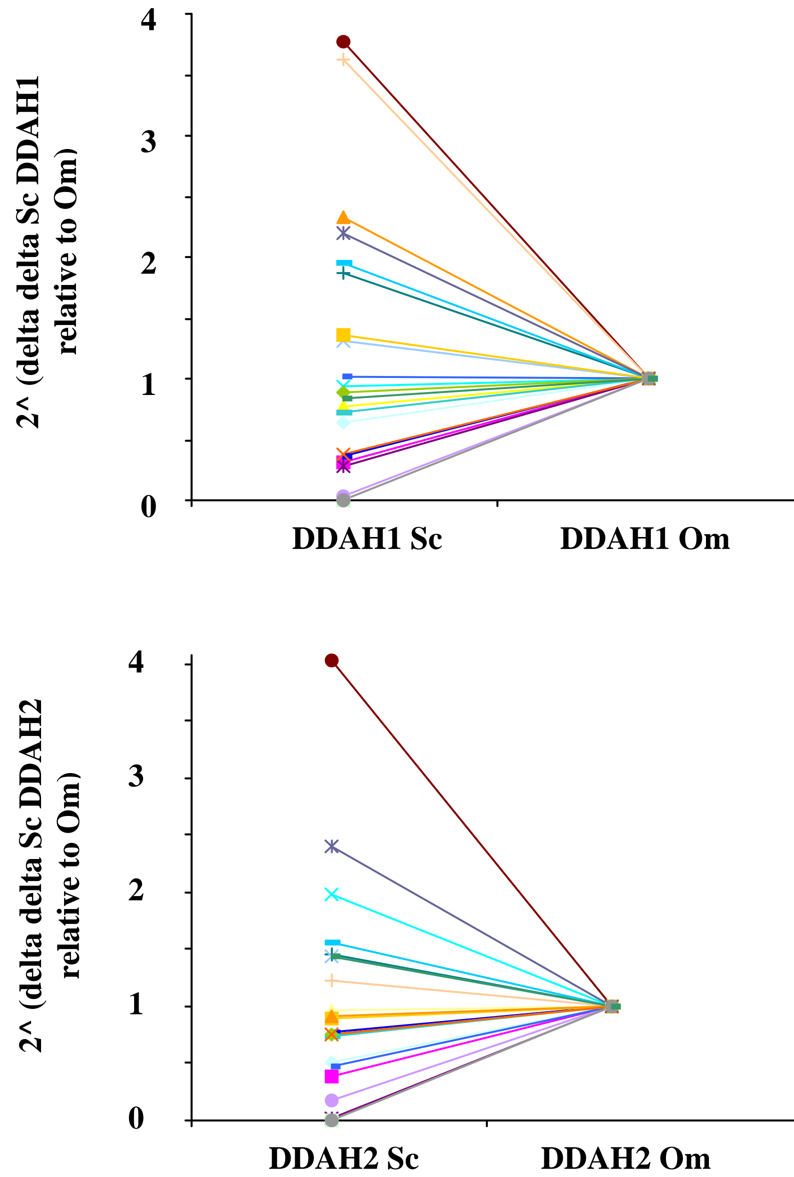


Figure 3.10: Comparison between subcutaneous and omental DDAH1and 2. No difference found between subcutaneous (Sc) and omental (Om) DDAH1 & DDAH2 expression. Formula explained in section 2.2.4(n =15, 2 missing)

DDAH 2 was expressed in higher amount compared to DDAH 1 in both depots (figure 3.11).

