Serum Osteoprotegerin, a Potential Novel Marker of Systemic Inflammation: The Influence of Obesity, Insulin Sensitivity and Oral Glucose Loading on its Circulating Concentrations

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abbreviations

ACC	acetyl CoA carboxylase
ACRP30	adipocyte complement related protein
ADD-1	adipocyte determination and differentiation factor-1 and
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
ADP	adenosine diphosphate
Akt	acute transforming retrovirus thymoma
AMPK	adenosine monophosphate-activated protein kinase
ANCOVA	analysis of covariance
apo-E	apolipoprotein E
ATP	adenosine triphosphate
AUC	area under the curve
bFGF	basic fibroblast growth factor
BMD	bone mineral density
BMI	body mass index
BMP	bone morphogenetic proteins
BP	blood pressure
CAD	coronary artery disease
CD36	cluster of differentiation 36
CD4	cluster of differentiation 4
c-Fos	immediate early gene transcript,
CVD	cardiovascular diseease
CVD	cardiovascular disease
DAG	diacylglyceride
DcR1	membrane-bound decoy receptor
DEXA	dual energy x-ray absorbitometry
DPP-4	dipeptidyl peptidase-4
ECG	electrocardiogram
ELISA	enzyme linked inmmunoasorbent assay
eNOS	endothelial nitric oxide synthase

FDCR-1	follicular dendritic cell-associated receptor 1
G-6-P	Glucose-6-phosphate
GIP	glucose-dependent insulinotrophic peptide
GLP-1	glucagon-like-peptide 1
GLUT-4	glucose transporter-4
Grb-2	growth factor receptor-bound protein 2 associated binder 2
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HMW	high molecular weight
HOMA-IR	homeostasis model assessment of insulin resistance
hsCRP	high sensitivity c-reactive protein
ICAM1	intracellular cell adhesion molecule 1
IFG	impaired fasting glucose
IgG	Immunoglobulin G
IGT	impaired glucose tolerance
IKK	Ikβ kinase
Ikβ	inhibitor of nuclear factor kβ
IL-1	Interleukin-1
IL-6	interleukin-6
IRS-1	Insulin receptor substrate 1
JNK	c-Jun NH2-terminal kinase
Kir6.2	member of inwardly rectifying potassium channel superfamily
LCCoAs	long-chain acyl-coenzyme A
LDL	low density lipoprotein
LMW	low molecular weight
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
MITF	microphthalmia-associated transcription factor
MKK6	mitogen-activated protein kinase kinase 6
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
mtGPAT1	mitochondrial acyl-CoA:glycerol-3-phosphate acyltransferase 1

NAD	reduced form of NADH
NADH	nicotinamide adenine dinucleotide
NEFAs	non-esterified fatty acids
NFATc1	c-myc, calcineurin/nuclear factor of activated T cells
NF-κβ	nuclear factor-κβ
NGT	normal glucose tolerance
NO	nitric oxide
OCIF	osteoclastogenesis inhibitory factor
OGIS	oral glucose insulin sensitivity
OGTT	oral glucose tolerance test.
OPG	osteoprotegerin
OPG-Fc	OPG fusion protein
PAD	Peripheral Artery Disease
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3-kinase	phosphatidylinisitide 3-kinase
PKC	protein kinase C
PPARa	peroxisome proliferator-activated receptor-alpha
PPARγ	peroxisome proliferator-activated receptor gamma
QUICKI	quantitative insulin sensitivity index
RANK	receptor activator of NF-kβ
RANK-Fc	RANK fusion proteins
RANKL	receptor activator of NF-kβ ligand
RBP4	retinol binding protein-4
SEM	standard error measurement
SI Units	Système international d'unités
siRNA	short interfering RNA
Src	steroid receptor coactivator
SREBP-1C	sterol regulatory element–binding protein-1c
SUR1	sulfonylurea receptor 1
TACE	TNF-α converting enzyme

TG	triglycerides
TGFβ1	transforming growth factor β1
TNF-R1	TNF receptor-1
TNF-α	tumour necrosis factor – α
TR1	TNF receptor like molecule 1
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis Inducing ligand
TZD	thiazolidinedione
VCAM1	vascular cell adhesion molecule 1
VEC	vascular endothelial cell
VO2max	maximal oxygen uptake
VSMC	vascular smooth muscle cell
WHO	World Health Organisation
WPBs	Weibel-Palade bodies

Abstract

Circulating osteoprotegerin (OPG) promotes bone formation in vivo and correlates with the presence of type 2 diabetes, severity of vascular calcification and coronary artery disease. Obesity is a risk factor for diabetes and cardiovascular disease but little is known about the impact of body weight on circulating OPG. The purpose of these experiments was to evaluate the impact of body mass index, vascular dysfunction and insulin sensitivity on circulating concentrations of OPG. This thesis investigated; (i) The effect of obesity and insulin sensitivity on circulating OPG levels; (ii) The effect of type 2 diabetes and vascular dysfunction on OPG levels; (iii) The influence of glycaemic status on circulating OPG concentrations. Briefly, our findings were as follows (i) obese subjects who have normal glucose tolerance and are free from cardiovascular disease have lower circulating levels of OPG than their lean age matched counterparts. (ii) Osteoprotegerin is inversely correlated with insulin sensitivity, adiponectin and indicators of total body and visceral adiposity and positively correlated with aerobic fitness. (iii) TNF receptor apoptosis inducing ligand (TRAIL) is positively correlated with both fat mass and waist circumference, independent of age, gender and BMI. (iv) OPG is significantly higher as is IL-6 and hsCRP and adiponectin significantly lower in type 2 diabetics than in age and gender matched normoglycemic controls, while there is no difference in TNF-α, TRAIL or sRANKL concentrations. (v) Osteoprotegerin is higher in type 2 diabetics after excluding patients with previously diagnosed vascular disease, a distinction which could not be made using traditional inflammatory markers such as IL-6, hsCRP or TNF-α. (vi). There is no difference in OPG concentrations between those with prediabetes and overt type 2 diabetes, however both conditions appear to have significantly higher levels than age and BMI matched obese normoglycemic controls. (Sipos *et al.*, 2008) Lean subjects have OPG concentrations which are similar to that of both prediabetic and type 2 diabetic patients but significantly higher than their matched lean counterparts. Circulating OPG is lower in obese, but otherwise healthy subjects, and correlates with indices of insulin sensitivity. OPG (but not RANKL or TRAIL) was found to be elevated in type 2 diabetes. OPG may have a protective effect on vascular cells and the observed decrease in circulating concentrations with increasing BMI could be an early biomarker of vascular dysfunction. It remains to be determined whether an increase in insulin secretion, insulin resistance, adiposity or systemic inflammation is the main regulatory factor.

List of Publications

DT Ashley, EP O'Sullivan, C Davenport, D Smith, DJ O'Gorman. Osteoprotegerin and its relationship with adiponectin and Insulin Sensitivity in overweight and obese adults. **International Journal of Obesity**. *Submitted.*

EP O'Sullivan, **DT Ashley**, C Davenport, DJ O'Gorman, D Smith. Osteoprotegerin is elevated in type 2 diabetes even in the absence of microvascular complications, and lacks the relationships observed with metabolic parameters seen in healthy individuals. **Diabetes Metabolism Research**. *Submitted.*

DT Ashley, EP O'Sullivan, N Devlin, R Crowley, A Agha, CJ Thompson, D O'Gorman, D Smith Osteoprotegerin and RANKL activity in patients with type 2 diabetes. *In Preparation.*

Chapter I Introduction

Obesity poses a major threat to the health of the developed world. The latest figures from the World Health Organisation estimate that globally 1.6 billion adults are overweight and as many as 400 million are obese, furthermore it is predicted that this will rise to 2.3 billion and 700 million respectively by 2015 (World Health Organisation, 2000). One of the most devastating and insidious conditions associated with obesity is type 2 diabetes. At the turn of the century it was estimated that 171 million people worldwide had type 2 diabetes and this is expected to rise to 366 million by 2030 (Wild *et al.*, 2004).

Type 2 diabetes mellitus occurs when there is a concordance of insufficient secretion of the hormone insulin from the pancreatic β-cells superimposed upon a background of a reduced effectiveness of insulin to stimulate cellular glucose uptake (insulin resistance). In addition to its role in glucose disposal in a variety of tissue types, insulin is an important vasoactive hormone that has pleiotropic actions in skeletal muscle, adipose tissue and vascular endothelium (Cavaghan & Polonsky, 2005). The exponential increase in the prevalence of obesity and type 2 diabetes mellitus is largely due to behavioural and lifestyle changes, with an increased intake of high fat foods and lower levels of physical exercise being the main causes (Zimmet & Thomas, 2003). In addition to these lifestyle factors our genetic heritage has likely influenced the progression of these conditions. The mammalian genome has evolved to cope with a constant flux of nutrient availability and has allowed for the development of a highly efficient mechanism that permits the long-term storage of energy during times of nutritional oversupply. Humans and other mammals achieve this by sequestering excess calories to the adipose tissue. A number of clinical studies have demonstrated the importance of the distribution of this adipose tissue, in particular the contribution of visceral fat accumulation to the development of cardiovascular co-morbidities such as congestive heart failure, myocardial infarction and stroke (Lakka *et al.*, 2002), (Kenchaiah *et al.*, 2002). Many cross sectional studies have shown a strong association between obesity and type 2 diabetes. This relationship is in part due to increased insulin resistance, which is a clear predisposing factor in the development of type 2 diabetes (Olefsky & Kolterman, 1981). Insulin resistance is the result of a progressive decrease in receptor and post-receptor biological processes in a number of tissue types. These include decreased insulin-mediated glucose disposal in skeletal muscle, impaired glucose disposal, increased hepatic glucose production and increased lipolytic turnover with reduced fat oxidation in adipose tissues (Miller, 2003), (Shuldiner *et al.*, 2001). In obesity there is an increased volume of

subcutaneous adipose tissue and a greater accumulation of fatty tissue around the organs in the visceral cavity. It has been repeatedly shown that greater total body adiposity and a preferential accumulation of visceral adiposity are independently associated with insulin resistance (Frayn, 2000), (Kissebah *et al.*, 1982), (Bjorntorp, 1997). It is now well established that increased visceral fat is associated with increased morbidity independent of age, ethnicity and gender (Okosun *et al.*, 2000), (Nicklas *et al.*, 2004). Up until recently scientists and physicians have considered the adipose tissue to be a simple energy storage depot. However the alarming rise in obesity and type 2 diabetes towards the end of the last century has resulted in a wave of intense scientific study of this tissue type. The adipose tissue secretes a number of proteins and cytokines with autocrine, paracrine and endocrine functions. These cytokines exercise profuse metabolic influence. In addition to regulating fat mass and nutrient homeostasis, these "adipocytokines" are involved in the regulation of glucose and lipid metabolism. They exert anti- and pro-inflammatory effects, are involved in blood pressure control, haemostasis, bone mass turnover, and thyroid and reproductive regulation (Trayhurn, 2005), (Rosen & Spiegelman, 2006), (Ahima & Flier, 2000). Increasing evidence suggests that these adipocytokines are intrinsically involved in the pathophysiology of obesity-related insulin resistance, inflammation and atherosclerosis. In the early 1990s Hotamisligil et al. (1993), observed that increased production of tumour necrosis factor– α (TNF-α) was present in several models of animal obesity. Two years later the same group found that TNF-α was also present in human adipose and muscle tissue and was positively related to insulin resistance and obesity (Hotamisligil *et al.*, 1995). Adiponectin is another important adipocytokine that was discovered in the mid 1990s by three different research groups (Scherer *et al.*, 1995), (Shapiro & Scherer, 1998), (Hu *et al.*, 1996), (Maeda *et al.*, 1996). Circulating adiponectin is inversely related to body mass index (Zhao *et al.*, 2007) and its expression is increased in response to weight loss (Reinehr *et al.*, 2004), (Brichard *et al.*, 2003). Interestingly, the globular head of adiponectin is structurally homologous to TNF-α and its mRNA expression in 3T3L1 adipocytes is substantially decreased in response to incubation with TNF-α (Ruan *et al.*, 2002). A reduction in adiponectin and an increase in TNF-α synthesis have been shown to reduce insulin sensitivity (Yamauchi *et al.*, 2001), (Valverde *et al.*, 1998), (Feinstein *et al.*, 1993), (Halse *et al.*, 2001) and increase vascular dysfunction (Wang *et al.*, 1994).

Significant epidemiological data has now accrued suggesting that cardiovascular disease and osteoporosis often coexist, implying that there may be a potential a link between bone and vascular tissue (Burnett & Vasikaran, 2002), (Koshiyama *et al.*, 2006). Interestingly, several proteins such as osteocalcin, osteopontin and bone morphogenic protein, which were once thought to be bone-specific in their biological action, have been identified in atherosclerotic lesions (Abedin *et al.*, 2004). Such observations have given rise to the suggestion of the existence of an interdependent set of connections between cytokines which interact on multiple levels and in multiple tissue types, an "Osteo-adipose-vascular" network as it were (Koshiyama *et al.*, 2006). One such protein that has garnered considerable interest in recent years is the novel molecule osteoprotegerin (OPG). OPG shares an interesting connection with TNF-α, not only is it a member of the TNF receptor superfamily (Simonet *et al.*, 1997) but it also appears to be upregulated *in vitro* by TNF-α (Olesen *et al.*, 2005) and downregulated both *in vivo* (Jorgensen *et al.*, 2009) and *in vitro* (Olesen *et al.*, 2005) by insulin. It is released into the circulation as a soluble glycoprotein (Yun *et al.*, 1998) and binds to receptor activator of nuclear factor κβ Ligand (RANKL) where it reduces bone resorption by blocking osteoclastogenesis. It also binds to another TNF-associated molecule, namely (TNF)-related apoptosis inducing ligand (TRAIL) (Corallini *et al.*, 2008), (Emery *et al.*, 1998), the binding of which may lead to the preservation of the integrity of the vascular wall by reducing vascular endothelial cell (VEC) apoptosis. However, the precise mechanism by which this is accomplished is poorly understood. Circulating OPG is significantly higher in patients with type 2 diabetes (Yaturu *et al.*, 2008), (Secchiero *et al.*, 2006), (Olesen *et al.*, 2005), (Rasmussen *et al.*, 2006) and coronary artery disease (CAD) (Jono *et al.*, 2002), (Schoppet *et al.*, 2003). It is also an independent predictor of silent CAD in type 2 diabetes (Avignon *et al.*, 2005) and cardiovascular mortality (Browner *et al.*, 2001), (Kiechl *et al.*, 2004), (Ueland *et al.*, 2004), (Omland *et al.*, 2008). OPG-deficient mice exhibit severe aortic and renal calcification in addition to profound osteoporosis (Bucay *et al.*, 1998) suggesting an important link between OPG, vascular dysfunction and bone metabolism. All of these novel cytokines and circulating factors signify a thorough integration of what were once considered to be insular tissue types with isolated physiological pathways contributing independently to preserve metabolic homeostasis.

The overall goal of this thesis is to investigate and evaluate the impact of body mass index (Zhao *et al.*, 2007) and insulin sensitivity on circulating concentrations of OPG, TRAIL and

RANKL and to investigate how they relate to an established adipose tissue-derived indicator of insulin sensitivity; adiponectin, in a healthy cohort free from cardiovascular disease. We shall also attempt to examine the impact of diabetes and vascular disease on their circulating concentrations while probing how these novel markers relate to other traditional inflammatory adipocytokines. Finally we hope to consider the influence of glycaemic status and adiposity together on serum levels of OPG and to interrogate if a worsening glycaemic status can influence its relationship with adiponectin and systemic inflammation.

Experiment I

An investigation of serum OPG, TRAIL and sRANKL levels and their relationship with indicators of adiposity and insulin sensitivity in a healthy, representative Irish cohort.

Aims

i. The purpose of this study will be to determine if BMI and insulin sensitivity influence the concentrations of serum OPG and TRAIL in subjects who do not have cardiovascular or metabolic disease.

Hypotheses

i. In a healthy cohort, in the absence of an inflammatory process, OPG may be differentially regulated in obesity and.

ii. OPG will be related to fasting insulin and Oral Glucose Insulin Sensitivity (OGIS).

Experiment II

The relationship between osteoprotegerin, TRAIL, sRANKL and markers of inflammation in type 2 diabetes and vascular disease.

Aims

i. To measure serum OPG/RANKL/TRAIL in a cohort of well controlled type 2 diabetic patients with no evidence of underlying metabolic bone disease and compare them to a healthy age and BMI control group.

ii. To determine whether any differences that may arise can be attributed to the presence of underlying vascular disease or inflammation.

Hypotheses

i. OPG, along with other traditional inflammatory markers will be higher in type 2 diabetic patients.

ii. OPG will be a sensitive marker of inflammation that can distinguish between diabetics and normoglycemic controls irrespective of prior history of vascular disease in these patients.

Experiment III

The effect of glycaemic status and the underlying inflammatory state on circulating levels of OPG and adiponectin.

Aims

i. To examine changes in OPG levels across the typical pattern of the pathogenesis of type 2 diabetes, examining how OPG relates to insulin resistance and hyperinsulinaemia in the obese, pre-diabetic, and type 2 diabetic state

ii. To investigate the influence of adiposity in combination with the developing inflammatory state of associated with the progression from lean – obese – prediabetes – type 2 diabetes states.

Hypotheses

i. The deteriorating inflammatory state coupled with the sharp rise in hyperinsulinaemia observed over the spectrum of glycaemic dysfunction will induce a break in continuity in the relationship between OPG, markers of inflammation and indicators of insulin sensitivity that have been observed in previous experiments.

ii. Acute hyperinsulinaemia associated with an oral glucose load may act to suppress OPG secretion and that this will be differentially regulated depending of glycaemic status.

Chapter II Literature Review

Insulin has emerged as an important regulator of OPG production both *in vivo* and *in vitro* (Xiang *et al.*, 2007), (Jorgensen *et al.*, 2009), (Olesen *et al.*, 2005). Insulin resistance has also been demonstrated to correlate with circulating OPG (Gannage-Yared *et al.*, 2006), (Ugur-Altun *et al.*, 2005). Therefore this review will consider in detail, the role of insulin in metabolic control and glucose homeostasis, furthermore its perturbations in obesity and type 2 diabetes will be interrogated. Since there is evidence that TNF-α is an important player in OPG regulation and as a result of the structural homology of OPG and TRAIL to the TNF-receptor superfamily (Simonet *et al.*, 1997), TNF-α and its role in insulin resistance will be discussed in greater detail. Additionally, as adiponectin has been shown to correlate with OPG in healthy cohorts (Gannage-Yared *et al.*, 2006);(Gannage-Yared *et al.*, 2008) and has been demonstrated to stimulate RANKL and inhibit OPG production it will be subject to further consideration in this chapter. Finally, the literature exploring the interaction of OPG, RANK, RANKL and TRAIL will be subject to a detailed examination.

2.1 Insulin

Insulin is the primary endocrine regulator of glucose metabolism. It was originally identified and extracted by Banting and Best from pancreatic islet cells in 1921 (Banting & Best, 1922c). Its biological activity began to be unravelled when it was used to maintain pancreatectomized dogs (Banting & Best, 1922a) and treat human patients with type 2 diabetes (Banting & Best, 1922b). Islet cells are made of four principal cell types, the glucagon-producing α-cell, the somatostatin-producing δ-cell and the polypeptide-producing PP-cell. Insulin is released by the β-cells which make up 60-80% of all cells of the islets of Langerhans (Figure 2.1), the most important purpose of which is the synthesis, storage, and controlled secretion of insulin. When functioning correctly the β-cell ensures that there is an immediately available reservoir of insulin that can be quickly released in response to increased blood glucose levels. An increase in insulin secretion is compensated by augmented insulin biosynthesis, ensuring that insulin levels within the β-cell are continually preserved. Thus the biosynthesis and processing of the insulin molecule along the secretory pathway of the β-cell is a highly regulated and dynamic process (Rhode *et al.*, 2005). Insulin is a 6 kDa peptide formed by the C chain cleaved from proinsulin, the precursor of which is preproinsulin. Insulin is made up of two polypeptide chains, an "α" chain 21 amino acids in length, and a 30 amino acid "β" chain. The quaternary structure of insulin is primarily

enabled by two disulphide bonds, its secondary structure is mostly alpha helical (Rhodes, 2000). It has a half-life of 5 – 8 min and is degraded primarily in the liver and kidneys. Insulin secretion is stimulated during nutrient absorption, particularly in response to carbohydrate, by release of the neurotransmitter acetylcholine from the vagus nerve. Insulin secretion can also be stimulated by gastrin, secretin and specific amino acids such as arginine and leucine and lycine, free fatty acids, many pituitary hormones and some steroid hormones. Epinephrine and norepinephrine and the neuropeptide galanin also inhibit its secretion (Ferrannini & Mari, 1998).
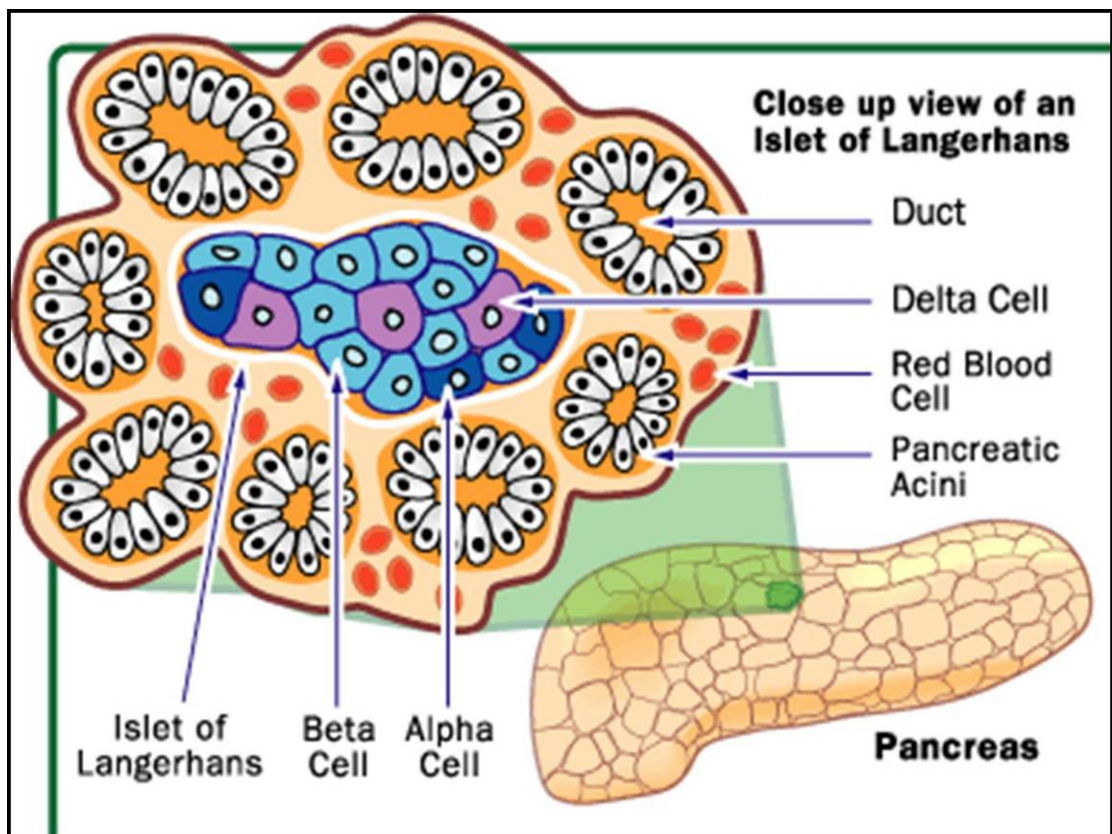
Figure 2.1 Structure of the Pancreas showing different types of cells present (Freudenrich *et al.*, 2009)

2.1.1 Regulation of Insulin secretion by Glucose

Glucose is the most significant physiological molecule involved in the regulation of insulin secretion (Porte, Jr. & Pupo, 1969), (Chen & Porte, Jr., 1976), (Ward *et al.*, 1984a). The first phase of this process involves glucose entering the β-cells by means of the GLUT-2 transporter. The first enzyme in the glycolytic pathway, glucokinase acts as an efficient sensor of ambient glucose concentrations. The subsequent metabolism of glucose and an increase in the

ATP:ADP ratio depolarises the cell membrane and triggers exocytosis of insulin secretory vesicles Insulin release under these circumstances occurs in a biphasic manner, with the first phase representing the release of stored insulin in granules primed at the plasma membrane and the second phase occurring as a result of further nutrient stimulus (Mayer *et al.*, 2007). Glucose stimulated insulin production from the β-cell is dose-dependent with as littlie as 1.4 units (~50 µg) being secreted in response to an oral glucose load of only 12 g (Waldhausl *et al.*, 1979), (Eaton *et al.*, 1983), (Nauck *et al.*, 1986). Ordinarily in response to glucose, insulin release from the β-cell does not appear to have a linear relationship with glucose concentration, instead the relationship appears to be best represented by a sigmoidal curve (Cavaghan & Polonsky, 2005), with a threshold corresponding to normal fasting blood glucose concentrations and with a rapid increase in the slope for that portion of the dose-response curve corresponding to the glucose levels normally found during the postprandial period (Pagliara *et al.*, 1974), (Gerich *et al.*, 1974), (Cavaghan & Polonsky, 2005). This sigmoidal pattern of the insulin secretory response to glucose has been attributed to a Gaussian distribution of thresholds for activation of insulin production among individual β-cells (Salomon & Meda, 1986), (Schmitz *et al.*, 1997). A constant rate infusion of intravenous glucose triggers a biphasic release of insulin from the β-cell characterised by a rapid initial peak (0-10 min) followed by a second phase in which the slope is much less steep and continues to a second peak (180-mins) (Porte, Jr. & Pupo, 1969), (Cerasi & Luft, 1967). The importance of first phase insulin kinetics are still uncertain but it could be representative of the rapid secretion of the reservoir of immediately available insulin stored within the β-cell as previously discussed or may be indicative of a temporary increase and subsequent reduction of some metabolic signal for insulin secretion (Grodsky, 1972).

