PERIVASCULAR ADIPOSE TISSUE AND VESSEL CONTRACTILITY IN HEALTH AND OBESITY

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School of medicine

Institute of Cardiovascular Sciences

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Abstract

PERIVASCULAR ADIPOSE TISSUE AND VESSEL CONTRACTILITY IN HEALTH AND OBESITY

Reza Aghamohammadzadeh (PhD candidate, Faculty of Medical and Human Sciences), September 2013

White adipocytes surround almost all blood vessels in the human body. It was thought previously that these cells merely provide mechanical support for the adjacent small vessels and are little more than fat storage units. Recent studies have identified these cells as metabolic and vasoactive engines that produce and secrete molecules that can affect the function of their adjacent small vessels. The adipocytes and a number of other cell types (including inflammatory cells) surrounding the vessels are collectively termed the PeriVascular Adipose Tissue (PVAT). Work from our group has shown previously that, in health, PVAT conveys a vasorelaxant effect on adjacent small arteries and that this effect is not observed in obesity thus the vessels must exist at an elevated level of basal tone. It is plausible that increased basal vessel constriction can explain the elevated blood pressure amongst the obese population and a better understanding of the obesity-induced PVAT damage may lead to clues to a new approach in the treatment of the condition which burdens its sufferers with a greater cardiovascular risk profile.

In this thesis we have studied individuals with morbid obesity at baseline and six months following surgery and observed that PVAT function following dramatic weight loss restores the PVAT vasorelaxant effect close to that observed in lean patients. Moreover, we have concluded that inflammation plays a significant role in this process and indeed using protocols with antioxidant enzymes we were able to restore the damaged PVAT function at baseline. We have have shown also that in health, PVAT vasorelaxant function is independent of the endothelium, and that obesity-induced PVAT damage and its reversal following weight loss and ex-vivo anti-oxidant treatment are both independent of the endothelium and at least in part due to nitric oxide bioavailability. Finally, we have observed that in sleep apnoea, which often coexists with morbid obesity and hypertension, there is a greater degree of PVAT inflammation.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university.

Contribution

Chapter 3: I was involved at all stages of this project. I helped write the ethics application and its amendments, I recruited the patients and personally performed all the biopsies and harvested the tissue. I dissected all the samples, was involved with the design of the protocols and performed wire myography in each case. I preserved the samples for immunohistochemistry which was performed by Dr Jeziorska. I was involved with all the data analysis for immunohistochemistry and personally performed all the analysis for myography data. The protocols for measurement of lipids and circulating biomarkers were primarily performed by Phil Pemberton and our collaborator in the lipid study group. I performed all the analysis on this data and wrote the manuscript.

Chapter 4: The human biopsies were performed by Dr Greenstein, however I performed all the analysis. The Proteomics protocols were outsourced to Oxford Genomics and I performed all the analysis on the data. All the animal protocols were designed, performed, and analysed by me. I have written the manuscript.

Chapter 5: I was involved with the ethics application, patient recruitment and biopsies. I was involved with tissue preparation and analysis of the data as well as preparing the manuscript. Dr Maria Jeziorska performed the immuhistochemistry protocols. All the lipid oxidation laboratory protocols were performed by Rahul Yada, Salam Hama and Yifen Liu.
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I am very grateful to all the study participants who voluntarily and without remuneration underwent gluteal fat biopsies and provided blood samples for the purposes of the protocols detailed in this thesis.

The nursing and administrative staff at the Manchester NIHR/ Wellcome Trust Clinical Research Facility provided much needed support in coordinating patient scheduling and collating demographic data.

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Lastly, I would like to thank all members of the Heagerty laboratory for providing technical assistance with research methodology in the laboratory and for being great friends throughout the process.

DEDICATION

This thesis is dedicated to my father Dr Hossein Aghamohammadzadeh, mother Akram Akhtari and brother Dr Soheil Aghamohammadzadeh who provided me with support, both
emotionally and scientifically, and for putting up with my endless moans and rants during
the three year process.

LIST OF THE MOST FREQUENTLY USED ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AdipoR1</td>
<td>Adiponectin Receptor 1</td>
</tr>
<tr>
<td>ADRF</td>
<td>Adventitium Derived Relaxing Factor</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BP</td>
<td>Blood Pressure</td>
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<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<td>HDL</td>
<td>High Density Lipoprotein</td>
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<td>hsCRP</td>
<td>High sensitivity C-Reactive Protein</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>K+</td>
<td>Potassium</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-Arginine Methyl Ester</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
</tr>
<tr>
<td>MS/ MetS</td>
<td>Metabolic Syndrome</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PVAT</td>
<td>Perivascular Adipose Tissue</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive Sleep Apnoea</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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THE AUTHOR

Reza Aghamohammadzadeh graduated from the University of Manchester with an Honours degree in Medicine in 2006. He was appointed to an Academic Foundation Training Programme in Manchester as a junior doctor. He was awarded his full membership of the Royal College of Physicians in 2009 and subsequently took time out of his clinical training programme to pursue research towards this thesis. In 2009, he was awarded a Clinical Research Fellowship from NIHR Manchester Biomedical Research Centre to start work on this thesis and in 2010 he was awarded further personal funding in the form of a Clinical Research Training Fellowship from the British Heart Foundation to support this body of work. In 2012 he was awarded an NIHR Manchester Biomedical Research Research Centre International Scholar award to pursue a 6 month research project at Harvard Medical School (Boston, USA) in the field of vascular biology in relation to pulmonary hypertension. This work was carried out during his thesis pending year. Currently, he is an NIHR Academic Clinical Fellow in Cardiology and an honorary research fellow at the University of Manchester and continues to pursue his research endeavours alongside his cardiology training.
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- Effects of bariatric surgery on human small artery function: evidence for reduction in
perivascular adipocyte inflammation, and restoration of normal anticontractile activity
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- Protection from development of obesity in high fat diet rats is associated with preservation
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PH Park, AS Greenstein, AM Heagerty. *Heart* 2010;96:e17
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5. The obesity-induced perivascular adipose tissue damage can be rescued by both free radical scavengers and weight loss following bariatric surgery. R Aghamohammadzadeh*, Adam Greenstein, R Yadav, S Hama, P Pemberton, H Soran, B Ammori, AM Heagerty. *Manchester Medical Society (section of medicine) April 2012 (Oral presentation)*

6. Damage to the anticontractile capacity of perivascular adipose tissue in obesity is in keeping with reduced nitric oxide bioavailability and strongly correlates with blood pressure elevation. R Aghamohammadzadeh*, B Park, AS Greenstein, AM Heagerty.


10. There is reduced nitric oxide production in perivascular adipose tissue (PVAT) of diet-induced obese rats, and the loss of PVAT function is endothelium-independent and is rescued by superoxide dismutase and catalase. R Aghamohammadzadeh*, AS Greenstein, A Mastan, AM Heagerty. *International Symposium on Resistance Arteries, Skørping, Denmark May 2011- Winner of best poster award

11. Damage to the anticontractile capacity of perivascular adipose tissue strongly correlates with elevation of blood pressure: Results from an environmental model of obesity, R Aghamohammadzadeh*, PH Park, AS Greenstein, AM Heagerty. *International Society of Hypertension, Vancouver Sep 2010 (poster) Selected as highest ranking abstract

A Khavandi, A Mastan, A Greenstein, R Malik, R Khattar, AM Heagerty. *American College of Cardiology April 2011 (Poster presentation)*

13. Protection from development of obesity in high fat diet rats is associated with preservation of the anticontractile function of perivascular adipose tissue. R Aghamohammadzadeh*, PH Park, AS Greenstein, AM Heagerty. *British Cardiovascular Society, Manchester 2010 (Poster presentation)*

CHAPTER 1: Introduction

Perivascular adipose tissue

from human systemic and coronary vessels:

The emergence of a new pharmacotherapeutic target

Running title: Signals from PVAT

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**Summary**

Fat cells or adipocytes are distributed ubiquitously throughout the body and are often regarded purely as energy stores. However, recently it has become clear that these adipocytes are engine rooms producing large numbers of metabolically active substances with both endocrine and paracrine actions. White adipocytes surround almost every blood vessel in the human body and are collectively termed PeriVascular Adipose Tissue (PVAT). It is now well recognised that PVAT not only provides mechanical support for any blood vessels it invests, but also secretes vasoactive and metabolically essential cytokines known as adipokines, which regulate vascular function. The emergence of obesity as a major challenge to our healthcare systems has contributed to the growing interest in adipocyte dysfunction with a view to discovering new pharmacotherapeutic agents to help rescue compromised PVAT function. Very few PVAT studies have been carried out on human tissue. This review will discuss these and the hypotheses generated from such research, as well as highlight the most significant and clinically relevant animal studies showing the most pharmacological promise.

**Keywords**

Perivascular adipose tissue; PVAT; adipocytes; adipokines; obesity; metabolic syndrome; adiponectin; leptin; epicardial adipose tissue; coronary vessels
Abbreviations

PVAT: perivascular adipose tissue; MetS: metabolic syndrome; BMI: body mass index; NO: nitric oxide; LNAME: NG-nitro-L-arginine methyl ester; CAD: coronary artery disease; TNF: tumour necrosis factor; IL-6: interleukin-6; PPAR: peroxisome proliferator-activated receptor; ROS: reactive oxygen species; ADRF: adventitium derived relaxing factor; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1

Introduction: the clinical problem

Obesity, defined as a body mass index (BMI) of greater or equal to 30kg/m² is a major problem in acculturated and developing societies. It often co-exists with a number of other diseases including hypertension, dyslipidaemia and insulin resistance. Such a constellation has been labelled the metabolic syndrome (MetS). The international obesity taskforce (IOTF) estimates that approximately 1 billion adults are currently overweight (BMI 25-29.9 Kg/m²), and of these 475 million are obese (≥30kg/m²) (IOTF, 2010).

The enormity of this epidemic highlights the need for novel approaches to obesity management and a furthering of our knowledge of the mechanisms responsible for the consequences of being overweight.

A number of reports has indicated that the distribution of fat around the body determines not only the obese phenotype but its consequences. For example, intra-abdominal and visceral fat depots have been linked with an increased cardiometabolic risk and the mortality associated with obesity (Fox et al., 2007; Gesta et al., 2007). The total amount of internal fat rises with increasing subcutaneous adiposity, but even individuals classed as
thin may have more visceral fat than some obese individuals. In addition increased
gluteofemoral fat mass has been negatively linked to levels of inflammatory cytokines and
positively linked to raised concentrations of adipokines resulting in decreased metabolic
and cardiovascular risk (Manolopoulos et al., 2010).

Our current understanding of this problem focuses on our ancestors and the fact that fat
was the energy store developed in times of plenty which could then be burned during
famine. Therefore genes which predisposed to obesity would confer survival benefits and
such individuals would live long enough to reproduce. Several reviews have suggested
that it is the breakdown of this system that is responsible for the contemporary problems
associated with obesity, where susceptible individuals no longer have periods of famine
and are instead constantly over-eating readily available high energy foods (Diamond,
2003; Neel, 1962). Whilst in hibernating mammals, short-term obesity and insulin
resistance have the beneficial effect of directing glucose to the brain, only man has
developed chronic obesity with its associated cardiovascular morbidity and mortality
(Scott et al., 2006).

Yudkin et al. (2005) originally postulated that perivascular adipose tissue might hold the
key to linking obesity with the development of metabolic syndrome and diabetes as a
result of an adverse influence upon the vasculature (Yudkin et al., 2005). In health, PVAT
could produce adipokines that profoundly influence metabolism and the control of local
vascular tone via vasocrine actions. It was suggested that the loss of such substances
would result in a change in vascular function and development of insulin resistance. These
authors suggested that the effect of circulating insulin on NO-mediated vasodilatation,
which is of paramount importance in modulating the postprandial increase in nutritive flow, could be challenged by the paracrine action of adipokines released from local fat stores in obesity. They further highlighted the role inflammation may play and suggested elevated levels of TNF-α in obesity could disrupt the crosstalk between fat and blood vessels.

In this review we intend to focus on the vasoactive properties of perivascular adipose tissue as mediated by adipokines.

**What are the principle adipokines released from adipocytes?**

The recent interest in adipose tissue as a dynamic endocrine organ has resulted in a number of studies examining the shared, and in cases distinct, properties of different fat depots. Perivascular adipose tissue (PVAT) surrounds subcutaneous small arteries, coronary vessels (peri-coronary and epicardial fat), aorta and systemic vessels and secretes a number of important adipokines (Figure1).

Amongst the adipokines, leptin and adiponectin have been subject of recent reviews (Kadowaki et al., 2005; Kadowaki et al., 2006; Ren, 2004; Sweeney, 2010). Here, we review briefly the most significant findings on leptin, adiponectin and adrenomedullin. A more comprehensive list of adipokines and their roles with regards to the metabolic syndrome has been published recently (Deng et al.).
Leptin

In rats leptin has a direct vasodilator effect acting via an endothelium and nitric oxide dependent mechanism (Lembo et al., 2000). However, it has an endothelium-independent vasodilator effect on segments of human subclavian vein and internal mammary arteries harvested during surgery, thus highlighting the need for careful selection of vascular tissue in order to design clinically relevant studies (Momin et al., 2006).

The role of leptin as an indirect vasoconstrictor has also been studied. Fruhbeck et al have shown that leptin has a sympathoexcitatory effect leading to a rise in BP in Wistar rats (Fruhbeck, 1999). They demonstrated a significant rise in both systolic and diastolic BP when rats were given an intravenous bolus of the nitric oxide inhibitor NG-nitro-L-arginine methyl ester (L-NAME) followed by leptin, demonstrating the role NO plays in facilitating the vasorelaxant effects of leptin. In the same experiment they showed that leptin administration, following post ganglionic blockade using chlorisondamine, resulted in a reduction in BP, which was abolished by NO inhibition.

Weight gain is often associated with increased insulin resistance and resultant hyperinsulinaemia. Interestingly, Vecchione et al have demonstrated that insulin potentiates leptin-induced NO release and even at physiological levels, insulin enhances the vasodilator effects of leptin (Vecchione et al., 2003).

That leptin, a proinflammatory adipokine, possesses vasodilator properties, and that obesity is associated with leptin resistance, implies its role in obesity is not understood fully. In MetS leptin levels are increased within the epicardial fat adjacent to the coronary
vessels (Payne et al., 2010). In dogs, hyperleptinaemia has been associated with endothelial dysfunction (Knudson et al., 2005), and work on a swine model of MetS has shown that leptin exacerbates endothelial dysfunction via a protein kinase C – beta dependent pathway (Payne et al., 2009).

It is unclear whether elevated levels of leptin in epicardial fat are of benefit to the adjacent coronary vessels, or whether they may contribute to endothelial dysfunction in human arteries.

**Adiponectin**

Adiponectin is a 30kDa protein made up of 244 amino acids. It is the most abundant adipokine (Dridi et al., 2009; Liu et al., 2010) and exists in two forms, full length, or a smaller globular fragment (Dridi et al., 2009; Kadowaki et al., 2005; Kadowaki et al., 2006). The full length form acts via the R2 receptor and the globular form via R1 (Yamauchi et al., 2003a). In human adipocytes, the expression of Adipo R1 is ~15 fold higher than that of Adipo R2 (Rasmussen et al., 2006). Circulating adiponectin levels are reduced in obesity (Sowers, 2008), diabetes (Kadowaki et al., 2006), and there is a down regulation of adiponectin receptors in the adipose tissue of obese individuals (Rasmussen et al., 2006). Given its anti-diabetic (Berg et al., 2001; Davis et al., 2008; Maeda et al., 2002; Pajvani et al., 2003; Yamauchi et al., 2003b; Yamauchi et al., 2001), anti-atherosclerotic (Han et al., 2009; Yamauchi et al., 2003b) and vasodilator (Fesus et al., 2007; Greenstein et al., 2009) properties, adiponectin is believed to be the link between obesity and metabolic syndrome. Its mechanism of action has yet to be determined fully.
In the liver, full-length adiponectin activates adenosine monophosphate-activated protein kinase (AMPK) and the trimeric form is known to activate AMPK in adipose tissue and muscle. Activation of AMPK leads to phosphorylation of Acetyl-Coenzyme-A Carboxylase (ACC) which results in fatty acid β-oxidation and inhibition of triacylglycerol and fatty acid synthesis (Liu et al., 2010); (Kadowaki et al., 2005).

Deng et al. have demonstrated that adiponectin improves endothelial dysfunction by increasing nitric oxide (NO) production via phosphorylation of endothelial NO in the aorta of high-fat-fed obese Sprague-Dawley rats (Deng et al., 2010). Moreover, in adiponectin receptor-KO mice, there is a significant attenuation of endothelium-dependent vasodilatation in response to ACh (Ouchi et al., 2003). Of relevance, adiponectin suppresses both basal and oxidised-LDL induced superoxide generation in bovine endothelial cells (Motoshima et al., 2004), and also suppresses excess ROS production under high-glucose conditions via a cAMP/PKA-dependent pathway (Ouedraogo et al., 2006). Given the elevated levels of oxidised-LDL (Holvoet et al., 2008) and plasma glucose in patients with metabolic syndrome, the concomitant reduction in adiponectin levels may explain partly the endothelial dysfunction observed by our group (Greenstein et al., 2009) in the cohort of patients with MetS.

Incubation of healthy human vessels with a blocking peptide for the adiponectin R1 receptor almost abolished completely the anticontractile effect of PVAT in response to cumulative noradrenaline doses (Greenstein et al., 2009). Our unpublished proteomic analysis of adipose tissue from obese patients shows a significant reduction in adiponectin levels as compared with lean individuals. Therefore we suspect that the absence of the
anticontractile function of PVAT in obesity is accounted for by a reduction in adiponectin levels in PVAT at least in part. It has been suggested that the high levels of adiponectin in some disease states may be a compensatory response to the development of ‘adiponectin resistance’ (i.e. a dysfunction in the adiponectin signalling pathway) (Sam et al., 2009). Clearly, further research is required to describe the exact mechanisms of action of adiponectin in health and to explain how these pathways become affected in disease.

**Adrenomedullin**

Amongst the adipokines, adrenomedullin (AM) has received the least attention in recently published literature. It is a 52 amino acid peptide first isolated from a sample of human phaeochromocytoma, but later shown to be synthesised by adrenal, heart, and vascular endothelial and smooth muscle cells as well as white adipocytes of both rodents and humans (Fukai et al., 2005; Silaghi et al., 2007). Its dose-dependent vasodilator effect on the rat mesenteric vessels was first reported in 1993 (Nuki et al., 1993). Its direct and potent vasodilatory action on blood vessels would suggest that it plays an important role in the control of vessel tone. Human studies have shown that intravenous infusion of the peptide leads to significant vasodilatation of pulmonary vessels providing a potential therapeutic strategy for pulmonary hypertension (Nagaya et al., 2003; Nagaya et al., 2000). Also there is evidence of AM-induced human coronary (Terata et al., 2000) and skeletal artery (Nakamura et al., 1997) vasodilatation via a nitric oxide dependent pathway.
The exact role of adrenomedullin in obesity needs further evaluation, but we know that AM reduces levels of reactive oxygen species (ROS) in vascular smooth muscle cells (Yoshimoto et al., 2005), and AM knockout mice express higher levels of ROS (Shimosawa et al., 2003).

Catecholamine stimulation of beta3 adrenoceptors on adipocytes results in lipolysis and release of stored adipokines (Robidoux et al., 2006). Adrenomedullin inhibits lipolysis via a nitric oxide dependent mechanism (Harmaneey et al., 2005) which in theory, would affect vessel tone indirectly by blocking the release of vasoactive adipokines such as adiponectin (Figure 2). Gettys et al. have reported that stimulation of the beta3-adrenergic receptor on white rat adipocytes by the selective agonist CL316,243 leads to the inhibition of the release of leptin (Gettys et al., 1996). The balance between the potential lipolysis-induced release of adiponectin and the inhibition of leptin release and their relevance to vascular tone needs further clarification.

Epicardial adipose tissue from patients with CAD displays higher levels of adrenomedullin than those without CAD. Given its vasorelaxant properties, one would assume this may serve as a protective mechanism for the diseased coronary vessels (Shibasaki et al., 2010). Plasma AM concentrations are also elevated in disease states, which may provide further protection against oxidative stress and vasoconstrictors. Despite its antioxidant and vasorelaxant properties, it is possible that elevated AM levels in disease states may actually contribute to the vascular dysfunction.
PVAT and control of local vascular tone

Perivascular adipose tissue function has been assessed in canine, swine and rodent models and demonstrated different functional and structural properties of PVAT which vary both between species and anatomical site. Examples of structural differences include the fact that PVAT from rat aorta comprises smaller adipocytes compared with mesenteric vessels (Galvez-Prieto et al., 2008). Whilst the murine thoracic aorta is surrounded by brown adipocytes, peri-abdominal fat is comprised of white adipocytes (Police et al., 2009). From a functional perspective, coronary artery PVAT in healthy dogs attenuates acetylcholine induced relaxation (marker of endothelial function) (Payne et al., 2009; Payne et al., 2008), but does not affect bradykinin-mediated dilatation in healthy pig arteries (Payne et al., 2010).