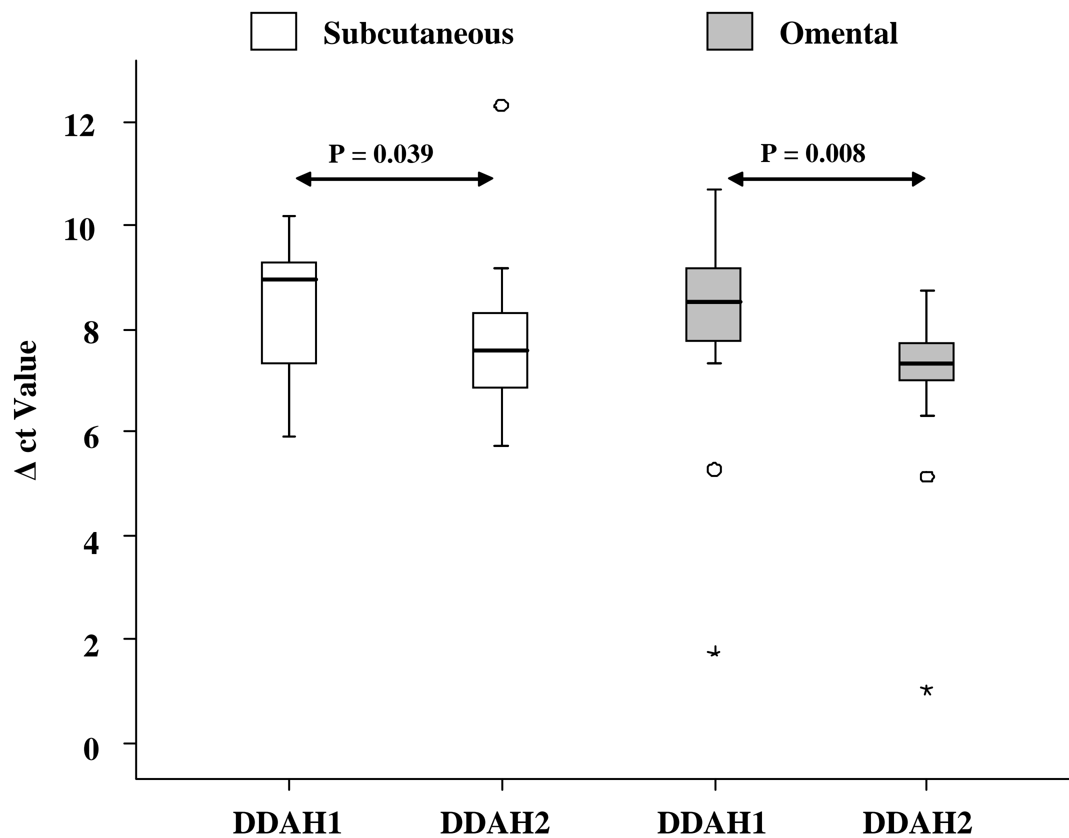


Figure 3.11: Comparison of DDAH1 and DDAH2 in subcutaneous and omental depots. There were significantly higher levels of DDAH2 compared to DDAH1 in both omental ($p = 0.008$) and subcutaneous ($p = 0.039$) depots. Graph shown as box plot and data were median +/- IQR ($n = 15$). Comparisons were made by Wilcoxon.

There were no significant differences in the expression of DDAH1 or DDAH2 from the stromavascular fraction between depots either. There was higher expression of DDAH2 compared to DDAH1 in the stromavascular fraction of both subcutaneous (p = 0.0046) and omental (p = 0.018) depots but not in the whole, unfractionated adipose tissue of each depots (figure 3.12).

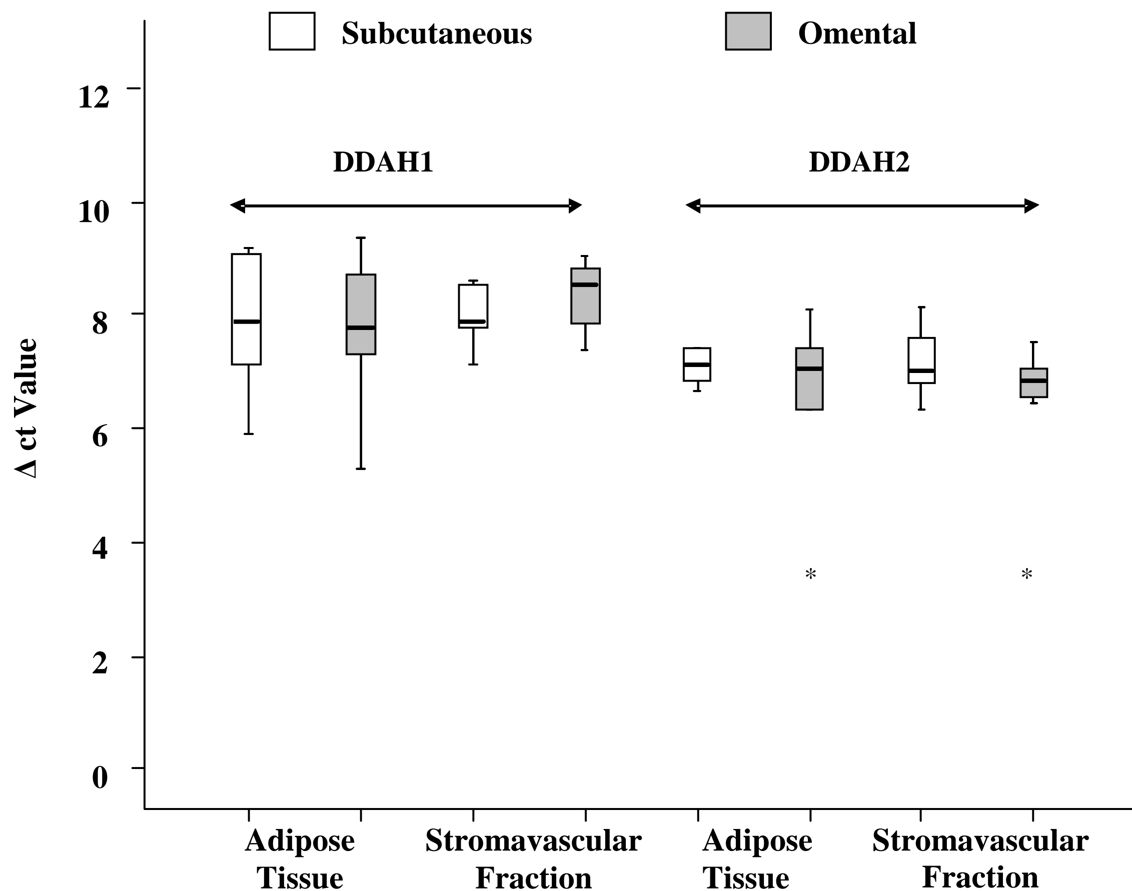


Figure 3.12: Comparison of DDAH1 and DDAH2 expression between adipose tissue and stromavascular fraction of subcutaneous and omental depots.
No depot specific differences were seen of DDAH1 or DDAH2 in adipose tissue or the stromavascular fraction. There was higher expression of DDAH2 compared to DDAH1 in the stromavascular fraction of both subcutaneous (p = 0.0046) and omental (p = 0.018) depots but neither in subcutaneous adipose tissue (p = 0.859), nor in omental adipose tissue (p = 0.086). Graph shown as box plot of Δ ct values and data as median +/- IQR (n = 9). Comparisons were made by Wilcoxon.