2.1.2 Effect of Amino Acids on Insulin Secretion

Several essential amino acids have been shown to increase insulin secretion without the presence of glucose. The most effective secretagogues are leucine, arginine and lysine (Levin *et al.*, 1971) with the latter two being the most potent stimulators of β-cell insulin secretion. Even though the effects of these amino acids on insulin release are not related to concomitant fluctuations in glucose concentrations, the effects are enhanced by glucose (Ward *et al.*, 1984b), (Kadowaki *et al.*, 1984). β-cell-insulin production has also been assessed in response

to a number of amino acid metabolites. α-ketoisocarporate, α-keto-β-methylvalerate, Phenylpyruvate and α-ketocaproate appear to increase insulin output from islet cells, and are effective without the presence of glucose (Pagliara *et al.*, 1974).

2.1.3 Effect of Lipids and Lipid Metabolites on Insulin secretion

Several studies have demonstrated that the infusion of lipids, fatty acids and their metabolites do not acutely raise insulin secretion in the presence of low glucose *in vivo* (Campillo *et al.*, 1979), (Conget *et al.*, 1994), (Warnotte *et al.*, 1994). This has been explained by the fact that although β-cells are quite capable of oxidizing fatty acids (Berne, 1975), (Tamarit-Rodriguez *et al.*, 1984), this does not lead to an increase in the ATP:ADP ratio and as a result does not polarize the plasma membrane and increase $[Ca^{2+}]$ (Warnotte *et al.*, 1994). When there is a significant concentration of glucose present, it is possible for fatty acids to increase Ca^{2+} influx by opening Ca^{2+} ion channels. This amplification in the trigger signal can add to the insulin secretory potency of fatty acids under such conditions of elevated glucose (Henquin, 2005). However other studies have shown a direct stimulatory effect of free fatty acids on insulin secretion. Crespin *et al.*, (1973) who infused long-chain fatty acids into the pancreatic arteries of dogs and found an acute increase in insulin in pancreatic venous blood (Crespin *et al.*, 1973). Moreover, Hennes *et al.*, (1973) demonstrated in healthy, young women that raising plasma free fatty acid concentrations from 0.5 to 1.1 $mmol \cdot l^{-1}$ led to a 17% increase in insulin secretion under euglycaemic conditions with no change in insulin clearance. Interestingly, when blood glucose concentrations were increased to 7 $mmol \cdot l^{-1}$ and plasma and free fatty acids to 1.1 $mmol \cdot l^{-1}$, the resultant increase in insulin levels was as a result of an increase in insulin secretion but also to a decrease in metabolic clearance of insulin. When glucose was raised to 11 $mmol \cdot l^{-1}$, the rise in insulin was almost entirely due to a decrease in the clearance of insulin clearance (Hennes *et al.*, 1997). Although meals high in carbohydrate potently stimulate insulin release, carbohydrate-free fatty meals have little immediate effect on β-cell function (Muller *et al.*, 1971). However, interestingly ketone bodies and short and long-chain fatty acids have been shown to stimulate insulin secretion from islet cells *in vitro* and *in vivo* in humans (Goberna *et al.*, 1974), (Crespin *et al.*, 1973), (Boden & Chen, 1999), (Paolisso *et al.*, 1995). The influence of elevated free fatty acids on glucose-stimulated-insulin secretion is related to the length of the

treatment. Zhou et al., (1994) examined the effects of long-term exposure of pancreatic islets to free fatty acids and found that after 48 hours of co-culture with basal glucose (3.3 mmol·l⁻¹), insulin secretion had increased several fold. However, during stimulation with a supraphysiological glucose concentration of 27 mmol·l⁻¹, secretion was reduced by 30-50% and proinsulin synthesis was also decreased by 30-40% (Zhou & Grill, 1994). Carpentier *et al.*, (1999) demonstrated that the insulin resistance as a result of a 90 min increase in free fatty acids was met by a suitable rise in insulin secretion. However the compensatory insulin secretion-response of the β-cell was not sufficient to cope with the insulin resistance accompanying 48 hours of elevated fatty acids (Carpentier *et al.*, 1999). Additional studies have demonstrated that the adverse effects of prolonged elevations in free fatty acids on glucose induced insulin secretion are not seen in individuals with type 2 diabetes. On the basis of these results, it appears that elevated free fatty acids may contribute to the failure of β-cell compensation in insulin-resistance (Cavaghan & Polonsky, 2005).

2.1.5 Gut Hormones and the Regulation of Insulin Secretion

Interestingly, the insulin secretory response is higher after oral than intravenous glucose infusion (Tillil *et al.*, 1988), (Faber *et al.*, 1979), (Madsbad *et al.*, 1983), (Shapiro *et al.*, 1987). This phenomenon has been known as the incretin effect (Nauck *et al.*, 1986), (Creutzfeldt & Ebert, 1985) as the amplified reaction to oral glucose indicated that absorbance of glucose by the gut causes (i) an endocrine response or (ii) promotes other intermediary mechanisms that lead to improved sensitivity of the β-cell to an equivalent glucose load. Shapiro et al., (1987) studied nine healthy volunteers who received a glucose bolus intravenously at a rate designed to elicit glucose concentrations which had been previously achieved by an oral glucose load. The authors found that the insulin release after an intravenous load was 26% lower than that released in response to an oral glucose load (Shapiro *et al.*, 1987). Glucose dependant insulinotrophic polypeptide (GIP) and glucagon-like peptide (GLP-1) are two such intestinal hormones that increase the release of insulin following glucose ingestion. These hormones are released from the intestinal endocrine cells postprandially and travel through the bloodstream to reach the β-cells where they act through secondary messengers to increase the sensitivity of the islet cells to glucose (Cavaghan & Polonsky, 2005). GLP-1 also inhibits glucagon secretion, slows the release of nutrients from the intestine and regulates post-meal satiety. However,

although GLP-1 effectively lowers blood glucose, it is rapidly degraded in the circulation by dipeptidyl peptidase 4 (Ranganath *et al.*, 1996), (Vilsboll *et al.*, 2002). A decrease in GLP-1 expression has been implicated in the development of weight gain and obesity. The principle reason for this is because of its effect on appetite (Verdich *et al.*, 2001). It has been shown that obese subjects have decreased GLP-1 after a meal when compared to lean healthy controls but this is reversed after weight loss (Verdich *et al.*, 2001). The mechanism that leads to reduced GLP-1 production upon weight gain is not fully understood, but may be related to the insulin resistance that accompanies weight gain (Rask *et al.*, 2001). Type 2 diabetes is also characterized by impaired gut hormone production which may contribute to the altered rates of insulin release evident in the disease (Nauck *et al.*, 1986). A large part of this incretin defect is due to the loss of the insulin stimulating effect of GIP (Nauck *et al.*, 1993) even though the secretion of GIP is normal in type 2 diabetes, there is a substantial reduction in GLP-1 release after a meal but the insulin stimulating effect is retained (Nauck *et al.*, 1993).

2.1.6 Insulin Secretion in Obesity

Obesity is characterized by compensatory hyperinsulinaemia (Kissebah *et al.*, 1982) resulting from increased insulin production (Meistas *et al.*, 1982), (DeFronzo, 1982) and reduced insulin clearance (Meistas *et al.*, 1982), (Faber *et al.*, 1981), (Savage *et al.*, 1979), (DeFronzo, 1982), (Rossell *et al.*, 1983). Despite a reduction in clearance rates it appears that hypersecretion is the predominant contributor to elevated levels of basal insulin (Polonsky *et al.*, 1988a), (Jones *et al.*, 1997). It also appears that 24 hour insulin secretion rates are 3 or 4 times higher in the obese and are strongly correlated with BMI (Polonsky *et al.*, 1988b). Polonsky et al. (1988) reported that the temporal pattern of insulin secretion was similar in lean and obese subjects. They also found that obese subjects do not have significantly elevated postprandial plasma glucose levels and that basal insulin secretion accounted for 50% of total 24 hr insulin secretion, interestingly, when the postprandial insulin release was expressed relative to basal insulin release, the insulin response was identical between obese and lean subjects, suggesting that the elevated secretion of insulin observed in the obese population may be due to an enlarged β-cell mass rather than hypersensitivity (Cavaghan & Polonsky, 2005). This conclusion is in line with the much earlier findings of Ogilvie et al. (1933), who described a pathology of increased numbers of islet cells in obese subjects (Oglivie, 1933). This is also in a agreement with finding

of a compensatory mechanism of increased β-cell mass by Pick et al. (1998) to maintain glucose tolerance in insulin resistant Zucker fatty rats (Pick *et al.*, 1998). Evidence to date would therefore suggest that obese subjects exhibit moderate insulin resistance and tend to be hyperinsulineamic but have normal regulatory mechanisms controlling insulin secretion (Cavaghan & Polonsky, 2005).

2.1.6 Insulin Secretion in Type 2 Diabetes

Type 2 diabetes is characterized by hyperinsulinaemia, nevertheless even these elevated levels of insulin are too low to compensate for the levels of ambient circulating glucose (Cavaghan & Polonsky, 2005). Despite this, many patients in the early stages of type 2 diabetes have enough β-cell-insulin-secretory capacity to maintain glucose control with appropriate diet and exercise, with or without the use of oral therapeutic agents. The traditional aetiology of overt type 2 diabetes has been the development of defective β-cell function against a background of deteriorating insulin resistance (Weir, 1982), (Reaven, 1984), (Cahill, Jr., 1988), (Polonsky *et al.*, 1996), (Kahn, 1998). Pathological investigations of deceased diabetics give further weight to this observation (Kloppel *et al.*, 1985), (Clark *et al.*, 1988), (Stefan *et al.*, 1982) underlining that inadequate growth of β-cell mass is a mitigating factor in the development of the condition (Pick *et al.*, 1998). However, this viewpoint has been questioned and evidence to support a simultaneous decrease in secretion and resistance has also been proposed (Weyer *et al.*, 2001b). The acute insulin and C-peptide response to glucose is blunted or absent and the second phase response is significantly impaired (Pfeifer *et al.*, 1981), (Garvey *et al.*, 1985), (Ferner *et al.*, 1986), (Nesher *et al.*, 1987). The blunted acute insulin response to glucose remains even following improvements in glucose control (Pfeifer *et al.*, 1981), (Garvey, 2006) These results suggest the presence of an inherent flaw in the β-cell in type 2 diabetes. Several studies have reported that circulating proinsulin is significantly elevated and that this increase occurs in tandem with an increased ratio of proinsulin to insulin in circulation (Duckworth & Kitabchi, 1972), (Mako *et al.*, 1977), (Ward *et al.*, 1987) supporting the theory that the propensity of the β-cell to release a surplus of immature insulin (proinsulin) is an important defect in type 2 diabetes. The concentration of proinsulin produced in these patients is related to their glycaemic control rather that to the duration of diabetes (Saad *et al.*, 1990). In support of this, insulin specific assays report lower insulin concentrations in lean compared with obese

subjects with or without diabetes. However, when using a non-specific assay with cross-reactivity for proinsulin the differences are not apparent (Temple *et al.*, 1989), (Saad *et al.*, 1990), (Reaven *et al.*, 1993b). Abnormalities in the temporal pattern of insulin secretion in patients with type 2 diabetes mellitus have also been demonstrated. Patients with type 2 diabetes secrete a greater proportion of their daily insulin under basal conditions compared to obese, insulin resistant but non diabetic subjects (Cavaghan & Polonsky, 2005), This reduction in the proportion of postprandial insulin secretion appears to be related in part to a reduction in the amplitude of the secretory pulses of insulin that occur after meals rather than a reduction in the number of pulses (Cavaghan & Polonsky, 2005). The rapid oscillatory pattern of insulin production by the β-cells is also altered in patients with type 2 diabetes mellitus, who exhibit cycles that are shorter and more irregular than the persistent, regular, rapid oscillations present in healthy subjects (Lang *et al.*, 1979).. Various therapeutic strategies that improve glycaemic control in type 2 diabetics also appear to improve the β-cell secretory response (Garvey *et al.*, 1985), (Turner & Holman, 1978), (Kosaka *et al.*, 1980), (Hidaka *et al.*, 1982), (Shapiro *et al.*, 1989). However, even with enhanced glycaemic control, the kinetics of β-cell insulin secretion in type 2 diabetics do not become normalized with therapeutic intervention (Garvey *et al.*, 1985), (Hidaka *et al.*, 1982), (Shapiro *et al.*, 1989), (Cavaghan & Polonsky, 2005) implying that there is likely to be a continued inherent defect in the β-cell.

2.1.7 Insulin Regulation of Glucose Metabolism

The physiological significance of insulin is mediated through its effect on glucose metabolism in the liver, skeletal muscle and adipose tissue. These tissues are largely responsible for the control of whole body glucose homeostasis under physiological conditions. Brain cells and erythrocytes depend on glucose as their predominant fuel source and metabolise it at the same rate during overnight fasting as they do during the postprandial state. Consequently maintaining normoglycaemia despite oscillations in endogenous glucose production requires a careful and synchronized equilibrium between the regulation of glucose disposal and endogenous glucose production (DeFronzo, 1988). The liver, and to a lesser extent the kidneys, release glucose to compensate for whole body glucose disposal during rest or overnight fast. Under these conditions glucose utilisation by skeletal muscle and adipose tissue is relatively low and lipids are oxidised as the primary fuel source. After a meal, glucose and insulin levels are elevated

and glucose is transported from the circulation into the skeletal muscle, adipose tissue and liver (DeFronzo, 1988), (Konrad *et al.*, 2006). In addition to promoting glucose uptake in muscle and adipose tissue, insulin promotes glycogen synthesis in muscle and the liver and the conversion of excess carbohydrate to lipid. Insulin concurrently inhibits the breakdown of these molecules through glycogenolysis and lipolysis. In a similar fashion, endogenous glucose production is curbed by insulin in the liver and kidney through the inhibition of glycogenolysis and gluconeogenesis (Gerich *et al.*, 2001), (Moller *et al.*, 2001). More recent studies using gene-specific knockout animals have confirmed the direct effects of insulin on glucose metabolism. Liver insulin receptor knockout mice exhibit decreased endogenous glucose production during a hyperinsulinaemic clamp (Michael *et al.*, 2000). Similarly a decrease in insulin-stimulated glucose transport and glycogen synthesis in skeletal muscle is evident in mice that have undergone ablation of the gene encoding the muscle insulin receptor. Surprisingly glucose uptake in adipose tissue is elevated in this model implying perhaps that the hormone's target tissues are coordinated and can adapt in order to maintain whole body glucose homeostasis (Kim *et al.*, 2000).

2.2 Insulin Action

2.2.1 Skeletal Muscle

Ingested glucose has a number of potential fates. It can be oxidized, stored as glycogen in the muscle and liver, converted to gluconeogenic precursors or converted to fat by de novo lipogenesis. Early studies using indirect calorimetry during a euglycaemic-insulin clamp showed the primary role of nonoxidative glucose disposal in normoglycaemic healthy humans (DeFronzo *et al.*, 1981), (DeFronzo, 1992). *Ex vivo* glycogen concentrations, measured during muscle biopsy studies in the presence of high plasma glucose demonstrated that more than half of an intravenously administered glucose load was stored as glycogen (Bergstrom & Hultman, 1967), (Nilsson & Hultman, 1974). Shulman *et al.* (1990) used ^{13}C magnetic resonance spectroscopy (MRS) to directly measure changes in muscle glycogen during hyperglycaemic-hyperinsulinaemic clamps in conjunction with indirect calorimetry to assess non oxidative glucose disposal (Shulman *et al.,* 1990). They demonstrated for the first time that skeletal muscle accounted for the majority of insulin-mediated glucose disposal and that greater than

80% was subsequently stored as glycogen. ^{13}C MRS has also been used to measure glycogen synthesis in normoglycemic individuals following a meal where it was found that skeletal muscle was responsible for the disposal of approximately 30% of ingested glucose (Woerle *et al.*, 2003). Several other studies which measured similar characteristics in type 2 diabetic and insulin resistant offspring of diabetics have found that muscle glycogen levels were reduced by 30% in type 2 diabetics when compared to matched healthy controls (Carey *et al.*, 2003), (Shulman *et al.*, 1990). It was also noted that the rate at which glycogen was synthesised in skeletal muscle was 50 % lower in diabetics than in matched controls during hyperglycaemic-hyperinsulinaemic clamps. In addition it was found that postprandial increases in skeletal muscle glycogen concentrations were also significantly lower than those found in controls (Carey *et al.*, 2003).

Facilitated by insulin, glucose is transported into the myocyte by glucose transporter 4 (GLUT4) where it is phosphorylated by hexokinase to Glucose-6-phosphate (G-6-P). G-6-P can then undergo anaerobic glycolysis or be converted to glycogen by glycogen synthase.

Extracellular glucose → intracellular glucose → G-6-P → Glycogen (Savage *et al.*, 2007)

In order to assess the rate limiting steps in glucose transport Rothman et al., (1992) used ^{13}C and ^{31}P MRS to monitor intracellular G-6-P concentrations and intramuscular glycogen synthesis simultaneously during a hyperinsulinaemic-hyperglycaemic clamp in type 2 diabetics (Rothman *et al.*, 1992). The lower concentration of G-6-P in the diabetic subjects despite a decreased rate of nonoxidative glucose metabolism suggested that glucose transport or phosphorylation, and not glycogen synthesis, were the rate-controlling step in skeletal muscle insulin-stimulated glucose (Shulman, 2000). This suggests that a defect in the glucose transport mechanism manifests itself before the development of type 2 diabetes in both offspring of diabetics and nondiabetic obese females, who together are at increased risk of developing type 2 diabetes (Savage *et al.*, 2007). Glucose transport into the myocyte is predominantly facilitated by the insulin responsive GLUT-4. Glucose phosphorylation however is enabled by hexokinase. If hexokinase were the rate controlling step in diabetes then intracellular glucose levels would be expected to rise significantly. To investigate this mechanism further and to determine whether glucose transport or phosphorylation was the rate controlling step, ^{13}C MRS was used to assess intracellular free glucose in muscle (Cline *et al.*, 1999). The authors concluded that

intracellular glucose was 1/25 what they might have expected had hexokinase been the primary rate controlling enzyme (Cline *et al.*, 1999), These findings give further support to the contention that insulin stimulated GLUT-4 translocation to the plasma membrane is the crucial rate limiting step in regulating insulin stimulated muscle glycogen synthesis in type 2 diabetes

2.2.2 The Liver

The liver plays an important role in preserving glucose homeostasis during the constant switching between the fed and fasted state. Although peripheral tissues such as the skeletal muscle are responsible for the majority of postprandial insulin stimulated glucose disposal, the liver also plays an important balancing role by reducing hepatic glucose output and increasing the retention of glucose by hepatic glycogenesis (DeFronzo, 1992). While fasting, liver glycogen stores are readily used to maintain blood glucose levels. The breakdown of glycogen in the liver contributes approximately half of the endogenous glucose production in the initial hours of fasting (Petersen *et al.*, 1998), while gluconeogenesis accounts for the other half.

Net Glycogen synthesis is determined by the enzymes, glycogen synthase and glycogen phosphorylase. The production and breakdown of hepatic glycogen (glycogen cycling) occurs simultaneously (David *et al.*, 1990), (Magnusson *et al.*, 1994), (Petersen *et al.*, 1998). The effect of glucose and insulin signalling on glycogen turnover under hypeglucagonaemic conditions was investigated using ^{13}C MRS. It was demonstrated that hyperglycaemia decreases net hepatic glycogenolysis by inhibiting glycogen phosphorylase. In contrast hyperinsulinaemia inhibits net hepatic glycogenolysis primarily by upregulating glycogen synthase (Petersen *et al.*, 1998). The net rate of glycogen synthesis is dependant on portal vein insulin concentrations. Insulin levels in the region of 130 – 170 pmol·l^{-1} are required for half maximal stimulation of glycogen synthesis. Under basal insulin concentrations glucagon was shown to strongly regulate net hepatic glycogen synthesis (Roden *et al.*, 1996a).