Three studies have shown that healthy human PVAT exerts an anticontractile effect on adjacent vessels. Rodent (both mouse and rat) mesenteric and aortic vascular beds have been the most frequently studied models of PVAT function. Data from these models have matched closely the data obtained from limited human studies.

In 1991, Soltis and Cassis were the first to report that vessels with intact PVAT were less responsive to noradrenaline than naked vessels (Soltis et al., 1991). Later studies used solution transfer protocols to demonstrate the existence of a transferrable adventitium-derived relaxing factor (ADRF) (Gao et al., 2005b; Greenstein et al., 2009; Lohn et al., 2002; Malinowski et al., 2008). The solution transfer experiments involved using a small volume of solution from a tissue bath with PVAT, adding it to a pre-constricted vessel and
measuring the vascular response. These suggest that the observed anticontractile property of PVAT is not merely a consequence of it acting as an obstacle to diffusion for vasoconstrictors, but as a dynamic tissue which secretes adipokines with anticontractile properties.

There are likely to be a number of substances which account for the anticontractile effects of PVAT. Indeed, both endothelium dependent and endothelium independent mechanisms have been demonstrated. Protocols using rat mesenteric vessels have shown that both PVAT and exogenous adiponectin exert anticontractile effects on preconstricted small arteries (Greenstein et al., 2009); the effects of PVAT have been corroborated by experiments on rat aorta and its surrounding PVAT, which mostly consists of brown adipocytes (Gao et al., 2007; Lohn et al., 2002). Gao has shown that the endothelium independent mechanism involves hydrogen peroxide (H$_2$O$_2$) and subsequent activation of soluble guanylyl cyclase. At low concentrations (10-100µM) of H$_2$O$_2$, mesenteric vessels preconstricted with phenylephrine undergo further constriction, but higher concentrations of H$_2$O$_2$ (0.3-1mM) result in a biphasic response, with an early constriction followed by relaxation. Hydrogen peroxide released from both adipocytes and macrophages can act via the raf/MEK/ERK pathway and influence the phosphorylation of contractile apparatus in vascular smooth myocytes (Figure 2).

Interestingly, exposure of rat mesenteric arteries to electric field stimulation (EFS) leads to a contractile response via stimulation of $\alpha_1$-adrenoceptors by the perivascular sympathetic nerves. PVAT enhances this contractile response by stimulating superoxide generation and activation of tyrosine kinase and MAPK/ERK pathway (Gao et al., 2006) (Figure 2).
recently it has been shown that Angiotensin II derived from adipocytes potentiates the contractile response to EFS (Lu et al.), thereby highlighting the role of the renin-angiotensin system within PVAT with regards to local vascular tone modulation.

**Physiological release and action of ADRF: Possible role of the potassium channel**

The exact mechanisms by which adipocytes exert their effects on adjacent arteries are not well understood. Figure 2 shows a number of the potential mechanisms involved. Downstream in the PVAT anticontractile pathway are vascular potassium (K) channels, which occupy a central position in the maintenance and regulation of vascular tone. Early experiments on potassium channels have identified roles for a number of K channels. In rat mesentery the anticontractile effect is attenuated by blockade of the delayed rectifier K channel (K\textsubscript{v}) (Verlohren et al., 2004). Studies of rat aorta suggest roles for adenosine triphosphate (ATP)-sensitive K channels (K\textsubscript{ATP}) (Lohn et al., 2002) as well as small and intermediate conductance calcium-sensitive potassium channels (SK\textsubscript{Ca} and IK\textsubscript{Ca}) (Gao et al., 2007). In human internal mammary artery, the relaxing factor appears to work via large conductance calcium-sensitive K channels (BK\textsubscript{Ca}) (Gao et al., 2005b).

More recent experiments have highlighted the pivotal role of the BK\textsubscript{Ca} channel in mediating the PVAT effect. Work from our group supports the role of the BK\textsubscript{Ca} channel in the vasodilator function of PVAT. Pharmacological inhibition of the channel using paxilline or using mesenteric arteries from BK\textsubscript{Ca} knockout mice (Slo\textsuperscript{−/−}, BK\textsubscript{Ca}−/−) results in a significant impairment of the response (Lynch et al., 2010b). This highlights the role of BK\textsubscript{Ca} channels in facilitating the ADRF effect, but further work is needed to clarify
whether \( \text{BK}_{\text{Ca}} \) channels are also present on adipocytes as well as the vascular myocytes. Additionally the removal of endothelium or inhibition of NOS can significantly attenuate the response in these mouse models (Lynch et al., 2010a).

Elegant micro-electrode studies of de-endothelialised rat mesenteric arteries have shown that in non-constricted vessels the hyperpolarisation to exogenous adiponectin is inhibited by selective blockade of \( \text{BK}_{\text{Ca}} \) (Egner et al., 2010). This group has reported also that stimulation of \( \beta_3 \) adrenoceptors releases a factor which indirectly activates myocyte \( \text{BK}_{\text{Ca}} \) channels. They have suggested that this factor is adiponectin working via myocyte adipoR1 receptors to activate AMPK. These protocols were performed in the absence of endothelium. However, there are \( \text{BK}_{\text{Ca}} \) channels present on the endothelium (Hughes et al., 2010) and stimulation of these channels by circulating ADRF could result in hyperpolarisation of endothelial cells and subsequent hyperpolarisation of vascular myocytes via the myoendothelial junction, thus leading to their relaxation (Figure 2).

Obesity is a state of adrenergic overdrive with increased circulating noradrenaline levels (Prezio et al., 1964) and an overactive sympathetic nervous system (Lambert et al.) releasing noradrenaline at nerve terminals which are present in PVAT. Noradrenaline can bind to \( \beta_3 \) adrenoceptors, although with lower affinity than to \( \beta_1 \) and \( \beta_2 \) adrenoceptors. In obesity one would expect that, overstimulation of the \( \beta_3 \) adrenoceptor by noradrenaline would result in an increase in adiponectin release from adipocytes thus lowering vessel tone and enhancing metabolic homeostasis. However in obesity, there are reduced blood and adipocyte adiponectin levels (Asayama et al., 2003; Kern et al., 2003) as well as downregulation of adiponectin receptors (Kadowaki et al., 2005). Further research is
required to explain the reduced levels of adiponectin and its receptors. There may be an increase in breakdown of adiponectin secondary to oxidative stress and inflammation or a reduction in its production due to obesity-induced damage to the intracellular mechanisms involved in production of the protein. We shall discuss further evidence for inflammation-induced PVAT damage later in this review.

**PVAT and human vessels**

Harvesting human arteries for PVAT studies is fraught with difficulty, which may explain the limited number of published studies. The most accessible human blood vessel is the internal thoracic artery, obtained during coronary artery bypass graft operations (Gao et al., 2005b; Malinowski et al., 2008) and small arteries from gluteal fat biopsies (Greenstein et al., 2009). Both are clinically relevant vascular beds. However, the atraumatic harvest of saphenous vein grafts, via the ‘no-touch’ technique has been shown to result in vessels with intact PVAT and immunohistochemical evidence of the potent vasodilator nitric oxide (Dashwood et al., 2007; Dashwood et al., 2009). Venous PVAT has been shown to exert an anticontractile effect on adjacent tissue by releasing Ang-(1-7) which activates Kv channels and relaxes vascular myocytes through eNOS release (Lu et al., 2011).

Gao et al first studied the anticontractile properties of human PVAT using segments of human internal thoracic artery. It was shown that human PVAT exerted an anticontractile effect on its adjacent vessel upon exposure to contractile agents including U46619, which is a thromboxane A2/prostaglandin H2 receptor antagonist (Gao et al., 2005b). This is a
significant finding because thromboxane A$_2$ and its stable metabolite thromboxane B$_2$ (TxB$_2$) are known to be potent vasoconstrictors and the levels of TxB$_2$ are known to be increased during cardiopulmonary bypass (Davies et al., 1980). Perioperative spasm of the internal thoracic artery is a commonly encountered issue (He et al., 1994), therefore leaving the perivascular fat intact may help counteract the vasoconstricting effects of the increased plasma thromboxane levels. In this study, the anticontractile effect of PVAT was shown to be due to a transferrable relaxing factor (adipose derived relaxing factor or ADRF) that activated BK$_{Ca}$ channels.

Malinowski et al. also used segments of human internal thoracic artery and investigated whether it is perivascular adipose tissue per se, or adipose tissue in general, that is capable of exerting an anticontractile effect (Malinowski et al., 2008). Following a coronary artery bypass operation, the in situ graft (taken from the internal thoracic artery) heals in close proximity to pleural tissue. Dose responses to serotonin were constructed for skeletonised vessel segments with and without incubation with pleural fat. The study showed that, despite the presence of white adipocytes in pleural fat, there was no anticontractile effect. The study also demonstrated clearly that adipokines released by PVAT are able to affect the blood vessel even if the fat tissue has been loosely added to the tissue bath and is not in direct contact with the artery.

In order to assess whether ADRF acts via the endothelium, Malinowski et al. incubated their vessels with inhibitors of nitric oxide synthase and cyclo-oxygenase (COX). It is well known that nitric oxide and prostacyclin produced in the endothelium have vasodilatory effects. This study showed that inhibition of NO and COX did not abolish the
anticontractile effect of PVAT. This does not mean that PVAT function is endothelium-independent; rather that it can exert its effect independently of the NO and COX pathways.

The most recent study of human PVAT has been carried out on small arteries harvested from adipose tissue samples obtained by gluteal biopsies in patients with metabolic syndrome (MetS). Figure 3A demonstrates the anticontractile function of PVAT in healthy individuals whilst figure 3B shows that PVAT did not exhibit an anticontractile function in the group with MetS (Greenstein et al., 2009). Incubating healthy PVAT intact human vessels with a fragment of the human type 1 adiponectin receptor completely abolished PVAT anticontractile function, thus identifying adiponectin as an adipose-derived relaxing factor (ADRF) in human PVAT. Also the presence of nitric oxide in human PVAT was demonstrated by incubating PVAT intact healthy vessels with L-NMMA which resulted in attenuation of the anticontractile property of PVAT. This study will be discussed further in the next section.

**PVAT, obesity and inflammation**

The effect of obesity on PVAT function was first reported in 2005. It was shown that, prenatal exposure of rats to nicotine caused postnatal obesity and an increased amount of PVAT. However, the rise in PVAT volume which was associated with weight gain resulted in a reduction in the anticontractile function of PVAT surrounding the aorta. It was thought that this may be due to a change in the nature of PVAT or a reduction in secretion of relaxation factor(s) from PVAT(Gao et al., 2005a).
Often obesity is associated with hyperglycaemia and hyperinsulinaemia in the context of the metabolic syndrome. The role of circulating insulin on vascular tone has been well documented (Eringa et al., 2004), but its effect on PVAT mediated vascular responses has not been established. However, both acute and chronic hyperglycaemia enhance the PVAT anticontractile effect (Lee et al., 2009b). This highlights the complex nature of the metabolic syndrome and the difficulty of controlling for variables in ex-vivo experiments.

Adipocytes undergo significant hypertrophy in obesity. The cross-sectional area of adipocytes in obese individuals with metabolic syndrome is up to 1000 μm$^2$ larger than that of healthy individuals (Greenstein et al., 2009). Given that the diffusion limit of oxygen is thought to be around 100μm (Hosogai et al., 2007), the hypertrophied adipocytes are likely to be subjected to a decreased oxygen tension. In obese individuals, there is no increase in blood supply to match the increase in adipocyte size, and the postprandial increase of blood flow that occurs in lean subjects is also absent in obesity (Bulow et al., 1987; Coppack et al., 1992). This implies that hypertrophied adipocytes exist in a state of relative hypoxia. This has been confirmed by pimonidazole staining (a 2-nitroimidazole which is activated at low oxygen concentrations) of adipocytes from obese mice (Hosogai et al., 2007), as well as measurements of partial pressures of oxygen in abdominal subcutaneous adipose tissue of obese humans (Pasarica et al., 2009). In the context of hypoxia, vasa vasorum in the adipose tissue surrounding vessels play an important role in providing oxygen to the hypoxic PVAT. Recent work has shown that PVAT induces the formation of vasa vasorum (Manka et al. 2010) which would be beneficial in obesity.
Hypoxia reduces PVAT anticontractile effects in rat mesenteric arteries (Greenstein et al., 2009), however in aorta the opposite occurs, namely an enhancement of PVAT function (Maenhaut et al., 2010). Clearly, these regional differences merit further evaluation.

From a therapeutic perspective, experimental hypoxia-induced damage to PVAT function can be rescued using free radical scavengers (Greenstein et al., 2009) and Eplerenone (Withers et al., 2011). Future clinical trials can explore the possibility of using such drugs in the treatment of patients with obesity.

The chronic low-grade inflammatory state which develops as a consequence of hypoxia in obesity is marked by an increase in blood levels of reactive oxygen species and pro-inflammatory cytokines such as CRP, IL-6 and TNF-α (Trayhurn et al., 2008). Incubation with TNF reduces the anticontractile effect of PVAT in vessels harvested from healthy individuals, suggesting that the higher levels of the inflammatory cytokine in obesity may partly account for the reduced anticontractile function of PVAT. Similar results were shown in healthy rat tissue where incubation with TNF and IL6 resulted in a reduction of the anticontractile function of PVAT (Greenstein et al., 2009).

Further human studies have shown a link between high levels of IL6 and increased risk of CAD (Pai et al., 2004). Epicardial adipose tissue IL6 mRNA levels have been shown to be higher in CAD than non-CAD patients, with higher levels correlating with greater degrees of angiographically defined vascular injury (Eiras et al., 2008). The upregulation of inflammatory cytokines in the adipose tissue suggest a role for oxidative stress as a mechanism for damage to PVAT function. Proteomic analysis of epicardial adipose tissue
and subcutaneous adipose tissue obtained from CAD patients has demonstrated higher levels of reactive oxygen species (ROS) in epicardial adipose tissue, and mRNA analysis has revealed lower levels of the antioxidant enzyme catalase (Salgado-Somoza et al.).

**The role of the macrophage**

Increased macrophage numbers in adipose tissue of obese animals (Rausch et al., 2008) and humans (Weisberg et al., 2003) also support the hypoxia theory, as hypoxic cells secrete chemokines to attract macrophages (Pasarica et al., 2009). Further data from our group suggest that when PVAT from mouse vessels is rendered hypoxic, the presence and activation of macrophages is the key modulator of increased vascular contractility. Moreover, following the conditional ablation of macrophages, hypoxic insult has no effect on contractility of the vessels (Figure 4), further implicating a role for macrophages in mediating the response to inflammatory stimuli (Withers et al., 2011).

The levels of monocyte chemotactic protein-1 (MCP-1) are raised in plasma and adipose tissue of both genetically obese and diet-induced obese mice. In MCP-1 KO diet-induced obese mice, there is a significant reduction in numbers of macrophages in the adipose tissue as well as improved insulin sensitivity (Kanda et al., 2006). Paradoxically however, while it is likely that activated macrophages in obese patients contribute to PVAT damage, in health, perivascular macrophages enable structural remodelling of the small artery wall in response to flow. Bakker et al have shown that in mouse mesenteric arteries, inactivation of macrophages by clodronate reduces flow-induced remodelling (Bakker et
which is observed in arteries from diabetic animals (Belin de Chantemele et al., 2009).

We propose that reduced levels of adiponectin in obesity enhances macrophage effects on PVAT function as adiponectin suppresses macrophage phagocytosis and TNF-α production (Yokota et al., 2000). Moreover, it has been shown that hypoxia and ROS decrease adiponectin production from 3T3-L1 adipocytes (Chen et al., 2006).

A major process in the development of atherosclerosis is the recruitment of leucocytes to the endothelium and their subsequent migration into the subendothelial space. VCAM-1 and ICAM-1 are two of the adhesion molecules expressed on vascular endothelial cells and are responsible for facilitating the recruitment of the white blood cells. In patients with coronary heart disease and carotid artery atherosclerosis, there are increased levels of intercellular adhesion molecule-1 (ICAM-1) (Hwang et al., 1997). Resistin is produced by adipocytes and is also expressed in macrophages which are present in larger numbers in the PVAT of obese animals and humans. It has been shown that resistin induces the expression of ICAM-1 and VCAM-1 in human endothelial cells and that adiponectin can block this effect of resistin (Kawanami et al., 2004). PPARγ agonists have been shown to reduce the expression of resistin mRNA in macrophages (Patel et al., 2003) whilst increasing plasma levels of adiponectin (Long et al., 2010). Ouchi et al have also reported that adiponectin can dose-dependently inhibit the expression of VCAM-1 and ICAM-1 in human aortic endothelial cells (Ouchi et al., 1999).
The RAS within PVAT

The Renin-Angiotensin System (RAS) is an important regulator of blood pressure and vascular function. Circulating angiotensinogen is cleaved by renin to produce angiotensin I (Ang I). Angiotensin converting enzyme ACE) converts this to Ang II (Engeli et al., 1999) which binds to two receptors, AT1 and AT2. Adipocytes secrete products of the renin-angiotensin system (RAS) and the functional significance of this has been reviewed in depth (Engeli et al., 2003; Gorzelniak et al., 2002). RAS products such as ACE, angiotensinogen and AT 1 and AT2 receptors have been identified on human and rodent adipocytes (Cassis, 2000; Cassis et al., 1988; Crandall et al., 1999; Harp et al., 1995; Schling et al., 1999). However it is becoming apparent that the adipocyte RAS system influences tissues beyond its local environment and that it plays a role in the development of vascular diseases such as obesity and hypertension. Much is known about adipose RAS however this section will focus on the RAS component of perivascular adipose tissue.

There appears to be differential distribution of components of the RAS system expressed in both white and brown perivascular adipose tissue (Campbell et al., 1995; Campbell et al., 1993; Cassis et al., 1988; Engeli et al., 1999; Galvez et al., 2006). Angiotensinogen and Ang I expression is similar between white and brown tissue (Galvez et al., 2006); however Ang II expression appears to vary between brown and white perivascular adipocytes (Engeli et al., 1999; Giacchetti et al., 2002; Jonsson et al., 1994; Schling et al., 1999). There is conflicting evidence of renin expression in perivascular brown or white adipose tissue with some groups reporting no detectable levels of the protein and others reporting its presence (Engeli et al., 1999; Galvez-Prieto et al., 2008; Giacchetti et al., 2002)
There is a growing body of evidence to suggest that elements of adipocyte-derived RAS play vital roles in the normal and pathogenic responses of blood vessels. Ang1-7 has recently been identified as an important PVAT diffusible RAS product (Lee et al., 2009a). It is released by PVAT and acts on the endothelium to stimulate the release of nitric oxide which in turn hyperpolarises smooth muscle cells through $K_{Ca}$ channels. Ang 1-7 is thought to counterbalance the contractile influence of Ang II (Ferrario et al., 2005; Reudelhuber, 2006; Yagil et al., 2003). Increased expression of Ang II and decreased expression of Ang 1-7 has been reported in hypertensive models. Indeed treatment of hypertensive models with ACE inhibitors lowers Ang II and increases Ang 1-7 (Yagil et al., 2003). Ang I antagonism using Losartan restored PVAT induced anticontractility in fructose fed rats (Huang et al.). Ang II type 1 receptor antagonism using olmesartan can reduce blood pressure and increase adiponectin levels in a rat model of metabolic syndrome (Mizukawa et al., 2009). Lu et al have recently shown that PVAT-induced anticontractility is impaired in spontaneously hypertensive rats and that the anticontractile response could be inhibited with an Ang 1-7 antagonist in normotensive rats (Lu et al.). Interestingly Ang 1-7 is also thought to be vital for the PVAT-induced anticontractile response observed in venous rings (Lu et al.).

**Epicardial adipose tissue and its clinical correlates**

Given its proximity to the coronary vessels, epicardial and pericoronary fat present an attractive therapeutic target for coronary artery disease and the treatment of its consequent symptoms. Epicardial adipose tissue thickness correlates well with waist circumference, visceral adipose tissue mass, fasting insulin and diastolic blood pressure (Iacobellis et al.,
2003a; Iacobellis et al., 2003b) and has been shown to be significantly greater in patients with MetS than those without (Iacobellis et al., 2008).

In man, Chatterjee et al have shown that PVAT surrounding coronary vessels is made up of smaller, more irregularly shaped adipocytes which exhibit a reduced differentiation state as compared with visceral and subcutaneous fat depots. They also report that PVAT from the coronary vessels secretes lower levels of the anti-inflammatory cytokine adiponectin and higher levels of proinflammatory cytokines IL-8 and IL-6 as compared with subcutaneous and visceral adipocytes (Chatterjee et al., 2009). Moreover, exposure to IL6 has been linked with a reduction in adiponectin production by human adipocytes (Simons et al., 2007). There is a high level of macrophage infiltration in the epicardial fat tissue of CAD patients (Baker et al., 2006); indeed there is a greater pro-inflammatory profile of the epicardial adipose tissue as compared with the subcutaneous adipose tissue of individuals with CAD (Mazurek et al., 2003). Also it has been shown that peri-coronary adipose tissue contains higher levels of monocyte chemotactic protein-1 (MCP-1) as compared with visceral and subcutaneous tissue (Chatterjee et al., 2009).