3.4.3 Adiponectin

In chapter 3.1.1 no correlations were found between any of the adipokines, including serum immunoreactive adiponectin, and insulin or the indices of obesity, perhaps because this is a morbidly obese population. As adiponectin may circulate in different multimeric structures, serum samples were re-analysed measuring the total adiponectin, as well as the levels of high molecular weight (HMW), medium molecular weight (MMW), low molecular weight (LMW) and HMW+MMW isoforms.

There was a trend towards a negative correlation between insulin and total adiponectin ($r = -0.47$, $p = 0.06$), MMW ($r = -0.42$, $p = 0.092$) and LMW ($r = -0.47$, $p = 0.07$). BMI also was negatively associated with total adiponectin ($r = -0.52$, $p = 0.05$), HMW ($r = -0.52$, $p = 0.031$), LMW ($r = -0.57$, $p = 0.016$) and MMW+HMW ($r = -0.48$, $p = 0.05$). Serum total adiponectin was positively associated with HDL-cholesterol ($r = 0.61$, $p = 0.01$), HMW ($r = 0.58$, $p = 0.02$), MMW ($r = 0.61$, $p = 0.02$), HMWMMW ($r = 0.6$, $p = 0.01$), but not with LMW.

Both systolic and diastolic blood pressures were negatively associated with total serum adiponectin ($r = -0.53$, $p = 0.03$; $r = -0.60$, $p = 0.02$), HMW ($r = -0.67$, $p = 0.005$; $r = -0.62$, $p = 0.008$), MMW ($r = -0.50$, $p = 0.05$; $r = -0.48$, $p = 0.05$), MMWHMW ($r = -0.63$, $p = 0.001$; $r = -0.61$, $p = 0.01$). There was no correlation between circulating adiponectin and CRP.

Adiponectin release in explants did not correlate with BMI, glucose or insulin levels. However subcutaneous explant adiponectin showed a positive trend with total adiponectin ($r = 0.44$, $p = 0.09$) and with HMW ($r = 0.45$, $p = 0.07$). Subcutaneous and omental explant adiponectin were also closely related ($r = 0.56$, $p = 0.02$).

Correlation between ADMA, DDAH and adiponectin

Although neither the serum ADMA, nor SDMA correlated with circulating adiponectin levels, the ADMA/SDMA ratio was negatively associated with total adiponectin ($r = -0.51$, $p = 0.04$), HMW ($r = -0.54$, $p = 0.03$), LMW ($r = -0.60$, $p = 0.01$), MMW+HMW ($r = -0.50$, $p = 0.04$) and MMW ($r = -0.44$, $p = 0.08$). There were no significant associations between serum ADMA/SDMA or explant ADMA release and adiponectin explant secretion from either depot.

Depot ADMA release was not related to serum adiponectin levels but there was a significant positive correlation between subcutaneous SDMA and total adiponectin (r = 0.94, p = 0.005), HMW (r = 0.94, p = 0.005and MMWHMW (r = 0.94, p = 0.005). Subcutaneous Δct DDAH2 correlated negatively with serum ADMA (r = -0.50, p = 0.05) and serum SDMA (r = - 0.73, p = 0.001).

Subcutaneous Δct DDAH1 correlated with subcutaneous adiponectin levels (r = 0.70, p = 0.002), and with subcutaneous Δct DDAH2 (r = 0.49, p = 0.057) and omental Δct DDAH1 (r = 0.69, p = 0.004). Omental Δct DDAH1 and omental Δct DDAH2 were also closely related (r = 0.55, p = 0.035). Δct DDAH1 and Δct DDAH2 from neither depot was related to serum adiponectin levels. All correlations were made by Spearman's Rho.

3.4.4 Effect of insulinaemia on ADMA, DDAH and adiponectin

Patients were divided into two groups depending on their fasting systemic insulin levels and data were compared (table 3.4). Circulating total adiponectin, HMW, MMW and HMWMMW levels were all significantly higher in the normoinsulinaemic group but depot specific release of adiponectin was not different between the two groups.

Both the adipose tissue content and release of ADMA and SDMA were only available in 1 normoinsulinaemic patient and therefore analysis was not possible. Other data were available in 2 of normoinsulinaemic obese patients and therefore all comparisons below should be considered rather preliminary. All comparisons were made by independent t-test. Serum ADMA was lower in the normoinsulinaemic group (p = 0.046); mean [1.9(0.1) vs 1.7(0.55) μM/l}. There were no differences in subcutaneous and omental DDAH 1 and DDAH 2 expression between the groups.

	Normoinsulin-aemia	Hyperinsulin-aemia	P value
Number	4	13	
Age (years)	43.75 (4.35)	42.9 (8.65)	NS (0.9)
Sex	All Female	All Female	
Body fat (%)	52.78 (10.64)	51.37 (5.4)	NS (0.8)
BMI (kg/m^2)	43.2 (16.1)	42.5 (9.7)	NS (0.9)
Glucose (mmol/l)	4.6 (4.45 – 4.78)	5.3 (4.9 – 5.8)	0.009
Insulin (MU/l)	5.11 (4.4 – 5.43)	9.8 (7.6 – 17)	0.023
Total Adiponectin (µg/ml)	7.0 (2.1 – 11.1)	3.21 (2.3 – 4.5)	0.031
HMW-Ad (µg/ml)	3.3 (0.7 – 6.1)	1.1 (0.69 – 1.96)	0.03
MMW-Ad (µg/ml)	1.43 (0.48 – 2.5)	0.83 (0.6 – 1.14)	0.03
LMW-Ad (µg/ml)	1.97 (0.9 –2.28)	1.01 (0.75 – 1.64)	NS (0.12)
HMW+MMW-Ad (µg/ml)	5.05 (1.2 – 8.8)	2.02 (1.3 – 2.96)	0.03
Sc Adiponectin (ng/ml/hr)	31.97 (13.66 – 49.45)	21.1 (11.2 – 29.8)	NS (0.12)
Om Adiponectin (ng/ml/hr)	23.1 (11.82 – 101.56)	19.4 (10.2 – 28.51)	NS (0.89)

Table 3.4: Comparison of the circulating and tissue release of adiponectin in normoinsulinaemic and hyperinsulinaemic individuals. Insulin levels > 7.0 MU/l were recorded as hyperinsulinaemia. Data are shown in mean (SD) of median (IQR). Comparisons were made by independent T Test, equal variance assumed. HMW: High Molecular Weight, LMW: Low Molecular Weight, MMW: Medium Molecular weight.