2.2.3 Adipose Tissue

Intravenous infusion of insulin results in an immediate and substantial reduction in circulating concentrations of non-esterified fatty acids (NEFAs). The effect on NEFAs can be more prominent than insulin's ability to lower blood glucose (Frayn & Karpe, 2006). NEFAs are

secreted into the circulation mostly from the breakdown of triglycerides within adipocytes. Glycerol is a by-product of this process and its release from adipose tissue is considered to be a good indicator of total body lipolysis, particularly because adipocytes express little or no glycerol kinase activity (Frayn & Karpe, 2006). The insulin-mediated decrease in circulating NEFA levels is an essential part of the synchronization of metabolic processes that occur in the postprandial period. After a typical meal, glucose becomes the preferred fuel source for skeletal muscle and therefore it is advantageous for substrate competition from fatty acids to be reduced as much as possible. In addition, NEFAs provide a very effective stimulus for hepatic gluconeogenesis (Boden *et al.*, 1994), (Chen *et al.*, 1999). However, this stimulus would not be helpful in the period following a meal when hepatic glucose production must be reduced in order to maintain blood glucose at appropriate concentrations. As a result NEFA levels have significant diurnal variation. They tend to follow a pattern which is the opposite of insulin secretion with reduced postprandial concentrations and an obvious peak prior to the next meal (Frayn & Karpe, 2006). Insulin plays an important role in almost all aspects of adipocyte biology. In fact, adipocytes are among the most insulin-responsive cells (Kahn & Flier, 2000). Triglyceride storage in adipocytes is promoted by insulin in several ways, such as the initiation of the differentiation of preadipocytes to adipocytes, upregulation of glucose transport and lipogenesis in more mature adipocytes and the inhibition of lipolysis (Kahn & Flier, 2000). Insulin also increases the uptake of fatty acids from lipoproteins by enhancing the action of lipoprotein lipase in the adipocyte (Fielding & Frayn, 1998). The physiological effects of insulin on adipose tissue are brought about by a wide variety of tissue-specific actions, involving both changes in protein phosphorylation and function, as well as sudden changes in gene expression (Collins *et al.*, 2005). Insulin can also affect gene transcription in the adipocyte. The transcription factors adipocyte determination and differentiation factor-1 and sterol regulatory element–binding protein-1c (ADD-1 / SREBP-1C) also have an important function regulating insulin-mediated adipocyte gene expression (Kim *et al.*, 1998), (Shimomura *et al.*, 1999a), (Foretz *et al.*, 1999) by activating genes that promote lipogenesis and suppressing those implicated in fatty acid oxidation. Other transcription factors, such as those belonging to the forkhead group have also been show to play an important role in translating insulin actions to the nucleus (Kops & Burgering, 1999).

2.2.4 Non-Classical Tissues

Although the liver, skeletal muscle and adipose tissue account for the majority of insulin-mediated glucose metabolism, almost all tissue types have insulin receptors. Tissue specific knockout models of the insulin receptor have provided further insight into the action of insulin in non-classical target tissues such as pancreatic β-cells, the central nervous system and vascular endothelial cells. These tissues may also have a crucial role in controlling whole body insulin sensitivity. It has been demonstrated that β-cell-insulin receptor deficient mice have impaired glucose tolerance and decreased insulin secretion (Kulkarni *et al.*, 1999). Mice lacking the insulin receptor in neural cells demonstrate increased nutrient intake and become obese as a result, suggesting that the binding of insulin in the central nervous system may produce an anorexogenic effect (Bruning *et al.*, 2000). The counter-regulation of hypoglycaemia involves a myriad of hormones and neurotransmitters that are released to provide glucose for the brain, while decreasing glucose need in peripheral tissues (Hileman & Bjorbaek, 2006). Increases in counter-regulatory hormones such as glucagon, epinephrine, norepinephrine and cortisol take place when glucose levels reach $\sim$ <3.6 mM. Symptoms of hypoglycaemia that are neural in origin such as sweating, hunger, tingling, weakness, dizziness and cognitive dysfunction begin to appear at glucose levels $\sim$ < 3 mM (Mitrakou *et al.*, 1991), (Schwartz *et al.*, 1987). Decreasing glucose concentrations also lead to the release of epinephrine from the adrenal medulla which stimulates glucose production and limits glucose utilisation through a β2-adrenergic-receptor-mediated mechanism. Epinephrine can also stimulate the mobilization of free fatty acids and inhibit pancreatic β-cell insulin production and secretion (Woods & Porte, Jr., 1974). In addition vascular endothelial cells insulin regulates vasodilation and capillary recruitment which in turn increase glucose uptake to the muscle bed (Clark *et al.*, 2003). As these non classical tissues only make a small contribution to whole body glucose disposal, it has been suggested that they play a secondary role in the control of whole body glucose metabolism. (Konrad *et al.*, 2006)

2.3 Insulin Resistance

Insulin resistance is an important precursor in the pathogenesis of the metabolic syndrome and type 2 diabetes mellitus. It can be defined as a state of diminished responsiveness to normal circulating concentrations of insulin. In the initial stages pancreatic β-cells secrete an adequate

amount of insulin to compensate for insulin resistance and preserve euglycaemia. Eventually, however, relative or absolute insulin deficiency ensues leading to hyperglycaemia and type 2 diabetes (McGarry, 2002). Healthy humans respond to excess energy intake by storing the net energy surplus as triglyceride in adipose tissue, predisposing them to weight gain and ultimately obesity. This also results in ectopic lipid accumulation in sites such as the liver and skeletal muscle, and possibly in pancreatic β-cells and the kidney (Shulman, 2000), (Unger, 2003). One explanation for the deposition of intracellular lipid in these tissues is that excess energy availability exceeds the storage capacity of the adipose tissue leading to energy overflow (Unger, 2003), (Danforth E Jr, 2000). This is further evidenced by the occurrence of ectopic lipid accumulation in mice and humans with generalised lipodystrophy (Adams *et al.*, 2004), a severe example of insufficient adipose tissue storage capability with excess calorie ingestion. One method by which ectopic lipid deposits in lipodystrophic mice can be reduced is to transplant adipose tissue from wild type mice. This technique considerably improves insulin sensitivity (Gavrilova *et al.*, 2000). Ectopic lipid deposits can also be reduced in lipodystrophic mice and humans through the administration of the anorexogenic adipocytokine, leptin (Oral *et al.*, 2002), (Shimomura *et al.*, 1999b) leading to a reduction in energy intake and significantly improved insulin-stimulated liver and muscle carbohydrate metabolism (Petersen *et al.*, 2002). The concept of energy overflow is further supported by the finding that significant weight loss as a consequence of liposuction does not improve the metabolic characteristics of obese individuals (Klein *et al.*, 2004). The procedure simply reduces adipose tissue storage capability in the face of unchanged caloric intake (Savage *et al.*, 2007). However even relatively small reductions in weight through diet and exercise can substantially improve insulin sensitivity (Petersen *et al.*, 2005), (Tamura *et al.*, 2005).

Metabolic derangements of type 2 diabetes have traditionally been viewed as glucocentric, however in recent years a more lipocentric approach to the problem has become accepted (McGarry, 2002). As well as hyperglycaemia most type 2 diabetics have abnormal lipid storage, secretion or metabolism, resulting in elevated circulating free fatty acids and triglycerides as well as increased ectopic fat deposits in a variety of tissues including the muscle bed (Reaven, 1995), (Schalch & Kipnis, 1965). It is less clear if the change in lipid homeostasis is a cause or consequence of diabetes (Figure 2.2). There is growing evidence to suggest that excessive fat accumulation in muscle and other tissues contributes to the development of insulin resistance

and pancreatic β-cell dysfunction in type 2 diabetes (McGarry, 2002). Intravenous lipid infusion designed to increase the concentration of plasma fatty acids have been shown to impair both oral glucose tolerance (Felber & Golay, 2002) and insulin stimulated glucose disposal (Roden *et al.*, 1996b), (Roden *et al.*, 1996a), (Boden, 1997). Interestingly the reduction in insulin sensitivity observed during such infusions only seems to manifest itself between 3-5 hours after circulating levels of fatty acids begin to increase (Boden, 2001).
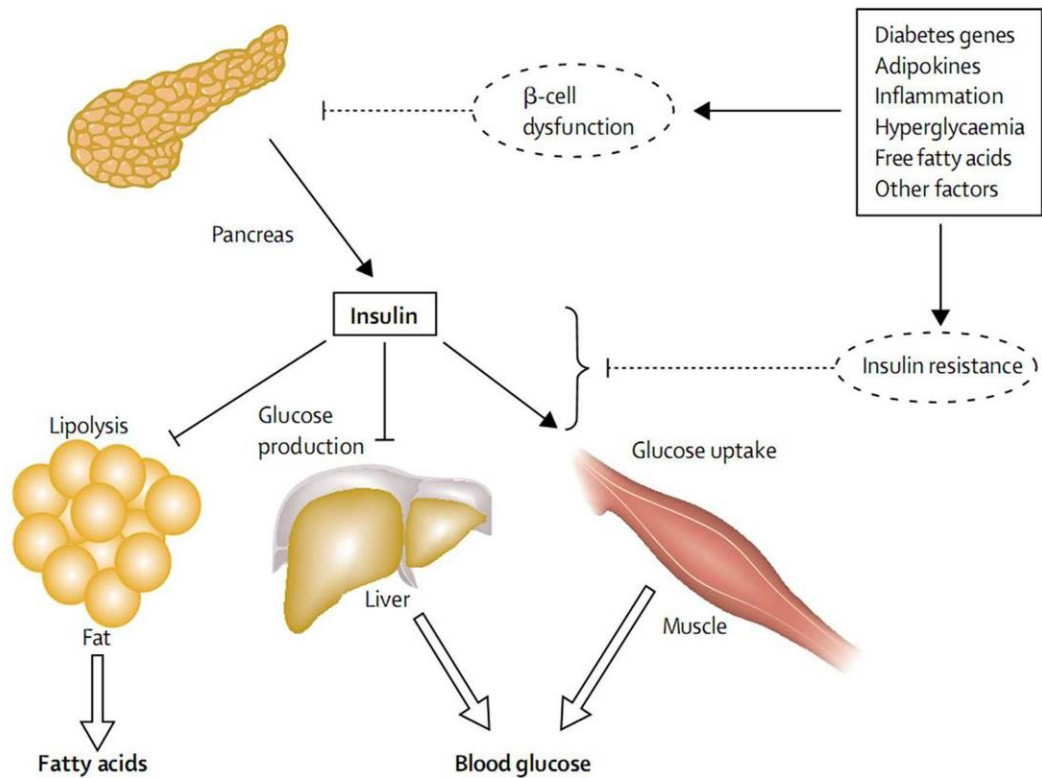
Figure 2.2 Pathophysiology of hyperglycaemia and increased circulating fatty acids in type 2 diabetes Insulin secretion from the pancreas normally reduces glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid release from fat tissue. The various factors shown that contribute to the pathogenesis of type 2 diabetes affect both insulin secretion and insulin action. Decreased insulin secretion will reduce insulin signalling in its target tissues. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycaemia of diabetes. In turn, the raised concentrations of glucose and fatty acids in the bloodstream will feed back to worsen both insulin secretion and insulin resistance (Stumvoll *et al.*, 2005).

2.3.1 The Glucose Free Fatty Acid Cycle – Randle / Reverse Randle Hypothesis

Randle et al. (1963) demonstrated that fatty acids compete with glucose for substrate selection in rodent heart muscle (RANDLE *et al.*, 1963). They hypothesized than an increase in lipid oxidation might be responsible for insulin resistance by increasing mitochondrial acetyl CoA: CoA and NADH: NAD ratios. These biochemical changes would inactivate pyruvate dehydrogenase, increase citrate concentrations and inhibit phosphofructokinase and G-6-P accumulation. As G-6-P inhibits hexokinase activity there would be an accumulation of intracellular glucose and decreased glucose uptake. However this hypothesis has been challenged recently (Roden *et al.*, 1996a), (Boden *et al.*, 1994), (Dresner *et al.*, 1999), (Griffin *et al.*, 1999). When free fatty acid concentrations were maintained at high concentrations for 5 h during a euglycaemic-hyperinsulinaemic clamp the decrease in glucose disposal was accompanied by a decrease in G-6-P concentrations (Roden et al., 1996) and not an increase as would have been predicted by the Randle Cycle These findings were consistent with those observed in type 2 diabetic patients (Rothman *et al.*, 1992). Furthermore, Sidossis et al., (1996) challenged the traditional view by proposing a "reverse Randle cycle". They found that,, contrary to the predictions of Randle's glucose-fatty acid cycle, that the intracellular availability of glucose and not fatty acids was the primary determinant of substrate selection (Sidossis & Wolfe, 1996).

2.3.2 Influence of Circulating Free Fatty Acids on Glucose Uptake and Glycogen Synthesis

The cellular mechanism responsible for free fatty acid mediated insulin resistance may be an inhibition of insulin signalling to GLUT-4 containing vesicles or a decrease GLUT4 trafficking between the intracellular compartment and the cell surface (Savage *et al.*, 2007). To investigate this mechanism in more detail Dresner *et al.* (1999), examined various elements of the insulin signalling pathway in skeletal muscle biopsies obtained from subjects infused with high levels of free fatty acids for 5 h before and during a hyperinsulinaemic-euglycaemic clamp (Dresner *et al.*, 1999). Glucose oxidation and glycogen synthesis were 50 - 60% lower after free fatty acid infusion when compared to a glycerol control trial. The lipid infusion trial was associated with a decrease in intramuscular G-6-P of approximately 90%, suggesting that there had been a significant decrease in glucose transport or phosphorylation. Intracellular glucose was

decreased in the lipid infusion trial, suggesting that glucose transport is the rate controlling step (Savage *et al.*, 2007). Insulin receptor substrate 1 (IRS-1) associated phosphatidylinositol-3 kinase activity was also decreased under these conditions. Later studies carried out in rodent models have suggested that this might be as a result of increased serine phosphorylation of IRS-1 (Dresner *et al.*, 1999), (Griffin *et al.*, 1999), (Morino *et al.*, 2005), (Yu *et al.*, 2002). The specific nature of the lipid product responsible for fatty acid induced insulin resistance has been the subject of some debate but long-chain acyl-coenzyme A (LCCoAs) and diacylglyceride (DAG) are thought to be important players. Neschen *et al.*, (2005) found that mitochondrial acyl-CoA:glycerol-3-phosphate acyltransferase 1 (mtGPAT1) knockout mice have increased hepatic insulin sensitivity and LCCoAs but decreased DAG and TG. Their results suggest that DAG may mediate fatty acid-induced hepatic insulin resistance (Neschen *et al.*, 2005). Protein Kinase C (PKC) is a serine/threonine kinase, activated by DAG and may offer a potential link between lipid accumulation and serine phosphorylation of IRS-1 (Griffin *et al.*, 1999), (Schmitz-Peiffer *et al.*, 1997). Itani *et al.*, (2002) noted that an accumulation of DAG in skeletal muscle during lipid infusions was correlated with an increase in PKC-β11 and PKC-δ expression and activity (Itani *et al.*, 2002). Therefore the breakdown in lipid dynamics in type 2 diabetes that lead to an increase in LCCoAs and DAG in hepatic and muscle tissue would lead to lipid induced insulin resistance (Shulman, 2000).

2.3.3 Skeletal Muscle Insulin Resistance – Defects in Signalling Pathways

Skeletal muscle metabolic dysfunction occurs early on in pathogenesis of insulin resistance as healthy first degree relatives of type 2 diabetics exhibit decreased insulin-stimulated glucose uptake (Eriksson *et al.*, 1989), (Vaag *et al.*, 1992). GLUT4 protein content is similar in diabetic and non-diabetic skeletal muscle (Eriksson *et al.*, 1989), (Kahn *et al.*, 1992) but the translocation of GLUT-4 containing vesicles is impaired (Dohm *et al.*, 1988); (Zierath *et al.*, 1996). Therefore much of the research has focused on characterising the insulin signalling cascade and identifying loci with impaired kinase activity. Tyrosine phosphorylation of the insulin receptor has been shown to be decreased (Goodyear *et al.*, 1995) or unchanged in diabetic skeletal muscle (Arner *et al.*, 1987), (Krook *et al.*, 2000). Research has consistently demonstrated a decrease in IRS-1 tyrosine phosphorylation and IRS-1 associated PI3-K activity (Bjornholm *et al.*, 1997), (Kim *et al.*, 1999), (Krook *et al.*, 2000). As well as tyrosine

phosphorylation IRS-1 can be phoshorylated at multiple serine theronine residues , which serve to either reduce or improve insulin signalling (Gual *et al.*, 2005). Many circulating factors related to the insulin resistant state such as free fatty acids and TNF-α, appear to augment IRS-1 serine phosphorylation therefore inhibiting its function (Gual *et al.*, 2005), (Hotamisligil *et al.*, 1994b). As previously mentioned, accumulation of DAG may impair insulin action by PKC-mediated serine phosphorylation of IRS-1.

2.3.4 Hepatic Insulin Resistance

In insulin resistant type 2 diabetes, elevated fasting blood glucose concentrations are heavily influenced by the presence of increased rates of endogenous glucose production (Bogardus *et al.*, 1984), (DeFronzo, 1992), (Fery, 1994). This is likely to result from increased gluconeogenesis and decreased glycogenolysis. Magnussen *et al.* (1992) examined net hepatic glycogenolysis in poorly controlled type 2 diabetic patients and found that fasting hepatic glycogen concentrations were sharply decreased in the diabetics when compared to matched controls. Therefore, as well as reduced insulin stimulated muscle glycogen synthesis (Shulman *et al.*, 1990), it appears type 2 diabetics also have a reduced capacity to store and/or synthesise liver glycogen leading to an increased incidence of elevated postprandial hyperglycaemia. These reductions in hepatic glycogen synthesis were linked to a decrease in hepatic glycogenolysis and a further 60 % upregulation in the rate of gluconeogenesis (Shulman *et al.*, 1990).

2.3.5 Insulin Resistance in Adipose Tissue

Insulin resistance in adipose tissue leads to an increased rate of lipolysis which is accompanied by a rise in plasma free fatty acids. The increased circulating free fatty acids impair hepatic and skeletal muscle insulin action and β-cell insulin release, leading to the development of type 2 diabetes mellitus (Reaven, 1988). A degree of mitochondrial dysfunction also contributes to insulin resistance (Richardson *et al.*, 2004) by limiting lipid oxidation and promoting intracellular accumulation of fatty acyl-CoA and other lipid by-products. Fatty acid oxidation is also impaired by the accumulation of intracellular malonyl-CoA, and the downregulation of carnitine palmitoyltransferase activity. This accumulation of acyl-CoA impairs insulin action in skeletal

muscle which results in even greater insulin resistance (Ferrannini & DeFronzo, 2004), (Schling & Loffler, 2002).

2.4 Insulin Resistance and Atherosclerosis

Haffner *et al.* (1998) found that the 10-year risk of myocardial infarction (MI) in a normoglycemic cohort with no prior history of infarction was less than 4% and increased to 19% for those with a previous MI. However, in diabetic subjects who had never had an MI the relative risk was 20% and the risk of a second MI was 45% in this cohort (Haffner *et al.*, 1998). Several epidemiological studies have also demonstrated that insulin resistance measured using both the euglycaemic-hyperinsulinaemic clamp (Bokemark *et al.*, 2001) and the frequently sampled intravenous glucose tolerance test (Howard *et al.*, 1996) is an independent risk factor for CVD using carotid intima-media thickness as a surrogate measures of atherosclerosis. The European Group for the Study of Insulin Resistance is currently conducting a large, multi centre study to investigate insulin resistance as an independent risk factor for cardiovascular event rate (Cleland & Connell, 2006).

2.5 Hemodynamic Action of Insulin

In order for insulin to dock with its target receptor on the plasma membrane it must first cross the endothelial monolayer (Figure 2.3). Before it can reach the perivascular space, insulin is internalized by the vascular endothelial cells (Wiernsperger, 1994) in a poorly understood receptor-mediated process (Cersosimo & DeFronzo, 2006). This process can delay insulin-stimulated glucose uptake in insulin resistance (Bonadonna *et al.*, 1998), (Laakso *et al.*, 1990), (Olefsky *et al.*, 1973), (Sjostrand *et al.*, 2002). Studies assessing insulin-mediated glucose disposal have demonstrated a substantial delay in obese (Laakso *et al.*, 1990), and type 2 diabetic patients (Olefsky *et al.*, 1973), (Cersosimo & DeFronzo, 2006) healthy lean controls (Bonadonna *et al.*, 1998). Baron *et al.* (1995) and others (Steinberg *et al.*, 2000) (Baron & Brechtel, 1993) have shown that there is a significant increase in leg blood flow during a euglycaemic-hyperinsulinaemic clamp at physiological levels in lean,, obese and diabetic individuals. When insulin was administered at a rate of 40 or 120 $\mu U \cdot ml^{-1}$, leg blood flow increased after 240 min by 100% at both infusion rates. The physiological importance of the

insulin-mediated vasodilatory response is still unclear (Cersosimo & DeFronzo, 2006), (Yki-Jarvinen & Utriainen, 1998), as a significant vasodilatory response only begins to happen after 40-50 min of insulin infusion at supraphysiological concentrations (Ueda *et al.*, 1998). However, in patients with coronary artery disease, insulin stimulated glucose uptake in the myocardium is significantly impaired (Paternostro *et al.*, 1996). One theory that may be teleologicaly attractive is that insulin is a chronic regulator of blood flow in the vascular beds of metabolically active tissues (Cleland & Connell, 2006). Such a mechanism would be advantageous as insulin would potentially direct fuel substrates such as glucose and fatty acids to important tissues before stimulating their cellular uptake. This link is supported by Cleland et al., (1999) who showed a positive correlation between insulin sensitivity and insulin-mediated vasodilation in healthy normotensive men (Cleland *et al.*, 1999). The mechanism by which this insulin-induced vasodilation occurs has become clearer in recent years. It is now recognized that the integrity of vascular endothelium must be intact and that the endothelial-derived vasodilator, nitric oxide (NO) is an important facilitator of the vascular actions of insulin (Scherrer *et al.*, 1994), (Zeng & Quon, 1996), (Landry & Oliver, 2001). In the intact vascular endothelium, arginine is converted enzymaticaly to NO by endothelial nitric oxide synthase (eNOS). The activity of NOS is increased several fold by insulin responsive cytokines such as IL-1β, IL-6, TNF-α, interferon-γ and adenosine (Landry & Oliver, 2001) There is also evidence that nitric oxide synthase is directly upregulated in response to a pharmacological dose of insulin in cultured endothelial cells (Zeng & Quon, 1996). As previously stated, insulin increases eNOS activity in the vascular endothelial cells (VEC) (Zeng & Quon, 1996), (Aljada & Dandona, 2000), (Kuboki *et al.*, 2000), In fact all of the components of the insulin signalling cascade are present in endothelial cell culture and higher concentrations of insulin will increase the activation of IRS-1, PI3 kinase and PKB/AKT (Cleland & Connell, 2006). Adding insulin to VEC cultures leads to an immediate increase in NO release and the uptake of L-arginine by it's specific receptor transporter (Sobrevia *et al.*, 1996). Insulin stimulated NO production can be achieved at physiological concentrations (Cleland & Connell, 2006), (Cleland *et al.*, 1999).