There are lower adiponectin mRNA levels in the epicardial adipose tissue of patients with coronary artery disease as compared with samples from those undergoing thoracic operations for non-CAD related indications (Eiras et al., 2008; Iacobellis et al., 2009; Iacobellis et al., 2005). Lower levels of adiponectin in epicardial tissue have also been associated with hypertension (Ohashi et al., 2006; Teijeira-Fernandez et al., 2008) and increased risk of myocardial infarction (Pischon et al., 2004).
Greif et al (Greif et al., 2009) have studied the relationship between epicardial adipose tissue and coronary atherosclerosis in 286 consecutive patients with an intermediate pretest likelihood for CAD using dual-source multi-slice CT coronary angiography. Interestingly, they found no correlation between BMI and coronary atherosclerosis, but those with atherosclerotic lesions were found to have higher volumes of pericardial adipose tissue (226+/−92 cm³) than those without lesions (134 +/-56 cm³; P<0.001). Those with larger volumes of pericardial adipose tissue had lower levels of plasma adiponectin and HDL, but higher levels of the pro-inflammatory cytokine TNF and highly-sensitive CRP.

A recent comprehensive post-mortem study on 41 human cadavers carried out by Spiroglou et al. has shown that adiponectin is present in both peri-aortic and peri-coronary human fat depots and its levels inversely correlate with the degree of atherosclerosis (Spiroglou et al., 2010). In animal models of vascular injury, adiponectin knock-out mice exhibit more severe neointimal thickening and increased proliferation of vascular smooth muscle cells than wild type mice (Matsuda et al., 2002).

**Conclusions and perspectives**

In this review we have discussed clinically significant PVAT studies and highlighted a number of potential mechanisms via which PVAT may exert its anticontractile effect in health and how these can become disordered in obesity (figure 2).

Our current understanding of the intricate nature of interactions between adipocytes, vascular myocytes and endothelial cells is not sufficient to explain precisely the role adipokines play and the receptors and signalling pathways involved in facilitating their
actions. We can conclude that in response to vasoconstrictor challenges, adipocytes release adipokines with anticontractile effects on the smooth myocytes of adjacent vessels. The mechanism of action of the ADRF(s) may involve direct action on potassium channels of vascular myocytes. Circulating ADRF(s) can also work via potassium channels on the endothelium and there is also the possibility that the potassium channels present on white adipocytes may be involved in facilitating the ADRF action. ADRF action on its receptors can also lead to nitric oxide release from adipocytes and endothelial cells. We have also discussed the contribution of macrophages and ROS in compromising the anticontractile effect of PVAT in hypoxia.

It is clear that animal studies have contributed significantly to our current understanding of the mechanism of action of PVAT. However the studies have been conducted using different vascular beds in different animal models using different pharmacological approaches. Also it has become apparent that PVAT property varies amongst species and vascular beds, and not all animal studies are clinically relevant thus highlighting the paramount importance of research using human tissue.

Obesity and its associated co-morbidities pose a huge threat to public health and to our increasingly fragile economies. Clearly, prevention of obesity by effective educational programmes needs to be coupled with treatments for those currently suffering from the consequences of the disorder. Currently, the most radical and arguably the only effective intervention in treatment of obesity is bariatric surgery which is available only to a limited number of patients worldwide. The recent appreciation of the contribution of perivascular adipose tissue to metabolic homeostasis and control of local vessel tone, as well as its
involvement in disease states such as obesity, hypertension and atherosclerosis has generated great interest in the possibility of reversing the PVAT damage and rescuing its pre-morbid properties. This field of research will no doubt continue to provide new challenges and opportunities in the years to come.

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References


Figure 1
**Figure 1:** PVAT is the source of a number of vasoactive and metabolically significant adipokines. IL: Interleukin; PAI-1: Plasminogen Activator Inhibitor-1; ROS: Reactive Oxygen Species; TNF-α: Tumour Necrosis Factor-α.
Figure 2: Potential mechanisms via which perivascular adipocytes, vascular smooth muscle cells and endothelial cells interact. Dotted lines represent unproven pathways.

Figure 3

A

B

% constriction of KPSS

Log [NA] (M)

No PVAT

PVAT

No PVAT

PVAT
**Figure 3:** Effect of obesity and the metabolic syndrome on anticontractile capacity of PVAT on small arteries from subcutaneous gluteal fat *p*<0.01 (Greenstein *et al.*, 2009)

**A:** In healthy control participants PVAT exerts a significant anticontractile effect (p=0.009, Multiple ANOVA) when compared with contractility of arteries without PVAT (n=10).

**B:** In patients with obesity and metabolic syndrome, presence of PVAT has no effect on contractility. (n=10).
Figure 4

A  Wild-type

![Graph showing the effect of NE on KPSS constriction in Wild-type with symbols indicating Fat and Fat + Hypoxia conditions. The x-axis represents [NE] (mol/L) ranging from -9 to -5, and the y-axis represents % of KPSS constriction ranging from 0 to 200.]
Figure 4: The presence of macrophages is the key modulator of increased vascular contractility in vessels with hypoxic PVAT (Withers et al., 2011)

A: When PVAT from mouse vessels is rendered hypoxic, there is increased sensitivity of the vessel to cumulative doses of noradrenaline

B: In CD11b–diphtheria toxin (DT) receptor (DTR) transgenic mice (DT administration selectively kills monocytes/macrophages), hypoxia has no effect on vascular contractility
Obesity-related hypertension: epidemiology, pathophysiology, treatments and the contribution of perivascular adipose tissue

Running title: Obesity-related hypertension

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Abstract

The advent of the obesity epidemic has highlighted the need to re-assess more closely the pathophysiology of obesity-related hypertension with the aim of identifying new therapies. In this article, we review the role of the renin-angiotensin-aldosterone system, sympathetic nervous system and inflammation in relation to the pathophysiology of this condition. We also discuss the potential role of the perivascular adipose tissue in the context of obesity-related hypertension.

Key words

Adiponectin, bariatric surgery, hypertension, inflammation, obesity, oxidative stress, perivascular adipose tissue, sympathetic nervous system

Key messages

1) Obesity-related hypertension is a major and growing public health concern.
2) The pathophysiology of obesity-related hypertension is complex and involves renin-angiotensin-aldosterone system, sympathetic nervous system, oxidative stress and adipokine dysregulation.
3) Perivascular adipose tissue damage in obesity may be a contributing factor to the development of hypertension in obesity and rescuing its function may offer therapeutic potential.
**Abbreviations**

ACE: Angiotensin converting enzyme

ADRF: Adipose derived relaxing factor

AMPK: 5' adenosine monophosphate-activated protein kinase

BMI: Body mass index

BP: Blood pressure

CNS: Central nervous system

DEXA: Dual-energy x-ray absorptiometry

eNOS: Endothelial nitric oxide synthase

G6PD: Glucose-6-phosphate dehydrogenase

IRS: Insulin receptor substrate

MCP-1: Monocyte chemotactic protein-1

MetS: Metabolic syndrome

MR: Mineralocorticoid receptor

NADPH: Nicotinamide adenine dinucleotide phosphate

NO: Nitric oxide

NOS: Nitric oxide synthase

OSA: Obstructive sleep apnoea

PAME: Palmitic acid methyl ester
PVAT: Perivascular adipose tissue
RAAS: Renin-angiotensin-aldosterone system
ROS: Reactive oxygen species
SHR: Spontaneously hypertensive rat
SNS: Sympathetic nervous system
WC: Waist circumference

**Trends in obesity and hypertension**

The obesity epidemic has emerged as a major challenge to our increasingly stressed healthcare systems. Globally, obesity has more than doubled in the past 30 years. In 2008 there were 1 billion overweight and 500 million obese adults worldwide (1). In England nearly a quarter and in the US close to a third of all adults has been classified as obese (2, 3). Public health initiatives and education have had an impact on diet in England as less saturated and trans fats were consumed in 2008/09 compared to 10 years ago, but despite this, 38% of adults had a raised waist circumference in 2009 as compared to 23% in 1993, and those with a raised waist circumference were at least twice as likely to have high blood pressure (2).

Whilst it is widely accepted that obesity and its associated disorders are major risk factors for cardiovascular events, a recent study has reported that BMI, waist circumference and waist-to-hip ratio do not improve cardiovascular disease risk prediction substantially when information regarding other aspects of the medical history such as blood pressure, lipid profile and a history of diabetes are available (4). However this does not understate the
importance of obesity and metabolic syndrome as predictors of future cardiovascular events. The heterogeneity of body fat distribution has prompted the American Heart Association to stress the importance of measuring waist circumference (WC) as well as BMI. In the case of those with disproportionately high WC for a given BMI, assessment of further cardiometabolic risk factors is encouraged (5). A more streamlined assessment of body composition is fraught with difficulties as there are no internationally agreed cut-offs for measurements such as waist circumference for a given BMI, or DEXA and bioelectric impedance values (5).

Hypertension affects nearly one third of the US population (6), and globally around 40% of adults had raised BP in 2008 (7). The co-occurrence of obesity and hypertension has focused minds on understanding the pathophysiology of obesity-related hypertension. Data from the Framingham Heart Study implicate obesity as a contributory factor in 60-70% of essential hypertension (8) and obese individuals have a 3.5 fold increase in the likelihood of suffering from hypertension (9).

According to a 2011 National Health Service survey in England, high blood pressure was recorded in 48% of men and 46% of women in the obese group, compared with around 30% of those in the overweight and 15% of those in the normal weight category (2).

In this review, we aim to discuss briefly the pathophysiology of obesity-related hypertension and highlight the role of perivascular adipose tissue in this context.

**What causes hypertension in obesity?**

There are many mechanisms via which obesity can lead to hypertension (Figure 1). Gosmanov et al have reported the acute effects of high fat ingestion by normotensive
obese individuals (10). Their work suggested that both bolus oral ingestion and the intravenous infusion of fat result in a significant rise in systolic blood pressure, attenuated endothelial function (assessed by flow-mediated dilatation), increased oxidative stress markers, and activation of the sympathetic nervous system as measured by heart rate variability.

Clearly, obesity is a chronic disorder with a complex aetiology. Genetic causes of obesity are rare and the majority of cases encountered in clinics is a consequence of indulgence in readily available and calorie-rich food which provides a large amount of fat as well as a significant proportion of the recommended daily allowance of salt. High salt diets accelerate the development of hypertension in diet-induced obese rats without raising the ceiling of the systolic blood pressure beyond that observed in diet-induced obese rats fed a low salt diet (11). This effect may be propagated by an increase in oxidative stress levels in the vasculature as the same study also demonstrated a significant increase in superoxide levels within aortic rings of high-fat and high-salt diet fed animals.

There are numerous facilitators of obesity-related hypertension. These include the renin-angiotensin-aldosterone system (RAAS), the over-active sympathetic nervous system, metabolic dysregulation including hyperinsulinaemia, adipokine imbalance, and potentially PVAT damage, defined as a disturbance in the normal metabolic and vasoactive function of the adipocytes surrounding blood vessels (Figure 1). There is currently no direct evidence to suggest that a loss of PVAT anticontractile function leads to systemic hypertension, but the theory behind such a proposition will be discussed in this review.
The contribution of the renal system is significant and manifold; these include sodium retention and impaired pressure natriuresis (12). The kidneys contribute to the disease process only to fall victim to its detrimental effects as end organ damage ensues in the form of chronic kidney disease, thus leading to a vicious cycle.

There are three major factors that highlight the involvement of the renal system in obesity-related hypertension: increased activity of the renal sympathetic nervous system (SNS), activation of the RAAS and physical compression of the kidneys by intrarenal fat deposition and extracellular matrix modifications (13).

Obesity has a differential effect on local SNS activity. A significant increase in renal sympathetic activity is observed in obese individuals, but compared with lean normotensive subjects the obese normotensive individuals exhibit suppression of their cardiac sympathetic activity, whilst the hypertensive obese individuals show an increased sympathetic activity in both cardiac and renal nerves (14). The degree of renal sympathetic stimulation in obesity is similar in both normotensive and hypertensive cohorts, thus emphasising the significance of other contributory factors such as the suppression of cardiac sympathetic drive in the normotensive individuals (15). A lower cardiac sympathetic tone in obesity can be viewed as a protective factor, and it may be it is over-activity that tips the balance in favour of the development of hypertension, but it is not clear which factors dictate the differential activity of local SNS. Also there is evidence of central stimulation of the SNS by reactive oxygen species in obesity. There are raised levels of oxidative stress markers such as the superoxide anion and animal studies suggest
that NADPH oxidase-dependent oxidative stress in the brain may be a cause of increased sympathetic tone leading to hypertension in high fat fed animals (16).

In obesity, the full compliment of the RAAS components is raised. In part this is as a consequence of the intrinsic RAAS system within the adipocyte and includes ACE, angiotensin type 1 and type 2 receptors. In addition adipocytes secrete angiotensinogen, the levels of which are raised in obesity (17), as well as renin; though the source of the adipocyte renin activity remains controversial (18). Raised plasma aldosterone levels also seen in obesity correlate with the degree of visceral adiposity and waist: hip ratio (19-21). The elevated aldosterone levels will not only contribute to increased blood volume by increasing sodium reabsorption, but also lead to the generation of reactive oxygen species (ROS) (Figure 2). Aldosterone activates NADPH oxidase thus increasing ROS levels leading to oxidative posttranslational changes to guanylyl cyclase rendering it NO-insensitive (22). The generated ROS also react with nitric oxide to reduce its bioavailability by forming molecules such as peroxynitrite, thus contributing to endothelial dysfunction. There is also evidence of a vicious cycle with ROS stimulating the mineralocorticoid receptor (MR) (23), thereby theoretically contributing to further elevations in ROS levels. Aldosterone also decreases endothelial glucose-6-phosphate dehydrogenase (G6PD) activity. G6PD is a cytosolic enzyme and the main source of intracellular NADPH which functions to limit ROS activity (24). There are two aldosterone receptor antagonists in clinical use; Spironolactone is a nonselective aldosterone receptor antagonist, whereas Eplerenone is a selective aldosterone receptor antagonist which has a lower degree of cross reactivity with sex-steroid hormones and a longer half life than Spironolactone (25). Spironolactone leads to increased expression of G6PD and its activity, as well as elevated NADPH levels leading to diminished ROS
generation in aortas of aldosterone-treated mice (24). Eplerenone, a mineralocorticoid receptor antagonist, attenuates hypertension associated with diet-induced obesity in dogs, despite only modestly elevated levels of plasma aldosterone. It is not clear to what extent the blood pressure reduction is a consequence of reductions in blood volume and cardiac output secondary to reduced sodium reabsorption, or due to a reduction in sympathetic activity through the direct CNS effect of aldosterone (26, 27).

Sodium reabsorption in the kidneys is a major contributor to disease progression. The raised Angiotensin II levels in obesity raise blood pressure by direct vasoconstrictor action and also by increasing sodium reabsorption either via direct action on the kidneys or by stimulation of aldosterone release. Insulin also acts on renal tubules to increase salt reabsorption leading to retention of water and blood volume expansion (28, 29). Insulin resistance in obesity leads to a state of hyperinsulinaemia and there is a suggestion that renal tubular cells may possess a different insulin receptor substrate (IRS) to that of adipocytes and skeletal muscle cells (IRS1), thus renal tubular cells may well remain sensitive to the increased levels of plasma insulin in obesity via IRS1-independent stimulation (30).

Another important contributing factor in the pathophysiology of obesity related hypertension is vessel stiffness. Obesity has long been associated with arterial stiffness and increased pulse wave velocity independently of age, blood pressure and ethnicity. However, the association is stronger for waist circumference and visceral adiposity, rather than global obesity as measured by BMI (31). Obesity is a complex multifaceted disorder and dysregulation of any number of factors can affect vascular stiffness. Leptin has been
linked with impairment of arterial distensibility and its raised levels in obesity may be a significant contributing factor in arterial stiffness (32). Hyperinsulinaemia is another contributor to vascular stiffness. In lean individuals, insulin reduces central arterial stiffness before it exerts its slow vasodilatory effect on peripheral small arteries. In obese individuals, its effect on arterial stiffness is severely blunted; this attenuation correlates with the degree of obesity (33).

Inflammation, oxidative stress and monocyte recruitment all play their part in initiating endothelial dysfunction in obesity. There is also disruption to the fine balance between the vasoconstrictor action of endothelin-1 and the vasodilator effect of NO in endothelial cells. In health, insulin activates phosphoinositide 3-kinase leading to increased NO production secondary to eNOS phosphorylation(29). Postprandial physiological surge in insulin concentrations leads to dilatation of precapillary arterioles, thus improving blood flow and delivery of nutrients to tissues; a process known as nutritive flow (34). In obesity, NO mediated vasorelaxation is impaired, leading to vasoconstriction via unopposed endothelin-1 action (29, 34). Reduced endothelial nitric oxide bioavailability in obesity is a significant consequence of the reactions between free radicals and the vasodilator gas. Reactive oxygen species such as the superoxide anion react with nitric oxide to produce peroxynitrite and deplete endothelial NO levels (figure 2). The role of nitric oxide in vessel tone modulation and its fate in inflammatory diseases have been extensively reviewed by Jin and Loscalzo (35).

There is a close correlation between obesity, Obstructive sleep apnoea (OSA) and hypertension. OSA has been identified as both a cause and a consequence of a number of
the physiological and metabolic sequelae of obesity. A prospective study of 709
individuals with a follow up period of four years has reported a dose-response relationship
between sleep-disordered breathing and hypertension, independent of confounding factors
(36). There is a significant degree of OSA in about 40% of obese individuals and nearly
70% of OSA individuals suffer from obesity (37). Fat deposition around the upper airway
in obesity is thought to be the most significant contributor to the development of OSA in
obesity. Almost half of all hypertensive patients suffer from sleep apnoea and half of all
sleep apnoea patients are hypertensive (19). There are a number of potential mechanisms
linking OSA with hypertension, including endothelial dysfunction, CNS stimulation,
oxidative stress and inflammation (37). It is hypothesised that the most significant causal
factor is the elevated oxidative stress levels initiated by intermittent hypoxia, coupled with
hyperleptinaemia (38) with its direct stimulatory effects on the sympathetic nervous
system. There is also the suggestion that elevated levels of aldosterone exist in OSA and
that this correlates with severity of OSA. Clearly, elevated aldosterone levels would lead
to blood pressure elevations and there is a suggestion that it may even worsen upper
airway resistance by contributing to pharyngeal oedema via fluid retention (39).

**PVAT function and damage in relation to obesity-related hypertension**

Adipocytes surround almost every blood vessel in the body and secrete a number of
adipokines with metabolic and vasoactive properties. These predominantly white
adipocytes form the perivascular adipose tissue or PVAT (Figure 2).
Healthy PVAT exerts an anticontractile effect on adjacent small arteries when subject to vasoconstrictors (40). The exact mechanism via which PVAT has this effect has been the subject of many recent publications and yet remains controversial. Experimental protocols have identified both endothelium-dependent and –independent (41) mechanisms, and a number of molecules have been implicated which will be briefly discussed here. It is important to highlight that white and brown adipocytes have slightly different secretion profiles (42), but the anticontractile property of PVAT has been documented in both tissues (43, 44).

Adiponectin is the most abundant adipokine with a significant vasorelaxant effect on small arteries and is able to reverse endothelial dysfunction in diet-induced obese rats via the AMPK-eNOS pathway (45). Clinical studies have shown its levels to be low in hypertension and to improve with antihypertensive treatment (46). It has been shown that adiponectin secreted from murine PVAT modulates the tone of the adjacent vessel by functioning as an adipose tissue derived relaxant factor (47). Moreover, data from our group have clearly demonstrated that adiponectin receptor type-1 blockade abolishes PVAT anticontractile effect on adjacent small arteries from healthy human tissue (48); clearly demonstrating that adiponectin is also an ADRF in humans. Recently we have reviewed in detail the properties of this adipokine and its role as an ADRF (49).

Apart from adiponectin, PVAT secretes a number of other molecules with vasorelaxant properties; these include Angiotensin 1-7 (Ang 1-7), nitric oxide (NO), leptin, hydrogen sulphide and palmitic acid methyl ester (PAME).
Angiotensin 1-7 is secreted from PVAT and exerts its anticontractile effects by stimulating the release of endothelial NO thus activating calcium dependent potassium channels (43) in arteries, and voltage dependent potassium channels in veins (50). In support of this finding, Ang 1-7 receptor antagonists have been shown to attenuate PVAT anticontractile function (51). Ang 1-7 is also able to function via AT2 and Mas receptors to decrease the nerve stimulated overflow of noradrenaline(52). This may be of particular therapeutic interest as sympathetic over-activity contributes to pathophysiology of obesity-related hypertension. Recently, an oral preparation of Ang 1-7 has been prepared to assess its cardioprotective effects in infarcted rats (53); the effect of this product on small vessel tone remains to be assessed.