Serum ADMA and adiponectin levels were analysed in 25 patients, including male, lean and diabetic patients but excluding the 6 Afro-Caribbean individuals. As the numbers were small adequate comparisons could not be made. However patients were divided into the five groups shown in figure 3.13 to assess any interesting trends that may later be followed up in greater number of patients. Serum ADMA levels seemed comparable and elevated in the obese individual, whether normoinsulinaemic or hyperinsulinaemic, compared to lean individuals. Circulating ADMA was also lower in the obese normoinsulinaemic and hyperinsulinaemic subjects on Metformin compared to obese subjects not on Metformin (figure 3.13).

Plasma adiponectin was, as expected, higher in the lean compared to the obese hyper-insulinaemic patients. Patients on Metformin had elevated plasma adiponectin compared to their counterparts not on Metformin (figure 3.14).

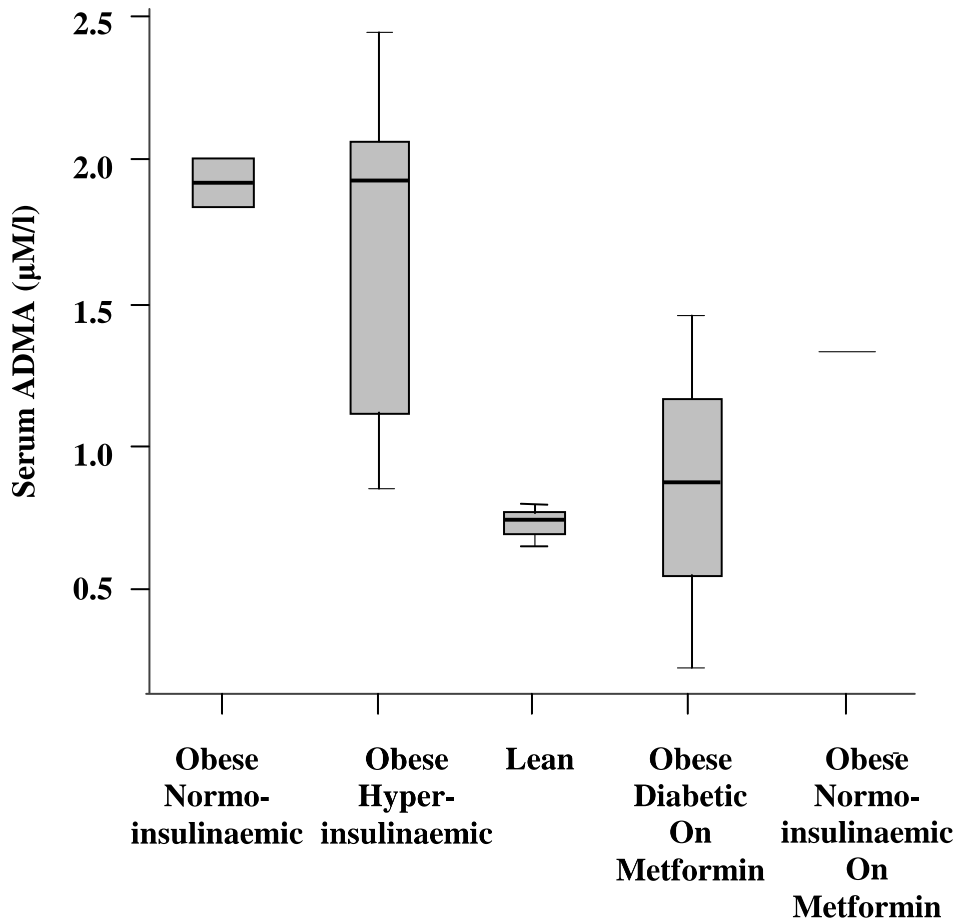


Figure 3.13: Comparison of serum ADMA in lean, normoinsulinaemic obese and hyperinsulinaemic obese and diabetic on Metformin and obese normoinsulinaemic on Metformin. Although comparison test were not performed, because of small number and sex miss match, but serum ADMA in lean and diabetic patients on Metformin seems to be lower. The obese normoinsulinaemic patient on Metformin had a lower serum ADMA compared to patients not on Metformin. Graph shown as box plot and data as median +/- IQR. Obese normoinsulinaemic n=2 (All female), Obese hyperinsulinaemic n=16 (14F/2M), Lean n=3 (1F/2M), Diabetics on Metformin (n=3, 2F/1M), Obese normoinsulinaemic on Metformin (n=1, F).