The insulin-mediated pathway that regulates eNOS activity/synthesis may be similar in adipose tissue and skeletal muscle. The similar regulation of the insulin signalling cascade in the vascular endothelium and adipocytes suggests that insulin resistance and endothelial dysfunction may be linked. A direct association between insulin resistance and eNOS

availability in healthy males (Petrie *et al.*, 1996), type 2 diabetic or hypertensive subjects (Cleland *et al.*, 2000) has been observed. There is also evidence from animal studies for a direct vasoactive function of insulin. Defective endothelium-dependent vasodilation has been shown in IRS-1 knockout mice (Abe *et al.*, 1998) and insulin signalling is impaired in the vascular endothelium of the Zucker fatty rat (Jiang *et al.*, 1999). The fact that insulin signalling is essential for the normal function of most metabolically active tissues, such as skeletal muscle, adipose tissue and the vascular endothelium means it is reasonable to assume that there is significant insulin-mediated crosstalk between these tissues that may influence the development of endothelial dysfunction. In addition, insulin resistance and endothelial function can improve in response to a variety of interventions and treatments, such as exercise, weight loss and pharmacotherapy. The adipose tissue secretes a multitude of adipocytokines that can act to regulate insulin sensitivity and endothelial function. TNF-α is one such adipocytokine that can induce insulin resistance by disruption of the insulin signalling cascade (Valverde *et al.*, 1998), (Feinstein *et al.*, 1993), (Halse *et al.*, 2001), while simultaneously promoting endothelial dysfunction (Wang *et al.*, 1994). This TNF-α-mediated effect on endothelial function may be the result of nuclear factor-κβ (NF-κβ) activation (Ruan *et al.*, 2002). Cytokines such as IL-1β and TNF-α, that stimulate NF-κβ, can also be directly activated by the NF-κβ pathway, demonstrating some auto-regulation in this signalling cascade (Yamamoto & Gaynor, 2001). Insulin has been shown to have a potent anti-inflammatory effect *in vitro* by inhibition of the NF-κβ pathway and up-regulation of the inhibitor of nuclear factor κβ (Iκβ) (Dandona *et al.*, 2001) Thiazolidinediones (TZDs) which reduce NF-κβ production, block TNF-α induced insulin resistance (Peraldi *et al.*, 1997). Additionally aspirin, which is known to up regulate Iκβ, reduces insulin resistance and supports the belief that it is an important molecule, linking insulin signalling, endothelial dysfunction and the inflammatory process (Hundal *et al.*, 2002), (Brandstrom *et al.*, 1998).
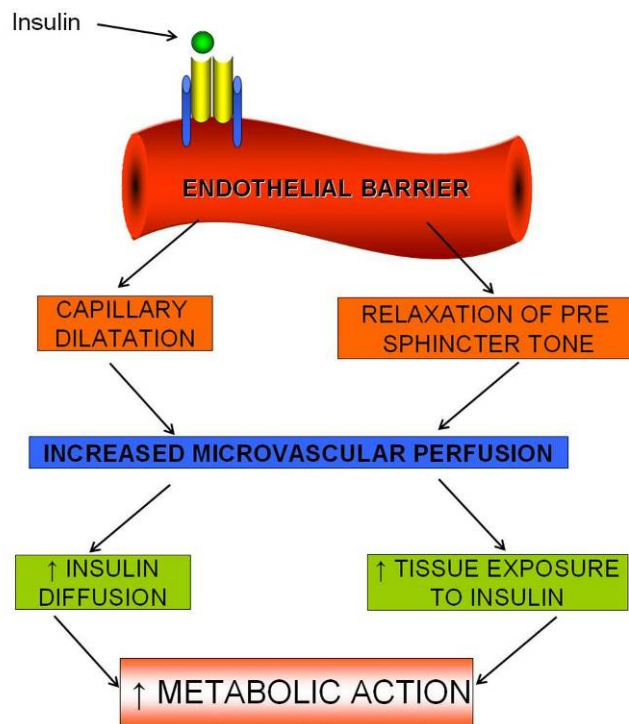
Figure 2.3 Hemodynamic actions of insulin. The time-course of insulin's hemodynamic action is closely integrated with its metabolic effects. Following its passage through the endothelial barrier, insulin promotes precapillary sphincter tone relaxation with capillary dilatation. As a result, more microvessels are recruited, the capillary network is expanded, and peripheral microvascular perfusion increases. Insulin then diffuses into the interstitial fluid more readily, and the exposure of the target tissues to insulin is magnified, resulting in an increase in insulin-mediated glucose metabolism, (reproduced from (Cersosimo & DeFronzo, 2006).

2.6 Adipocytokines

The discovery of adipose tissue-derived cytokines (adipocytokines), which regulate skeletal muscle and hepatic glucose metabolism and insulin sensitivity (Figure 2.4) (Fruhbeck *et al.*, 2001), (Havel, 2004) has given new insight into the important endocrine function of adipose tissue. Adiponectin increases insulin sensitivity by suppressing hepatic glucose production as well as increasing glucose uptake by skeletal muscle (Combs *et al.*, 2001), (Tomas *et al.*, 2002). Adiponectin also acts on the vascular endothelium and shows significant promise as a mediator of both vascular and metabolic function (Yamauchi *et al.*, 2001). Leptin, signals through its receptors in the arcuate nucleus of the hypothalamus to decrease food intake and increase energy expenditure (Leibel, 2002). It has been suggested that leptin may also have a role in regulating peripheral insulin sensitivity (Ceddia *et al.*, 2002). Insulin positively regulates the gene expression and secretion of leptin from adipose tissue (Fasshauer *et al.*, 2002), (Stefan & Stumvoll, 2002). Changes in the plasma concentrations of these adipocytokines provide evidence of a secondary regulator of glucose metabolism that complements insulin action. In addition, several other adipocytokines have been implicated in metabolic and endothelial

dysfunction (Stears & Byrne, 2001), (Saltiel & Kahn, 2001), (Shulman, 2000), IL-6, a powerful inducer of hepatic CRP production, has been associated with diabetes and CVD in many longitudinal and cross-sectional epidemiological studies (Bermudez *et al.*, 2002), (Han *et al.*, 2002), (Festa *et al.*, 2002) and there is also good support for a connection between IL-6 synthesis and insulin resistance (Bastard *et al.*, 2002). It has also been demonstrated that leptin is an active regulator of insulin sensitivity and acts directly on the vascular endothelium (Mantzoros, 1999), (Shimomura *et al.*, 1999b) (Steppan *et al.*, 2001).
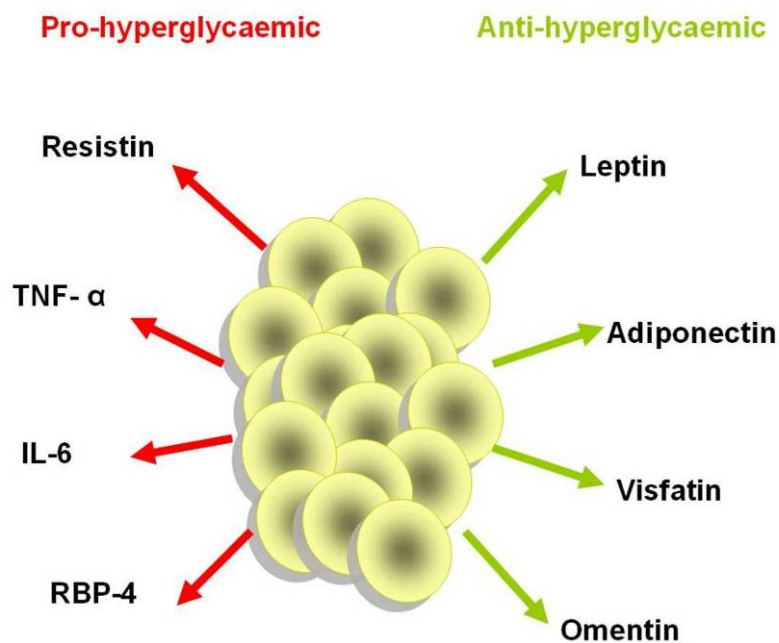
Figure 2.4 Adipocytes secrete proteins with varied effects on glucose homeostasis. Adipocyte-derived proteins with anti-diabetic action (green arrows) include leptin, adiponectin, omentin and visfatin. Other factors tend to raise blood glucose (Lincz *et al.*, 2001), including resistin, TNF-α and RBP4. TNF-α and human resistin are probably secreted by non-adipocytes within the fat pad. IL, interleukin. (adapted from (Rosen & Spiegelman, 2006)

2.6.1 TNF-α Structure and Function

TNF-α was initially depicted as an endotoxin-induced serum factor that promoted necrosis in tumours (Carswell *et al.*, 1975). It has more recently been perceived as an important regulatory cytokine of inflammatory processes, cell survival and apoptosis, production of secondary cytokines, such as IL-1 and IL-6, and induction of insulin resistance in a variety of clinical settings. TNF-α is synthesized as a monomeric 26 kDa molecule which is bound to the plasma

membrane (Kriegler *et al.*, 1988). Proteolytic cleavage by the TNF-α converting enzyme (TACE) of the membrane-bound precursor protein leads to the release of the TNF-α molecule into the circulation as a biologically active 17 kDa protein (Collins *et al.*, 2005), where it multimerizes to form a 51 kDa homotrimer (Kriegler *et al.*, 1988), (Beutler, 1995), (Xu *et al.*, 1999), (Clarke & Mohamed-Ali, 2006). (Maskos *et al.*, 1998) (Black *et al.*, 1997). Even before the molecule was specifically named, TNF-α had long been associated with insulin resistance (Pekala *et al.*, 1983). Many studies have demonstrated both an epidemiological and mechanistic link between adipose tissue-TNF-α secretion, obesity and insulin resistance in human and animal models. (Hotamisligil *et al.*, 1995), (Hotamisligil *et al.*, 1994a).

2.6.2 The role of TNF-α in Insulin Resistance

A negative role for TNF-α in insulin resistance is evidenced by the fact that knockout of TNF-α functionality leads to an improvement in insulin sensitivity and maintenance of glucose homeostasis in obese mice (Uysal *et al.*, 1997). In addition, TNF-α infusion in healthy humans leads to skeletal muscle insulin resistance, impaired insulin signalling and decreased glucose uptake (Plomgaard *et al.*, 2005). The biological activity of TNF-α is mediated by two specific receptors, TNF receptor-1 (Chen & Goeddel, 2002) and TNF receptor-2 (TNF-R2) that are present on the membranes of almost all cell types. Both TNF-α and its membrane receptors are significantly elevated in obesity and insulin resistance (Hofmann *et al.*, 1994). There is substantial data demonstrating its capacity to negatively regulate components of the insulin signalling pathway (Clarke & Mohamed-Ali, 2006). TNF-α reduces adipocyte mRNA expression and secretion of GLUT-4 (Stephens & Pekala, 1991), (Ohsumi *et al.*, 1994), (Hauner *et al.*, 1995) and reduces insulin-mediated glucose uptake by the adipocyte. Feinstein et al. (1993) demonstrated one mechanism by which TNF-α reduces insulin sensitivity. Incubating insulin-sensitive rat hepatoma cells with TNF-α for 1 hour led to a 65% decrease in insulin-induced tyrosine phosphorylation of the insulin receptor beta-subunit and IRS-1, and an upregulation in serine phosphorylation, thus preventing the tyrosine phosphorylation cascade (Feinstein *et al.*, 1993). *In vitro*, TZDs lead to a considerable reduction in TNF-α expression in adipocytes (Okuno *et al.*, 1998). In this model TZD administration also inhibits TNF-α-induced decreases in GLUT-4 expression, (Ohsumi *et al.*, 1994) and can augment IRS-1 tyrosine phosphorylation. (Peraldi *et al.*, 1997), (Iwata *et al.*, 2001), (Solomon *et al.*, 1997), (Shibasaki *et al.*, 2003). This

mechanism seems to be mediated by downstream actions on PPARγ (Clarke & Mohamed-Ali, 2006). TZDs have also been demonstrated to have positive effects on secondary mechanisms of TNF-α induced-insulin resistance, such as reduced levels of systemic free fatty acids and an increase in lipoprotein lipase expression (Shibasaki *et al.*, 2003), (Porat, 1989), (Kroder *et al.*, 1996). TNF-α has also been shown to inhibit insulin signalling and reduce insulin action in skeletal muscle (Lang *et al.*, 1992), (Nolte *et al.*, 1998), (del Aguila *et al.*, 1999), (Halse *et al.*, 2001), (Li & Reid, 2001). In a similar fashion TNF-α induces insulin resistance in hepatic tissues by reducing tyrosine phosphorylation (Solomon *et al.*, 2001), (Lang *et al.*, 1992). Hence the three most important tissues involved in insulin-stimulated glucose disposal are targets for TNF-α induced insulin resistance. In addition, TNF-α considerably augments the expression of IL-6, (Stephens *et al.*, 1992), (Fasshauer *et al.*, 2003) decreases the expression of adiponectin (Maeda *et al.*, 2001) and resistin (Fasshauer *et al.*, 2001), (Shojima *et al.*, 2002), and is correlated with elevated leptin concentrations (Bullo *et al.*, 2002). TNF-α can also reduce the sequestering of lipid in adipocytes because it upregulates premature adipocyte apoptosis, thus reducing space for excess lipid storage. This results in pre-existing adipocytes being burdened by the excess lipid and in turn diverting it to other tissues, such as skeletal muscle and the liver (Prins *et al.*, 1997), (Niesler *et al.*, 1998). TZDs also appear to ameliorate the TNF-α-induced adipocyte apoptosis. This leads to an increased number of smaller fat cells, without reducing the total mass of adipose tissue (Okuno *et al.*, 1998).

2.6.3 Downstream Signalling of TNF-α

TNF-α binding to the TNF-R1 on the cell surface results in the activation of two important transcription factors (c-Jun and NF-κβ) that consequently activate several genes implicated in the inflammatory pathway, the stress response and cell growth and development. TNF-α binding causes structural alterations to the receptor, which result in the recruitment of the adaptor protein; TNF receptor-associated death domain (TRADD) (Chen & Goeddel, 2002). TRADD then promotes the ligation of other adaptor proteins, such as receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (Kanazawa & Kudo, 2005), leading to the eventual activation of c-Jun NH2-terminal kinase (JNK) and NF-κβ pathways (Chen & Goeddel, 2002) (Figure 2.5).
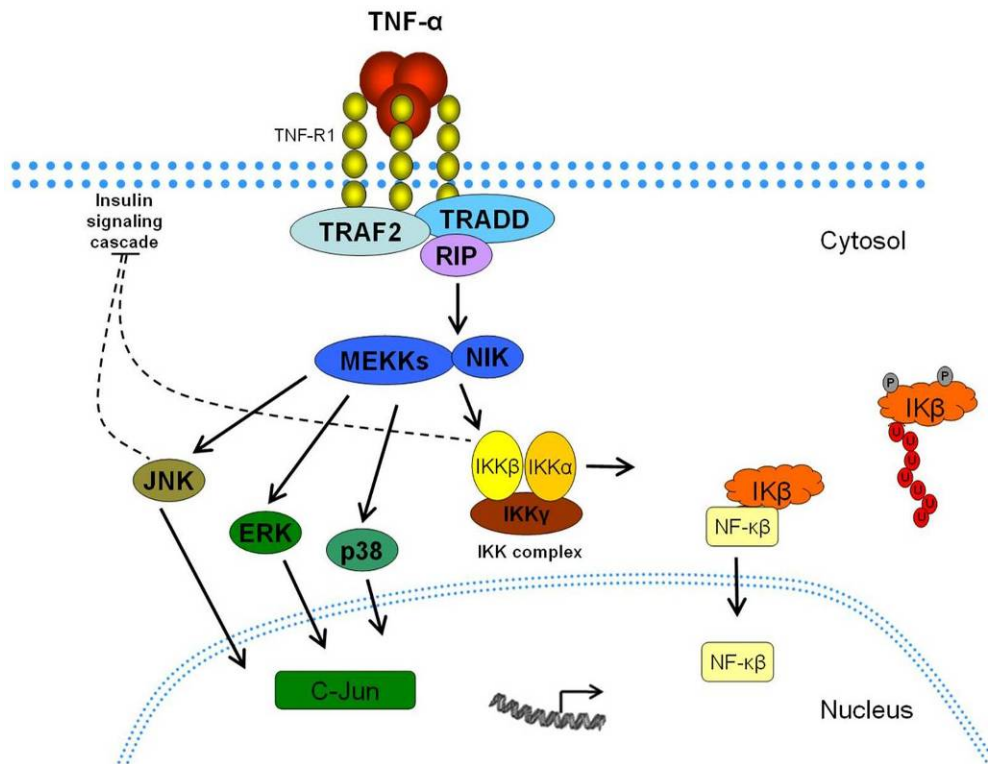
Figure 2.5 TNF-α signal transduction pathway. Initiation of TNF-α signaling leads to activation of two major pathways; JNK and NF-κβ. Both of which have been implicated in the negative regulation of insulin signalling Engagement of TNF with its cognate receptor TNF-R1 results in the formation of a receptor-proximal complex containing the important adaptor proteins TRADD, TRAF2, RIP. These adaptor proteins in turn recruit additional key pathway-specific enzymes. Activation of the IKK complex leads to phosphorylation of IKβ, which marks it for ubiquitination and proteasomal degradation. This sequence of events permits NF-κβ to enter the nucleus and regulate gene expression.

2.6.4 Adiponectin Structure and Function

Recent research indicates that the adipocytokine, adiponectin regulates insulin sensitivity and has a role in carbohydrate and lipid metabolism (Kralisch *et al.*, 2005), (Jazet *et al.*, 2003) as well as anti-atherogenic and anti-inflammatory processes (Havel, 2004), (Trujillo & Scherer, 2005). Adiponectin was discovered almost simultaneously by four different laboratories in 1995. It was first identified as a protein synthesised and secreted by cultured 3T3-L1 adipocytes (Scherer *et al.*, 1995) and was named adipocyte complement related protein 30kDa (ACRP30) because of it's sequence homology to complement C1q and structural homology to TNF-α (Shapiro & Scherer, 1998). It was also named adipoQ (Hu *et al.*, 1996), adipose most abundant gene transcript 1: APM1 (Maeda *et al.*, 1996) and gelatine-binding protein 28 kDa: GBP28 (Nakano *et al.*, 1996). It is 247 amino acids long and is secreted by adipocytes (Scherer *et al.*, 1995). Structurally adiponectin consists of 3 distinct domains; a globular domain at the C terminus, a signal sequence near the N terminus, and a collagenous domain. Three of these

collagen like domains bind together to form a trimer and four to six of these trimers bind together to form a multimer. In circulation adiponectin exists in three forms (i) as a hexamer (two bound trimers) called low molecular weight (LMW), (ii) as an oligomer of high molecular weight (HMW, and (iii) in the globular form. Although all of these are present in plasma, HMW adiponectin is considered to have the most potent biological activity (Pajvani *et al.*, 2003), (Tsao *et al.*, 2003). Adiponectin is the most abundant protein secreted by the adipose tissue. Plasma concentrations range from 5 – 30 µg/ml in humans. Unusually by comparison to most other adipocytokines, adiponectin decreases concurrently with obesity and increases in response to weight loss (Reinehr *et al.*, 2004), (Brichard *et al.*, 2003).

2.6.5 Adiponectin in Obesity, Type 2 Diabetes and Cardiovascular Disease

Plasma concentrations of adiponectin are negatively correlated with body mass index (Hu *et al.*, 1996), (Kern *et al.*, 2003), (Matsubara *et al.*, 2003), (Milan *et al.*, 2002), (Yang *et al.*, 2002). Concentrations are also lower in type 2 diabetes mellitus patients than in age and BMI matched controls (Hotta *et al.*, 2000) and have been shown to correlate strongly with insulin sensitivity, implying that low levels of adiponectin are linked to insulin resistance (Stefan *et al.*, 2002). As adiponectin expression is specific to the adipocyte a changes in adipose tissue mass can alter serum adiponectin levels. Weight loss significantly increases the expression of adiponectin (Lazzer *et al.*, 2005) but weight gain has the opposite effect (Weyer *et al.*, 2001a). Plasma adiponectin levels are approximately 40% higher in women than in men. This is thought to be a result of androgenic suppression (Combs *et al.*, 2003), (Nishizawa *et al.*, 2002). Additionally women have higher ratios of HMW adiponectin than men (Pajvani *et al.*, 2003). Adiponectin is reduced in cardiovascular disease (Ouchi *et al.*, 1999) and in type 2 diabetes mellitus (Hotta *et al.*, 2000). Evidence from longitudinal studies suggest that low levels of adiponectin are predictive of later development of type 2 diabetes (Spranger *et al.*, 2003), (Lindsay *et al.*, 2002) and myocardial infarction (Pischon *et al.*, 2004)

2.6.6 Adiponectin Signalling

Adiponectin signals through two recently cloned receptors, AdipoR1 and AdipoR2 which are predominantly expressed in skeletal muscle and hepatic tissue respectively (Yamauchi *et al.*, 2003) but have also been found in the brain, macrophages and atherosclerotic lesions. AdipoR1

has a high affinity for the globular form of adiponectin, which acts on skeletal muscles to increase glucose uptake and oxidation as well as lipid oxidation (Lihn *et al.*, 2005), (Lara-Castro *et al.*, 2006). The activation of these receptors leads to the phosphorylation and activation of AMP-activated protein kinases (AMPK) and peroxisome proliferator-activated receptor-gamma (PPARγ) (Yamauchi *et al.*, 2003). This results in an upregulation of proteins involved in fatty acid transport and oxidation such as CD36, acetyl-coenzyme A oxidase, uncoupling protein-2, and PPARα, leading to an increase in β-oxidation (Kadowaki & Yamauchi, 2005). Consequently there is a reduced concentration of intramuscular triglycerides and a decrease in plasma free fatty acids and FA influx in the liver.

Elevated intramuscular triglyceride concentrations or decreased IMTG turnover impede insulin signalling and GLUT-4 translocation by increasing serine phosphorylation of the insulin receptor and IRS-1. Therefore reductions in fatty acid concentrations improvement insulin signalling and insulin sensitivity (Jequier, 1998), (Frayn, 2003). In the liver, AdipoR2 has a greater affinity for full-length adiponectin. Adiponectin activated AMPK reduces the expression of enzymes involved in gluconeogenesis such as glucose-6-phosphase. This in turn reduces hepatic glucose production and output, which contributes to whole body glucose homeostasis and thus enhances insulin sensitivity (Kralisch *et al.*, 2005), (Jazet *et al.*, 2003), (Havel, 2004), (Yamauchi *et al.*, 2003) (Figure 2.6).
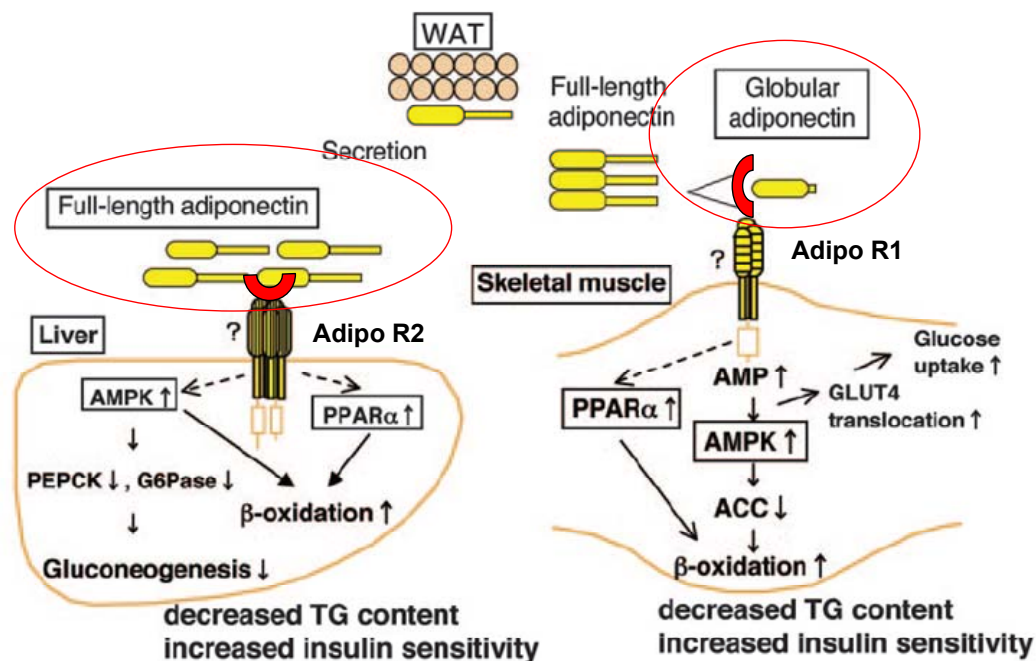
Figure 2.6 Adiponectin can activate AMPK and PPARα in the liver and skeletal muscle. In skeletal muscle, both globular and full-length adiponectin activate AMPK, thereby stimulating phosphorylation of ACC, fatty-acid oxidation, and glucose uptake. Adiponectin activates PPARα, thereby also stimulating fatty-acid oxidation and decreasing tissue TG content in muscle. In the liver, only full-length adiponectin activates AMPK, thereby reducing molecules involved in gluconeogenesis and increasing phosphorylation of ACC and fatty-acid oxidation. Adiponectin activates PPARα, thereby stimulating fatty-acid oxidation and decreasing tissue TG content in the liver (Kadowaki & Yamauchi, 2005).