In health, white adipose tissue (54) and PVAT itself (55) are known to be sources of Nitric Oxide (NO). Insulin (56) and leptin (57) have been shown to stimulate NO production in adipocytes and, in theory, the elevated levels of leptin and insulin in obesity should enhance NO levels in PVAT. In early diet-induced obesity, there is evidence of improved NO bioavailability in mesenteric PVAT of rats (55), but factors such as elevated superoxide levels in chronic obesity would lead to a reduction in NO bioavailability via mechanisms already discussed in this review.

Leptin is secreted from white adipocytes and its plasma levels are increased in obesity. It acts centrally on the hypothalamus to reduce appetite and also to increase SNS activity (58), and locally, it has a direct endothelial nitric oxide dependent vasorelaxant effect in health. It is proposed that leptin plays a major role in pathophysiology of obesity-related hypertension. Leptin deficient ob/ob mice develop severe obesity, but remain
normotensive (59). In the vasculature, leptin stimulates the release of endothelial NO, so an acute rise in leptin levels does not significantly affect BP despite SNS activation. However, in obesity, its plasma levels are chronically raised, and endothelial dysfunction means a reduction in NO bioavailability (60), thus the vasopressor effects of leptin become more apparent.

Hydrogen sulphide and palmitic acid methyl ester (PAME) are the most recent additions to the list of potential ADRFs. *Hydrogen sulphide* is thought to function via KCNQ, and at least partly contribute to the PVAT anticontractile effect (61); whilst *PAME* functions via Kv channels, independent of nitric oxide and endothelium (62) and its release is calcium-dependent. PAME is implicated in the pathophysiology of hypertension. There is a reduction in its release from PVAT of 20 week old SHR as compared with pre-hypertensive SHR and normotensive Wistar Kyoto Rats, and exogenously applied PAME has a reduced vasorelaxant effect on de-endothelialised aortic rings of SHR as compared with its significant vasorelaxant effect on preconstricted vessels from pre-hypertensive SHR and normotensive rats (62).

There may be more than one substance released from PVAT that satisfies all the criteria for ADRF. We have shown that adiponectin is the ADRF from human subcutaneous PVAT (48). The identity of the relaxant factor may vary according to species and site of the PVAT compartments being studied.

In obesity, the anticontractile function of PVAT is attenuated or lost completely.
A number of explanations and theories exist as to the cause of this loss of function. Amongst the most likely are the effects of oxidative stress and inflammation, as well as adipokine dysregulation and increased sympathetic nervous system action.

We have shown that incubation of healthy PVAT with TNF-α and IL-6 leads to significant attenuation of PVAT anticontractile function similar to that observed in the obese phenotype (48). Macrophages secrete a number of inflammatory cytokines including TNF-α, IL-6 and free radicals such as the superoxide anion. Following experimental hypoxia, which approximates obesity-induced PVAT damage, macrophage recruitment and activation in adipose tissue is an essential step leading to the loss of PVAT anticontractile function (63). Interestingly, the PVAT surrounding rat thoracic aorta expresses brown adipose tissue genes and appears to resist inflammation and macrophage infiltration in diet-induced obesity (64).

The contribution of aldosterone to obesity-related hypertension has been discussed previously in this article. It has been shown that aldosterone increases the expression of TNF-α from macrophages. Moreover, activation of the mineralocorticoid receptor results in generation of reactive oxygen species (ROS), and blockade of the receptor, using Eplerenone, leads to a reduction of ROS and increased levels of adiponectin in obese and diabetic mice (65). The superoxide anions generated by macrophages and MR stimulation also contribute to PVAT damage. Work by Gao et al has shown that superoxide generated by NADPH oxidase in response to electric field stimulation enhances the contractile response of adjacent small arteries (66). It has been shown that Candesartan (angiotensin II type 1 receptor antagonist) reduces this PVAT-mediated potentiation of EFS-induced
contractile response, thus providing another potential explanation for the increased vascular resistance in obesity where there is both increased sympathetic nerve activity and increased angiotensin II levels (67).

In view of the significant role macrophages play in PVAT damage, their recruitment from blood to the perivascular adipose tissue is of paramount importance. Monocyte chemotactic protein-1 (MCP-1) levels are increased in adipose tissue and plasma of genetically obese and diet-induced obese mice (68), as well in obese humans (69). Insulin increases the secretion of MCP-1 in insulin resistant 3T3-L1 adipocytes and in ob/ob mice (70), thus the hyperinsulinaemic state in obesity leads to macrophage recruitment in PVAT and subsequent release of cytokines which attenuate its anticontractile function. Fractalkine (CX3CL1) is a recently identified chemokine secreted from adipocytes that promotes monocyte adhesion to human adipocytes (71). Its levels are increased in inflammatory states such as diabetes, HIV and rheumatoid arthritis as well as in obesity (72). There is also direct evidence for involvement of fractalkine in hypertension. The expression of CX3CL1 receptor gene in blood leukocytes from patients with arterial hypertension has been shown to be significantly increased (73). The increased levels of MCP-1 and fractalkine in obesity can be considered as facilitators for the initiation of the macrophage-induced loss of PVAT anticontractile function.

Adipose tissue depots have unique inflammatory profiles. PVAT from murine aortic arch expresses lower levels of adipocyte-associated genes when compared to subcutaneous and visceral fat and this becomes even more pronounced after 2 weeks of high fat feeding whilst proinflammatory genes become upregulated (74). Visceral adipose tissue exhibits a
more inflammatory profile with a higher macrophage content than subcutaneous fat (75). This may somewhat explain the stronger link between central obesity and hypertension than between BMI and raised blood pressure (76).

**Treatments**

There is no robust guidance with regards to the use of antihypertensive drugs in the treatment of obesity related hypertension and we will not cover the topic in this review. An important way to tackle the growing problem of obesity-related hypertension is to treat obesity using therapies that result in sustained weight loss. Weight reduction in obese hypertensive patients does indeed lead to an improvement in blood pressure (77).

*Diet and exercise* are the first line in both prevention and treatment of obesity. Studies have shown that the combination of intense exercise and moderate caloric restriction can lead to major reductions in markers of inflammation such as high sensitivity C-reactive protein as well as improving insulin sensitivity and enhancing adiponectin levels (78). Exercise and weight loss can also individually help lower BP. A study of 133 overweight and sedentary individuals with either unmedicated high normal BP or stage 1 to 2 hypertension has reported a reduction of 7 mm Hg in systolic BP of those assigned to behavioural weight management strategies and a 4 mm Hg reduction in systolic BP of those who took up aerobic exercise 3 to 4 times per week over the 6 month study period (79); thus clearly demonstrating that simple lifestyle measures should not be ignored.
Apart from antihypertensive treatment and lifestyle modifications, there are a number of therapeutic interventions that have either been shown to be effective or show promise in the treatment of obesity-related hypertension.

*Bariatric surgery* was first performed in 1953 and with the advent of the obesity epidemic the procedure has become increasingly popular (80). In the US the number of procedures has risen from 16,200 in 1992 to 220,000 in 2008 (81).

Lifestyle changes including diet and exercise are often the first line in the treatment of obesity. However, for the morbidly obese, bariatric surgery is able to achieve a significantly greater degree of sustained weight loss. The remission rate of hypertension is also significantly higher after bariatric surgery than after lifestyle changes (82). A one year follow up of 88 hypertensive obese patients who underwent gastric banding has shown that 59% were normotensive and 33% had reduced BP with a reduction in doses of medication (83). A recent systematic review of literature has shown that out of 16,867 patients, 49% had hypertension before the operation and 68% of these cases were either improved or resolved completely after an average follow up period of 34 months (80).

A review of 18 studies looking at bariatric surgery outcomes has shown that blood adiponectin levels increased by nearly 70% post gastric bypass and by around 36% post gastric banding procedures. The greatest increase was achieved after losing 35% of the original body weight. They also demonstrated a strong correlation between percentage increase in adiponectin levels and percentage decrease in BMI (84). This is not true of weight loss by liposuction where despite a 10% weight reduction, no improvements in adiponectin or insulin resistance have been noted (85). This is likely due to the differing
qualities of adipose tissue depots with visceral fat exhibiting a more inflammatory profile as compared with subcutaneous adipose tissue (86).

Bariatric surgery has also been shown to improve the inflammatory profile of obese individuals (87). In subcutaneous adipose tissue, the expression of IL-6 and TNFα mRNA decreases significantly and expression of adiponectin and its receptors increase after dramatic weight loss post surgery (88). The significant degree of weight loss, together with improvements in adipokine and inflammatory cytokine profile, as well as resolution or improvement in diabetes status (89) make this an invaluable procedure in those suffering from morbid obesity and its sequelae.

The over-activity of the renal SNS has already been discussed in this review. In 1995, Kassab et al had shown that renal denervation leads to a reduction in sodium retention and lower blood pressures in dogs fed a high-fat diet (90). More recently, the Symplicity HTN-2 has shown that catheter-based renal denervation can reduce BP by 32/12 mmHg in treatment resistant hypertension (91), and has already been performed in the private sector in England in November 2010 (92).

The oxidative stress-induced damage to the endothelium and PVAT in obesity would suggest that antioxidants should serve as suitable therapeutic agents to reverse this damage and possibly lower blood pressure in obesity. Indeed, administration of desmethyltirilazad (Lazaroid), a potent antioxidant, to SHR rats for three weeks has been shown to
significantly ameliorate blood pressure in these animals whilst having no effect in control animals (93).

We have already described that MR activation by aldosterone results in the generation of reactive oxygen species. Data from our group have also shown that MR blockade using Eplerenone is able to reduce macrophage activation and rescue aldosterone-induced and hypoxia-induced PVAT damage (63).

Well designed and large scale clinical trials are required to assess the potential antihypertensive actions of traditional antioxidants such as vitamin C or scavengers of ROS such as superoxide dismutase. It has been recently proposed that prevention of ROS generation using NADPH oxidase (NOX1 and NOX 2) inhibitors may be a better way of tackling the oxidative stress problem rather than attempting to scavenge the free radicals once they have been generated (94).

_Vitamin D_ is known to function as a negative modulator of RAAS by suppressing renin transcription via a vitamin D receptor (VDR) - mediated mechanism. It has been shown that VDR-null mice have increased renin and angiotensin II levels and develop hypertension and cardiac hypertrophy (95), thus vitamin D analogues can potentially serve as antihypertensive agents in the context of obesity. However, vitamin D is also implicated in energy expenditure and adipocyte biology. Compared with WT animals, VDR-null transgenic mice, fed a high fat diet remain lean and exhibit smaller adipocytes and lower leptin and triglyceride levels, as well as higher energy expenditure and oxygen consumption leading to lower fat mass (96). These findings are a result of global ablation of the vitamin D receptor and do not identify the organ system or tissues responsible for the effects. A recent publication has shown that over-expression of VDR in adipocytes
leads to suppression of lipolysis, reduction in fatty acid beta-oxidation and thermogenesis and overall reduction in energy expenditure leading to obesity (97).

The current evidence suggests that use of vitamin D analogues to treat obesity-related hypertension would be fraught with difficulties. Vitamin D is a highly fat-soluble compound and is readily deposited within adipose tissue leading to lower plasma levels of the vitamin. This leads to attenuation in the negative regulation of renin expression. Higher levels of vitamin D deposited in adipocytes would also lead to reduced lipolysis and energy expenditure and contribute to further weight gain (98).

Conclusion

Obesity-related hypertension has a complex aetiology and pathophysiology. Development of new therapies to treat this disorder requires a better understanding of the physiological dysfunction and the intricate interplay between obesity and hypertension. Rescuing the damaged PVAT function in obesity may be a new target for treatment of hypertension associated with obesity.

Sources of funding

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References


Figure 1: The complex pathophysiology of obesity-related hypertension

OSA: Obstructive sleep apnoea, RAAS: renin-angiotensin-aldosterone system, SNS: sympathetic nervous system.
Figure 2: Perivascular adipose tissue as relevant to vessel tone and adipokines implicated in obesity-related hypertension

eNOS: endothelial nitric oxide synthase, $K_v$: voltage gated potassium channel,

cNQ: A member of the $K_v$ family, MR: mineralocorticoid receptor, NO: nitric oxide,

$O_2^-$: superoxide anion, ONOO$^-$: peroxynitrite, PAME: palmitic acid methyl ester,

VSMC: vascular smooth muscle cell
CHAPTER 2: METHODS AND MATERIALS

Methods

Introduction

Aim

The overarching aim of this thesis was to use wire myography to study the effects of perivascular adipose tissue on vessel contractility in obesity.

The major focus was to ascertain how PVAT anticontractile function is altered in obesity, are the changes endothelium-dependent and can they be reversed following weight loss surgery. In addition, further protocols using immunohisotchemistry and proteomics contributed to findings from our functional protocols. These are detailed in the methods sections of the corresponding papers.

Methods

The chapter contains details of the methods used in this thesis. Tissue collection and wire myography is described in detail in the first part of the introduction. In the second part, the clinical assessment and examination procedures for patients are described, and finally, statistical methods used to analyse data are described in section three.
Tissue collection

There are two arms to this thesis (animal and human) and they will be discussed in separate sections.

Animal protocols

The animal studies were carried out on murine mesenteric arteries. I have attended the Home Office animal handling course and have been trained in schedule 1 sacrifice of rodents.

The animals were sacrificed according to the aforementioned regulations and a midline laparotomy incision was used to visualise the intestines and mesenteric bed.

The whole mesenteric bed was removed, placed in a petri dish containing chilled PSS, and prepared for dissection using micro-dissection tweezers and scissors.

Figure 1: Dissected rat mesentery

1. Small intestine - covered in a thin layer of visceral peritoneum
Human protocols

Study participants were approached at the Bariatric surgery outpatient clinic at Salford Royal Hospital (Manchester, UK). New patients attending the clinic were invited to take part in the study. On the day of the clinic I explained the study in detail and those willing to take part in the study were contacted after a few days (minimum 48 hours in accordance with our local ethics guidelines) to arrange a visit to the Wellcome Trust Clinical Research Facility (WTCRF) where further assessments including the gluteal fat biopsy were performed. The same patients were invited back 6 months following weight loss surgery to undergo the same set of investigations.

In each case, the patient was prepared, lying on their front, on a bed. The area extending from their lower back to their upper thighs was exposed. The area between the natal cleft and the outer aspect of the buttock was identified. The area was cleaned with Betadine and then a sterile field delineated using drapes. Local anaesthesia was achieved by infiltration of 2% Lignocaine. An elliptical incision was made measuring 2cm in length and just less than 1cm in width at its widest point. A sample of skin, subcutaneous tissue and fat was removed (Figure 2). The wound was closed using mattress sutures and dressed using a waterproof dressing.
The sample was immediately immersed in chilled PSS and processed into 3 sections:

a) small samples of the adipose tissue were snap frozen by immersion in liquid nitrogen

b) a cross-section of tissue was cut vertically (skin down to the deepest adipose tissue), processed and stored for immunohistochemistry.

c) the remaining part of the biopsy sample was dissected using micro-dissection equipment to identify arteries for myography (figure 3).
Isolation of arteries

In the case of the rat mesenteric bed, there is always a vein running alongside the artery and the first step is the correct identification of the artery using its distinct characteristics which only become apparent with practice.

In the human samples, there may be arteries without a corresponding vein, but the issue of the correct identification of vessel is again of paramount importance.

In the humans samples, the sample stored in PSS, was transferred to the laboratory and pinned out on a dissection dish. Arteries were identified and dissected out under a light microscope as demonstrated in the diagram below.

![Figure 4: Dissection of an artery from a gluteal fat biopsy](image)

Artery for wire myography (250-400μm in diameter)
Wire Myography

Preparation of arteries

Arteries of internal diameter 250-400μm and approximately 6mm in length were identified by blunt dissection whilst the tissue was placed in ice cold PSS and oxygenated. Perivascular adipose tissue (PVAT) was removed from a 3mm section using dissection scissors, whilst PVAT was left intact in the adjacent 3mm segment of artery, thus harvesting paired vessel rings both with and without PVAT intact. Care was taken to avoid contact of dissection equipment (scissors and tweezers) with the adventitia or the endothelium of segments.

Mounting and Normalisation

The arteries were mounted in two separate baths of a multi-chamber myograph (Danish Myotechnology, DMT) and set up as illustrated by figure 5.

Figure 5 A: Vessel mounted onto wire myograph with perivascular adipose tissue removed. B: Vessel mounted onto wire myograph with perivascular adipose tissue intact.
The baths were filled with 6ml of PSS solution and the length of the mounted vessel was manually measured using eye piece calibration. Baths were oxygenated (95% O₂ and 5% CO₂) via an attachment to the myograph and the bath solution was warmed to 37°C. At this point, the vessel was left to equilibrate for one hour before a normalisation procedure was performed. This procedure enables arteries mounted in a wire myograph to be stretched to such an extent that the transmural stretch approximates to 90mmHg and therefore reproducing a similar environment to that present \textit{in vivo}, thereby providing a standard and clinically relevant degree of tension on each vessel segment.

![Diagram of wire myograph](image)

\textbf{Figure 6}: The technique of wire myography. Taken from Mulvany \textit{et al} (Physiological Reviews 1990)².

\textbf{Assessment of contractility}

Each vessel was stimulated with KPSS three times prior to commencing dose response studies with Noradrenaline. To do this, all 6ml of the PSS in the myograph bath was aspirated and replaced with 6ml of KPSS. Once stable contraction was achieved, the KPSS was replaced with PSS and the vessel left to relax to its baseline tension. In addition to
establishing arterial viability, the contraction in response to KPSS established a baseline force in contraction to standardise dose response contractions across different preparations. Thus, the data presented for agonist induced vascular contractility in this thesis is as a percentage of the KPSS constriction in keeping with previously published literature.

Cumulative dose response curves were constructed using agonist-induced contractions elicited by the addition of increasing concentrations of Noradrenaline to the myograph bath (10^{-8} to 3 \times 10^{-5} M). Successive doses were only added once the previous contraction had reached a stable tension.

On analysis, all data regarding tension generated, whether by KPSS or agonist induced were taken from the highest, stable point of contraction. All data are expressed as mean ± standard deviation (SD).
Concentrations used to construct dose response curves:

Stock solution: Noradrenaline (Sigma-Aldrich, Dorset, United Kingdom)

$10^{-2}$M: 0.0319g in 10ml of PSS

<table>
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Table 1: Doses used for noradrenaline dose response curves
**Krebs solution: Physiological Saline Solution (PSS)**

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<td>Glucose</td>
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</tr>
<tr>
<td>KH$_2$PO$_4$</td>
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Table 2: PSS

**Modified Krebs solution: Depolarising (high potassium) kerbs solution**

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Table 3: KPSS
Clinical studies

Ethical considerations

Ethical approval for the study was initially granted in July 2009 (REC reference number 09/H1011/22) with further amendments granted during the study period to accommodate the changing requirements for specific protocols. Study participants were recruited from outpatient clinics at Salford Royal Infirmary and all assessments and procedures were performed at the Manchester Wellcome Trust Clinical Research Facility.

Clinical Procedures

Anthropomorphic measurements

All examinations were performed by nurses at the Wellcome Trust Clinical Research Facility. These included height, weight, waist and hip circumference, skin fold thickness (scapula, triceps and biceps).

Blood pressure

Blood pressure was measured after 15 minutes of rest by a semiautomatic machine (OMRON 75). Three readings were taken with the last of the three recorded as final pressure.

Bioimpedance

Bioelectrical impedance analysis is a means of measuring body composition by measuring movement of an electrical current through the body. Fat within the body allows virtually no electricity to pass through, while electricity passes quite easily through water, much of which is found in muscles. The degree of difficulty with which electricity passes through a substance is known as the electrical resistance and thus the percentage of body fat and
water can be calculated by measurement of this resistance. For the purposes of the studies in this thesis, bioimpedance was measured using a Tanita Body Composition Analyser (Model: BC-418 MA).

**Blood tests**

Protocols were performed both at the Clinical Research Facility, University of Manchester and at the specialist laboratories at Manchester Royal Infirmary

**Statistical analysis**

Data are presented as mean ±SD. The data were assessed for normality in each case and the appropriate t-test (parametric/non-parametric) was used to assess statistical significance. In order to assess the difference between vessels with intact PVAT and vessels without PVAT, a 2-way Analysis of Variance (ANOVA) test was used (dose-response experiments). The software Graphpad Prism5 (version 5.03) is used to analyse the data.

**References**

CHAPTER 3

The Effects of Bariatric Surgery on Human Small Artery Function: Evidence for Reduction in Perivascular Adipocyte Inflammation, and the Restoration of Normal Anticontractile Activity Despite Persistent Obesity

Short title: Obesity and Perivascular Adipose Tissue

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Abstract

Objectives: The aim of this study was to investigate the effects of bariatric surgery on small artery function and the mechanisms underlying this.