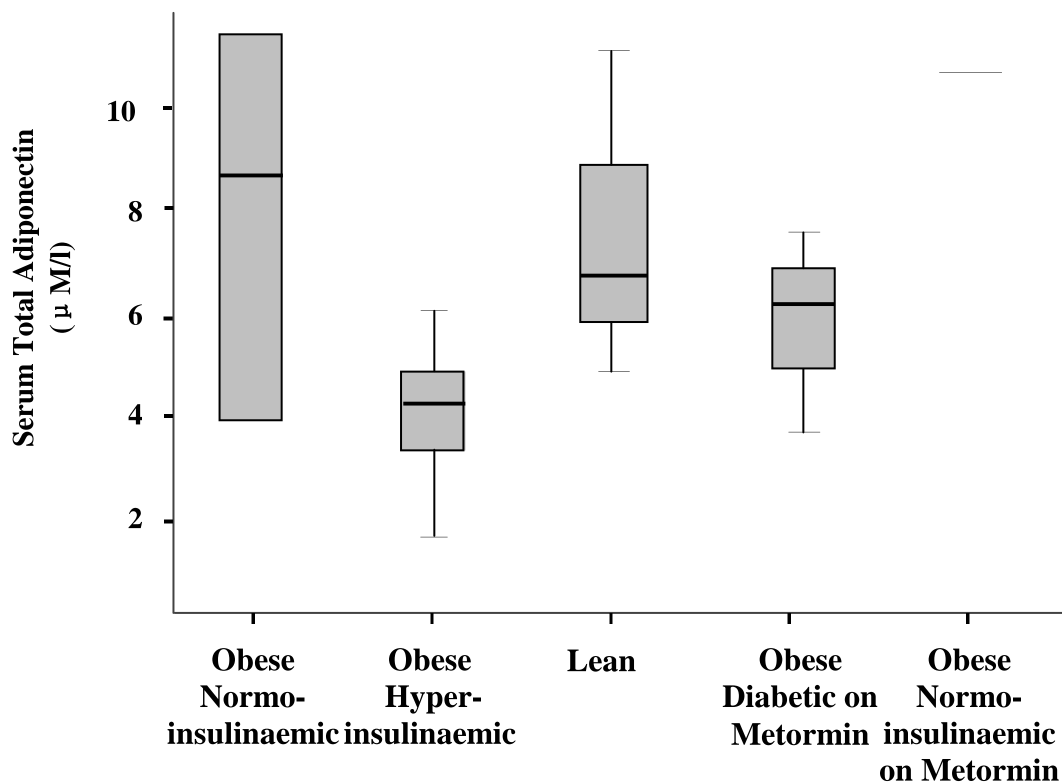


Figure 3.14: Comparison of serum adiponectin in lean, normoinsulinaemic obese and hyperinsulinaemic obese and diabetic individuals. Although comparison test were not performed, because of small number and sex miss match. However, serum adiponectin in diabetic patients on Metformin seems to be higher compared to obese hyperinsulinaemic patients not on Metformin. This may be the case for obese normoinsulinaemic on Metformin as well compared to the same group not on Metformin. Graph shown as box plot and data as median +/- IQR. Obese normoinsulinaemic n=2 (All female), Obese hyperinsulinaemic n=16 (14F/2M), Lean n=3 (1F/2M), Diabetics on Metformin (n=3, 2F/1M), Obese normoinsulinaemic on Metformin (n=1, F).

CHAPTER 4: DISCUSSION

4.1 ADIPOKINES

Several proinflammatory cytokines and chemokines, including RANTES, MCP-1, adiponectin, leptin and IL-6, were released *in vitro* from the subcutaneous abdominal and two visceral depots studied: the omental fat and the gastric fat pad. However, there were significant differences only in RANTES secretion between the different adipose tissue depots studied in this population of morbidly obese patients undergoing surgery. These subjects secreted significantly greater amounts of RANTES from their gastric fat pad than from adipose tissue of abdominal subcutaneous or omental origin. Wu *et al.* reported higher expression of RANTES in visceral compared with subcutaneous adipose tissue of morbidly obese humans (Wu *et al.,* 2007). However, this study is the first to compare two visceral depots, the gastric fat pad and the omental. Previous studies have assumed all visceral depots were similar.

The gastric fat pad lies in close anatomical proximity to the stomach, sitting in the angle of His. This depot is highly vascularised and may become thickened in morbidly obese subjects and it is thought that its resection may prevent obstruction after gastric banding (Ren & Fielding, 2003; Sacks *et al*. 2007). The increased release of RANTES from the gastric fat pad may be due to local regulation by gastrointestinal factors. The close association between omental and subcutaneous RANTES, but not with that from the gastric fat pad, may also imply that this depot is independently regulated. These differences in RANTES secretion between visceral adipose tissue depots could be explained by possible variations in the proportions of the components that make up each tissue depot, with particular respect to the stromavascular fraction, which accounts for the majority of chemokine and cytokine release (Clement *et al*., 2004). It could be that different depots are more susceptible to the inflammatory cell infiltration seen in obesity or conversely that some have a greater tendency for the adipocyte fraction to undergo greater hypertrophy, therefore causing greater release of chemokines, depending on which cell is responsible for their secretion. This would further enhance our understanding of the 'apple and pear' affect of obesity and may provide some evidence to suggest that the increased risk of co-morbidity associated with visceral fat may be refined to the type of visceral depot involved. It has been reported that IL-6 and MCP-1 are predominantly secreted by the stromavascular cells (Trujillo & Scherer, 2006) and it is likely that the same could apply for RANTES, since it is has long been recognized as being a product of immune cells. Although