Emerging evidence suggests that weight loss not only increases total adiponectin but possibly, also influences the ratio of the different adiponectin isoforms. A recent study has reported that obese subjects who participated 6 month dietary restriction weight loss programme had significantly greater HMW adiponectin (0.37 ± 0.07 *vs.* 0.49 ± 0.08 µ.ml^{-1}), MMW (2.3 ± 0.2 *vs.* 2.9 ± 0.3 µ.ml^{-1}) but not LMW adiponectin (Bobbert *et al.*, 2005). Several other studies have demonstrated a similar increase in HMW adiponectin after biliopancreatic bypass in obese subjects (Salani *et al.*, 2006) and after hypocaloric diet-induced weight loss in obese and overweight postmenopausal women (Polak *et al.*, 2007). The expression of HMW isoforms is also increased in murine adipocytes following treatment with the PPARγ agonist, pioglitazone (Bodles *et al.*, 2006). Indeed many of the inverse relationships identified between total adiponectin and measures of insulin resistance (Hara *et al.*, 2006), (Katsuki *et al.*, 2006) obesity (Araki *et al.*, 2006) and other markers of metabolic dysregulation (Aso *et al.*, 2006) are stronger with the HMW isoform. Studies have consistently demonstrated an inverse relationship between adiponectin and insulin resistance (Cnop *et al.*, 2003), (Matsubara *et al.*, 2003). Hotta *et al.*, (2001) generated a genetically modified model of type 2 diabetes in rhesus monkeys, and found that plasma adiponectin decreased with the onset and development of insulin resistance. Lihn

et al., (2003) found that first degree relatives of subjects with type 2 diabetes were characterised by reduced adiponectin mRNA expression in the adipose tissue (Lihn *et al.*, 2003a).

Interestingly, insulin resistance is a feature of obesity but also lipodystrophy, a condition where adipose tissue is partially or totally depleted (Carr *et al.*, 1998). Both lipodystrophy and HIV-associated dystrophy syndrome is correlated with reduced plasma adiponectin and mRNA expression in adipose tissue (Lihn *et al.*, 2003b), (Tong *et al.*, 2003) This suggests that reduced adiponectin may play an important role in the pathogenesis of insulin resistance in lipodystrophy. Yamauchi *et al.*, (2001) administered recombinant adiponectin to lipodystrophic, insulin resistant mice with no detectable plasma adiponectin. Adiponectin reduced blood glucose and insulin concentrations by suppressing hepatic glucose production and increased the expression of genes involved in lipid transport and fatty acid oxidation. The content of muscle and liver triglyceride was reduced and insulin resistance was almost completely reversed. These results suggest that insulin resistance in lipodystrophy may result from a lack of adiponectin production (Yamauchi *et al.*, 2001).

2.6.7 Adiponectin and Atherosclerosis

The antiatherogenic properties of adiponectin have also been shown in animal models. Adiponectin knockout mice develop more severe intimal thickening in response to endothelial injury than wild-type mice (Okamoto *et al.*, 2002). Additionally, increased expression of adiponectin by adenovirus transfection reduces the formation of atherosclerotic plaques in apolipoprotein E knock out mice (Matsuda *et al.*, 2002). As adiponectin circulates in large quantities, it comes in contact with the vascular endothelium all over the body. Immunohistochemical staining of vascular cells with antibodies to adiponectin show no adiponectin protein in normal rabbits. However immunohistochemical staining with adiponectin antibodies revealed high levels of adiponectin in balloon injured vascular walls, indicating that adiponectin may play a restorative role in endothelial vascular injury (Okamoto *et al.*, 2002).

Investigations of its cellular actions have revealed that adiponectin exerts many important antiatherogenic effects. When the vascular endothelium is injured by aggravating factors such

as oxidised low density lipoprotein (LDL), chemical substances and mechanical stress, adiponectin accumulates in the subendothelial intima by binding to collagen in the extracellular matrix. Adiponectin suppresses monocyte binding by downregulating the production of endothelial adhesion molecules such as vascular cell adhesion molecule 1 (VCAM1), intracellular cell adhesion molecule 1 (ICAM1) and E-selectin through inhibition of Nuclear Factor κβ (Ouchi *et al.*, 2000). It appears that adiponectin also attenuates the proliferation of smooth muscle cells into the intimal space (an important step in the development of atherosclerotic plaques) by inhibiting mitogen-activated protein kinase (Arita *et al.*, 2002). Furthermore, adiponectin appears to directly stimulate nitric oxide in endothelial cells (Shimada *et al.*, 2004) which is a vasodilator and facilitates normal endothelial function.

2.6.8 Adiponectin and Exercise

Exercise mediated weight loss significantly increases serum adiponectin, which is also accompanied by increases in insulin sensitivity (Yatagai *et al.*, 2003). However it is not known if increased adiponectin is a cause or consequence of exercise-mediated insulin sensitivity. Yatagai *et al.*, (2003) studied twelve non-obese sedentary men before and after a 6-week training programme that involved stationary cycling at lactate threshold for 60 minutes per day, 5 days per week. Following the training programme VO_{2max} and lactate threshold increased, BMI and body fat mass remained unchanged, fasting glucose and insulin decreased indicating increased insulin sensitivity, but adiponectin concentrations either remained the same or showed a slight decrease. These results suggest that increased insulin sensitivity following exercise training is not due to increased serum adiponectin and also implies that adiponectin concentrations may only increase following exercise training that induces weight loss (Yatagai *et al.*, 2003), (Hulver *et al.*, 2002).

These results seem to contradict other studies examining the effect of exercise training on adiponectin levels. Fatouros *et al.*, (2005) examined the effect of a 6-month resistance training programme on adiponectin in overweight inactive elderly adults. The subjects were randomly assigned to a control group, a low intensity group (45-50% 1RM), a moderate intensity group (60-65% 1 RM), or a high intensity group (80-85% 1 RM) group where they completed 3 sets of 10 exercises, 3 days per week for 6 months. Strength, maximal oxygen consumption, and

insulin sensitivity increased following the intervention and this was accompanied by a decrease in BMI. Circulating adiponectin increased in the moderate and high intensity groups but not in the low intensity group, although this was associated with increased insulin sensitivity and decreased BMI (Fatouros *et al.*, 2005). Monzillo *et al.* (2003) showed weight loss induced by exercise and caloric restriction increased adiponectin levels similarly in diabetic and non-diabetic groups (Monzillo *et al.*, 2003).

Hara *et al.*, (2005) investigated the effect of exercise training on young obese men who were divided into either an aerobic training group, an aerobic and resistance training group, or a control group. The aerobic exercise group underwent 8 weeks of training, 3 times per week, for more than 30 minutes at ventilatory threshold. The resistance and endurance exercise group underwent the same aerobic training in addition to 2 or 3 resistance training sessions consisting of 3 sets of 10 repetitions of 14 exercises at 80% of 1 RM. Adiponectin levels did increase but this increase was due to decreased body composition associated with exercise training (Hara *et al.*, 2005). Current evidence suggests that increases in adiponectin, which occur with exercise training, are related to changes in adiposity rather than the exercise training itself. This is supported by the fact that serum adiponectin remained unchanged following a single bout of aerobic exercise despite increased insulin sensitivity that occurred immediately after exercise (Jamurtas *et al.*, 2006).

2.7 Osteoprotegerin, RANKL and TRAIL

2.7.1 Structure and Function of OPG

OPG is a soluble glycoprotein and member of the TNF-receptor superfamily that is characterized by its ability to bind to RANKL and TRAIL (Corallini *et al.*, 2008), (Emery *et al.*, 1998). It exists as a 60-kd monomeric structure or as a disulfide linked 120-kd homodimer and is encoded on chromosome 8q (Yun *et al.*, 1998). In contrast to other members of the TNF receptor superfamily, OPG does not have specific transmembrane or cytoplasmic domains. It is instead secreted into the circulation as a soluble receptor (Yun *et al.*, 1998), (Corallini *et al.*, 2008). OPG consists of 401 amino acids, however the cleaving of a 21-amino acid signal peptide leads to the formation of a mature 380 amino acid form (Simonet *et al.*, 1997). OPG

also distinguishes itself from other members of the TNF-receptor superfamily because it maintains biological activity in its soluble, circulating form. It was identified in 1997-1998 simultaneously by two separate groups (Simonet *et al.*, 1997), (Tsuda *et al.*, 1997) and has had a number of synonyms including osteoclastogenesis inhibitory factor (OCIF), TNF receptor like molecule 1 (Kwon *et al.*, 1998), and follicular dendritic cell-associated receptor 1 (FDCR-1). However, OPG is now the accepted term for this glycoprotein. At the time of discovery both groups demonstrated an important role for OPG in the regulation of bone turnover as a result of its direct inhibition of osteoclastogenesis (Simonet *et al.*, 1997), (Tsuda *et al.*, 1997), (Reid & Holen, 2009). It consists of 4 amino-terminal cysteine rich domains that are structurally similar to the extracellular portions of other associates in the TNF receptor superfamily. The carboxy-terminal incorporates portions 5 and 6, that are death domain homologous regions (Baker & Reddy, 1998) (Figure 2.7)
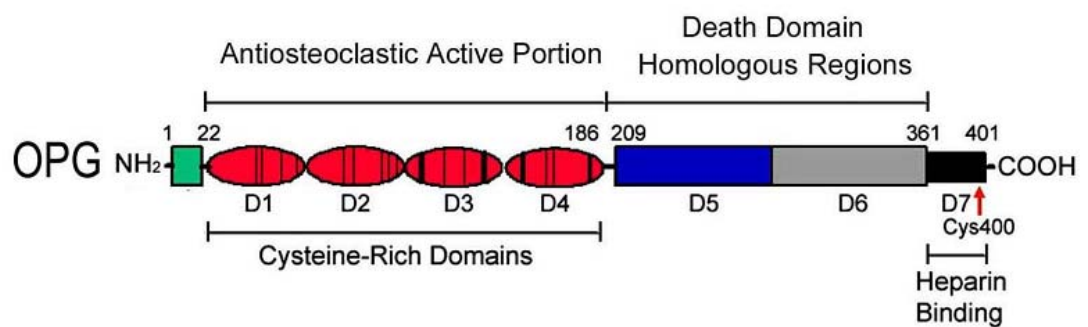
Figure 2.7 Schematic representation of the structure of OPG. Main domains and their biochemical and/or functional properties are indicated. NH2 indicates amino-terminus; COOH, carboxy-terminus (reproduced from (Corallini *et al.*, 2008).

2.7.2 Role of the OPG/RANK/RANKL axis in Bone Turnover: Evidence from mouse studies

A physiological role for OPG in regulating bone formation and resorption was initially demonstrated when OPG deficient mice, produced by targeted disruption of the gene were viable and fertile but developed profound bone loss, marked destruction of growth plates and reduced trabecular femur bone mass (Bucay *et al.*, 1998). In this study the authors further noted that the elevated mortality of these adolescent mice was related to an increased occurrence of vertebral or endochondral fractures. Interestingly the offspring of surviving, female double knockout mice gave birth to histologicaly normal double knockout offspring, suggesting that OPG is not essential for normal foetal development. Besides the effect on bone quality and elevated alkaline phosphatase, mice who survived to 6 months appeared to have no untypical

haematological or biochemical characteristics. In a similar study Mizuno et al., (1998) also created an OPG homozygous mouse: they found no histopathological abnormalities in the femurs of these mice at 5 weeks. However there was a marked increase in osteoclast size, number and proliferation, coupled with a progressive loss of trabecular femoral bone found between 8 and 13 weeks, suggesting that the early osteoporotic phenotype observed in these adolescent mice is likely due to an increase in osteoclastogenesis. (Mizuno *et al.*, 1998). A putative role for OPG in this process was first elucidated in a classical study by Simonet et al. (1997) who created OPG-overexpressing mice. At 10 weeks, other than an enlarged spleen (~38%), these mice were phenotypically no different from their normal littermates; however they showed signs of profound osteopetrosis characterized by significant radio-opacity of the long bones, vertebrae, and pelvis when compared to their ordinary littermates. Mice that highly express the OPG transgene displayed obvious signs of osteopetrosis by x-ray at birth, the severity of which increased significantly into adolescence and adulthood. Despite this increase in radio-density, there was no irregularity in tooth eruption, a symptom commonly observed in ostepetrotic mice (Yoshida *et al.*, 1990), (Soriano *et al.*, 1991). In order to investigate the effect of OPG on healthy mice, Simonet et al. (1997) administered recombinant OPG to 4 week old wild-type mice and found that after 7 days they had a 3 fold increase (31.1% versus 12.0%) in trabecular bone of the proximal tibial metaphysis when compared to controls (Simonet *et al.*, 1997). The authors further clarified a role for OPG in the regulation of bone formation demonstrating that the administration of recombinant OPG blocks differentiation of precursor cells into osteoclasts in a dose dependant manner *in vitro*. Additionally the authors underlined a possible clinical application of recombinant OPG by illustrating the potential for OPG therapy to ameliorate the bone loss that one would expect in ovariectomized rats, where bone volume in the proximal tibial metaphysis was increased in OPG treated rats relative to controls (Simonet *et al.*, 1997).

2.7.3 The RANK/OPG/RANKL Axis

The mechanism of action for OPG has been well described. Osteoblasts and their precursor cells, stromal cells express the homotrimeric, transmembrane protein; RANKL, particularly in regions where there is active bone remodelling or inflammatory osteolysis (Hofbauer & Schoppet, 2004). RANKL is a 316 amino acids sequence that is abundantly expressed in

osteoblastic / stromal cells, and T cells in lymph tissue (Figure 2.8). RANKL appears in circulation after being secreted by T cells or following proteolytic cleavage from cell surfaces (Hofbauer & Heufelder, 2001), (Walsh & Choi, 2003), (Schoppet *et al.*, 2002). RANKL stimulates RANK, a transmembrane receptor, consisting of 616 amino acids that is found on the surface of cells with a monocyte/macrophage lineage, such as dendritic cells and osteoclasts and their precursors (Dougall *et al.*, 1999). RANKL binds to RANK on osteoclast precursors and more mature osteoclasts, upregulating intracellular pathways that increase proliferation and survival of osteoclasts leading to activation of osteoclastogenic processes, increased bone resorption and bone loss. Generally an increase in RANKL is associated with a decrease in OPG, such that the ratio of RANKL to OPG changes in favour of osteoclastogenesis. Many papers have given credence to the claim that the RANKL to OPG ratio is an important determinant of bone density (Hofbauer & Schoppet, 2004). Both stromal cells and osteoblasts secrete OPG as a homodimer, which acts as a decoy receptor, binding to RANKL, thus blocking the resultant inhibition of osteoclastogenesis and bone loss (Corallini *et al.*, 2008). *In vitro* investigations have demonstrated the importance of OPG dimerisation for this process. Homodimeric OPG binds strongly (K_D 10nM) with homotrimeric RANKL to form stable dimer-trimer compounds.
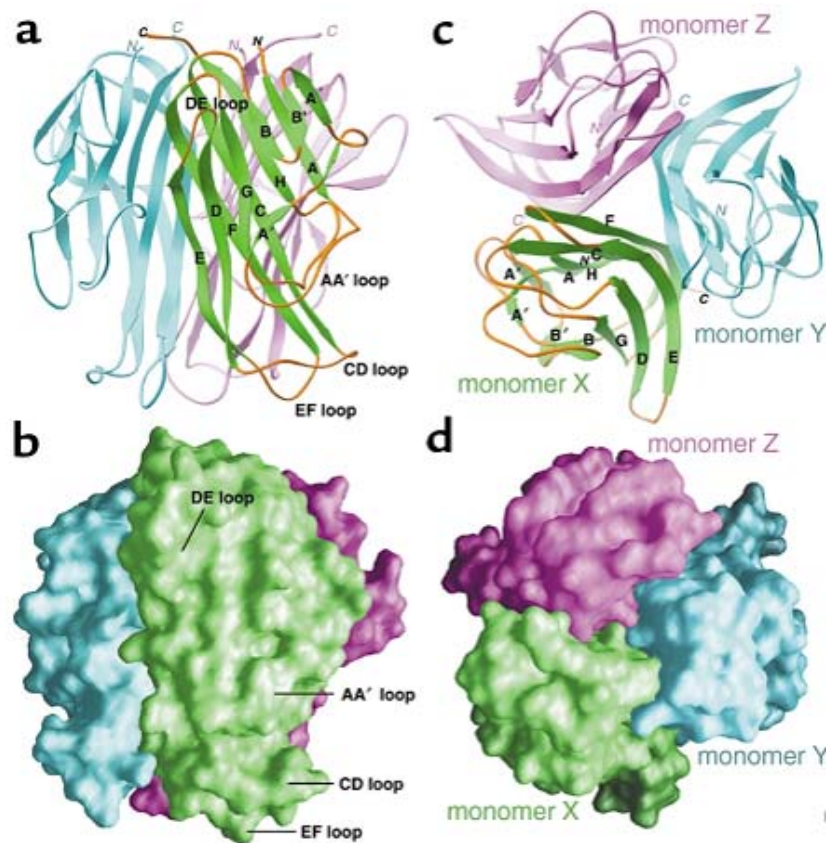
Figure 2.8 Crystal structure of RANKL. (a) Ribbon diagram of the RANKL trimer, shown with the β-strands (green) and connecting loops (orange) of one RANKL monomer. The other two RANKL monomers are cyan and magenta, respectively. (b) In this view, oriented identically to a, the RANKL transmembrane stalk projects to the top of the image, while the membrane-distal region is toward the bottom. The homotrimer exhibits the shape of a truncated pyramid, being slightly wider at the membrane proximal end. (c) Ribbon diagram of the RANKL trimer viewed down the axis of threefold symmetry, oriented with the membrane-distal face forward. The secondary structure of monomer X is labelled as in a. (d) The RANKL trimer, shown with the molecular surfaces of monomers X, Y, and Z colored in green, cyan, and magenta, respectively. The orientation of the molecule is identical to that in c. from (Lam *et al.*, 2001)

Schneeweis *et al.*, (2005) showed, using sedimentation velocity analysis that 1:2 OPG-RANKL complexes were not formed in mixtures containing a 2-fold molar excess of RANKL over OPG, implying that both of the OPG monomers in the homodimer cannot bind to a separate RANKL trimer simultaneously. However, 2:1 OPG-RANKL complexes did emerge when OPG was present at a 2-fold molar excess over RANKL. Moreover, the authors found that the second OPG dimer displayed a significant loss of affinity (K_D – 3µM). The authors concluded that the most likely explanation based on these findings was that the high affinity OPG-RANKL binding is dependant on avidity. Two of the OPG monomers in each dimer bind to two out of three of the RANKL monomers in each trimeric structure. Only one monomer in the second OPG molecule is able to weakly interact with the third and only available RANKL monomer (Schneeweis *et al.*, 2005).

2.7.4 RANKL / RANK Molecular Pathway Inducing Osteoclastogenesis

The regulatory role of RANKL in bone resorption and formation has also been shown *in vivo*, Baud'huin *et al.*, (2007) demonstrated that administration of RANKL to adult mice induces bone resorption, whilst mice deficient in functional RANKL develop osteoporosis (Baud'huin *et al.*, 2007). A crucial mechanism in promoting the resorptive action of osteoclasts is the binding of RANK to RANKL. As an affiliate of the TNF receptor superfamily, RANK does not have any kinase activity, therefore it is necessary for RANK to enlist the help of associated factors to transduce the signals after binding to its ligand (Leibbrandt & Penninger, 2008). Binding of RANK to its ligand leads to the translocation of TNF receptor-associated factors (TRAFs) to the intracellular surface of RANK. RANK has been shown to associate with TRAFs 1 – 6 during *in vitro* experiments (Darnay *et al.*, 1998), (Galibert *et al.*, 1998), (Wong *et al.*, 1998), (Leibbrandt & Penninger, 2008). The cytoplasmic domain of RANK has several TRAF binding sites that cluster in specific regions. The areas enclosed by the amino acids 235–358 and 359–531 bind the TRAF6 adaptor molecule and the 532–625 region contains several binding locations for TRAFs 2, 5, and 6 (Darnay *et al.*, 1998), (Wong *et al.*, 1998), (Wong *et al.*, 1999). However, only TRAF6 interacts with the membrane-proximal region of the RANK cytoplasmic domain which is distinct from other TRAFs. The functional significance of these TRAF binding domains is to initiate RANK-induced NF-κβ and c-Jun NH2-terminal kinase (JNK) activation. Deletion of the TRAF6 binding site of RANK almost completely blocked the RANK-dependent activation of NF-κβ (Galibert *et al.*, 1998). However, JNK activation was intact and demonstrating that interactions with TRAF6 are essential for NF-κβ but not JNK pathway activation (Darnay *et al.*, 1998), (Wong *et al.*, 1998), (Galibert *et al.*, 1998), (Lee *et al.*, 2000). Armstrong et al. (2009) used genetically modified gene constructs of RANK that selectively inhibited TRAF protein binding, to show that TRAF6 was the only functional TRAF protein downstream of RANK affecting osteoclast differentiation. The interaction of RANK with TRAF6 however was extremely important for the formation of cytoskeletal structures and the resorptive activity of osteoclasts (Armstrong *et al.*, 2002). Lomaga *et al.*, (1999) found that viable TRAF6 double knockout mice appeared phenotypically normal at birth but did not mature and died soon after birth. The TRAF6−/− animals that lived longer than 14 days had a 20 - 30% reduction in body mass and length. In addition, they had modest enlargement of the heart and liver which was accompanied by significant splenomegaly, represented by an increase in organ size of 2 – 6 fold compared to wild type littermates. X-ray examination of these mice showed that their long bones and

vertebral bodies were radio-opaque. The long bones, especially the femur, were reduced in length and exhibited a distinct broadening at the ends attributable to a failure in bone modelling, indicative of osteopetrosis. Molars and incisors of the double knockout animals had failed to erupt which is, again, common in osteopetrotic mice (Popoff & Marks, Jr., 1995), as bone resorption allows for the opening of a channel through the jawbone for teeth to grow. Peripheral quantitative computed tomography analysis of the proximal tibial bone metaphyisis showed a significant increase in bone mass in double knockout compared to the transgenic mice (Lomaga *et al.*, 1999). These findings were strengthened by Naito et al. (1999) who also found that in addition to premature mortality and runting, TRAF6 double knockout mice had limited bone marrow cavities consisting of mostly spongy bone. Further histological analysis highlighted abnormal bone formation and thickened epiphyseal growth plates. Like Lomaga *et al.*, (1998), the authors attributed this profound osteopetrosis to a failure of osteoclast precursors to differentiate into mature osteoclasts in response to RANKL. (Lomaga *et al.*, 1999), (Naito *et al.*, 1999).