Background: In lean healthy humans, perivascular adipose tissue (PVAT) exerts an anticontractile effect on adjacent small arteries, but this is lost in obesity-associated conditions such as the Metabolic Syndrome and Type II Diabetes where there is evidence of adipocyte inflammation and increased oxidative stress.

Methods: Segments of small subcutaneous artery and perivascular fat were harvested from severely obese individuals before (n = 20) and 6 months after bariatric surgery (n = 15). Small artery contractile function was examined in-vitro using wire myography and perivascular adipose tissue (PVAT) morphology was assessed using immunohistochemistry.

Results: The anticontractile activity of PVAT was lost in obese patients before surgery when compared with healthy volunteers, and was restored 6 months after bariatric surgery. In-vitro protocols using superoxide dismutase and catalase rescued PVAT anticontractile function in tissue from obese individuals prior to surgery. The improvement in anticontractile function following surgery was accompanied by improvements in insulin sensitivity, serum glycaemic indices, inflammatory cytokines, adipokine profile, and systolic blood pressure together with increased PVAT adiponectin and nitric oxide bioavailability and reduced macrophage infiltration and inflammation. These changes were observed despite the patients remaining severely obese.

Conclusions: Bariatric surgery and its attendant improvements in weight, blood pressure, inflammation and metabolism collectively reverse the obesity-induced alteration to PVAT
anticontractile function. This reversal is attributable to reductions in local adipose inflammation and oxidative stress with improved adiponectin and nitric oxide bioavailability.

**Introduction**

Obesity has become a global public health challenge affecting almost half a billion adults \(^1\) and an estimated 40 million children\(^2\). Large artery disease is very common in obese patients, and manifests clinically as myocardial infarction, stroke and hypertension\(^3\). Small artery disease is also common in obesity and contributes to the development of hypertension and microvascular disease due to changes in peripheral resistance and local autoregulation\(^4\). Historically, the small artery dysfunction in obesity has been attributed to damage to the endothelium\(^5\), most notably to generation and release of nitric oxide\(^6\). However, more recently appreciation has grown for an additional mechanism by which vascular damage occurs in obesity; the function of fat surrounding arteries, or Perivascular Adipose Tissue (PVAT). PVAT surrounds the majority of blood vessels in the body and in addition to adipocytes contains inflammatory cells, stem cells, and microvasculature. In health, PVAT modulates the contractile tone of adjacent small arteries by secreting vasodilatory molecules. These adipose derived vasodilators act independently of the endothelium and include adiponectin\(^7\), nitric oxide\(^7\), hydrogen sulphide\(^8\) and palmitic acid methyl ester\(^9\). In 2009, we performed the first human small artery study of PVAT and showed that subcutaneous gluteal PVAT from lean healthy individuals reduced adrenergic constriction in adjacent arteries, an ‘anticontractile’ effect. However, in patients with metabolic syndrome the vasodilatory effect of the PVAT was entirely lost, due to dual processes of adipose tissue hypoxia and inflammation, both of which are established
sequelae of obesity in fat depots\textsuperscript{7}. More recently, we have seen that macrophage activation in adipose tissue contributes to the attenuation in PVAT anticontractile effect\textsuperscript{10}.

Bariatric surgery has been performed for nearly 60 years and is established as the most effective clinical intervention to achieve both significant and sustained weight-loss in severely obese individuals. There are three types of bariatric surgical procedures: restrictive, malabsorptive and combined operations\textsuperscript{11}. Gastric bypass surgery is a combination of restriction and malabsorption and has been shown to achieve a significantly higher degree of weight loss than restrictive bariatric surgery\textsuperscript{12}.

Bariatric surgery also dramatically improves cardiovascular risk profiles in obese patients and reduces overall mortality\textsuperscript{13,14}. The mechanisms which underlie these cardiovascular improvements remain unclear, however. In the present study we investigated the effects of bariatric surgery achieved by the gastric bypass method on the vasodilatory properties of PVAT. We report amelioration of inflammation in PVAT with complete restoration of anticontractile activity as a consequence of improved adiponectin and nitric oxide bioavailability, despite persisting obesity.
Methods:

Study population

Patients with severe obesity (BMI > 35) who were awaiting gastric bypass surgery (n=15) and lean healthy volunteers (n=7) were recruited following full informed written consent in accord with Local Research Ethics Committee approval. All participants provided a fasting venous blood sample for the measurement of inflammatory markers and adipokines.

The study participants also provided gluteal subcutaneous fat samples (1.5 x 1.5 x 1.5 cm) by undergoing a surgical biopsy under local anaesthesia\(^7\). The sample was immediately processed in three sections. One part was stored for immunohistology, the second was snap frozen for estimation of NO levels, and the remainder was used to harvest small subcutaneous arteries using micro-dissection.

Blood pressure was recorded as a mean of three recordings measured using a semiautomated machine (OMRON 705CP, White Medical, Clifton-Upon-Dunsmore, United Kingdom) whilst the participants were seated at rest for 15 minutes.

Further measurements including body mass index (BMI) and waist circumference were also recorded.

The obese patients were invited to return for a follow-up assessment, including a biopsy, six months after bariatric surgery.
Biochemical analyses

High-sensitivity CRP (hs-CRP) was measured in serum by an in-house, antibody sandwich ELISA technique using anti-human CRP antibodies, calibrators and controls from Abcam (Cambridge, UK). IL-6, TNFα, adiponectin, leptin and resistin were measured in serum, and E-selectin in plasma, all using DuoSet® ELISA development kits from R&D Systems (Abingdon, UK).

Wire Myography

One section of the gluteal fat biopsy was placed in chilled physiological saline solution (PSS, composition in mmol/L: NaCl 118, KCl 3.4, MgSO₄ 1.2, CaCl₂ 1, NaHCO₃ 25, Glucose 11, KH₂PO₄ 1.2) and oxygenated. The dissection dish was placed on ice during microdissection to preserve the integrity of the tissue whilst arterial segments 250-350 μm in diameter were harvested, one segment with PVAT intact and the adjacent segment devoid of PVAT. Both segments came from the same artery.

Endothelium denuded vessels were mounted on 40 μm wires and studied using wire myography (Danish MyoTech, Aarhus, Denmark).

The mounted vessel segments were oxygenated and maintained at a temperature of 37°C before vessel diameter and wall tension was normalised as previously described⁷,¹⁵. Vessels were challenged with a 60mM KPSS solution to establish viability and baseline constriction.
Each vessel segment was stimulated with cumulative doses of norepinephrine (Sigma-Aldrich, Dorset, UK) at the following doses: $10^{-9}$, $10^{-8}$, $3 \times 10^{-8}$, $10^{-7}$, $3 \times 10^{-7}$, $5 \times 10^{-7}$, $10^{-6}$, $2 \times 10^{-6}$, $3 \times 10^{-6}$, $5 \times 10^{-6}$, $10^{-5}$, $2 \times 10^{-5}$, $3 \times 10^{-5}$ mol/L. Contractile responses to norepinephrine are presented as a percentage of KPSS constriction, consistent with published literature\(^7,16-18\).

**Pharmacological Assessment**

Pharmacological protocols were applied to study the effect of PVAT on adjacent small arteries. In each case two segments of the same artery were prepared with and without PVAT attached as previously described\(^7\).

The role of oxidative stress was evaluated by incubation of samples from pre-operative patients with superoxide dismutase and catalase (superoxide dismutase, Sigma-Aldrich, 100u/ml incubation period 45 minutes and Catalase, Sigma-Aldrich, 100u/ml incubation period 45 minutes; \(n=4\)).

Further protocols assessed the contribution of nitric oxide and adiponectin to PVAT function in samples taken from patients after weight loss. Arteries with PVAT were incubated with blocking peptide for adiponectin receptor 1 (5μg/ml, 1.6 $\times 10^{-4}$ mol/L, Enzo Life Sciences; 45 minutes; \(n=7\)) and L-NMMA (5 $\times 10^{-5}$ mol/L, Sigma; 45 minutes; \(n=4\)).
Nitric oxide assay

Five paired pre-operative and post-operative frozen PVAT samples were homogenised by suspending the tissue in 400μl of lysis buffer (50 mmol/l Tris base, 150 mmol/l NaCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 40 mmol/l β-glycerophosphate, 50 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 10% glycerol, 1% Triton X-100 (pH 7.4)) and protease inhibitor (Complete Mini EDTA-Free; Roche Diagnostics) on ice, and utilizing a Dounce (glass/glass) tissue grinder set (Sigma-Aldrich, Dorset, UK). Upon complete homogenisation, the solution was incubated at 4°C for 10 minutes then centrifuged for 10 minutes at 16,000g. The resulting supernatant was then removed for application of the assay.

Total nitrate/nitrite concentration was measured with a nitric oxide colorimetric assay kit according to the manufacturer’s instructions (Abcam, Cambridge, UK).

Immunohistochemistry

Macrophage markers and TNF-α.

Immediately after dissection, tissue from each gluteal biopsy consisting of skin and subcutaneous fat was placed in 4% paraformaldehyde in phosphate buffered saline for 24 hours and subsequently processed to paraffin wax blocks. Consecutive 5μm sections were de-waxed, rehydrated and immunostained for two macrophage markers: CD68 [KP1], (Dako, Glostrup, Denmark; dilution 1:100), and CD68 [PGM1] (Biocare Medical, Concord, CA; dilution 1:100) and the pro-inflammatory cytokine TNF-α (Abcam, Cambridge, UK; dilution 1: 100). Antigen retrieval was performed followed by blocking of endogenous peroxidase and nonspecific protein binding with Dako blocking solutions.
Tissue sections were incubated with primary antibodies for 18 hours followed by anti-rabbit/anti-mouse EnVision-HRP (Dako) and finally by Vector SG chromogen kit (Vector Laboratories, Burlingame, CA), used to disclose the presence of the macrophages and TNF-α. Colour images were captured with a Go-3 QImaging camera (QImaging Corp., Vancouver, Canada) mounted on a Leitz Diaplan microscope. Cells stained with macrophage markers present in adipose tissue were counted and the results expressed as cells/mm². For assessment of TNF-α quantitative analysis of immunostaining was obtained converting colour images to greyscale and using a macro subroutine in an ImagePro version 6.2 image analysis programme (MediaCybernetics UK, Marlow, UK). The extent of staining was expressed as a percentage of the entire area photographed.

Adipocyte size was quantified on microphotographs obtained on Zeiss Axio Imager M2 microscope equipped with AxioCam camera and AxioVision Rel. 4.8 programme by free-hand tracing the margins of 100 consecutive cells (on average) per case to avoid selection bias (total: 3000 cells).

**Immunohistochemistry for Adiponectin receptor 1 (AdipoR1)**

Five µm deparaffinised formalin-fixed tissue sections were microwaved in citrate buffer pH 6.0, treated with 0.3% H₂O₂, blocked with Protein Block (Dako), and incubated with one of the anti-AdipoR1 antibodies, goat polyclonal to AdipoR1 (abcam), or a rabbit monoclonal to AdipoR1 (Epitomics), followed by anti-rabbit/anti-mouse EnVision-HRP (Dako) and Vector SG chromogen kit (Vector Laboratories, Burlingame, CA). The results of immunostaining were compared and the monoclonal rabbit antibody was selected for further use. Slides were examined and images captured on a Zeiss Axio Imager M2 microscope.
Statistical Analysis

The statistical presentation includes paired and unpaired tests. In the case of ordinal tests, medians and quartiles are used and for parametric tests means and standard deviation (SD) is used. Cumulative concentration-response curves were constructed using data obtained by wire myography and analysed using a two-way ANOVA and a Bonferroni post hoc test for each dose. A $P$ value of less than 0.05 was considered statistically significant. Analyses were performed using the GraphPad Prism 5 software.

Results

Study design and participants

Six months after surgery and weight loss, the patients had a significantly lower waist circumference ($n = 15; P < 0.0001$), body mass index ($P < 0.0001$) and systolic blood pressure ($P = 0.0025$) (Table1).

Post-operatively there was a significant reduction in insulin ($P = 0.0042$), fasting glucose ($P = 0.0312$), HbA1c ($P < 0.0071$) and an improvement in pancreatic $\beta$-cell function (HOMA-B; $P < 0.01$) and insulin resistance (HOMA-IR; $P = 0.0023$). After surgery serum levels of adiponectin increased significantly ($P = 0.0197$), leptin decreased ($P = 0.0014$) but resistin levels did not change significantly ($P = 0.0796$).

Weight-loss surgery restores PVAT anticontractile function
In severely obese patients before surgery and weight-loss, PVAT did not significantly alter the norepinephrine-induced contractility of the small arteries in comparison to skeletonised segments of the same vessels (n = 15, P = 0.96; Figure 1A).

In samples taken from patients 6 months following surgery and weight-loss, PVAT had a significant anticontractile effect on vessels when compared to vessels without PVAT (n = 15, P< 0.01; Figure 1B). The vessels with intact PVAT following surgery had a very similar response to cumulative doses of norepinephrine as the PVAT-intact vessels harvested from healthy lean volunteers (P = 0.52, n = 7, Figure 1B). The maximal degree of constriction is lower in PVAT-intact vessels in healthy and post-surgery samples as compared with skeletonised vessels, but the degree of constriction of skeletonized vessels to NE is similar in segments from pre and post surgery (Supplementary figure 1).

The anticontractile property of PVAT after weight loss is abolished by adiponectin blockade

Samples taken from patients six months following surgery were incubated with blocking peptide for adiponectin receptor 1. This had no effect on segments without PVAT, but in vessels with PVAT there was a significant increase in vessel contractility (P < 0.0001, n= 7; Figure 2A).

Free radical scavengers can rescue PVAT anticontractile function in obesity

In patients with severe obesity, the presence of perivascular adipose tissue had no effect on vessel contractility in comparison with vessel segments devoid of PVAT. Incubation of the vessels with the free radical scavengers superoxide dismutase and catalase resulted in a
shift in the curve to resemble that of vessels with intact PVAT taken from healthy individuals \( P < 0.001, n = 7, \text{Figure 2B} \)

**Increased NO bioavailability after surgery**

There was a significant increase in nitric oxide in PVAT of severely obese patients 6 months after surgery \( 1.016 \text{ vs } 1.196 \text{ nmol/μl, } P = 0.029, n = 5 \).

In post surgery samples, de-endothelialised vessel segments were incubated with L-NMMA. The incubation had no effect on vessels without PVAT, but in vessels with PVAT, there was a significant increase in contractility to cumulative doses of norepinephrine \( P < 0.001, n = 4, \text{Figure 2C} \).

**Reduced PVAT inflammation, adipocyte size and adipoR1 receptors following bariatric surgery**

In order to quantify the inflammation, the perivascular adipose tissue was stained for both CD68 staining macrophages (Figure 3) and the cytokine TNF-α (Figure 4A-C).

There was a significant reduction in the CD68 [KP1] staining macrophage numbers following surgery \( 7.3 \pm 1.1 \text{ vs. } 4.1 \pm 0.7, P = 0.0067, n =14; \text{Figure 4C&D} \), but the change in CD68 [PGM1] staining was not statistically changed. There was a significant reduction in the percentage of adipose tissue area staining for TNF-α \( 1.41 \pm 0.34 \text{ vs. } 0.68 \pm 0.09, P < 0.05; \text{Figure 4A-C} \) following surgery.
The average adipocyte area was 7672 ± 369.6 μm² pre-surgery and 3955 ± 207.5 μm² (P < 0.0001) post-surgery (Figure 4D). The change in average adipocyte area correlated with the change in BMI after surgery (R² = 0.356, P = 0.0243, n = 14; Supplementary Figure 2).

There was a significant reduction in circulating markers of inflammation including high-sensitivity C-reactive protein (P < 0.001), IL-6 (P = 0.013), MCP-1 (283.1 vs 258.3 pg/ml; P = 0.013) and the adhesion molecule E-selectin (12.7 vs 7.47 ng/ml; P < 0.001). However, there was no significant change in circulating TNF-alpha levels (P = 0.519) (Table1).

**Discussion**

The present study investigated the effect of bariatric surgery on the vasodilatory properties of PVAT. We designed the study following our earlier observation that in severely obese patients, whilst there is an accumulation of adipose tissue, there is also a paradoxical inhibition of the beneficial PVAT vasodilation. There are now three main findings presented here. First, bariatric surgery reverses the obesity-induced damage to PVAT anticontractile function. Second, the functional recovery of the PVAT is independent of the endothelium. Third, bariatric surgery restores the function of PVAT by reducing adipose inflammation and increasing local adiponectin and nitric oxide bioavailability. The observations advance our understanding of the mechanisms by which obesity disrupts the vasodilating function of adipose tissue and how this pathology may be reversed by clinical intervention.
The cardiovascular complications of obesity are undoubtedly amongst the most pressing issues facing clinicians today\textsuperscript{19}. To date, the most common recommended intervention has been the encouragement of dietary modification. Without doubt, effective and sustained dieting does lead to significant weight loss, but unfortunately the weight loss is not sustained\textsuperscript{20}. Furthermore, the impact of dietary modification on cardiovascular outcomes is unclear\textsuperscript{13, 21}. For patients with severe obesity, the most effective method of achieving significant and sustained weight reduction is by weight-reducing surgery\textsuperscript{14}. In addition, the weight loss following surgery also significantly correlates with improvements to blood pressure\textsuperscript{22, 23}, left ventricular mass\textsuperscript{24}, exercise capacity\textsuperscript{25} and glucose metabolism\textsuperscript{26}.

Changes to weight in patients is associated with profound modulation of the cytokine and inflammatory profiles of adipose tissue. In this regard, it is now established that in obese patients, adipose tissue undergoes dual processes of hypoxia and inflammation, leading to a reduction in the secretion of adipocytokines such as adiponectin. The hypoxia is thought to be due to adipocyte hypertrophy\textsuperscript{27} twinned with reductions to capillary density and angiogenic capacity\textsuperscript{28}. This results in up-regulation of hypoxia inducible factor-1 (HIF-1) and inflammatory differentiation of macrophages which subsequently secrete Tumour Necrosis Factor alpha (TNF)\textsuperscript{29, 31}. Differentiated macrophages in obesity also release superoxide anions which further contribute to local vascular dysfunction by diminishing availability of nitric oxide\textsuperscript{32}.

The functional damage we observe in PVAT and its recovery following bariatric surgery are entirely consistent with this hypoxia/inflammation in fat hypothesis. Thus, in our previous studies we observed adipocyte hypertrophy and increased TNF staining in
adipose tissue associated with loss of PVAT vasodilatory function. We now show that following bariatric surgery there is restoration of PVAT vasodilatory capacity to a degree which is similar to that observed in healthy non-obese participants. It is interesting to note that this functional restoration of the PVAT occurs even though the patients are still severely obese. The improvement in PVAT function after surgery is associated with smaller adipocytes (in the context of a dramatic weight loss), a reduction in PVAT TNF and increased local adipose tissue nitric oxide bioavailability. Our findings are consistent with other studies which have shown significant reductions in macrophage numbers as well as MCP-1 and HIF-1α in the stromal vascular fraction of white adipose tissue following bariatric surgery\textsuperscript{33}. Also, importantly, we show that the functional vasodilatory improvement in the subcutaneous fat is due to restitution of PVAT derived adiponectin and NO bioavailability.

The functional improvement of PVAT following bariatric surgery was independent of the vascular endothelium as all arteries in this study were denuded of endothelium. Also notably, there were no differences in constriction of arteries devoid of fat to Norepinephrine before and after surgery. Taken together, these observations indicate that the improvement in adipose-vascular coupling after surgery was due to improvements in the vasodilating capacity of the PVAT rather than changes to isolated arterial contractility or endothelial function.

There are a number of limitations to this study which need to be considered in parallel with the findings. With respect to our patient selection, we did not monitor the level of exercise performed by the participants at baseline or following surgery. At six months
after surgery, the patients had lost a significant amount of weight and were presumably more mobile. As such, we acknowledge that the improvement in PVAT function may be as a consequence of increased levels of mobility. Second, it would have been preferential to recruit participants who were losing weight as part of a calorie controlled diet in order to compare changes to PVAT function due to this intervention. This was not possible within the limitations of our faculty, but we hope to perform this study in the future. Similarly, a drawback of this study is that we were not able to perform a 6 month follow-up biopsy on severely obese patients who received no intervention. However, we assume that there would have been no change in PVAT function over this time. We have studied subcutaneous small arteries under the assumption that all small arteries and the surrounding PVAT share similar properties. A separate consideration is that we have detected changes only to subcutaneous gluteal PVAT following surgery. We did not study the function of PVAT from other anatomical sites (i.e. mesenteric or skeletal). As such, we acknowledge that changes to subcutaneous vascular function may not be reflected in other vascular territories, but previous studies have shown similar remodeling profiles of human cerebral and mesenteric arteries to those seen in arteries taken from gluteal subcutaneous biopsies. Finally, it should be noted that norepinephrine was the only contractile agent used. We acknowledge that it would have been preferable to study a variety of vasoconstrictors. However, it should be noted that studies from other groups have shown that PVAT antagonizes contraction to U46619, Angiotensin II, Phenylephrine and serotonin.