adipose tissue is known to express a constantly increasing number of chemokines and cytokines; however, not all of these are actively secreted into the circulation (Mohamed-Ali *et al*. 1997 (a)). Systemic levels of RANTES were approximately 100-fold higher than those being released from any of the adipose tissue depots studied. Therefore, it would be reasonable to suggest that while adipose tissue is able to produce this chemokine, its contribution to systemic levels may be less significant. Other cells or tissues within the body, such as T-cells and other immune cells, are likely to be more potent producers of RANTES, thereby contributing significantly to circulating levels. This was further confirmed by a lack of association between its circulating levels and indices of obesity. In contrast, IL-6 production from the subcutaneous (Mohamed-Ali *et al*. 1997 (a)), as well as from the visceral depot (Fontana *et al*., 2007), contributes significantly to the systemic circulation, with the potential to have endocrine effects on various organs, especially in obesity. We have also shown the *in vitro* release of RANTES from the epicardial and thoracic subcutaneous adipose tissue depots in individuals undergoing cardiac surgery for valve replacement and without coronary artery disease (Madani *et al*. 2009 (a), in press). In this group the RANTES release from epicardial adipose tissue, but not the thoracic subcutaneous, correlates positively with BMI and is enhanced in obese individuals. So while epicardial RANTES is related to obesity, neither systemic RANTES nor its release from the subcutaneous and the abdominal visceral adipose tissues are good markers of adiposity. The significant heterogeneity in the release of RANTES from the various depots suggests differential regulation by autocrine/paracrine factors. Given that in other cell populations RANTES affects intracellular pathways that are central to adipocyte biology (Zhang *et al*. 2002), it would be interesting in the future to evaluate adipose tissue as a target, rather than as a source, of RANTES.

The two pathways involved in mediation of the secretion of these adipokines namely; COX and NOS, were then investigated further and discussed in the following sections.

4.2 COX PATHWAY

In this morbidly obese, female group of patients there were no depot specific differences in the release of IL-6. The *in vitro* pharmacological inhibition of COX with low dose aspirin, a non-selective COX inhibitor, significantly reduced

subcutaneous IL-6 release, but this was not apparent at higher doses or in the omental depots. COX-1 selective inhibitors had no effect on IL-6 release from either depot. However there was a trend towards reduced IL-6 release by COX-2 selective inhibitors in subcutaneous but not the omental depots. Experiments performed on mice in our group (Ogston *et al*., 2008) found that the treatment of murine adipose tissue in organ cultures with aspirin resulted in a significant reduction of IL-6 release which was dose dependent in both subcutaneous and gonadal depots. COX-2 selective inhibitor also brought about a significant reduction in IL-6 release from both depots but this did not appear to be dose-dependent, with the maximal effect observed at the lower dose. This difference between human and murine results may be due to problems with storage of the inhibitors used, as the experiments with the human tissue were carried out a few years after the murine experiments but using aliquots of the same inhibitors. The relatively small number of patients used in this study (n=5) could have influenced results as well. Therefore these results need to be confirmed both using new reagents and in a larger cohort.

Endogenous PGI_2S and PGE_2 secretions were unrelated to IL-6 secretion in human tissue; however this was not the case in mice (Ogston *et al*., 2008). Experiments in mice revealed greater PGI_2S protein and activity in gonadal compared to subcutaneous adipose tissue. In human tissue PGI_2S activity, but not its protein expression, was found to be higher in the omental depots. However, in a subgroup of patients, in whom fractionated adipose tissue was available the omental stromavascular fraction showed higher PGI_2S expression compared to that of the subcutaneous depot. No depot specific differences were apparent for PGE_2S activity. The effect of ingested aspirin on the adipose tissue in mice using ultra-low doses was investigated (Cyrus *et al*., 2002). Low dose aspirin significantly reduced circulating IL-6 *in vivo* in mice. Specific inhibition of COX-2 *in vitro* also reduced adipose IL-6 and abolished the differences seen between subcutaneous and gonadal adipose tissue in mice. Even at these doses aspirin inhibited the release of 6-keto-$PGF_1\alpha$ and IL-6, though not PGE_2, from murine white adipose tissue. Given that both COX isoforms are constitutively expressed, it seems that in this tissue, as in endothelial and smooth muscle cells (Schildknecht *et al*., 2004), PGI_2 synthesis is more coupled to COX-2 activity and drives the IL-6 secretion. Furthermore, depot-specific differences in expression and activity of PGI_2S may explain the greater release of IL-6 from omental adipose tissue.

Dietary and pharmacological interventions that lower the activity of COX mediated PGI_2S in adipose tissue are therefore likely to lead to local inhibition of IL-6 production. The novel observations of the susceptibility of the adipose tissue COX/PG pathway to ASA inhibition, as well as the fat depot-specific expression of PGI_2S and production of PGI_2 has important implications not only for the control of IL-6 release, but perhaps also for other important PGI_2 regulated functions in adipose tissue, such as adipogenesis (Ailhaud, 1999) and local adipose tissue blood flow. The relevance of this pathway needs further clarification in human adipose tissue.

4.3 NOS PATHWAY

Serum ADMA, an endogenous NO inhibitor, levels have been shown to be elevated in several clinical syndromes associated with insulin resistance and increased cardiovascular disease risk (Vallance 2001; Kielstein *et al.*, 1999; Zavaroni *et al.*, 1999), including obesity (Eid *et al.* 2004). In this study ADMA levels were determined in the serum, organ culture of adipose tissue and whole adipose tissue, of both subcutaneous and omental depots, in morbidly obese females. Serum ADMA was positively associated with insulin but not with glucose and the ADMA/SDMA ratio was proportional to systolic blood pressure, perhaps suggesting endothelial dysfunction.