The contributions of TRAF2 and TRAF5 to osteoclastogenesis seem to be relatively small. TRAF2−/− liver derived progenitor cells have only marginally reduced multinuclear osteoclasts accumulation and the activation of NF-κβ and JNK by RANKL was comparable to normal controls. Similarly, TRAF5 deficient cells only had a mild defect in osteoclastogenesis, and NF-κβ and JNK activation was not affected by RANK stimulation. (Kanazawa & Kudo, 2005), (Kanazawa *et al.*, 2003). There are at least seven distinct pathways activated by RANK-induced protein kinase signalling; four of them directly induce osteoclastogenesis; inhibitor of NF-κβ kinase/NF-κβ, c-Jun amino-terminal kinase/activator protein-1, c-myc, calcineurin/nuclear factor of activated T cells (NFATc1). There are three others that directly mediate osteoclast activation (src and MKK6/p38/ MITF) and survival (src and extracellular signal-regulated kinase) (Boyce & Xing, 2007). These studies indicate that TRAF6 is the most important adaptor molecule linking RANK signalling to the NF−κβ osteoclastogenesis pathway and that other TRAFs may circumvent and compensate for TRAF6-deficiency (Leibbrandt & Penninger, 2008). In addition to TRAFs, there are other adapter molecules that bind to RANK to induce signalling in this pathway. This in turn results in the activation of the transcription factor NF-κβ. (Matsumoto *et al.*, 2000), (Xing *et al.*, 2002). Growth factor receptor-bound protein 2 (Grb-2) associated binder

2 (Wada *et al.*, 2005) is one of a family of adapter proteins phosphorylated at tyrosine residues that leads to the recruitment of a variety of signalling molecules with steroid receptor coactivator 2 (Src 2) homology domains. Loss of Gab2 results in reduced RANKL/RANK-induced osteoclast differentiation, decreased bone resorption, and mild osteopetrosis (Boyce & Xing, 2007), (Wada *et al.*, 2005), suggesting that it is an important player in RANKL-induced osteoclastogenesis (Wada *et al.*, 2005).

The vital role of NF-κβ/activator protein-1/ (NFATc1) signalling for osteoclast formation was revealed after genetic disruption of the p50 and p52 subunits of NF-κβ and of the immediate early gene transcript, c-Fos (Karsenty & Wagner, 2002). A subsequent study that transferred NFATc1-/- stem cells to cFos-/- mice resulted in osteoclast formation (Takayanagi *et al.*, 2002). Over expression of a constitutively active form of NFATc1 induces osteoclast formation by Macrophage-Colonly Stimulating Factor (M-CSF) treated Fos-/- or NF-κβ p50/p52-/- osteoclast precursors in the absence of RANKL (Yao *et al.*, 2005) indicating that it is downstream from NF-κβ and c-Fos (Figure 2.9). On the basis of all of these studies, NFATc1 has been described as a master regulator of osteoclastogenesis (Boyce & Xing, 2007), (Takayanagi *et al.*, 2002).
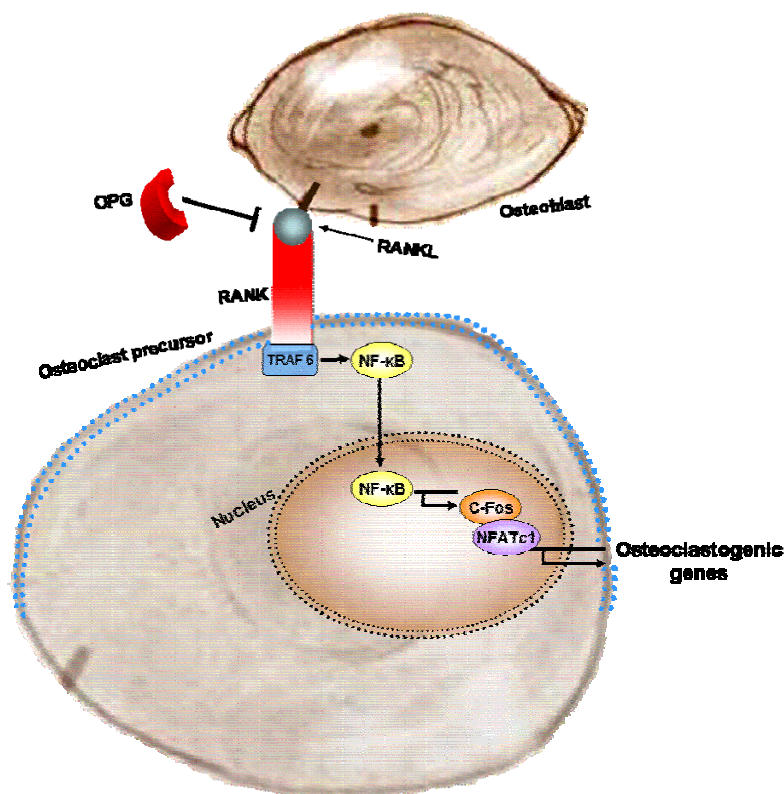
Figure 2.9 The essential signaling pathway for normal osteoclastogenesis. Under physiologic conditions, RANKL produced by osteoblasts binds to RANK on the surface of osteoclast precursors and recruits the adaptor protein TRAF6, leading to NF-κβ activation and translocation to the nucleus. NF-κβ increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes. OPG inhibits the initiation of the process by binding to RANKL. NFAT, nuclear factor of activated T cells; NF-κβ, nuclear factor-κB; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TRAF, tumor necrosis factor receptor associated factor. (adapted from (Boyce & Xing, 2007) and (Hofbauer & Schoppet, 2004)

2.7.5 Disorders of RANKL/OPG/RANK Signalling

Disequilibrium in the RANKL/OPG fraction or signalling contributes to the clinical pathology of many disorders of the skeleton, where increased bone resorption/formation, or inappropriate bone remodelling are a factor (Hofbauer & Schoppet, 2004). This is supported by Whyte el al. (2002) who demonstrated a loss in osteoprotective function for homozygous deletions of 100 kb of OPG in patients with the autosomal-recessive disorder; Juvenile Paget's Disease a condition in which increased resorption, severe osteopaenia, and persistent fractures are primary symptoms (Whyte *et al.*, 2002). It is further supported by the identification of an inactivating deletion in exon 3 of OPG in which idiopathic hyperphosphatasia, which is an autosomal-recessive disease typified by increased bone resorption, deformities of long bones, kyphosis, and acetabular protrusion (Cundy *et al.*, 2002), (Daroszewska *et al.*, 2004), (Boyce & Xing, 2007). The central role of defective OPG signalling and secretion in Juvenile Paget's Disease

was verified by Cundy *et al.*, (2005) where the weekly subcutaneous administration of recombinant OPG to two adult siblings with Juvenile Paget's Disease led to a decrease in the speed of bone resorption, a decrease in skeletal bisphosphonate retention by 37 and 55 % respectively and improved radio-density upon examination by x-ray (Cundy *et al.*, 2005).

In addition, *in vivo* models such as the T-cell-dependent model of rat adjuvant arthritis (Kong *et al.*, 1999) and collagen induced arthritis (Schett *et al.*, 2003) are both characterized by severe joint inflammation, bone and cartilage destruction and crippling. Blocking RANKL by osteoprotegerin treatment at the onset of disease prevents bone and cartilage destruction but interestingly not inflammation (Kong *et al.*, 1999). In addition, blockade of this pathway has been shown to prevent bone and tooth loss in an animal model of periodontal disease, without having any significant effect on the immune process (Teng *et al.*, 2000). More recent animal models have used combination therapy to block RANKL with the administration of OPG in conjunction with the blockade of various inflammatory agents, including; IL-1 and TNF-α and found that with the use of these two treatments in tandem significantly reduces bone loss and systemic inflammation (Zwerina *et al.*, 2004).

There have been several studies in postmenopausal women that have attempted to investigate the relationship between circulating OPG and Bone Mineral Density (BMD). However the findings from these studies are conflicting. Circulating OPG has been shown to increase (Rogers *et al.*, 2002) or decrease with osteoporosis and be negatively correlated to BMD. Mezquita *et al.*, (2005) studied a cohort of 206 postmenopausal women and found that lower concentrations of circulating OPG were positively related to low BMD as well as prevalence of vertebral fracture (Mezquita-Raya *et al.*, 2005). However a study by Yano et al. (1999) comparing serum OPG in Japanese men and women found that serum OPG was significantly increased in postmenopausal women who were osteoporotic (Yano *et al.*, 1999). A possible reason for the differences between these studies could be the difference in experimental design and different populations utilized. (Reid & Holen, 2009)

In addition to the severe osteoporosis observed in OPG deficient mice (Mizuno *et al.*, 1998), OPG knockout mice appear to exhibit significant renal and aortic calcification (Bucay *et al.*, 1998). Furthermore, administration of recombinant OPG to rodents appears to prevent the

onset of arterial calcification induced by warfarin treatment or high doses of vitamin D (Price *et al.*, 2001). Arterial calcification usually complicates chronic atherosclerosis and it appears to be accelerated in these mice, suggesting that OPG may play an important role in protecting large blood vessels from medial calcification and other complications of atherosclerosis (Bucay *et al.*, 1998). The relationship between osteoporosis and vascular calcification in these animal models of OPG deficiency is somewhat reminiscent of the clinical setting where these conditions often occur congruently (Hofbauer *et al.*, 2007). Longitudinal analysis of bone loss and vascular calcification over a 25-year period in the Framingham Heart Study showed that women with the greatest magnitude of bone loss had the most severe progression of abdominal aortic calcification (Kiel *et al.*, 2001). Furthermore a cross sectional study in 2,348 postmenopausal women revealed that aortic calcification strongly predicts low bone mineral density and occurrence of fractures. A subgroup of 228 women within this cohort who were longitudinally observed showed that the percentage yearly increase in aortic calcification accounted for almost half of the variance in the percentage rate of bone loss. Additionally a strong graded association was observed between the progression of vascular calcification and bone loss for each quartile. Women in the highest aortic calcification-quartile had four times greater yearly bone loss than women in the lowest quartile (Schulz *et al.*, 2004).

2.7.6 OPG Expression and Function in the Vascular System

Evidently the RANKL/RANK/OPG triad is an important player in the homeostatic control of the immune and skeletal systems. Research in recent years has also begun to shed light on an equally intriguing role for this axis in the homeostasis of the vascular environment. Many of the same signals that modulate RANKL and OPG, both immunomodulatory and osteogenic in origin, may also regulate their expression in the vascular endothelium. As well as the typical activity of OPG in boney tissues, OPG expression and secretion is also found at high concentrations in the arterial wall, where the content in aortic extracts is reported to be 500 – 1000 times greater than those found in the circulation (Olesen *et al.*, 2005), (Knudsen *et al.*, 2003), a similar concentration to that found in bone. It has also been demonstrated that both micro/macro vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) secrete OPG (Collin-Osdoby *et al.*, 2001), (Secchiero *et al.*, 2006), (Zhang *et al.*, 2002). A number of potential growth factors and inflammatory cytokines which are thought to be key

players in the pathogenesis of atherosclerosis and coronary artery disease have also been implicated in the regulation of OPG in the vascular wall. VEC-expression of OPG can be induced by the addition of the inflammatory cytokines; TNF-α, IL-1α, IL-1β, activated integrin $\alpha_v\beta_3$ and additionally porphyromonas gingivalis, an initiating activator of periodontal disease. (Kobayashi-Sakamoto *et al.*, 2004), (Secchiero *et al.*, 2006), (Ben-Tal *et al.*, 2007). Collin-Osdoby *et al.* (2001) demonstrated that human microvascular ECs express mRNA transcripts for both RANKL and OPG. In addition they showed that RANKL and OPG mRNA are significantly and dose-dependently upregulated in response to TNF-α and IL-1 as measured by semi-quantitative real time PCR. Further analysis of the time course of OPG and RANKL mRNA expression revealed that the rise in RANKL expression was first observed at 10 hours after the addition of TNF-α and by 24 hours, had risen to a peak of 3–6-fold in comparison to untreated VECs. These levels of expression continued between 48 and 72 hours when continuously co-cultured with TNF-α. Removal of the cytokine after 24 hours led to a sustained decline in RANKL expression, however levels were still elevated by as much as 2-fold after 48 hours. OPG mRNA levels in VEC rose more swiftly in response to TNF-α. Elevated OPG mRNA levels were apparent within 1 hour, reached their highest level by 10 hours, but declined to approximately half their maximum values at 24 hours, and thereafter fell more slowly up to 72 hours. Despite this, OPG mRNA levels were ten times higher than the unconditioned VECs. OPG mRNA levels quickly returned to concentrations similar to that of untreated VECs after withdrawal of TNF-α treated media. (Collin-Osdoby *et al.*, 2001).

2.7.7 Expression of OPG in Vascular Endothelial Cells

Zannettino *et al.*, (2005) have identified the site of OPG endothelial intracellular localisation to compartments known as Weibel-Palade Bodies (WPBs). They also observed that OPG was physically associated with von Willebrand Factor both in WPBs and in serum (Zannettino *et al.*, 2005). Following thrombogenic and inflammatory insult with cytokines such as TNF-α, and IL-1β, the contents of WPBs quickly translocate to the plasma membrane and extracellular space, where they promote migration of leukocytes and platelets to inflammatory sites and areas of thrombus formation (Arnaout, 1993), (Wagner, 1993), strongly suggesting a vasoactive role for OPG in maintaining haemostasis and possibly in the prevention of vascular injury and inflammation. In VECs, activation of integrin $\alpha_v\beta_3$ and porphyromonas gingivalis appear to

augment OPG expression levels via initiation of the NF-κβ transcription pathway (Kobayashi-Sakamoto *et al.*, 2004); (Malyankar *et al.*, 2000); TNF-α and IL-1α also activate signalling pathways that result in NF-κβ activation suggesting that activation of this transcription pathway may be an important step in modulating production of endothelial cell OPG (Baud & Karin, 2001), (Wesche *et al.*, 1997).

2.7.8 Expression of OPG in Vascular Smooth Muscle Cells

Within the general vasculature however, OPG is more highly expressed in VSMCs compared to ECs, with VSMCs secreting up to 20-30 times that of endothelial cells. (Zhang *et al.*, 2002). Interestingly the specific area of OPG activity in the arterial architecture seems to be important as higher concentrations have been found in the tunica media of diabetics relative to normoglycaemic controls, however no difference in OPG concentration was observed in the same cohort when intimal tissue was compared (Golledge *et al.*, 2004). In vascular smooth muscle cells, a number of cytokines have been shown to augment OPG expression, including TNF-α, IL-1β, insulin, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and angiotensin II (Collin-Osdoby *et al.*, 2001), (Olesen *et al.*, 2005), (Ben-Tal *et al.*, 2007), (Zhang *et al.*, 2002) (Figure 2.10).

Zhang et al. (2002) demonstrated that PDGF-induced OPG gene expression in VSMCs could be blocked by inhibition of the PI3-kinase/AKT and p38/MAPK signalling pathways but that inhibition of NF-κβ did not attenuate PDGF-mediated OPG increases in VSMCs (Zhang *et al.*, 2002). This contrast with the NF-κβ pathway that upregulates OPG in VECs and suggests that other pathways are important in OPG production in VSMCs. Olesen et al. (2005) also found that TNF-α increased the amount of OPG produced from the VSMCs (Olesen *et al.*, 2005) but OPG secretion was attenuated by the addition of insulin to the media. Recent work by this group demonstrated that the addition sRANKL to VSMC cultures led to a decrease in the activation of the insulin signalling pathway by reducing the activity of 16 of the 52 genes that were upregulated in by insulin. Interestingly knock down of OPG production by the addition of siRNA did not affect the insulin signalling pathway (Olesen *et al.,* 2009). OPG production in VSMCs has also been shown to be reduced by peroxisome proliferator-activated receptor gamma (PPARγ) antagonists (Fu *et al.*, 2002). The authors found that OPG expression was inhibited by

PPARγ ligands in human VSMCs and that this effect was completely abolished by a PPARγ antagonist. Moreover overexpression of PPARγ in these cells by transfection of an adenovirus considerably decreased OPG expression (Fu *et al.*, 2002).

There is now accumulating evidence to suggest a role for OPG in the regulation of VEC survival (Malyankar *et al.*, 2000), (Cross *et al.*, 2006), (Pritzker *et al.*, 2004). However the specific means by which, OPG reduces VEC apoptosis has not yet been fully elucidated. It is unlikely that this involves protection from apoptosis induced by OPGs second cognate ligand, TRAIL, as a number of studies have found that ECs are resistant to apoptosis induction by TRAIL, and are only sensitized to TRAIL-induced apoptosis under harsh conditions such as serum deprivation (Secchiero *et al.*, 2003), (Scatena & Giachelli, 2002). Only one group have implicated TRAIL inhibition in the OPG-mediated reduction in EC apoptosis (Pritzker *et al.*, 2004), TRAIL will be discussed in more detail later in this chapter. Other groups have not found TRAIL to be present in EC cultures at all (Cross *et al.*, 2006), (Zauli *et al.*, 2007). Malyankar et al. (2000) reported that ECs plated on osteopontin had increasing OPG mRNA and protein secretion and a resultant reduction in EC apoptosis (Malyankar *et al.*, 2000). In addition Cross et al. (2006) found that OPG enhanced EC growth and differentiation in addition to promoting the growth of cord-like arrangements on a matrigel base (Cross *et al.*, 2006)

Several studies have demonstrated that in chronic exposure to inflammatory cytokines such as in rheumatoid arthritis, multiple myeloma, diabetes, or hyperlipidaemia, OPG synthesis and storage in ECs has been shown to be low, or indeed, absent altogether (Browner *et al.*, 2001), (Giuliani *et al.*, 2001), (Wallin *et al.*, 2001). One possibility is that this may be as a consequence of the continued secretion of OPG leading to a significant depletion of vascular endothelial intracellular content after an extended time.
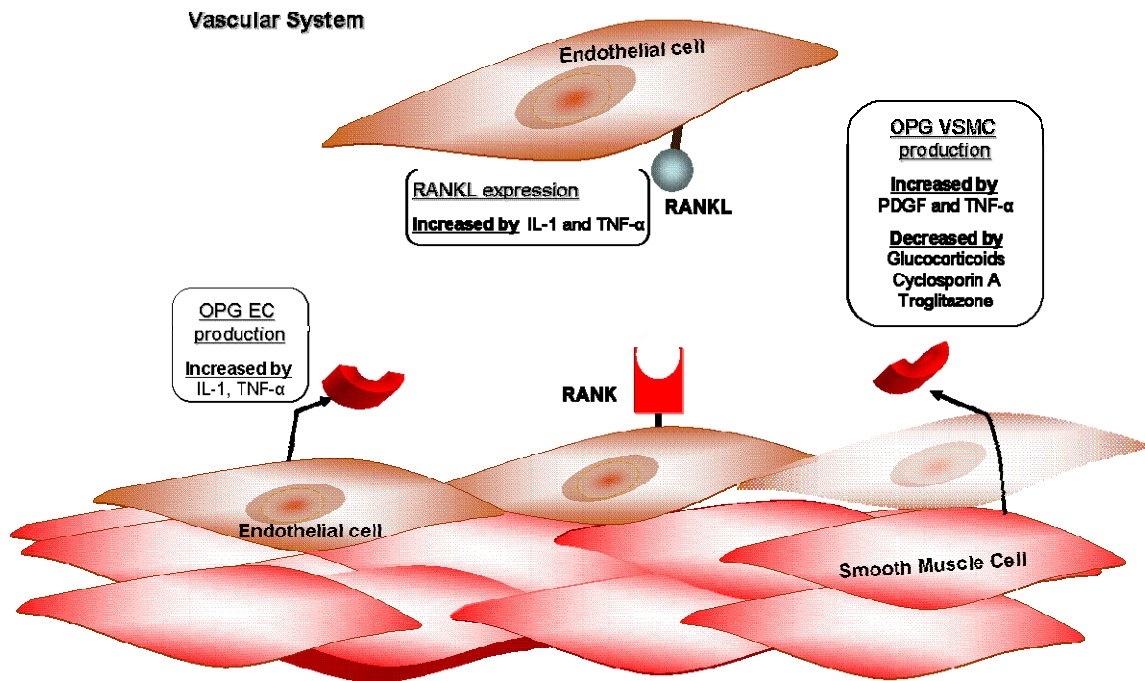
Figure 2.10 OPG, RANKL and RANK Expresion in the Vascular Endothelium. In the vascular system, RANKL and RANK are expressed by endothelial cells. RANKL /RANK interactions regulate endothelial survival and apoptosis. RANKL may be blocked by OPG, which is secreted by endothelial and smooth muscle cells. The physiological role of the OPG/RANKL /RANK system in the vascular wall and interactions with other ligands are currently under investigation. Adapted from (Hofbauer & Schoppet, 2004).

The specific area of OPG activity in the arterial architecture is important, as higher concentrations have been found in the tunica media but not intimal tissue of diabetics relative to normoglycaemic controls (Golledge *et al.*, 2004). This phenomenon of medial compared to intimal calcification was further studied by Schoppet et al. (2004) who found increased expression of OPG (but not RANKL) around areas of intimal and medial calcification in samples from patients with Monckeberg's sclerosis which is characterized by medial calcification but not in patients with atherosclerosis where intimal calcification is more common (Schoppet *et al.*, 2004). These findings were similar to those of Dhore *et al.*, (2001) and again suggest that OPG may be involved in the process of vascular calcification (Dhore *et al.*, 2001).

Subsequent to their earlier work (Olesen *et al.*, 2005), Olesen *et al.* (2007) showed that the addition of β-glycerophosphate to VSMC cultures led to significant calcification and as assessed by the measurement of total cellular calcium content was increased still further by the addition of insulin at a concentration of 1000 $\mu U \cdot ml^{-1}$. Interestingly the authors showed that there was a concomitant reduction in OPG expression, suggesting that this down-regulation of OPG may

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play some role in the increased calcification (Olesen *et al.*, 2007). Unlike their previous study (Olesen *et al.*, 2005), lower levels of insulin (200 µU.ml^{-1}) did not effect OPG secretion and the authors proposed this may have been due to the fact that the latter study was performed in the presence of serum or that the effects of insulin on OPG may be different depending on the degree of hyperinsulinaemia.

Induction of diabetes by streptozotocin led to an increase in detectable OPG levels and a fall in free RANKL concentration in apo-E null mice, and the addition of TNF-α (but not glucose or insulin) stimulated OPG release from human umbilical vein endothelial cells (Secchiero *et al.*, 2006). Using samples from human atherosclerotic plaque obtained at the site of rupture during an acute myocardial infarction and plaque from apoE knockout mice, Sandberg *et al.*, (2006) showed increased activity of the OPG/RANKL/RANK system. In addition they showed that RANKL increased the release of chemoattractant peptide-1 in mononuclear cells of patients with unstable angina and also stimulated matrix metalloproteinase activity in VSMCs (Sandberg *et al.*, 2006). Other factors influencing the secretion and expression of OPG and RANKL include the bone morphogenetic proteins BMP-2 and BMP-7 as well as transforming growth factor β1 (TGFβ1). All of these reduce OPG secretion and mRNA expression but BMP-2 and BMP-7 increase RANKL mRNA while TGFβ1 reduced RANKL. To address the question of whether OPG is elevated in states of atherosclerosis and vascular calcification as a compensatory mechanism or if it is playing a negative role in the pathogenesis of these conditions, Zauli *et al.*, (2007) examined the effect of OPG on adhesion of pro-inflammatory cytokines to endothelial cells (Zauli *et al.*, 2007). They found that OPG promotes the adhesion of primary polymorphonuclear neutrophills and leukaemic HL60 cells to endothelial cells *in vitro*, and they confirmed these findings *in vivo* in rat mesentery. The authors concluded that OPG may play a deleterious role in endothelial pathophysiology by instigating leukocyte adhesion to the endothelium which is thought to be an early step in the causation of endothelial dysfunction. On the other hand, Bennett et al. (2006) found that OPG-deficient ApoE$^{-/-}$ mice developed larger atherosclerotic lesions in addition to more vascular calcification than their OPG$^{+/+}$ littermates and that it acted as a survival factor for serum-deprived smooth muscle cells (Bennett *et al.*, 2006). The exact role of OPG in the process of atherosclerosis was further examined by Moroney *et al.*, (2008). They fed atherogenic LDL receptor knockout mice a high-fat diet and treated them with recombinant OPG or vehicle. The vehicle-treated mice developed

atherosclerosis with associated calcification and their OPG levels rose in parallel. The degree of calcification, but not atherosclerosis was significantly reduced in the mice given recombinant OPG. The authors concluded that these results supported the theory that OPG inhibits vascular calcification, and may act as a marker (rather than a mediator) of atherosclerosis progression (Morony *et al.*, 2008). It also appears that, in addition to slowing vascular calcification and possibly mediating atherosclerosis, OPG may be a pro-angiogenic factor (McGonigle *et al.*, 2009). When added *in vitro* to a rat aortic ring model of angiogenesis OPG increased neo-angiogenesis, an effect that was abrogated by pre-incubation with RANKL or TRAIL. Additionally, RANKL induced apoptosis on the endothelial cells. Circulating OPG has been shown to be significantly higher in patients with type 2 diabetes (Yaturu *et al.*, 2008), (Secchiero *et al.*, 2006), (Olesen *et al.*, 2005), (Rasmussen *et al.*, 2006), and is higher in the tunica media of type 2 diabetics than matched normal controls (Olesen *et al.*, 2005). Moreover, OPG is higher in individuals with severe Peripheral Artery Disease (PAD) than in those classified as having a mild to moderate PAD (Ziegler *et al.*, 2005), additionally it has also been shown that OPG can independently predict silent coronary artery disease in type 2 diabetic patients (Avignon *et al.*, 2005).