Despite these limitations however, we believe that we present convincing evidence that PVAT induced vasodilation of small arteries can be fully restored following bariatric surgery. Furthermore, this restoration of function is due to a reduction in inflammation.
within the adipose tissue which leads to increased adiponectin secretion. The data offer an insight into one mechanism by which bariatric surgery improves vascular function. We anticipate that further work will harness this observation to improve therapeutic options for the severely obese patient.

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**Disclosures**

The authors declare no conflict of interest.
References


Figure legends

**Figure 1: Background and Hypothesis**

**A:** In obesity there is an increase in PVAT levels of inflammatory cells and cytokines as well as increase in levels of leptin.

**B:** We hypothesize that following bariatric surgery, there will be an increase in adiponectin levels and nitric oxide bioavailability in PVAT and reduction this inflammation which would result in restoration of PVAT anticontractile function.

**Figure 2: The effect of perivascular adipose tissue on small artery tone before and after surgery**

**A:** In pre-surgery patients, the presence of PVAT did not affect vessel contractility as compared with skeletonised segments of the same vessel (n = 20, P = 0.95)

**B:** In post-surgery patients, the presence of PVAT had a significant anticontractile effect on the small artery contractility (n = 15, P < 0.01).
Figure 3: Pharmacological protocols on PVAT pre-surgery and post-surgery

A: Blocking peptide for PAb to adiponectin receptor 1 increases vessel contractility to norepinephrine (n = 7, P < 0.0001).

B: Incubation with SOD and catalase rescues the PVAT anticontractile effect in samples taken from pre-surgery patients (n = 7, P < 0.001).

C: Post-surgery, inhibition of nitric oxide synthase by incubation with L-NMMA leads to increased contractility of vessel segments with intact PVAT (n = 4, P < 0.001)

Figure 4: Subcutaneous adipose tissue before and after surgery. Inflammatory infiltrates composed mostly of macrophages were present either in wreath-like arrangements (A), in groups (B), or were scattered. Staining for macrophage marker (CD68 (KP1)) (C) allowed for performing cell counts. There was a significant reduction in the number of CD-68 staining macrophages post-surgery (n = 14, 7.3 ± 1.1 vs 4.1 ± 0.7, P < 0.01) (D). Scale bar = 20 μm.

Figure 5: Assessment of TNF-α staining and adipocyte size pre- and post-surgery. Immunostaining for TNF-α showed that adipocytes themselves were positive for this cytokine, to a higher extent pre-surgery (A) than post-surgery (B). Note positive staining of microvessels in the pre-operative biopsy. There is a significant reduction in the percentage of adipose tissue area that stains for TNF-α pre-surgery versus post-surgery (1.41 ± 0.34 vs 0.68 ± 0.09, P < 0.05; (C)). The average adipocyte area was 7672 ± 369.6 μm² pre-surgery and 3955 ± 207.5 μm² (P < 0.0001) post-surgery (D). Scale bar = 20 μm.
Table 1: Patient demographics and biomarker profile

Mean (SD) is indicated. *P* values compare the matched baseline and post-surgery values, and were determined using paired t-tests.
Figures

1A

1B
2A

\[ \text{PVAT (Post-surgery) + Adiponectin R1 blocker} \]

\[ \text{PVAT (Post-surgery)} \]

2B

\[ \text{PVAT (Pre-surgery)} \]

\[ \text{PVAT (Pre-surgery) + SOD & Catalase} \]

2C

\[ \text{PVAT (Post-surgery) + L-NMMA} \]

\[ \text{PVAT (Post-surgery)} \]
Pre-surgery  Post-surgery
0  2  4  6  8  10
*  
No. of macrophages/mm²

![Images of tissue samples](A, B, C)

![Graph showing comparison of macrophage counts](D)

Pre-surgery vs. Post-surgery: * indicates a statistically significant difference.
### Graphs

#### C

- **X-axis:** Pre-surgery, Post-surgery
- **Y-axis:** Fat stained for TNF-α/ frame (%)
- **Legend:**
  - Pre-surgery: Black bar
  - Post-surgery: Red bar
- **Note:** A significant difference is indicated by an asterisk (*)

#### D

- **X-axis:** Pre-surgery, Post-surgery
- **Y-axis:** Adipocyte area µm²
- **Legend:**
  - Pre-surgery: Black bar
  - Post-surgery: Blue bar
- **Note:** A significant difference is indicated by an asterisk (*)

### Diagram 5A

- **Left Segment:**
  - **Title:** Decreased Adiponectin
  - **Subtext:** Nitric oxide bioavailability, Free radical scavengers

- **Right Segment:**
  - **Title:** Increased Obesity
  - **Subtext:** Leptin, TNF α, MCP-1, Oxidative stress, Macrophages

- **Common Elements:**
  - Macroblades
  - Arrows indicating interactions

### Text

- **Key Terms:**
  - Adiponectin
  - Nitric oxide bioavailability
  - Leptin
  - TNF α
  - MCP-1
  - Oxidative stress

- **Relationships:**
  - Decreased Adiponectin
  - Increased Obesity

- **Notes:**
  - Increased Oxidative stress
  - Increased Macrophages
  - Decreased Adiponectin
  - Decreased Nitric oxide bioavailability
  - Increased Leptin
  - Increased TNF α
  - Increased MCP-1

- **Purpose:**
  - Illustrating the impact of obesity on various biological factors

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Healthy PVAT

Anticontractile

Obese PVAT

No anticontractile property

1) ↓ Adiponectin
2) ↓ Nitric Oxide
3) ↑ Inflammation

Post-surgery PVAT

Anticontractile

1) ↑ Adiponectin
2) ↑ Nitric Oxide
3) ↓ Inflammation
Supplementary materials

Supplemental figures

Figure 1

A

B
**Supplementary figure 1:** Comparison of vessel constriction pre- and post-surgery

A: There is no difference in constriction of skeletonised to NE before and after surgery (P = 0.07, n=15)

B: There is a significant difference in constriction of vessels with intact PVAT before and after surgery (P < 0.0001, n=15)
Supplementary figure 2: The change in average adipocyte area post-surgery as compared with baseline correlates with the change in BMI post-surgery (n = 14, $R^2 = 0.356$, $P = 0.024$)
CHAPTER 4

Obesity-induced damage to perivascular adipose tissue anticontractile function is nitric oxide dependent, independent of the endothelium and reversible

Short title: PVAT damage in obesity

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Abstract

Objective

White adipocytes are the main constituents of perivascular adipose tissue (PVAT) which surrounds most vessels in the human body. PVAT is the source of a number of molecules with vasoactive and metabolic properties. In health, PVAT exhibits a vasodilating effect on its adjacent vessel; however, this effect is not observed in obesity. The aim of this study was to investigate whether the obesity-induced damage to PVAT function is endothelium-dependent and assess the role of inflammation and nitric-oxide (NO) in obesity-induced PVAT damage.

Methods

Gluteal fat biopsies were performed on 10 obese and 10 control individuals. Adipose tissue was immediately snap frozen and stored for proteomic analysis. Small arteries were isolated from the biopsy samples and their function was assessed using wire myography (n=7 for obese and control). Sprague Dawley rats were fed a high fat diet (obese; n=16) or normal chow (control; n=6) and PVAT function was assessed using wire myography. Skeletonised vessel segments as well as segments with intact PVAT were prepared with both intact endothelium and as endothelium-denuded segments. Vessel segments were incubated with L-NNA (10^{-4}M, 45 minutes) or SOD (100 U/ml, 45 minutes) and Catalase (100 U/ml, 45 minutes), to assess NO bioavailability and the contribution of inflammatory-mediated damage to PVAT function respectively.
Results

*Human protocols:* In the control samples, presence of PVAT results in a significant attenuation in vessel contractility to cumulative doses of NE, but this effect is not observed in samples from obese individuals. Superoxide dismutase [Cu-Zn] (SOD1; fold change -2.4182, \( P = 0.018 \)), peroxiredoxin-1 (PRDX1; fold change -2.15, \( P = 0.048 \)), and adiponectin (fold change -2.1; \( P = 0.018 \)) were present in lower abundances in obese PVAT compared with controls.

*Animal protocols:* Obese rats developed significantly higher systolic (127 ± 3 mmHg vs 144 ± 3 mmHg; \( P < 0.01 \)) and diastolic (100 ± 5 mmHg vs 118 ± 3 mmHg; \( P < 0.01 \)) blood pressures (BP) as compared with controls. There were significant correlations between weight and both systolic (\( r = 0.5044, P = 0.0167 \)) and diastolic (\( r = 0.5329, P = 0.0107 \)) blood pressures. There was a significant correlation between weight and damage to PVAT function (\( r = 0.5059, P = 0.0163 \)), and PVAT function correlated with both systolic (\( r = 0.5324, P = 0.0108 \)) and diastolic (\( r = 0.4722, P = 0.0265 \)) blood pressures.

In healthy animals, there was preservation of PVAT anticontractile function after removal of the endothelium. In the obese animals, PVAT did not affect vessel contractility and presence of endothelium did not affect this. In endothelium-denuded vessels, incubation with L-NNA attenuated PVAT anticontractile function in control vessels (\( P < 0.0001 \)), but had no effect on vessel segments from the obese (\( P = 0.4 \)). Incubation with SOD and Catalase lead to an attenuation in PVAT-intact vessel contractility in the presence (\( P < 0.0001 \)) and after removal of the endothelium (\( P < 0.001 \)).
Conclusions

In keeping with our prior observations, obese PVAT has no effect on vessel contractility. The obesity-induced PVAT damage is independent of the endothelium, and in part, due to a reduction in NO bioavailability within PVAT.

There are significantly lower levels of SOD, Peroxiredoxin 1 and adiponectin in obese human PVAT as compared with healthy controls, and incubation with free radical scavengers restores PVAT function in obese animals, independently of endothelial presence.

Key words

Obesity
Vascular tone
Perivascular adipose tissue
Contractility
Endothelium
Nitric oxide
Abbreviations

PVAT: Perivascular adipose tissue

BP: Blood pressure

NE: Norepinephrine

SOD: Superoxide dismutase

CAT: Catalase

L-NNA: L-N\textsuperscript{G}-Nitroarginine; N\textsuperscript{G}-nitro-L-Arginine

NO: Nitric oxide
Introduction

Obesity has a profound effect on cardiovascular risk profile. Specifically with regards to hypertension, weight gain is associated with a rise in BP whilst weight loss is associated with a reduction in BP. Understanding this relationship has been substantially advanced by studies into the vasoactive properties of adipose tissue surrounding blood vessels, known as Perivascular Adipose Tissue (PVAT). Soltis and Cassis were the first to show that PVAT antagonised arterial vasoconstriction in 1991\(^1\). More recently, the vasorelaxant effect of PVAT has been demonstrated in both resistance and conduit arteries, and appears to utilise multiple physiological mechanisms. Thus, both endothelial independent and endothelial dependent pathways are present and nitric oxide, hydrogen sulphide, angiotensin 1-7, adiponectin and insulin have all been implicated in the vasorelaxant effect. We have shown that in subcutaneous human tissue from healthy participants, adiponectin release from PVAT acting via nitric oxide was the predominant mediator of the anticontractile effect on small arteries. We also observed that in obese patients with metabolic syndrome, there was complete loss of the vasoactive properties of PVAT. In this follow-on study, we have used a proteomic approach to examine, for the first time, molecular changes to subcutaneous PVAT in obese patients compared with healthy participants; and interpret these in tandem with the functional properties of the adipose tissue. We have used the findings to advance an existing hypothesis to account for damage to PVAT function in obesity and validated this in an animal model of diet-induced obesity. Overall, the project provides an insight into the changes of human adipose tissue which develop in obesity and how these affect the vasoactive function of PVAT.
Materials and Methods

Study population

10 patients with obesity and 10 control participants gave full written informed consent and participated in the study, which was approved by the Local Research Ethics Committee. Fasting venous blood samples were taken to assess glycaemic and lipid profiles and inflammatory markers. Blood pressure was measured sitting, after 15 minutes of rest, by a semiautomatic machine (OMRON 705CP, White Medical) with a mean of 3 readings recorded. Anthropometric measurements and bioimpedance were also measured.

Gluteal fat biopsy

A subcutaneous gluteal fat biopsy was obtained from each subject, under local anaesthesia, allowing tissue (2x1.5x1.5cm) to be harvested. Using microdissection, a small section of perivascular adipose tissue was dissected from the tissue and placed into liquid nitrogen for proteomic analysis. The remainder of the biopsy was then placed in physiological saline solution (PSS) with a composition (mmol/L) of: NaCl 119, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.17, MgSO₄ 1.17, EDTA 0.026, CaCl₂ 1.6 and glucose 5.5.

Biochemical analysis of serum

Highly sensitive-CRP, leptin, IL-6, TNF and adiponectin were assayed by an in-house ELISA technique (hs-CRP:Abcam: remainder:R&D Systems). Insulin levels were measured using a radiolabelling technique described previously². The homeostasis model
assessment (HOMA) was used to estimate beta cell function (HOMA-B) and peripheral insulin sensitivity (HOMA-S).

**Wire Myography**

Viable arterial segments were obtained from 7 control participants and 7 obese patients with metabolic syndrome. An arterial segment with an internal diameter of 250-350μm was dissected from each biopsy. One segment was cleaned of PVAT, whilst on the adjacent segment PVAT was left intact. Both arteries were mounted on 40μm wires in a wire myograph (Danish MyoTech). After an initial 30 minute incubation, vessel wall tension and diameter were normalized in a standardised procedure and stabilised for 1 hour.

Arteries were first challenged with 60mM KPSS to establish a baseline contractile response. A cumulative dose response to Norepinephrine was then constructed. Each vessel was stimulated by addition of Norepinephrine (Sigma) at the following concentrations: $10^9$, $3 \times 10^9$, $10^8$, $3 \times 10^8$, $10^7$, $3 \times 10^7$, $10^6$, $3 \times 10^6$, $10^5$ mol/L. Contractile responses to Norepinephrine are expressed as a percentage of KPSS contraction, consistent with other studies.

**Proteomic analysis**

Gluteal perivascular adipose tissue fat samples from the 10 healthy and 10 of the obese patients were immediately immersed in liquid nitrogen and stored at -80°C for analysis by Oxford Biomarker Services (Oxford BioTherapeutics Ltd). Protein levels were compared using a label free mass spectrometric approach. Given our previously published data.
highlighting the significance of adiponectin in PVAT anticontractile function\textsuperscript{9}, we established a targeted quantification method for adiponectin in these samples using Selected Reaction Monitoring (SRM) mass spectrometry to determine the relative levels of two adiponectin peptides: GDIGETGVPGAEGPR and IFYNQQNHYDGSTGK (Supplemental methods).

**Animal model development**

**Animals and Diets**

Four week old male Sprague Dawley rats were purchased from Charles River (UK). The rats were housed in groups of three per cage (changed to two per cage after two months) in a standard experimental animal laboratory, illuminated from 6.30 a.m. to 6.30 p.m., at a temperature of 22±1°C. The protocol was approved by the Animal Experimentation Committee of the Medical Faculty of University of Manchester. The animals had free access to food and water during the experiment.

After a 1-week acclimatization period, the rats were divided into 2 groups receiving either a high-fat diet (Western RD; 4.24 kcal/g (AFE); 35% of energy derived from fat; Special Diets Services n = 16) or standard laboratory chow (AFE: 3.29 kcal/g; n = 6) for 3 months.

Body weight and blood pressure was monitored once a week and the consumption of feed was monitored daily using a standard laboratory table scale. At the time of sacrifice, the animals were weighed, blood pressure was recorded (LE5002 Storage Pressure Meter from Panlab Harvard Apparatus) and blood glucose was analysed using a portable glucose monitoring system (Ascensia® CONTOUR® blood glucose monitoring system, Bayer HealthCare).
**Vessel preparation and myography**

The mesenteric bed was immediately removed after animal sacrifice and placed in chilled PSS (detailed above) for transfer to the laboratory. Isolated mesenteric vessels with average internal diameter of 250 μm were dissected and prepared as segments with intact perivascular (PVAT) and segments without PVAT. The vessels were studied using a multi wire myograph system (Danish Myo Technology; Model 610 M, version 2.2) and analysed on ADInstruments Chart™ 5 (version 5.5.1). The vessels were bathed in PSS (as above), maintained at 37°C and gassed with a mixture of (95% air, 5% CO₂). The vessels were normalised and their viability was checked by addition of PSS containing 60mM KCl following which concentration-response curves to NE (Sigma-Aldrich A0937) were constructed. To assess the effect of free radical scavengers on the obese PVAT, vessels from the obese cohort were incubated with superoxide dismutase (100 U/ml) and catalase (100 U/ml) for 45 minutes, following which another norepinephrine concentration-response curve was constructed. Similarly, vessels were incubated with L-NNA (10⁻⁴M, 45minutes) to assess NO bioavailability within PVAT.

**Results**

**Patient details and PVAT function**

The obese individuals had significantly greater waist circumference, body mass index, HDL, total cholesterol, HbA₁C, leptin, insulin, glucose and HOMA-S (P<0.05). However, triglycerides, blood pressure, resistin, TNF-alpha, IL-6, adiponectin and HOMA-B were not significantly different (table 1). As we have demonstrated previously ⁹, ¹⁰, in healthy
participants the presence of perivascular adipose tissue lead to an attenuation in contractility of adjacent small arteries (P < 0.05; figure 1A) while in obesity this PVAT effect was not observed (figure 1B).

**Animal characteristics, blood pressure, NO and PVAT function**

At the time of sacrifice, the high fat fed group weighed significantly more than the control group (511 ± 13g vs 622 ± 14g ; P <0.001), and developed both systolic (127 ± 3 mmHg vs 144 ± 3 mmHg; P<0.01) and diastolic (100 ± 5 mmHg vs 118 ± 3 mmHg; P <0.01) blood pressures that were significantly raised as compared to controls. Glucose was not significantly different (P = 0.66). PVAT from the control group attenuated vessel contractility to NE (P < 0.0001), but no such effect was observed in the obese group (P = 0.191). There were significant correlations between weight and both systolic (r = 0.5044, P = 0.0167; figure 2A) and diastolic (r = 0.5329, P = 0.0107; figure 2B) blood pressures of the rats at the time of sacrifice.

In order to quantify the PVAT anticontractile effect, at a given concentration of norepinephrine, constriction of the vessel segment with PVAT was compared to the constriction of vessel segment without PVAT, and this value was presented as a percentage calculation (constriction of vessel with PVAT / constriction of vessel without PVAT * 100). There was also a significant correlation between weight and damage to PVAT function (r = 0.5059, P=0.0163; figure 2C). The damage to PVAT function also correlated with both systolic (r = 0.5324, P = 0.0108; figure 2D) and diastolic (r = 0.4722, P = 0.0265; supplementary figure 1) blood pressures at the time of sacrifice.
Incubation of PVAT-intact endothelium-denuded vessels with the NO synthase inhibitor L-NNA resulted in a significant increase in vessel contractility in vessels segments from healthy animals (figure 3A), however, no such phenomenon was observed in obese PVAT (figure 3B).

**Downregulation of Superoxide Dismutase, Peroxiredoxin 1 and adiponectin in human PVAT**

Proteomic analysis revealed 932 peaks present in over half of the samples analysed and these were used for quantification. Following statistical analysis, 67 peptides were found to be significantly different in these tissues. Removal of peptides emanating from blood-derived proteins left 47 significant peptides (table 2). Levels of the free radical scavengers superoxide dismutase [Cu-Zn] (SOD1; fold change -2.4182, \( P = 0.018 \)) and peroxiredoxin-1 (PRDX1; fold change -2.15, \( P = 0.048 \)) were found to be significantly lower in the obese versus lean volunteers. Adiponectin (two peptides: GDIGETGVPGAEGPR and IFYNQQNHYDGSTGK) levels in PVAT were lower in obese patients compared with the lean volunteers with an average fold change of 2.1 (\( P < 0.05 \); supplementary figure 2).

**PVAT anticontractile function in obesity is damaged independently of the endothelium and incubation with free radical scavengers restores PVAT function in obese animals**
Cumulative dose response protocols with norepinephrine were performed both with intact endothelium and after removal of endothelium. Vessel segments from healthy animals constricted significantly less as compared with segments from obese animals, both when the endothelium was present (P < 0.05, n=8; figure 4A) and in endothelium denuded vessels (P < 0.05, n=8; figure 4B).

Incubation with SOD and catalase resulted in attenuation of vessel constriction to a similar degree whether the endothelium was intact (figure 5A) or removed (figure 5B).