Hypercholesterolemia is thought to directly reduce DDAH activity resulting in ADMA accumulation (Chan & Chan, 2002). However, in contrast to published literature (Stuhlinger *et al.*, 2002), there was a negative correlation between serum ADMA and triglyceride. The median serum ADMA in this morbidly obese group was 1.84 µM/l (1.03 – 2.06). Schulze *et al.* analyzed 500 healthy subjects, aged 19 to 75 years, with the aim of determining a reference value for ADMA and reported a mean concentration of 0.69µmol/l (± 0.2). In the Athero Gene Study on 1758 patients with subclinical coronary artery disease but without a recurrent cardiovascular event reported a median serum ADMA value of 0.63µmol/l (Schnabel *et al.*, 2005). In the present study, although statistical comparison could not be made accurately, circulating ADMA seemed lower in the lean individuals at 0.75 µM/l (0.65 – 0.8). This is in keeping with report by Kryzanowska *et al.*, showing elevated ADMA levels in morbidly obese subjects compared to normal weight individuals, and a significant reduction in systemic ADMA, along with improvement in several components of the metabolic syndrome, following gastroplasty (Kryzanowska *et al.*, 2004).

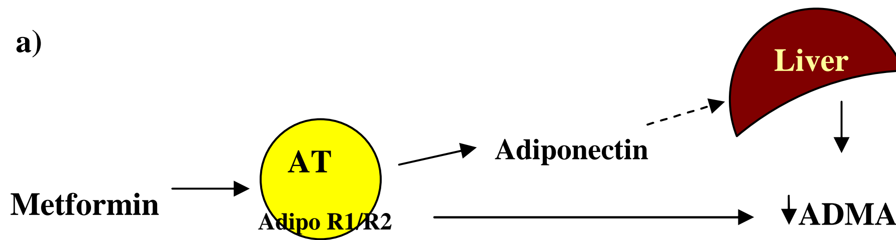
No depot specific differences in tissue ADMA was demonstrated here. However ADMA extraction from explants in thirteen obese patients showed higher levels being generated from the omental compared to the subcutaneous depot. The direct associations of omental ADMA release and BMI suggests a link between visceral obesity and endothelial dysfunction. Higher omental ADMA levels could reflect lower activity or expression of the catalysing enzymes, DDAH, or higher synthetic enzymes, PRMTs, in this depot. Published results have confirmed the expression of the whole gene set that codes for the enzymatic system responsible for the biosynthesis and degradation of ADMA in the human adipose tissue; PRMT1-6, DDAH1 and 2 and all forms of NOS (Spoto *et al.*, 2007). This is the only published study comparing subcutaneous and omental ADMA release, although tissue was obtained only from two healthy patients. Authors reported higher DDAH2 in visceral adipose tissue and higher DDAH1 in the subcutaneous depot (Spoto *et al.*, 2007). In our study, although DDAH2 was expressed in higher amounts compared to DDAH1 in both depots but there was no depot specific difference for DDAH1 or DDAH2. In order to assess whether the expression of DDAH reflects the stromavascular components of this tissue, in a subgroup of patients the stromavascular fraction was separated from adipose tissue. DDAH expression from adipocytes was not obtained due to poor RNA yields but DDAH2 was found to be higher compared to DDAH1, in the stromavascular fraction of both depots and once again no difference was seen between depots. We did not compare the expression of PRMTs between depots in this study but this would be interesting to evaluate in the future. Spoto *et al*. reported over expression of PRMT6 in the visceral adipose tissue but higher PRMT1-4 in the subcutaneous depot. Experiments on mice in our group found higher PRMT3 expression in gonadal adipose tissue compared to the subcutaneous depot (unpublished results).

Insulin-induced vasorelaxation in both humans and animals has been attributed to its ability to stimulate endothelium-dependent NO generation (Scherrer *et al.*, 1994; Bertuglia *et al.*, 1998). Reduced sensitivity to the metabolic actions of insulin and impaired NO mediated vasorelaxation has been demonstrated in obesity, type 2 diabetes, hypertension and dyslipidaemia (Pinkney *et al.*, 1997; Reaven, 1988).

In a small subgroup of patients obese normoinsulinaemic and hyperinsuliaemic patients both had raised circulating ADMA (figure 3.13). The raised ADMA, used as an index of endothelial dysfunction, was associated with insulin resistance in the

obese hyperinsulinaemic patients but not in the obese normoinsulinaemic group. The reason the later "fat and fit" group do not develop insulin resistance is not entirely understood. McLaughlin *et al*. found serum ADMA to be higher in insulin resistant obese women compared to their equally obese but insulin sensitive counterparts. They reported serum ADMA decreased after weight loss only in the former group (McLaughlin *et al*., 2006). The same group had treated healthy insulin resistant individuals with Rosiglitazone, an insulin-sensitizing drug, and showed enhanced insulin sensitivity and reduced plasma ADMA concentrations (Stuhlinger *et al*., 2002), concluding that insulin resistance is a potential mediator of ADMA concentrations. However work by Naderali *et al*. showed severely reduced insulin-induced vasorelaxation in diet induced fed rat but concentration-dependent relaxation by insulin (Naderali *et al*., 2001) suggesting that endothelial dysfunction comes prior to insulin resistance. A randomized placebo controlled study on 70 non-diabetic patients with metabolic syndrome demonstrated an independent association between improvement in flow-mediated dilation and reductions in ADMA in patients on Rosiglitazone (Wang *et al*., 2006). In our study the obese normoinsulinaemic and hyperinsulinaemic patients on Metformin had lower circulating ADMA compared to their counterparts not on Metformin. Metformin has been reported to decrease circulating ADMA both as monotherapy and as add on therapy to sulphonylurea in poorly controlled type 2 diabetic patients (Asagami *et al*., 2002). The modes of action of insulin sensitizing drugs are not well understood. Metformin may directly act on adipose tissue and enhance adiponectin release, which then inhibits ADMA release from the liver or it may have separate effects on adipose tissue and liver (figure 4.1). Other studies looked at endothelial vasodilatory function and had reported an inverse relationship between ADMA and endothelial function in the hypertensive (Perticone *et a*l., 2005) and hypercholestrolaemic patients (Boger *et al*., 1998). Weight loss has also shown to improve endothelial-dependent vasodilation in obese individuals (Sasaki *et al.,* 2002; Ziccardi *et al*., 2002). Interestingly adiponectin has been shown to reduce ADMA accumulation in human cultured endothelial cells (Eid *et al*., 2007). Serum adiponectin, an insulin sensitizing adipokine, was lower in the obese hyperinsulinaemic group compared to obese normoinsulinaemic individuals and circulating levels seemed to be higher in both groups when on Metformin. Plasma adiponectin have been reported to be higher after Metformin treatment in obese patients with polycyctic ovary syndrome (Jakubowska *et al*., 2008). Adiponectin acts