Many of the same signals that modulate RANKL and OPG in bone or immune cells may also regulate their expression in vascular cells. From an indirect perspective, it is likely that the RANKL/RANK/OPG axis exerts important effects on the vascular system through immunomodulatory and osteogenesis-related mechanisms. Despite the seemingly therapeutic effect, the exact mechanism by which OPG acts to protect the vascular wall remains elusive. However there is growing evidence to suggest that OPG may play a part in the regulation of EC survival/apoptosis in cell models (Pritzker *et al.*, 2004), (Scatena & Giachelli, 2002). It has been suggested that the pro-survival action of OPG on ECs may in part be due to inhibition of TRAIL-mediated apoptosis (Corallini *et al.*, 2008), although several studies have suggested that ECs are resistant to TRAIL-induced apoptosis under normal physiological conditions (Scatena & Giachelli, 2002), (Secchiero *et al.*, 2003). OPG can promote EC (Cross *et al.*, 2006), (Malyankar *et al.*, 2000) and VSMC survival by a mechanism which may involve the blocking of TRAIL-induced apoptosis in these cells lines. Furthermore, *in vitro* evidence shows that OPG can increase matrix metalloproteinase-9 activity in macrophages and smooth muscle cells and act as a survival factor for serum-deprived smooth muscle cells (Bennett *et al.*, 2006). A common

feature of atherosclerosis is the dysfunction and death of vascular cells (Littlewood & Bennett, 2003), (Reid & Holen, 2009). Therefore, the capability of OPG to improve survival of vascular smooth muscle and endothelial cells implies that it may play some protective role in this progression.

2.7.9 Serum OPG and Insulin Sensitivity / Resistance

There have been a number of studies which have attempted to elucidate the relationship between serum OPG and insulin sensitivity/resistance. In a study of 286 women with a mean age of 52 years, Oh et al. (2005) found that LDL, total cholesterol, follicle stimulating hormone as well as age and waist to hip ratio were positively correlated with OPG, but there was no relationship between OPG and fasting glucose, fasting insulin, or insulin sensitivity (Oh *et al.*, 2005). Ugur-Altun et al. (2004) also investigated the relationship between OPG and insulin resistance using the HOMA-IR model in 50 obese and 24 lean individuals who were not taking any medications. The authors found that OPG was lowest in the most insulin resistant obese group, and that OPG correlated negatively with insulin resistance, as measured by HOMA-IR (Ugur-Altun *et al.*, 2004). Gannage-Yared et al. (2006) had similar findings in a study of 151 older men where they found a weak positive correlation between OPG and insulin sensitivity using the Quantitative Insulin Sensitivity Index (QUICKI), in addition the authors found a, weak correlation with (positive) adiponectin and (negative) triglycerides (Gannage-Yared *et al.*, 2006). The same group subsequently investigated a relationship between OPG and insulin resistance in an obese cohort of patients undergoing bariatric surgery (Gannage-Yared *et al.*, 2008). Unlike the matched non-obese group, OPG showed a correlation with HOMA-IR even with adjustment for age and presence of diabetes. Multiple linear regression revealed that the acute phase reactant and marker of vascular in inflammation, CRP in addition to HOMA-IR were independent predictors of OPG concentration, a relationship which had not been observed in previous studies (Ugur-Altun *et al.*, 2004), (Browner *et al.*, 2001). This contrasted with the negative correlation seen between OPG and HOMA-IR in an obese population in an earlier study (Ugur-Altun *et al.*, 2004), and the positive correlation between OPG and QUICKI in the same group's previous work (Gannage-Yared *et al.*, 2006). The authors speculated that the small numbers in the HOMA paper (n=12 of obese with high HOMA) (Ugur-Altun *et al.*, 2004), and the different population studied in their previous paper (ie elderly males) (Gannage-Yared

et al., 2006) might account for the differences (Gannage-Yared et al., 2008). Considering these somewhat contradictory results some caution should be exercised when comparing findings from different studies. Several studies have used commercially available assays that measure unbound and uncomplexed forms of both sRANKL or OPG (Xiang et al., 2006), (Knudsen et al., 2003), (Rasmussen et al., 2006), (Jorgensen et al., 2009). The data which is the subject of this body of work refers to free soluble RANKL and total OPG. This OPG assay measures both monomeric and dimeric isoforms of OPG, including OPG bound to RANKL and TRAIL and has been used to measure total OPG in many cohorts (Gannage-Yared et al., 2006), (Gannage-Yared et al., 2008), (Anand et al., 2006), (Schoppet et al., 2003). In addition because of the non standard units of measurements used in other commercial ELISA assays and the difficulty in ascribing an exact molecular weight to the OPG-isoforms which they measure i.e. bound or unbound, monomeric or dimeric, the process of converting these values to SI units is somewhat complicated. Therefore previous studies that have exclusively measured uncomplexed OPG may unintentionally have excluded a large portion of the biologically active total circulating OPG which has either bound to TRAIL or RANKL or indeed has undergone some other unspecific binding. Recombinant OPG with a molecular weight of 19.9kD was used to calibrate the ELISA plates in these studies. This is identical to the extra cellular domain of RANK (TNF-receptor family). This in turn is identical to OPG. The precoated monoclonal anti-OPG capture antibody bound to the microtiter plate, binds to circulating OPG. The detection antibody is a biotinylated polyclonal anti-Osteoprotegerin antibody. The ELISA used in these experiments measures free OPG and complexed OPG-RANKL, since the binding site of the capture antibody lies outside of the binding site to sRANKL. The OPG ELISA detects the monomeric as well as the dimeric form of OPG. In the literature there is some discrepancy on the molecular weight of OPG. Molecular weights from 120 kD down to 35 kD can be found. The reason for this discrepancy are due to how the MW was determined either using isolation, SDS Page or DNA determination.

2.7.10 Tumour Necrosis Factor Receptor Apoptosis Inducing Ligand (TRAIL)

In addition to the essential role governing RANK–RANKL ligation in bone, OPG can also promote cell survival by binding to TRAIL. As OPG also acts as a soluble receptor for TRAIL (Emery et al., 1998) and since TRAIL is able to preferentially induce tumour cell apoptosis over normal cells, there has been much interest in its potential as a cancer chemotherapeutic (Smyth

et al., 2003), (Takeda *et al.*, 2002). The physiological importance of TRAIL-OPG connections is highlighted by the fact that OPG can bind to TRAIL with a similar affinity to that of RANKL under normal physiological conditions (Vitovski *et al.*, 2007). TRAIL is expressed and secreted by immune cells such as T cells that can penetrate the tumourous cellular environment (Reid & Holen, 2009). TRAIL that has been secreted by these T cells can bind to the death receptor-regions 4 and 5 expressed on the surface of tumour cells. These receptors enclose extracellular death domains that initiate apoptotic signalling cascades, leading to specific programmed tumour-cell apoptosis. Two other membrane-bound decoy receptors for TRAIL have been found, DcR1 and DcR2. However little is known about their biological activity and in this context they do not seem to play a major role. DcR1 does not have a cytoplasmic domain and the DcR2 cytoplasmic apoptotic region appears to be truncated and inactivated (Marsters *et al.*, 1999), (Sheridan *et al.*, 1997), (Reid & Holen, 2009). *In vitro* evidence suggests OPG may also promote survival in malignant tumours and cancer cell lines. (Wiley *et al.*, 1995). OPG may be involved in survival of a number of tumour cell types in this way (Holen *et al.*, 2002). Neville-Webbe *et al.*, (2004) demonstrated that OPG production from bone marrow stromal cells isolated from breast cancer patients was sufficient to increase survival of breast cancer cells that reach the bone microenvironment as part of the metastatic process. The authors suggested that OPG production may protect breast cancer cells from undergoing TRAIL induced apoptosis (Neville-Webbe *et al.*, 2004). Furthermore, Shipman *et al.*, (2003) demonstrated that TRAIL-induced apoptosis could be prevented in myeloma cells by the addition of recombinant OPG, an effect which seemed to be reversed by the addition of sRANKL (Shipman & Croucher, 2003). This may be important for tumour cells to escape apoptosis, since host immune cells present in the tumourous cellular environment produce TRAIL, and *in vivo* data suggests this to be important in promoting anti-tumour action (Almasan & Ashkenazi, 2003), (Griffith *et al.*, 1999), (Takeda *et al.*, 2002). Therefore, secretion of OPG by tumour cells may be a possible mechanism of defence by these cells to TRAIL-induced apoptosis (Holen *et al.*, 2002). However, there is still some doubt about this mechanism and some authors consider it unlikely to be related to its binding and neutralization of TRAIL, as TRAIL quickly induces apoptosis in a number of such cell lines and primary tumours but interestingly, it appears to demonstrates little or no toxicity to normal healthy cell lines (Corallini *et al.*, 2008), (Reid & Holen, 2009). However emerging evidence suggests that circulating TRAIL may be related to body composition and lipid status. Choi *et al.*, (2004) found that TRAIL was higher in individuals who had greater total

body fat and that it was positively correlated with LDL cholesterol (Choi *et al.*, 2004). TRAIL also exerts an effect at the level of the vascular wall with some studies suggesting it may contribute to plaque instability. Sato et al. (2006) found that the dominant plaque residing T cells, CD4 T's induce rapid apoptosis in cultured VSMCs by TRAIL expression which activates death receptors on the surface of VSMCs (Sato *et al.*, 2006). The addition of OPG appears to promote survival of cultured VEC (Cross *et al.*, 2006), (Malyankar *et al.*, 2000), (Pritzker *et al.*, 2004), though the exact means by which this is accomplished is still unclear. It may be related to a reduction in TRAIL-induced apoptosis (Pritzker *et al.*, 2004). However, other studies have not found TRAIL to be present in VEC cultures (Cross *et al.*, 2006), (Zauli *et al.*, 2007). These conflicting findings may suggest an alternative pathway by which OPG promotes survival of this cell type. Furthermore, healthy VECs seem to be resistant to TRAIL-induced apoptosis, which concur with the notion that non-malignant cells are unresponsive to TRAIL (Cross *et al.*, 2006). It is probable that OPG has other binding partners as well as TRAIL and RANKL. In this regard, it appears that OPG can promote leukocyte adhesion to the endothelial cell surface and this could be induced via interaction between the endothelial cell monolayer and the OPG heparin-binding domain. At this point however a binding associate for OPG in this process has yet to be identified. A potential mechanism that may mediate this activity is the interaction between proteoglycans containing heparan sulphate, such as syndecan-1, which can undergo ligation with several heparin-binding proteins, one of which is OPG (Borset *et al.*, 2000).

2.7.11 The Bone – Vascular Calcification Paradox

Vascular calcification is an active, cellularly controlled process in which mineral is ectopically deposited predominantly in the larger elastic and muscular arteries, such as the aorta, the coronary, carotid and iliofemoral arteries as well as in the cardiac valves more and more the advancement of this process has been recognised as a risk factor for cardiovascular disease (Arad *et al.*, 2005) and mortality (Budoff *et al.*, 2007). Interestingly, localized arterial mineralization is often observed in conjunction with a significant decrease in bone mineral density or increased bone turnover. This paradoxical inverse relationship, is best typified by the concomitant occurrence of osteoporosis and chronic kidney disease, and is often referred to as a "the calcification paradox" (Persy & D'Haese, 2009). Ectopic vessel mineralization can occur

in either in the tunica intima or in the tunica media of the artery. The idiosyncratic characteristics of both of these forms of vascular calcification are described in Figure 2.x
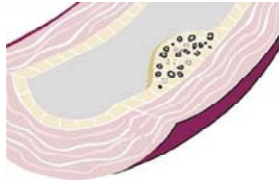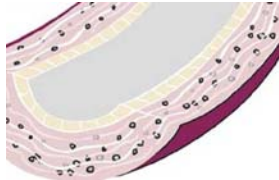
	Intima calcification	Media calcification
	Atherosclerosis	*Arteriosclerosis or Monckeberg's sclerosis*
Calcification pattern	*Focal, in plaques*	*Generalized*
Risk factors	Dyslipidemia, hypercholesterolemia	Aging, diabetes, renal failure, osteoporosis, hypertension
Molecular mechanisms	Lipid accumulation Foam cell formation Inflammation Oxidative stress Apoptosis	Transdifferentiation of VSMCs into bone-like cells (osteoblast-chondrocyte and osteoclast-like cells) Ca, P, vitamin D metabolism Loss of calcification inhibitors (pyrophosphate, MGP, fetuin)
Result	Plaque formation: stenosis Plaque calcification: controversial effect on plaque stability, possibly relating to the localization of calcification	Arterial stiffening: increased pulse pressure, elevated pulse wave velocity
Complication	Ischemia, infarction	Systolic hypertension, left ventricular hypertrophy

Figure 2.11 Ectopic vessel mineralization can be localized to either in the tunica intima or in the tunica media of the vessel. Intima calcification is associated with atherosclerosis and results in focal calcification of atherosclerotic plaques, whereas media calcification (arteriosclerosis or Monckeberg's sclerosis) is more generalized and is found mainly in the elderly and in patients with CKD, osteoporosis, hypertension or diabetes mellitus. Monckeberg's sclerosis leads to vessel stiffening, which is characterized by increases in pulse pressure and pulse wave velocity and is associated with increased cardiovascular risk. Adapted from (Persy & D'Haese, 2009)

In General, however these two structurally distinct forms of vascular calcification are not differentiated in cross-sectional or longitudinal epidemiological studies, an important factor which must be considered when interpreting results from such studies. London et al. (2003) delineated the two forms of calcification morphologically using plain x-ray images and found that the increasing severity led to an elevated mortality risk in patients undergoing haemodialysis (London *et al.*, 2003). As previously mentioned ectopic calcification in the vasculature is often occurs in conjunction with decreased bone mineral density or increased bone turnover. This conflicting association has been found in the general population (Hyder *et al.*, 2007), in osteoporosis and CKD patients (Raggi *et al.*, 2007;Toussaint *et al.*, 2008) and in more rare conditions such as Paget's disease (Laroche & Delmotte, 2005). An inverse correlation between BMD and vascular calcification has also been observed several cross-sectional and longitudinal studies using different techniques in postmenopausal osteoporosis, Additionally, several studies have reported that this calcification is in fact correlated with the occurrence of fragility fractures (Bagger *et al.*, 2006;Schulz *et al.*, 2004)

Moreover, in a large prospective study of over six thousand postmenopausal women Kado et al. (2000) using DEXA scanning found that for each decrease of one standard deviation in BMD, risk of cardiovascular mortality was amplified by 30% (Kado *et al.*, 2000). It appears this relationship is not merely a phenomenon exclusive to osteoporosis, it was also observed by Farhat et al. (2006) in a cohort of healthy perimenopausal subjects with a low prevalence of osteoporosis (Farhat *et al.*, 2006) as well as in a heterogeneous cohort of healthy men and women (Hyder *et al.*, 2007). Vascular calcification is a feature of the increased cardiovascular morbidity and mortality observed in patients with CKD (Schiffrin *et al.*, 2007). In addition to increased calcification of atherosclerotic plaques, patients on dialysis also show characteristic calcifications of the tunica, contributing to their elevated cardiovascular mortality (London *et al.*, 2003). Calcification quickly progresses in both dialysis (Goodman *et al.*, 2000) and end stage renal disease (Sigrist *et al.*, 2007). Increased circulating phosphate levels, elevated concentrations of calcium as well as high parathyroid hormone (PTH) concentrations have been acknowledged as risk factors for vascular calcification and mortality in patients with CKD, as well as the administration of calcium-containing phosphate binders and vitamin D metabolites (Ganesh *et al.*, 2001;Kestenbaum *et al.*, 2005) The decline in renal function associated with the progression of CKD often leads to the development of metabolic bone disease traditionally grouped under the name renal osteodystrophy. Monckeberg's sclerosis was first described in the mid nineteenth century and is a cell-mediated process (Steitz *et al.*, 2001) whereby VSMCs deposit hydroxyapatite in the tunica media (Wada *et al.*, 1999). The subject of bone mineralization and localized mineralization of the vasculature, with the transformation of vascular cells to bone-like cells that express a number of bone related proteins, such as bone sialoprotein, osteocalcin and alkaline phosphatase. Interestingly though, in vivo evidence suggests that these simultaneous expression patterns appear to have accumulate to contradictory results, leading to vascular calcification, whereas bone formation is actually impaired or disturbed.

Opposing regulation of the OPG–RANK–RANKL triad in bone and vasculature by transforming growth factor b fibrogenic hormone (TFG-β) might be one mechanism to explain this calcification paradox. TGF-β increases RANKL expression and reduces OPG expression in ECs but reduces RANKL in cells of an osteoblastic lineage (Hofbauer & Heufelder, 2001) leading to

an increase in the OPG/RANKL ratio in bone, inhibiting osteoclastic bone resorption, and decreases the OPG/RANKL ratio in blood vessels, decreasing the potential calcification inhibition possible with OPG.

2.7.12 Therapeutic Role for OPG

Simonet et al. (1997) first confirmed the role of OPG in the regulation of bone formation demonstrating that the administration of recombinant OPG blocks differentiation of precursor cells into osteoclasts in a dose dependant manner *in vitro*. The authors also outlined a possible clinical application of recombinant OPG by suggesting a potential for OPG therapy to ameliorate the bone loss that one would expect in ovariectomized rats, where bone volume in the proximal tibial metaphysis was increased in OPG treated rats relative to controls (Simonet *et al.*, 1997). Since this discovery there has been much interest in the use of OPG and or manipulation of the RANKL/RANK/OPG axis to treat bone related disorders. Evidence from both animal and *in vivo* studies have shown that RANKL expression can be reduced by 17β-estradiol (Eghbali-Fatourechi *et al.*, 2003) and that its biological activity can be ameliorated by introducing a barrier so it can not effectively bind to its receptor. This can be achieved by administration of peptides which mimic the function of OPG and include soluble RANK fusion proteins, (Hsu *et al.*, 1999), (Oyajobi *et al.*, 2001), OPG fusion proteins, (Simonet *et al.*, 1997), (Kong *et al.*, 1999), (Teng *et al.*, 2000), (Honore *et al.*, 2000), or antibodies to RANKL. In addition post-receptor signalling after RANK-RANKL binding to the c-Jun pathway can also be interrupted by the addition of estrogen to stromal cells (Shevde *et al.*, 2000). What's more, *in vitro* OPG secretion can also be upregulated in response to 17β-estradiol (Hofbauer *et al.*, 1999), the oral selective estrogen receptor modulator; raloxifene (Viereck *et al.*, 2003) and bisphosphonates (Hofbauer & Schoppet, 2004). Therapeutic efficacy has focused on OPG-Fc and RANK-Fc fusion proteins. Synthetic OPG fusion proteins do not have a heparin-binding region and as a result they are less prone to sequestration than naturally circulating OPG (Standal *et al.*, 2002). However, because OPG has the ability to bind RANKL and TRAIL, RANK-Fc fusion protein may be a more desirable modality for therapeutic inhibition of this pathway as it has no effect on TRAIL signalling (Oyajobi *et al.*, 2001). However OPG-Fc and RANK-Fc fusion proteins seem to be successful and without adverse side effects in animal models of arthritis (Kong *et al.*, 1999)

and osteoporosis (Simonet *et al.*, 1997), bone disease caused by multiple myeloma (Sezer *et al.*, 2003) and bone metastases (Honore *et al.*, 2000). One randomized controlled trial has investigated the acute effects of OPG-Fc fusion protein on markers of bone turnover. Bekker *et al.*, (2001) examined the effect of a single dose of OPG-Fc administered subcutaneously on biochemical markers of bone resorption in postmenopausal osteoporotic women. Participants were followed up after 85 days and it was found that OPG treatment led to a substantial and prolonged reduction of bone resorption as indicated by a reduction of 80% in urinary excretion of deoxypyridinoline concentrations and an increase in bone formation markers of 20% as indicated an increase in serum levels of osteocalcin (Bekker *et al.*, 2001). Another study which compared the effects of an OPG bolus against the bisphosphonate, pamidronate in 28 patients with myeloma related bone disease and in 26 women with multiple bone metastases as a result of breast cancer, with a follow-up of 6 months. The authors found that that urinary N-telopeptide (a marker of bone resorption) was reduced in OPG treated breast cancer patients by 74% and by 47% in the other group. This was similar to the outcomes observed in the bisphosphonate treated group (Body *et al.*, 2003). Administration of OPG-Fc fusion protein in such trials has only a few side effects, such as hypocalcaemia, and in only a few exceptional cases it has led to the production of anti-OPG antibodies, none of these side effects have been observed in patients who have had RANK-Fc fusion proteins (Bekker *et al.*, 2005) The potential for this mechanism as a therapeutic target has led to the development of a human monoclonal IgG2 antibody to RANKL, AMG162 (Denosumab). Denosumab selectively binds to RANKL but does not cross react with TNF-α, TNFβ, CD40 ligand, or TRAIL (Dougall & Chaisson, 2006). After binding to RANKL, denosumab blocks the interaction between RANKL and RANK, a mechanism similar to that of endogenous OPG. Denosumab is currently in Phase II clinical trials for postmenopausal women with osteoporosis (Bone *et al.*, 2008), breast cancer-related bone metastases (Lipton *et al.*, 2007) and structural damage in patients with rheumatoid arthritis (Cohen *et al.*, 2008). McClung *et al.*, (2006) showed that subcutaneous administration of denosumab at either 12 or 26 week intervals to more than four hundred postmenopausal women with low bone mineral density led to a continued reduction in bone resorption and a swift and significant increase in BMD. (McClung *et al.*, 2006). In a two year study conducted by Bone *et al.*, (2008) over three hundred postmenopausal patients with osteoporosis receiving 6-monthly subcutaneous administration of Denosumab showed significantly increased BMD and reduction in indicators of bone resorption both in early and late postmenopausal osteoporotic

females (Bone *et al.*, 2008). Similar positive results have been obtained in a study in patients with breast cancer and multiple myeloma who have radiographicly verified bone lesions. A one time dose of Denosumab led to an immediate and continued reduction in bone resorption (Body *et al.*, 2006). No data are currently available on whether this new therapeutic compound can also regress vascular calcifications, an issue that is worth investigating in view of the potential role of the OPG–RANK–RANKL triad in the development of vascular calcification.The possibility of using OPG or manipulating this axis for the treatment of vascular disease or calcification is somewhat more complex. OPG has been shown to act in an autocrine manner to reduce apoptosis and increase survival of endothelial cells (Malyankar *et al.*, 2000). However, it appears that RANK and RANKL are absent in tissue from arterial walls of wild-type mice and only appear to be present in calcified atherosclerotic plaques of OPG-deficient mice (Min *et al.*, 2000). There has been little evidence to date that RANK and its ligand play an important role in the biology of human vascular diseases. Furthermore, the molecular mechanisms involved and the mode of action by which OPG is engaged in the process of vascular disease and dysfunctions are still unclear.