Discussion

The present study investigated the effects of obesity on PVAT function and assessed the contribution of nitric oxide and endothelium in this process. We have shown previously that in healthy individuals PVAT exerts a vasorelaxant effect on its adjacent small vessel and that this is not observed in obesity\(^9\). Moreover, we have shown that this vasorelaxant effect is restored following significant weight loss\(^10\). We now present four novel findings here. First, using proteomic analysis, we show that levels of adiponectin and the free radical scavengers SOD and peroxiredoxin-1 are significantly lower in the PVAT from obese individuals as compared with healthy controls. Second, in healthy animals, there was preservation of PVAT anticontractile capacity after removal of the endothelium, confirming observations by Gao et al\(^6\). The obesity-induced damage to PVAT function is independent of the endothelium and the PVAT vasorelaxant effect is restored using free radical scavenger independent of the endothelium. Third, NO from PVAT is an important endothelium-independent contributor to PVAT vasorelaxant function in healthy tissue, but
not observed in obesity. Fourth, PVAT function correlates with systemic blood pressure and obesity-induced damage to PVAT vasorelaxant quality correlates with a rise in BP.

In keeping with increased inflammation in adipose tissue, our proteomic data show that levels of the free radical scavengers superoxide dismutase [Cu-Zn] and peroxiredoxin-1 are significantly lower in the obese patients as compared with the healthy lean volunteers. Macrophages present within PVAT contribute to the inflammatory environment by secreting superoxide anions. Superoxide dismutase converts the highly volatile superoxide anion into hydrogen peroxide which is then broken down into oxygen and water with the help of catalase, glutathione peroxidise (GTP) and peroxiredoxin (PRX) enzymes. Peroxiredoxin-1 resides within the cytoplasm and is the most abundant of the Prxs. The catalytic efficiency of Prxs is less than that of glucothione peroxidase and catalase, but a reduction in their levels may lead to a possible reduction in the rate of H₂O₂ catabolism and its accumulation in cells with its consequent effects on vascular tone. In the present study, we have shown that in obesity we can rescue the PVAT vasorelaxant effect by incubating the obese PVAT with the free radical scavengers superoxide dismutase and catalase, and that this is not endothelium-dependent.

Gil-Ortega et al have shown previously that after 8 weeks on a high fat diet, mouse mesenteric PVAT possesses greater NO bioavailability as compared with controls, and have described this as an adaptive overproduction of the vasorelaxant molecule in early diet-induced obesity. In keeping with their findings, we confirm that, in health, PVAT vasorelaxant function is partly dependent on NO present within the PVAT itself, independent of the endothelium. However, after 3 months on the high fat diet, our
functional data show a significant reduction in NO bioavailability within PVAT, thus suggesting that the proposed adaptive overproduction of NO is no longer observed once the animals remain on the diet for longer.

Finally, we have shown for the first time, that damage to PVAT vasorelaxant function correlates with a significant rise in BP and this remains to be explored further using human protocols.

**Conflict of Interest/Disclosure**

There are no conflicts of interest to declare.
References

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Figure Legends

Figure 1

In healthy participants the presence of PVAT lead to an attenuation in contractility of adjacent small arteries (P < 0.05, n=7; figure 1A) while in obesity, the presence of PVAT did not affect vessel contractility (n=7; figure 1B).

Figure 2

There were significant correlations between weight and both systolic (r = 0.5044, P = 0.0167; figure 2A) and diastolic (r = 0.5329, P = 0.0107; figure 2B) BP. There was a significant correlation between weight and damage to PVAT function (r = 0.5059, P=0.0163; figure 2C). The damage to PVAT function also correlated with systolic BP (r = 0.5324, P = 0.0108; figure 2D).

Figure 3

Incubation of PVAT-intact endothelium-denuded vessels with the NO synthase inhibitor L-NNA resulted in a significant increase in vessel contractility in healthy vessels (figure 3A), however, no such phenomenon was observed in obese vessels (figure 3B).

Figure 4

Vessel segments from healthy animals constricted significantly less as compared with segments from obese animals, both when the endothelium was present (P < 0.05, n=8; figure 4A) and in endothelium denuded vessels (P < 0.05, n= 8; figure 4B).
Figure 5

Incubation with SOD and catalase resulted in attenuation of vessel constriction to a similar degree whether the endothelium was intact (figure 5A) or removed (figure 5B).
Figures

Figure 1

**A**

![Graph showing the relationship between Log [NE] (M) and %constriction of KPSS for Healthy: P(-)E(+) and Healthy: P(+)E(+).](image)

**B**

![Graph showing the relationship between Log [NE] (M) and %constriction of KPSS for Obese: P(-)E(+) and Obese: P(+)E(+).](image)
Figure 2

A

Systolic BP (mmHg) vs Weight (g)

B

Diastolic BP (mmHg) vs Weight (g)
Figure 3

A

- P(+)E(-) Healthy
- P(+)E(-) Healthy + LNNA

% KPSS constriction

Log [NE] (M)

B

- P(+)E(-) Obese
- P(+)E(-) Obese + LNNA

% KPSS constriction

Log [NE] (M)
Figure 4

A

Log [NE] (M)

% KPSS constriction

PVAT(+)E(+) Healthy

PVAT(+)E(+) Obese

B

Log [NE] (M)

% KPSS constriction

PVAT(+)E(-) Healthy

PVAT(+)E(-) Obese
Supplementary figures

Figure 1

![Figure 1](image1)

Figure 2

![Figure 2](image2)
**Supplemental methods**

**Comparative profiling by label-free MS**

Frozen tissue was resuspended in a minimal volume of lysis buffer containing 8M Urea, 2M Thiourea, 4% (w/v) CHAPS, 65mM DTT. Lysate was clarified at 10,000rpm in a microfuge for 3 mins to remove particulates, and protein lysate was collected taking care to avoid the lipid layer in top of the sample. Protein was diluted 1:10, protein concentration determined using a Bradford assay, then further diluted to a final concentration of 1mg ml\(^{-1}\). Fifty µg of protein was then digested by addition of 50µL 200mM ammonium bicarbonate and 25µL of 0.5% (w/v) Rapigest (Waters). Proteins were reduced by addition of 10µL 75mM DTT and incubation at 80°C for 15 mins. Samples were cooled and alkylated using 10µL 150mM iodoacetamide in the dark for 30 mins. Trypsin was added in 50mM ammonium bicarbonate to a final enzyme:substrate ratio of 1:25, and incubated at 37°C overnight. Following digestions, peptides were cleaned up using a 96-well strong anion exchange plate (Bio-Rad) following the manufacturers instructions.

For each sample, an amount corresponding to 0.15µg of total protein was injected onto an HPLC Chip (High Capacity Loading, 150mm 300 Å C18 chip with 160 nL trap column; Agilent Technologies). Peptides were eluted using a gradient of 5-35% acetonitrile over 65 minutes at 300 nL min\(^{-1}\). Eluted peptides were detected using a 6510 Q-ToF mass spectrometer, with MS1 data collected in profile mode and MS2 data in centroid.

Mass, retention time and area of each peak in each sample were extracted using MassHunter (Agilent) and were analysed using GeneSpring (Agilent). Peaks of interest were identified by tandem MS using a targeted method where peptides were ordered by retention time and assigned sequentially to one of five target lists. LC-MS/MS methods
were built using these lists as MS/MS target lists. MS/MS data were searched against mouse and human protein databases using SpectrumMill software (Agilent).

**Selected Reaction Monitoring (SRM) determination of Adiponectin levels**

Adiponectin levels were determined by measuring the levels of two peptides in adipose tissue digests prepared as above. Peptide standards GDIGETGVPGAEGPR and IFYNQQNHYDGSTGK were synthesized with stable isotope labels on their terminal residues, using $^{13}\text{C}_6,^{15}\text{N}_4$-Arg and $^{13}\text{C}_6,^{15}\text{N}_2$-Lys respectively. A pool of aliquots from all samples in the study was used to optimise SRM transitions and calculate appropriate spiking volumes (GDIGETGVPGAEGPR at 1 fmol µl$^{-1}$, IFYNQQNHYDGSTGK at 55.5 fmol µl$^{-1}$). Digested samples were spiked and 4µL analysed using a Tempo chromatography system (AB Sciex) coupled to a 4000 QTrap hybrid triple quadropole/linear ion trap MS (AB Sciex) in random order. Peptides were separated using a PepMap 100-C18 150mm,75µm i.d. column using a gradient of water with 0.1% formic acid and an increasing concentration of acetonitrile from 5% to 40% over 20 minutes at 300 nL min$^{-1}$. Data was acquired using MRM transitions detailed in Supplementary Data Table 1. Data was extracted by manually integrating peaks to ensure veracity. Values were normalised to overall protein content using the total peak area from the label free quantification experiments. Data was then log transformed prior to statistical analysis.
<table>
<thead>
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<th>Transition (endogenous)</th>
<th>Transition (labelled)</th>
<th>Dwell time (ms)</th>
<th>Collision energy (eV)</th>
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<tr>
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**Table 1.** SRM transitions for quantification of adiponectin
CHAPTER 5

Adipose Tissue Inflammation is More Pronounced and High Density Lipoprotein Antioxidant Function is Impaired in Obese Patients with Obstructive Sleep Apnea

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Abstract

Background: Obstructive sleep apnea (OSA) is a common disorder in morbidly obese patients and is associated with oxidative stress and development of cardiovascular disease. Tumor necrosis factor α (TNFα), a mediator of vascular inflammation and paraoxonase 1 (PON1), an antioxidant enzyme associated with HDL may influence each other’s role inversely but their relation has not been studied in OSA. Moreover, the relation between obesity, inflammation (TNFα in particular) and PVAT function has been studied. However, PVAT function in relation to OSA has not been assessed.

Methods: Matched 41 morbidly obese patients were divided into two groups based on the severity of apnea (high or low apnea-hypo-apnea index (AHI) groups) and on presence of OSA (“OSA” and “no OSA or nOSA” group). HDL’s in vitro antioxidant function, serum PON1 activity, TNFα and intercellular adhesion molecule 1 (ICAM-1) were assessed. In a subset of 19 patients we immunostained gluteal subcutaneous adipose tissue (SAT) for TNFα expression, macrophages and measured adipocyte size. PVAT function was assessed in OSA (n= 5) versus no OSA (n= 5).

Results: The in vitro HDL lipid peroxide levels were higher and serum PON1 activity was lower in the high AHI group versus the low AHI group (p<0.05 & p<0.0001) and in the OSA group versus the “nOSA” group (p=0.005 & p<0.05). Serum TNFα and ICAM1 levels and TNFα expression in SAT increased with the severity of OSA. PON1 inversely correlated with AHI, whereas TNFα expression in SAT directly correlated with AHI. Presence of PVAT had no significant effect on vessel activity in OSA or nOSA groups (p>0.05)

Conclusion: Serum PON1 activity and HDL’s capacity to protect itself from in vitro oxidation in morbidly obese patients are impaired with the presence and severity of OSA. The differences in serum TNFα, ICAM-1 and TNFα expression in SAT suggest enhanced
inflammation due to OSA. Reduced PON1 activity in these patients could be a mechanism for HDL and endothelial dysfunction. Despite lower background inflammation, there is no difference in PVAT anticontractive function in morbidly obese individuals with no OSA.
Introduction

Early in atherogenesis, monocytes adhere to arterial endothelium [1, 2] via adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) [3, 4]. Mast cells, neutrophils, macrophages and adipose tissue release pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) that induce expression of adhesion molecules in endothelium and recruitment of leukocytes, which is essential for the pathogenesis of vascular inflammatory diseases [5]. Products of phospholipid peroxidation originating from oxidized low density lipoprotein (oxLDL) may propagate vascular inflammation by promoting increased production of TNFα and ICAM-1 [6]. Paraoxonase 1 (PON1) associated with high density lipoprotein (HDL) has anti-oxidant and anti-inflammatory potential mainly by protecting phospholipids on the surface of HDL [7] and LDL from peroxidation [8, 9]. These protective properties depend on the peroxidase and esterase activity of PON1 allowing the detoxification of oxidized molecules such as phospholipids and lipid hydroperoxides safely [10, 11].

HDL cholesterol (HDL-C) is inversely associated with ICAM-1 concentration in individuals with low plasma HDL-C [12]. HDL, incubated with human umbilical vein endothelial cells (HUVEC) before and after stimulation with TNFα, inhibits TNFα induced expression of ICAM-1 [13]. Jurek et al have examined the ability of HDL from dialysis patients to suppress the expression of adhesion molecules in endothelial cells and in monocytes and to inhibit the uptake of oxidized LDL by macrophages. This study showed that a decreased ability of HDL to suppress expression of adhesion molecules in endothelial cells was a potential risk factor for atherosclerosis development. This could be due to oxidative modification of HDL and the significantly reduced PON1 activity in these patients [14]. In vitro studies have demonstrated that purified PON1 had an anti-inflammatory effect in the presence of HDL, causing a 3- fold reduction in ICAM-1 levels.
PON1 also significantly decreased TNFα and oxidized phospholipid induced ICAM-1 expression [15]. This indicates PON1 associated with HDL is required for its anti-oxidant and anti-inflammatory functions. Apolipoprotein M (apoM) has also been proposed to assist HDL in its anti-oxidant and vasculo-protective functions [16, 17].

Perivascular adipose tissue conveys an anticontractile effect on adjacent small vessels, but this effect is not observed in obesity [30, 38]. This loss of function is attributed, in part, to the elevated inflammatory state in obesity. Given the heightened inflammatory state in OSA verus nOSA, it would be intuitive to compare PVAT function in these two groups.

In summary, there is evidence that oxidatively modified HDL has low PON1 activity and is unable to remove lipid peroxides originating from oxidized LDL, from the site of atheroma formation. This could lead to activation of monocytes and macrophages which release TNFα, promoting recruitment of monocytes from the circulation by inducing expression of adhesion molecules on the endothelial cell surface. This may initiate the process of atheroma formation and propagate a vicious circle as it has been suggested that TNFα also influences PON1 and HDL anti-oxidant function [18].

Many studies have reported an independent correlation between the presence and severity of obstructive sleep apnea (OSA) and cardiovascular diseases [19] like hypertension [20], coronary artery disease [21, 22] and stroke [23]. The chronic state of intermittent hypoxia due to OSA can lead to increased oxygen free radical stress [24] and endothelial dysfunction [25]. This process may propagate atherogenesis. Lipid peroxidation occurs in patients with OSA [26]. There is evidence that in patients with OSA, the HDL is dysfunctional [27] and the PON1 activity is reduced [28, 29]. The mechanisms that link oxidatively modified HDL with increased atherogenesis in these patients remain unclear. Furthermore, there is evidence that adipocytes influence local vascular tone but this
capacity is lost in obesity by the development of adipocyte hypertrophy, leading to hypoxia, inflammation, and oxidative stress [30].

In this study we showed that patients with OSA may have dysfunctional HDL due to low PON1 activity and ApoM component of HDL. Dysfunctional HDL is less able to protect itself from \textit{in vitro} oxidation and has reduced ability to protect the endothelium. We also showed adipose tissue inflammation increases with severity of OSA and may influence anti oxidant function of HDL.
Methods

Subjects
Forty one patients with body mass index (BMI) >40 kg/m² were recruited from Salford Royal Hospital NHS Foundation Trust (Salford, UK) obesity management clinic. All patients underwent overnight respiratory variable polysomnography to determine the apnea/hypo apnea index (AHI). The diagnosis of OSA was established if the AHI was \( \geq 5 \)/hour. Patients were divided into two groups based on the presence or absence of OSA (“OSA” and “nOSA” group) and were also divided into two groups around the median AHI (high or low AHI groups) in order to assess the effect of OSA and its severity on antioxidant function of HDL. We recruited morbidly obese patients without OSA (nOSA) that matched the OSA group for age, gender and BMI in order to avoid any bias.

Isolation of lipoproteins
Blood samples were collected between 9:00 and 11:00 after participants had fasted from 22:00 hours the previous day. Serum and EDTA-plasma were isolated by centrifugation at 2000 \( \times \) g for 15 min at 4°C within 2 hours of collection and were maintained at that temperature until further use. Serum was used for total cholesterol (TC), triglycerides (TG) and HDL-C assays and EDTA-plasma for apolipoprotein B100 (apoB).

HDL (density range 1.063-1.21g/mL) was isolated by density gradient ultracentrifugation without the addition of EDTA in a Beckman preparative M8-55M ultracentrifuge with a 50.4Ti fixed angle rotor at speeds of 34,000 rpm (144,361 \( \times \) g) for 22 h [31, 32]. The isolated HDL fraction was dialyzed against Tris buffered saline (TBS) overnight at 4 °C. HDL protein concentration was determined using bicinchoninic acid [33].

In vitro studies
Susceptibility of HDL to oxidation by copper in vitro was assessed immediately after isolation by incubating 0.25 mg protein/mL of HDL fraction with 5 µl copper for 3 hours
at 37°C in a Gallenkamp Economy Size 1 Incubator (Gallenkamp, Leicester, UK). Lipid peroxide (LPO) production was measured by spectrophotometry at 365 nm at baseline and 3 hours [34]. Group comparisons were made between OSA and ‘nOSA’ controls and between OSA patients with AHI above and below the median.

**Other laboratory analyses**

Cholesterol and triglycerides (TG) were determined using cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) methods respectively, using reagents from ABX Horiba-UK, Northampton, UK. HDL-C was measured by a direct second-generation homogeneous method (Roche Diagnostics, Burgess Hill, UK). Low density lipoprotein cholesterol (LDL-C) was estimated using Friedewald Formula. Apolipoprotein B100 (apoB) and apolipoprotein A1 (apo A1) were assayed immunoturbidimetrically. A Cobas Mira auto-analyzer (ABX Horiba-UK, Northampton, UK) was employed for all these assays. Serum PON1 was determined by a semi-automated micro-titre plate method using paraoxon (O,O-Diethyl O-(4-nitrophenyl) phosphate) as a substrate and read by spectrophotometer at 405 nm [35].

Small dense LDL apoB (sdLDL) (density range 1.044 – 1.063g/mL) was isolated from plasma adjusted to density of 1.044g/mL and at 100,000 rpm (435,680 x g) for 5h at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK, High Wycombe, UK). Glycated haemoglobin (HbA1c) and fasting blood glucose were measured using the standard laboratory methods of the Department of Clinical Biochemistry, Central Manchester University Hospitals NHS Foundation Trust. Insulin was determined in plasma using Mercodia ELISA kits from Diagenics Ltd, Milton Keynes, UK. Homeostatic model assessment was used to assess beta-cell function (HOMA-β) and insulin resistance HOMA-IR using formulas (HOMA-β)
= \frac{(20 \times \text{insulin (mU/l)})}{(\text{glucose (mmol/l)} - 3.5)} \text{ and HOMA-IR} = \frac{\text{insulin (mU/l)} \times \text{glucose (mmol/l)}}{22.5} [36]. \text{TNF}\alpha \text{ and adiponectin were measured in serum, and ICAM-1 in plasma all using DuoSet® ELISA development kits from R&D Systems (Abingdon, UK). Apo M was assayed in serum using Bluegene E01A0522 kits from Hölzel Diagnostika Handels GmbH, Köln, Germany.}

**Subcutaneous adipose tissue biopsies and immunohistochemistry**

Gluteal subcutaneous adipose tissue (SAT) biopsies were obtained from a subset of 19 individuals (13 with OSA and 6 without OSA) in order to assess the effect of apnea on adipose tissue inflammation. Tissues were routinely processed to paraffin wax blocks. The adipose tissue inflammation was assessed on 5µm tissue sections immunostained for TNF\alpha and the presence of macrophages. Immunostaining and adipocyte size were assessed using quantitative image analysis.

Adipocyte size was quantified on microphotographs captured on Zeiss Axio Imager M2 microscope equipped with AxioCam camera and AxioVision Rel. 4.8 programme by free-hand tracing the margins of 100 consecutive cells (on average) per case to avoid selection bias. Colour images were captured on the Leitz Diaplan microscope equipped with a digital G0-3 Q Imaging camera. Cells staining with the CD68 (KP1) macrophage marker were counted and the results expressed as cells/mm². Colour microphotographs of TNF\alpha immunostained adipose tissue were converted to greyscale assessed using a macro subroutine in an ImagePro version 6.2 image analysis programme (MediaCybernetics UK, Marlow, UK) and the results were expressed as a percentage of the entire area photographed.
**Assessment of PVAT function**

Small subcutaneous arteries measuring 250-400µm internal diameter were dissected from gluteal fat samples from 5 individuals with and 5 without OSA and vessels contractility was assessed using wire myography as previously described [30]. In brief, segments of vessel measuring approximately 6mm were isolated from each sample, and split into 2 segments. One segment was prepared with the perivascular adipose tissue intact, and the adjacent segment with PVAT removed. Vessel contractility to noradrenaline was assessed by constructing concentration-response curves in order to measure the effect of PVAT on contraction of adjacent vessels.