through two receptors, AdipoR1 and AdipoR2. It is thought that a decreased expression of these receptors could contribute to insulin resistance and diabetes. A study on Zucker diabetic rat found that Metformin induced the expression of these receptors in liver muscle and adipose tissue (Metais *et al*., 2008).



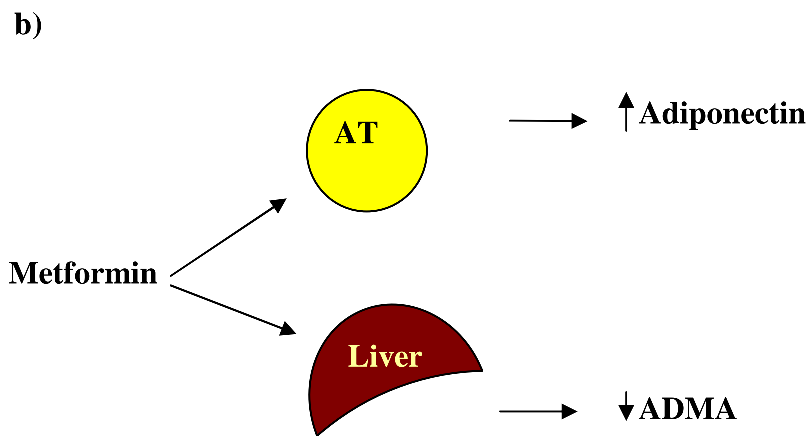


Figure 4.1: Proposal of the mechanism of action of Metformin.

Metformin may act by a) inducing adiponectin secretion from adipose tissue which then has inhibitory effect on ADMA release from the liver and on adiponectin receptors. b) having direct inhibitory effect on liver ADMA synthesis and release. AT: Adipose Tissue

The debate on whether insulin resistance is the result or the cause of endothelial dysfunction is not clear. Future longitudinal studies and myography of vessels from the adipose tissue may provide a clearer explanation for this. Given the pivotal role of ADMA in determining NO bioavailability and the large array of conditions associated with high ADMA concentrations, pharmacological modulation of circulating ADMA could lead to novel therapies for preventing cardiovascular disease in insulin resistant or high risk subjects. If endothelial dysfunction occurs prior to insulin resistance, and elevations in serum ADMA represent a reversible abnormality that contributes to the increased cardiovascular risk, ADMA modulation may be beneficial in preventing obesity associated cardiovascular dysfunction in insulin sensitive individuals.

4.4 LIMITATIONS OF RECRUITMENT

Two main limitations of this study were encountered during recruitment phase. While setting up the protocol it was decided that obese patients would be recruited from the patient group undergoing bariatric surgery and the lean patients would be a group undergoing reflux surgery. It was anticipated that 15 obese and 15 lean individuals would be enrolled by the end of the recruitment period for this study. However majority of the patients in the later group had BMI>28 (kgm^{-2}) and therefore were considered obese. Out of the total of 31 patients recruited only 4 were lean by WHO criteria. This meant that there were not enough lean patients to be used as the control group for this study. In order to address this short fall, ethical approval was obtained to include patients undergoing laparoscopic cholecystectomy. However after recruitment for few months it was evident that this group of patients were also mainly obese.

The second limitation to this study became evident midway during the recruitment period. This was the predominance of female, obese patients. Of the total 31 patients recruited, only 6 were male, of whom only 2 were obese. This meant that male obese individuals were not represented appropriately and therefore they had to be omitted from the study to avoid sex bias. The same applied to Afro-Caribbean individuals, who were excluded from all analysis.

4.5 LIMITATIONS IN ACQUIRING SAMPLES

Gastric fat pad biopsies were, at times, technically difficult or the sample not large enough. In patients undergoing laparoscopic cholecystectomy the field of operation was away from the gastric fat pad and therefore biopsies were not safe.

4.6 CONCLUSIONS

It is well proven that there are differences in the production/release of adipokines between the subcutaneous and visceral depots and visceral adiposity is directly linked to cardiovascular risk factors. However in this study we showed that there is also a difference in the release of at least one adipokine (RANTES) between different visceral depots which may suggest paracrine local factors mediating their release.

IL-6 and ADMA, an endogenous nitric oxide inhibitor, might also explain the link between obesity, type 2 diabetes mellitus and cardiovascular disease. PGI_2S seems to be more coupled to IL-6 production in omental adipose tissue and ADMA release was higher from the omental depot. Modulation of these adipokines may reduce the cardiovascular risks associated with obesity.

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