**Statistical analyses**

The distribution or normality of data was determined using Kolmogorov-Smirnov test, D’Agastino and Pearson omnibus normality test and Shapiro-Wilk normality test on GraphPad Prism version 5.03 (GraphPad Software Inc., California, USA). Statistical significance of differences between groups was determined using the Student’s t-test (for parametric data) or Mann Whitney test (for non parametric data), taking $p< 0.05$ as statistically significant. Statistical significance of correlations was determined using Spearman’s test, taking $p< 0.05$ as statistically significant. Results were expressed as mean ± standard deviation (SD) for parametric data and median (interquartile range) for non-parametric data. SPSS statistical software version 16.0 (SPSS, Illinois, USA) was used for all comparisons and correlations. We did not use the multiple regression models to study the strength of relationships between various parameters in this study due to the relatively small number of patients.

Vessel contractility to NA and PVAT function was analysed using two-way ANOVA analysis performed by GraphPad Prism v5.03, as above.
Results

Low and high AHI groups: demographics, lipid profile, HDL in vitro antioxidant function and factors affecting vascular inflammation

The two groups were well matched for age, gender, smoking history, body mass index (BMI), waist circumference, blood pressure, equivalent rosuvastatin dose and prevalence of diabetes, hypertension and cardiovascular disease (Table 1). There was no significant difference in the number of patients on insulin or insulin sensitizers like metformin and glitazones. There were no significant differences between the high and low AHI groups in TC, TG, HDL-C, LDL-C, apoB, apoA1, sdLDL apoB, HbA1c, HOMA α and HOMA-IR respectively (Table 1).

LPO concentration at 3 hours on incubating HDL with copper was higher in the high AHI group at 30 (14–43) nmol/mL compared to the low AHI group at 17 (6–31) nmol/mL; (p<0.05) (Figure 1). PON1 activity was significantly lower in the high AHI group compared with the low AHI group (101 ± 64 vs. 186 ± 68 nmol/mL/min; p<0.0001).

ApoA1 and apoM were lower in the high AHI group compared with the low AHI group but the difference was not statistically significant (121 vs. 129 mg/dl; p=0.3; 31 vs. 37 mg/l, p=0.2 respectively) (Table 2). TNFα and ICAM-1 were significantly higher in the high AHI group compared with the low AHI group {87 vs. 15.5 pg/mL, p<0.05; 267 vs. 218 ng/mL, p<0.0001 respectively). There was no significant difference in the levels of adiponectin between the two groups (high AHI vs. low AHI; 2.1 vs. 2.0 mg/l, p=0.5) (Table 2).

Subcutaneous adipose tissue (SAT) data (table 3)

To assess the effect of severity of apnea on adipose tissue inflammation, the subset of 19 patients from whom SAT biopsies were obtained, were divided into two groups based on median AHI. There was no significant difference in the adipocyte size and the number of
cells staining with CD68 (KP1) marker for macrophages in the two groups (7592 vs. 6139 μm²; p=0.12 and 7.3 vs. 6.6 per mm² respectively). On immunostaining the adipose tissue, the expression of TNFα as a percentage of the area examined when measured at the same grey pixel range in both groups revealed significantly greater staining in the high AHI group as compared with low AHI group (0.8 vs. 0.6; p=0.05) (figure 3).

Obese OSA and “nOSA” groups: demographics, lipid profile, HDL in vitro antioxidant function and factors affecting vascular inflammation

The two groups were matched for age, gender, smoking history, BMI, waist circumference, blood pressure, equivalent Rosuvastatin dose and prevalence of diabetes, hypertension and cardiovascular disease. There was no significant difference in the number of patients on insulin or insulin sensitizing drugs. The AHI scores were significantly higher in the OSA group compared to the nOSA group (13.5/hour vs. 3.8/hour; p<0.0001). There was no significant difference between the two groups in TC, TG, HDL-C, LDL-C, apoB, apoA1, HbA1c, HOMA β and HOMA-IR. SdLDL was significantly higher in OSA group compared to nOSA group (18.3 vs. 11.7 mg/dl, p<0.05).

The in vitro LPO concentrations at 3 hours on incubating HDL with copper in OSA group were significantly higher at 31 (15 – 38) nmol/mL compared with the nOSA group at 9 (2 – 19) nmol/mL; (p= 0.005) (Figure 2). PON1 activity and apoM were significantly lower in the OSA group compared with the nOSA group {127 vs. 187 nmol/mL/min; p<0.05 and 31 vs. 49 mg/l, p<0.05 respectively}. ApoA1 did not differ significantly between the OSA and nOSA group (123 vs. 128 mg/dl). PON1 activity was inversely correlated to AHI in the OSA group (Spearman’s correlation coefficient = -0.41, p=0.03). TNFα and ICAM-1 were significantly higher in the OSA group than in the nOSA group (66 vs. 15 pg/mL; p<0.05 and 255 vs. 163 ng/mL, p<0.0001 respectively). There was no significant
difference in the levels of adiponectin between the two groups (OSA vs. nOSA; 2.0 vs. 2.1 mg/l).

**Effect of PVAT on vessel contractility**

Vessel segments with intact PVAT constricted to a similar degree as compared with segments without PVAT (p>0.05, figure 4). This was true in vessels from both OSA and nOSA groups.
Discussion
In this study of morbidly obese patients with or without OSA we found impaired HDL antioxidant function, lower PON1 activity and apoM concentration associated with presence of OSA and increasing severity of OSA. The changes in serum TNFα and ICAM1 blood levels and TNFα expression in SAT suggest enhanced inflammation due to OSA and increasing AHI. Reduced PON1 activity in these patients, associated with enhanced oxidative stress and increased TNFα expression could be a mechanism for HDL and endothelial dysfunction. However, PVAT did not affect vessel contractility in either group.

We have shown that HDL’s in vitro antioxidant capacity is reduced in morbidly obese patients with OSA compared with matched patients without OSA. This function of HDL diminishes with increasing severity of OSA. Our results confirm findings from previous studies that the presence and increasing severity of OSA leads to reduction in PON1 activity [28, 29]. However, the previous studies were not matched for basic characteristics like BMI, gender and age. We recruited age, gender and BMI matched patients in order to avoid bias.

Others have demonstrated HDL dysfunctionality in OSA patients by its inability to prevent in vitro LDL oxidation or a lower PON1 activity in the OSA group. The degree of dysfunctionality of HDL and levels of oxidised LDL were directly related to AHI (which reflects the severity of OSA) [27]. We have shown that lower PON1 activity contributes to HDL dysfunction in these patients. PON1 was lower in the OSA and the high AHI groups compared to the obese nOSA and low AHI groups respectively. For the first time we have reported that the serum PON1 activity has an inverse relation to AHI. These results suggest that PON1 activity is increasingly affected by the oxidative stress associated with the presence and increasing severity of OSA. As PON1 is known to play an important role
in HDL’s antioxidant function, it is likely that the diminished PON1 activity due to OSA contributed to HDL’s reduced antioxidant function.

ApoM and apoA1 also play a role in HDL’s anti-oxidant function through distinct pathways. We show that apoM concentrations are lower in the OSA and high AHI groups, possibly contributing to the diminished anti-oxidant function of HDL. ApoA1 did not differ significantly between the two groups. Similar result was obtained in a previous study [27], suggesting that ApoA1 concentration may not be affected by the excessive oxidative stress in sleep apnea. OSA does not affect the levels of adiponectin and insulin sensitivity, consistent with a recent study showing an association between adiponectin and obesity but not OSA [37].

Obesity is a state of chronic inflammation where adipocytes hypertrophy faster than the rate of angiogenesis to oxygenate the cells, hence they exist in a state of relative hypoxia which leads to inflammatory differentiation of macrophages and secretion of TNF-α [38]. In animal studies it has been shown that in conditions that mimic OSA, there is a 2.5 to 3 fold increase in TNFα secretion by adipose tissue [39].

In the present study, we have shown greater levels of the circulating inflammatory biomarker TNFα in the OSA group as compared with the nOSA group, as well as higher levels in the high AHI group compared with low AHI. In keeping with this, adipose tissue staining for TNFα increased with severity of OSA, thus suggesting that within the obese population, higher AHI and OSA result in a more inflammatory state as compared with low AHI and without OSA. Adipose tissue macrophage numbers increase in obesity and macrophages are responsible for most of the TNFα within adipose tissue [40]. In this study we observed much stronger staining for TNFα around the macrophages with increasing AHI. There was no statistical difference in adipocyte size and macrophage number between the two groups. However, this may be due to the small numbers of patients from
whom gluteal SAT biopsy was obtained, which is a limitation of this study. Whilst CD68 is a widely used marker for macrophages [41] a more specific stain for activated macrophages may help explain this anomaly in macrophage numbers.

We have shown previously that, in health, PVAT exerts an anticontractile effect on adjacent small vessels and that in obesity this effect is not observed [30]. Moreover, we have shown that following weight loss surgery, PVAT exerts an anticontractile effect on its adjacent small vessels, in keeping with observations of a reduction in macrophage numbers and TNFα staining in PVAT and a reduction in adipocyte size [38]. Thus given a higher grade of background inflammation in OSA versus nOSA, we assessed PVAT function in the two groups and did not observe a difference in PVAT function between the two groups. This may be explained by the fact that despite the difference in OSA status, both groups are morbidly obese and adipocyte size in the two groups are similar and significantly larger than those in healthy individuals [38]. Also, there was not a significant difference in macrophage numbers between the two groups and whilst TNFα, secreted by macrophages has vasoactive effects [30], there may well be other inflammatory factors secreted by macrophages which result in increased vessel contractility contributing to the loss of PVAT anticontractile function that remain to be assessed. One limitation of this study is the small number of vessels studied.

We report a relation between PON1 activity and TNFα in OSA patients, which may be causative. Adenovirus based over-expression of human PON1 in apolipoprotein E knock-out mice is associated with reduced TNFα levels [42]. PON1 activity is correlated to serum TNFα levels in atheromatous patients with rheumatoid arthritis [43] and TNFα antagonist therapy leads to enhanced PON1 levels, heightened anti-oxidant capacity of HDL and a reduced inflammatory status [18]. Alternatively, a study showing low PON1
activity in HDL obtained from coronary disease patients suggests that reduced PON1 activity may lead to increased action of TNFα. This could result in activation of endothelial protein kinase C beta 2 (PKC β2) pathways and loss of endothelium’s ability to resist TNFα mediated expression of adhesion molecules [44].

Moreover, low PON1 activity may be one of the initial triggers for development of OSA. Reduced PON1 activity leads to accumulation of lipid peroxides and increased oxidative stress [8]. Free radicals can cause disruption of the blood brain barrier and reach the brain where they can damage the neurons [45]. It would be of interest to study the antioxidant capacity of HDL in CSF where it is the only lipoprotein present [46].

Finally we conclude that serum PON1 activity and capacity of HDL to protect itself from in vitro oxidation in morbidly obese patients are impaired with the presence and increasing severity of OSA. The differences in serum TNFα, ICAM-1 and TNFα expression in SAT suggest enhanced inflammation due to OSA. Reduced PON1 activity in these patients could be a mechanism for HDL and endothelial dysfunction. Further studies are needed to elucidate the interaction between PON1, adipose tissue inflammation and TNFα in this important group of patients.
References

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45. Lim D C, Pack A I. Obstructive sleep apnea and cognitive impairment: Addressing the blood-brain barrier. Sleep Med Rev
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<th>High AHI group (n=20)</th>
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<td>45 ± 9</td>
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<tr>
<td>Body Mass Index (kg/m²)</td>
<td>52 ± 6</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>143 ± 14</td>
<td>140 ± 18</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>139 ± 23</td>
<td>139 ± 19</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76 ± 13</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8 (4.5 – 5.5)</td>
<td>5.3 (4.7 – 5.7)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.2 – 2.0)</td>
<td>1.6 (1.1 – 2.1)</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 (1.1 – 1.5)</td>
<td>1.3 (1.2 – 1.5)</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.2 ± 1.3</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>Apolipoprotein B100 (mg/dl)</td>
<td>90 (81 – 106)</td>
<td>102 (89 – 123)</td>
</tr>
<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>121 (113 – 136)</td>
<td>129 (122 – 138)</td>
</tr>
<tr>
<td>Sd LDL apoB (mg/dl)</td>
<td>17.3 (10.9 – 31.4)</td>
<td>16.5 (11.2 – 22.6)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.2 (5.7 – 6.7)</td>
<td>6.0 (5.6 – 6.9)</td>
</tr>
<tr>
<td>HOMA – IR</td>
<td>6.6 (4.3 – 10.7)</td>
<td>6.9 (4.0 – 10.2)</td>
</tr>
<tr>
<td>HOMA – B</td>
<td>198.6 (95.5 – 390.4)</td>
<td>176.6 (104.7 – 299.9)</td>
</tr>
<tr>
<td>Equivalent rosvastatin doses in patients on statins (mg/day)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3. Characteristics of low AHI and high AHI groups. Values are mean ± SD or median (interquartile range). Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. ***p<0.0001. HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, SdLDL apoB – small dense LDL apolipoprotein B100, HbA1c – glycated hemoglobin, HOMA-IR – homeostatic model of assessment – insulin resistance, HOMAβ – homeostatic model of assessment – insulin sensitivity
Figure 1

LPO generated at 3 hours on incubating HDL with copper

High AHI group

Low AHI group

LPO (nmol/ml)

Figure 1. This figure demonstrates lipid peroxides (LPO) generated at 3 hours on incubating HDL with copper ions. LPO levels in HDL from high AHI group were significantly higher as compared to LPO generated in HDL from low AHI group at the end of 3 hours (p<0.05). Statistical analyses – Mann Whitney test used as data was non-parametric. LPO – lipid peroxides.
Figure 2. This figure demonstrates lipid peroxides (LPO) generated at 3 hours on incubating HDL with copper ions. LPO levels in HDL from obese OSA group were significantly higher as compared to LPO generated in HDL from obese no OSA group at the end of 3 hours (p = 0.005). Statistical analyses – Mann Whitney test used as data was non-parametric. LPO – lipid peroxides
<table>
<thead>
<tr>
<th></th>
<th>High AHI group (n=20)</th>
<th>Low AHI group (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A1 (mg/dL)</td>
<td>121 (113 – 136)</td>
<td>129 (122 – 138)</td>
</tr>
<tr>
<td>Paraoxonase1 activity (nmol/mL/min)</td>
<td>101 ± 64</td>
<td>186 ± 68 ***</td>
</tr>
<tr>
<td>Apolipoprotein M (mg/L)</td>
<td>31 (25 – 35)</td>
<td>37 (26 – 50)</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>87.2 (12.4 – 133.8)</td>
<td>15.5 (7.2 – 38.2) *</td>
</tr>
<tr>
<td>ICAM-1 (ng/mL)</td>
<td>267 (237 – 280)</td>
<td>218 (157 – 245) **</td>
</tr>
<tr>
<td>Adiponectin (mg/L)</td>
<td>2.1 ± 0.8</td>
<td>2.0 ± 0.7</td>
</tr>
</tbody>
</table>

Table 2. Apolipoproteins and enzymes associated with HDL that promote HDL’s antioxidant capacity (for both groups). This table also shows TNFα, ICAM-1 and adiponectin values in both groups. Values are mean ± SD or median (interquartile range). Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. Values are mean ± SD or median (interquartile range). * p<0.05, **p=0.01*** p<0.0001. TNFα – tumor necrosis factor alpha, IL6 – interleukin 6, ICAM-1 – intercellular adhesion molecule 1.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>SAT data for high AHI (n=10)</th>
<th>SAT data for low AHI (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte size (µm²)</td>
<td>7592 (7034 – 7971)</td>
<td>6139 (4743 – 7331)</td>
</tr>
<tr>
<td>Macrophages (per mm²)</td>
<td>7.3 (5.6 – 7.7)</td>
<td>6.6 (5.0 – 12.9)</td>
</tr>
<tr>
<td>TNFα expression</td>
<td>0.8 (0.6 – 1.3)</td>
<td>0.6 (0.2 – 0.8)*</td>
</tr>
</tbody>
</table>

Table 3. Gluteal subcutaneous adipose tissue (SAT) data from a subset of patients (n=19). Divided into two groups based on median AHI. Statistical analyses – Mann Whitney for non-parametric data. *p=0.05. TNFα expressed as a percentage of the area examined when measured at the same grey pixel range. Difference in adipocyte size and macrophage numbers between the two groups was not statistically significant.

Figure 3

Figure 3. Immunostaining for TNFα in adipose tissue of high AHI (A) and low AHI (B) patient. Panel A shows more intense expression of TNFα comparing to B. (Bar = 100 µm)
Figure 4

A

B

C

Figure 4: Presence of PVAT did not significantly alter vessel constriction in morbidly obese individuals without OSA (A) or with OSA (B) (P>0.05, n=5). Comparison of vessel contractility of segments with intact PVAT from those with OSA or No OSA (P>0.05, n=5)
Discussion

The overarching aims of the protocols comprising this thesis were to assess the effects of obesity on perivascular adipose tissue function, the contribution of inflammation in this process and whether the alterations in function could be reversed following weight loss surgery.

The principal observations were derived from the functional assessment of vessel segments harvested from human and rodent samples and studied using wire myography. Further collaborative and complimentary data were collated using immunohistochemistry, proteomics and lipid studies.

The main findings from each chapter are summarised here.

Effects of obesity and weight loss surgery on PVAT function

This study assessed the effect of weight loss (bariatric) surgery on the vasodilatory properties of PVAT. We confirmed that in severely obese patients, whilst there is an increase in adipose tissue volume, the beneficial PVAT anticontractile effect is abolished. There are three novel findings presented here. First, bariatric surgery achieves a dramatic degree of weight loss within only six months and reverses the obesity-induced damage to PVAT anticontractile function. Second, the functional recovery of PVAT is independent of the endothelium. This was confirmed by solely studying vessels denuded of endothelium, thus eliminating obesity-induced endothelial damage as a potential confounding factor. Third, bariatric surgery restores the function of PVAT by reducing adipose tissue inflammation and increasing local adiponectin and nitric oxide bioavailability. These findings advance our understanding of the mechanisms by which
obesity disrupts the vasodilating function of adipose tissue and confirm the relevance of enhanced adiponectin levels and nitric oxide bioavailability in the reversibility of PVAT anticontractile function following bariatric surgery. Future work needs to elucidate whether other factors including a change in the metabolic status following surgery plays a role.

**Contribution of inflammation and obesity-induced endothelial damage to PVAT function**

This study investigated the effects of obesity on PVAT function and assessed the contribution of nitric oxide and endothelium in more detail. We have shown previously that PVAT vasorelaxant effect is restored following significant weight loss. Here we observed four novel findings. First, using proteomic analysis, we show that levels of adiponectin and the free radical scavengers SOD and peroxiredoxin-1 are significantly lower in the PVAT from obese individuals as compared with healthy controls. We have reported previously that adiponectin secreted from PVAT has a major vasorelaxant effect on adjacent small arteries and the data presented in this study confirms that PVAT adiponectin levels are lower in the obese as compared with the healthy. Moreover, our proteomic data show that levels of the free radical scavengers are significantly lower in the obese patients as compared with the healthy lean volunteers. Macrophages present within PVAT contribute to the inflammatory environment by secreting superoxide anions. The accumulation of macrophages in the obese PVAT means a higher level of background superoxide levels; a reduction in free radical scavenger enzymes contributes to accumulation of inflammatory mediators such as hydrogen peroxide which has adverse vasoactive effects on adjacent vessels.

Second, in healthy animals, there was preservation of PVAT anticontractile capacity after removal of the endothelium, thus confirming observations by Gao et al that PVAT effect is not dependent on endothelial status. Third, Nitric oxide from PVAT is an important endothelium-independent
contributor to PVAT vasorelaxant function in healthy tissue which is not observed in obesity.

Fourth, PVAT function correlates with systemic blood pressure and obesity-induced damage to PVAT vasorelaxant quality correlates with a rise in BP.

Assessment of the effects of sleep apnoea in obesity on PVAT function

Obstructive sleep apnoea affects a large proportion of morbidly obese individuals and contributes to elevated cardiovascular risk factors. This has been attributed to increased prevalence of hypertension secondary to high circulating aldosterone and leptin levels, as well as increased oxidative stress and sympathetic nervous system overstimulation. Given the significant contribution of OSA to cardiovascular risk and its link to hypertension, this study assessed whether there was a difference in inflammation within the PVAT and if this translated to a difference in PVAT function between those with and without OSA. We observed higher levels of circulating and adipose tissue TNFα in those with OSA, however there was no significant difference in adipocyte size or macrophage numbers within the PVAT in those with high apnoeic scores as compared to those with low AHI scores. Similarly, no difference in circulating adiponectin was observed depending on the severity of apnoea. PVAT did not exert an effect on vessel contractility in either group and this can be explained by the fact that despite a lower level of TNFα in serum and adipose tissue in those without OSA, the individuals remain obese with high numbers of adipose tissue macrophages, low adiponectin levels and large adipocytes, thus eliminating the possibility of any discernible PVAT effect between the two groups using wire myography. A larger sample size may prove beneficial to assess this further.