2.8 General Summary

There has been a dramatic increase in the prevalence of obesity during the past two decades. Increased adiposity is associated with the development of insulin resistance and type 2 diabetes. Type 2 diabetes occurs when there is inadequate insulin secretion from the β-cells in tandem with increased insulin resistance in multiple tissues. In addition to its role in glucose disposal, insulin is an important vasoactive hormone that exerts pleiotropic actions in skeletal muscle, adipose tissue and vascular endothelium. In recent years there has been intense study of the biological activity of the adipose tissue, as a result of which it is now recognised that the adipose tissue is an active metabolic organ, releasing adipocytokines into the circulation, which influence insulin action and contribute to vascular dysfunction. Adiponectin and TNF-α are two such adipocytokines that which in addition to insulin also appear to regulate OPG production and secretion. Insulin resistance has also been demonstrated to correlate with circulating OPG. The role played by OPG, RANK and RANKL in bone turnover and bone-related disease has been the subject of extensive research. Most of these studies show that OPG exerts a protective effect on bone via inhibition of bone resorption. OPG also appears to be an important

player in the vasculature and may prevent processes involved in the pathogenesis of atherosclerosis. There is some evidence, albeit controversial to suggest that this may be achieved via its ability to promote VEC survival by acting as a decoy receptor for TRAIL. The interaction between these families of molecules and the evidence demonstrating significant cross-talk between their metabolic pathways provides support for the premise that they form a complex array of interconnected cytokines involved in physiological regulation in multiple tissue types.

Chapter III An investigation of serum OPG, TRAIL and sRANKL levels and their relationship with adiposity and indicators of insulin sensitivity in a healthy Irish cohort.

3.1 Introduction

Rationale

Circulating OPG has been shown to be significantly higher in patients with type 2 diabetes (Yaturu *et al.*, 2008), (Secchiero *et al.*, 2006), (Olesen *et al.*, 2005), (Rasmussen *et al.*, 2006), and is higher in the tunica media of type 2 diabetics than matched normal controls (Olesen *et al.*, 2005). In addition, OPG is higher in individuals with severe Peripheral Artery Disease (PAD) than those classified as mild to moderate PAD (Ziegler *et al.*, 2005). Indeed it has also been shown that OPG can independently predict silent coronary artery disease (Griffin *et al.*, 1999) in type 2 diabetic patients (Avignon *et al.*, 2005). Despite the higher circulating and tissue concentrations of OPG in metabolic and cardiovascular disease patients there has been little research on high risk obese subjects. Gannage-Yared et al. (2006) examined the relationship between OPG and components of the metabolic syndrome in 151 healthy ageing men. Contrary to many previous studies, they found that OPG was inversely correlated with fasting plasma glucose and insulin sensitivity and positively correlated with adiponectin. Despite their classification of this population as healthy, over 60% of this cohort had the metabolic syndrome, 28% had hypertension and 15% had previously diagnosed coronary artery disease (Gannage-Yared *et al.*, 2006). There has been significant research in diseased populations demonstrating that OPG is associated with the presence and severity of CAD and cardiovascular mortality (Browner *et al.*, 2001), (Jono *et al.*, 2002), (Kiechl *et al.*, 2004), (Omland *et al.*, 2008), (Schoppet *et al.*, 2003), (Ueland *et al.*, 2004). There have, however been few published papers that have examined the relationship between insulin sensitivity, adiposity and OPG in a normal population free from overt cardio-metabolic disease.

Aims

The purpose of this study was to determine if BMI and insulin sensitivity influence the concentrations of serum OPG and TRAIL in subjects who do not have cardiovascular or metabolic disease.

Hypothesis

We tested the hypothesis that in a healthy cohort circulating OPG would be lower in obese subjects and inversely related to insulin resistance.

3.2 Materials and Methods

3.2.1 Experimental Design Overview

One hundred and thirty six subjects volunteered to participate in the study. Of these thirty six were excluded because of undiagnosed hypertension, impaired glucose tolerance, and abnormal ECG. Subjects visited the laboratory on two separate occasions, separated by at least 4 days. On the first occasion they reported to the laboratory in the morning following an overnight fast. Subjects were interviewed by a physician, had anthropometric measurements and a 2 hr oral glucose tolerance test. On the second visit, subjects reported to the laboratory approximately 3 hr following a meal. They had a resting 12-lead ECG followed by an ECG stress test with oxygen consumption (Figure 3.1).
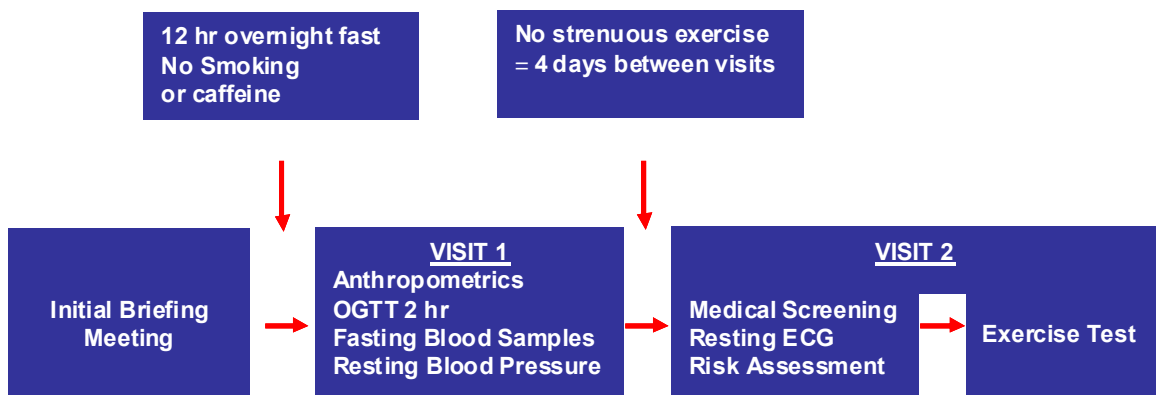
Figure 3.1 Schematic of experimental design for experiment I

3.2.2 Participant Recruitment

Participants were recruited by means of an open call for volunteers who were free from cardiovascular and metabolic disease. A plain language statement was given to those expressing an interest in the study, after which a briefing meeting was scheduled to allow for questions to be asked in relation to the study and written informed consent was provided by individuals wishing to participate. In total 136 subjects volunteered to participate in the study. Of these 36 were excluded because of undiagnosed hypertension, impaired glucose tolerance, and

abnormal ECG. The final cohort that met the inclusion criteria consisted of 100 subjects, aged 22-74 yrs. This group comprised a similar number of males (n=51) and females (n=49) and the distribution of normal weight (n=36), overweight (n=41) and obese (n=23) subjects is similar to the Irish adult population (Morgan *et al.*, 2008). The study was approved by the Dublin City University Research Ethics Committee and conformed to the Declaration of Helsinki. Finally, each participant completed a health history questionnaire and underwent medical screening examination see (Appendix 2, 3, 4).

3.2.3 Exercise Stress Test and Maximal Oxygen uptake

Following the medical examination all subjects underwent a multistage exercise treadmill test using a modified Bruce protocol. All exercise tests took place under standard laboratory conditions (19-21°C, 40-55% relative humidity). Expired oxygen, carbon dioxide, ventilatory volume, respiratory exchange ratios and VO_{2max} were determined by indirect calorimetry (Sensormedics Vmax 229, Sensormedics Corp., Yorba Linda CA). Systolic and diastolic blood pressure was measured using a sphygmomanometer and recorded when the subject was standing immediately before testing and during the last minute of each exercise stage. Electrical activity of the heart was also recorded at rest and at the end of each stage. Subjects exercised until reaching volitional fatigue. The test was deemed to be maximal if two or more of the following criteria were satisfied (i) plateau of oxygen consumption (increase of less than 2 $ml^.kg^{-1.}min^{-1}$), (ii) heart rate within 10 beats of the subjects' age predicted maximum heart rate (220 bpm – age in years) and (iii) respiratory exchange ratio > 1.10. VO_2 max was determined to be the highest minute average recorded for oxygen uptake during the test.

3.2.4 Anthropometric and body composition measurements

Height and body mass were measured to the nearest 0.1 cm and 0.1 kg respectively (SECA, Hamburg, Germany). Subjects were weighed barefoot and with minimal clothing. Harpenden Skinfold Callipers (British Indicators, 15 9LB. England) were used to measure double thickness subcutaneous adipose tissue on the right side of the body at seven sites. Waist and Hip circumferences were measured to the nearest 0.1 cm. Waist circumference was taken midway between the lowest rib (laterally) and the iliocristale landmark. Hip circumference was measured at the greatest protrubence of the gluteals. Body density was calculated by the method of

Jackson & Pollock (1985) (Jackson & Pollock, 1978) based on the sum of seven skinfolds (tricep, subscapular, mid-axillary, pectoral, suprailiac, abdominal, thigh). Percentage body fat was calculated from the equation of Siri (Suzuki *et al.*, 2004).

3.2.5 Glucose Tolerance and Insulin Sensitivity

In order to rule out previously undiagnosed type 2 diabetes, impaired fasting glucose or impaired glucose tolerance, subjects underwent a standard 2 hr Oral Glucose Tolerance Test. (OGTT) (Reinauer *et al.*, 2002). The 75 g (113 ml) anhydrous glucose equivalent (Polycal; Nutricia Clinical, Trowbridge, United Kingdom) was consumed in 300 ml of water within 5 min. Blood samples were taken prior to and at 30, 60, 90 and 120 min after the glucose load. Total area under curve (AUC) for glucose and insulin was determined by the trapezoidal method (Tai, 1994) and HOMA-IR was used as an indicator of insulin resistance (Matthews *et al.*, 1985). Insulin Sensitivity was estimated using the validated Oral Glucose Insulin Sensitivity (OGIS) predictive model (Mari *et al.*, 2001)

3.2.6 Collection of Blood Samples

Prior to the OGTT subjects had a 20 or 22 GA indwelling cannula (BD VialonTM, Biomaterial, Spain) introduced into a prominent forearm vein for blood sampling. Samples for glucose analysis were collected in grey top plasma tubes (BD Vacutainer®, 10 mg sodium fluoride, 8 mg potassium oxalate). Samples for insulin determination and other analytes were collected in red top serum tubes (BD Vacutainer®). Blood samples were collected 10 min before the oral glucose load and, 30, 60, 90 and 120 min after. Lines were flushed with saline solution after each blood draw and approximately 2.5 ml of blood was evacuated as waste at each time point before collection of analytical samples. Serum was allowed to stand for 30 min before centrifugation at 3000 $r\dot{p}m^{-1}$ (Dovio *et al.*, 2007) for 15 min at 4ºC at which point aliquots were stored at -80°C for further analysis.

3.2.7 Biochemical Analysis and Assays

Plasma glucose was measured using the glucose oxidase method (YSI 2300 Stat Plus, Yellow Springs, Ohio). Serum insulin was measured with a commercially available fluoroimmunoassay

(Delphia; Perkin Elmer, Wallac, Turku, Finland). Serum OPG, total sRANKL (Biomedica, Vienna, Austria), TRAIL and adiponectin (R&D Systems Inc., Minneapolis, MN), were measured using commercially available ELISA kits. The minimal detectable limit for OPG was 0.014 pmol$^{.}$l^{-1}, 0.02 pmol$^{.}$l^{-1} for total sRANKL, 0.246 ng$^{.}$ml^{-1} for adiponectin and 2.86 ng$^{.}$ml^{-1} for TRAIL. The intra and inter assay coefficients of variance were <6 % for OPG and total sRANKL and <5 % for Adiponectin and TRAIL. High sensitivity C-Reactive Protein (hsCRP), triglycerides, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and total cholesterol were measured with Randox reagents on the Randox-Daytona automated analyser using a spectro-photometric method (Randox, Antrim, Northern Ireland).

3.2.8 Statistical Procedures

SPSS 15.0 for Windows (SPSS Inc., USA) was used for statistical analysis. Data are reported as means ± SEM. Normally distributed variables were explored using simple bivariate or partial regression. Non-normally distributed variables including fasting glucose, insulin, 2 hr insulin, AUC glucose, AUC insulin, HOMA IR, adiponectin, sRANKL, low density lipoprotein and high density lipoprotein were log-transformed. The degree of relationship was calculated using Pearson's product moment (r). Participants were classified as normal weight, overweight or obese based on their BMI. A one-way analysis of covariance (ANCOVA) was used to examine differences between BMI categories with age and gender as covariates. Bonferroni's post hoc test was applied to determine differences among means. Statistical significance was set at $p<0.05$.

3.2.9 Subject Characteristics

Physical and metabolic characteristics for male and female subjects are presented in Table 3.1 and for the normal weight, overweight and obese subjects are presented in Table 3.2.

Table 3.1 Selected Characteristics of Subjects broken down by gender.

	Male	Female
	(53)	(57)
Age (years)	44.5 ± 1.5	47.7 ± 1.8
BMI (kg·m^{-2})	27.0 ± 0.4	25.7 ± 0.5
Waist Circumference (cm)	92.4 ± 1.3	84.2 ± 1.9 *
Waist to Hip Ratio	0.90 ± 0.01	0.83 ± 0.01 *
Body fat (%)	21.6 ± 0.9	29 ± 1.2 *
VO$_{2\ max}$ (ml·kg·min^{-1})	41.6 ± 1.5	32.0 ± 1.2 *
Systolic BP (mmHg)	123.2 ± 1.6	119.5 ± 1.9
Diastolic BP (mmHg)	77.8 ± 1.2	75.2 ± 1.5

BMI (Body mass index), BP (blood pressure), VO$_{2\ max}$, (maximal oxygen consumption).Values are mean ± SEM. * $p < 0.05$ vs. Normal weight. † $p < 0.05$ *vs.* Overweight.

Table 3.2 Selected Anthropometric and Cardiovascular Characteristics of Subjects

	Normal weight	Overweight	Obese
	(36)	(41)	(23)
Gender (male/female)	19/17	19/22	11/12
Age (years)	44.4 ± 1.5	46.7 ± 2.0	47.2 ± 2.8
BMI (kg·m^{-2})	22.8 ± 0.2	26.7 ± 0.2 *	31.4 ± 0.3 * †
Waist Circumference (cm)	78.5 ± 1.2	89.8 ± 1.2 *	102.2 ± 2.0 * †
Waist to Hip Ratio	0.82 ± 0.01	0.87 ± 0.01 *	0.93 ± 0.02 * †
Body fat (%)	19.6 ± 1.15	27 ± 1.16 *	31.9 ± 1.5 * †
VO$_{2\ max}$ (ml·kg·min^{-1})	41.5 ± 1.9	37.5 ± 1.5	29.6 ± 1.7 * †
Systolic BP (mmHg)	117.1 ± 2.2	120.4 ± 1.6	130.3 ± 2.2 * †
Diastolic BP (mmHg)	74.0 ± 1.5	76.5 ± 1.4	80.6 ± 2.1 *

BMI (Body mass index), BP (blood pressure), VO$_{2\ max}$, (maximal oxygen consumption).Values are mean ± SEM. * $p < 0.05$ vs. Normal weight. † $p < 0.05$ *vs.* Overweight.

3.3 Results

3.3.1 Physical Characteristics

Age and gender distribution was similar for the three groups but there were significant differences in BMI, % body fat, waist circumference and waist-to-hip ratio. In addition, the obese group had significantly higher systolic and diastolic blood pressure and lower aerobic capacity compared with the other two groups Table 3.2. Glucose and Insulin kinetics during the OGTT are presented in Figure 3.1.
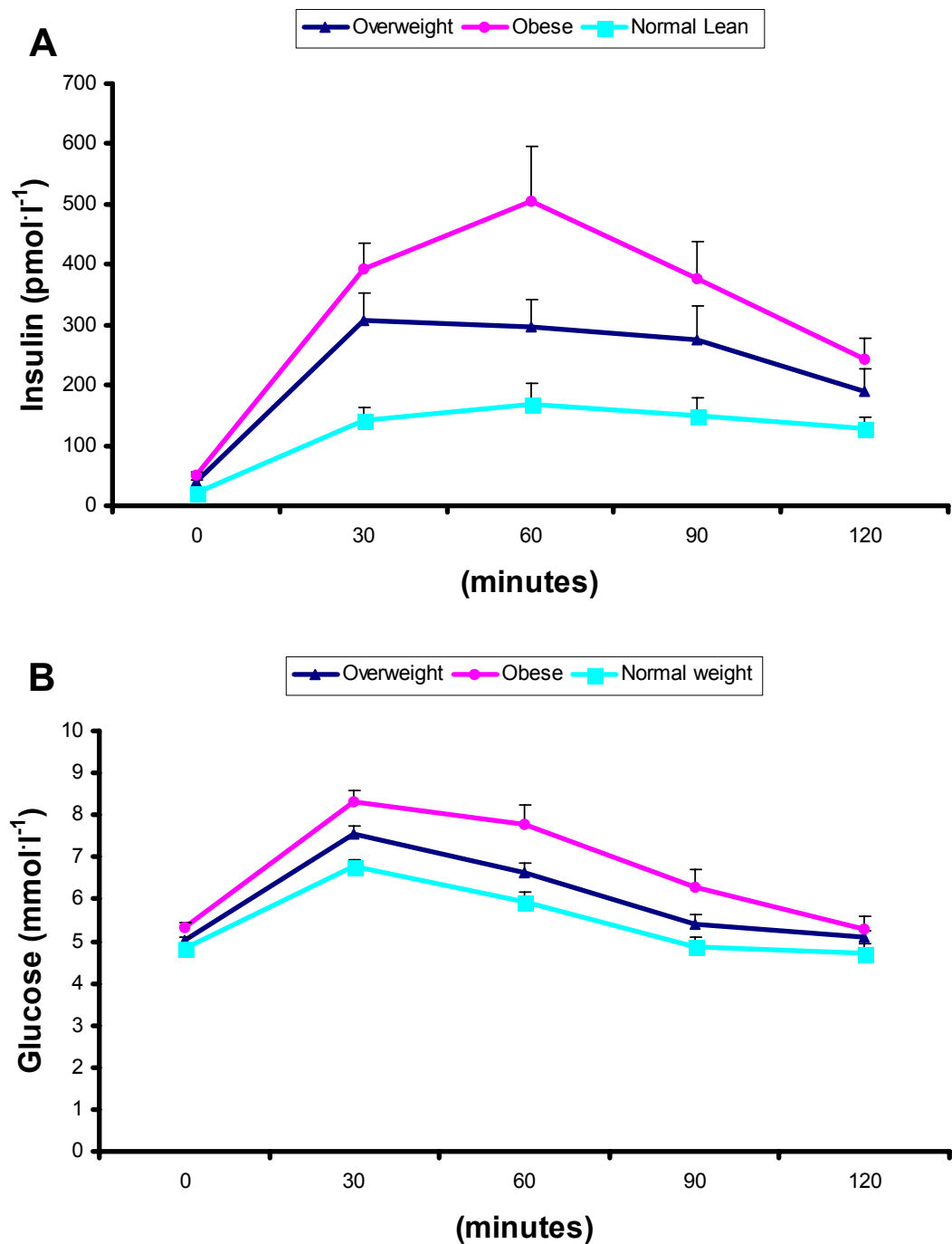
Figure 3.1 Insulin (A) and Glucose (B) kinetics in response to a 75 g Oral Glucose Tolerance Test in age and gender matched; Obese ♦, overweight ▲ and lean subjects ■.

3.3.2 Metabolic Phenotype

All subjects had normal glucose tolerance but the obese group had significantly higher fasting glucose, insulin and triglycerides compared to the other groups. They also had a greater glucose and insulin response to the OGTT and had lower insulin sensitivity, as determined by OGIS and HOMA-IR. Circulating adiponectin was significantly lower in males compared to females (4.99 ± 0.35 *vs.* 10.06 ± 0.71 µg·ml^{-1}, $p < 0.001$) and in overweight and obese subjects compared to controls (Table 3.3).

Table 3.3 Metabolic Markers and Indicators of Insulin Sensitivity.

	Normal weight	Overweight	Obese
Fasting Glucose (mmol·l$^{-1)}$)	4.8 ± 0.1	5.0 ± 0.1	5.3 ± 0.1 * †
Fasting Insulin (ρmol·l^{-1})	26.4 ± 3.5	38.2 ± 4.2 *	51.3 ± 5.6 *
Triglycerides (mmol·l^{-1})	0.97 ± 0.05	1.32 ± 0.10 *	1.55 ± 0.15 *
HOMA-IR	0.83 ± 0.12	1.25 ± 0.14 *	1.8 ± 0.2 * †
OGIS (ml·min·m^{-2})	533 ± 11	512 ± 9	451 ± 11 * †
AUC Glucose (mmol·l·min)	671 ± 18	738 ± 19 *	831 ± 36 * †
AUC Insulin (pmol·l·min)	20487 ± 2444	29585 ± 4285	42336 ± 4979 * †
hs-CRP (mg·L^{-1})	0.92 ± 0.18	0.92 ± 0.08	1.16 ± 0.15
Adiponectin (µg·ml^{-1})	9.9 ± 0.9	6.6 ± 0.5 *	4.8 ± 0.5 *
TRAIL (ρg·ml^{-1})	72.2 ± 5.4	81.6 ± 3.9	82.4 ± 6.9
sRANKL (ρg·ml^{-1})	3.4 ± 0.6	3.0 ± 0.4	2.9 ± 0.7

HOMA-IR (Matthews *et al.*, 1985), OGIS (Mari *et al.*, 2001), AUC Glucose (area under the glucose curve after 2 hr), AUC Insulin (area under the insulin curve after 2 hr), hs-CRP (high sensitivity C-Reactive Protein), TRAIL (TNF-related apoptosis inducing ligand), sRANKL (soluble receptor activator of NF-κβ ligand). Values are mean ± SEM. *$p < 0.05$ *vs.* Normal weight. †$p < 0.05$ *vs.* Overweight.

3.3.3 Osteoprotegerin

Circulating OPG was lower in males than in females (5.13 ± 0.20 vs. 6.07 ± 0.23 ρmol·l^{-1}, $p = 0.003$). There was a significant decrease in OPG in the obese compared with normal weight and overweight groups (Figure 3.2). Neither TRAIL nor sRANKL were significantly different

between BMI categories (Table 3.3). There was no significant relationship between OPG and age, however as previous studies have consistently shown a correlation between age and OPG (Khosla *et al.*, 2002), (Szulc *et al.*, 2001), (Kudlacek *et al.*, 2003), we controlled for age in addition to gender in all subsequent analysis. For correlation analysis, this was achieved in SPSS using the partial correlation function, which, in addition to examining the relationships between variables of interest, allows the user to control for the potential bias that may be caused by confounding variables such as gender, age, ethnicity etc. A similar method whereby potential covariates are adjusted for in SPSS is used when comparing between three or more groups (ANCOVA)
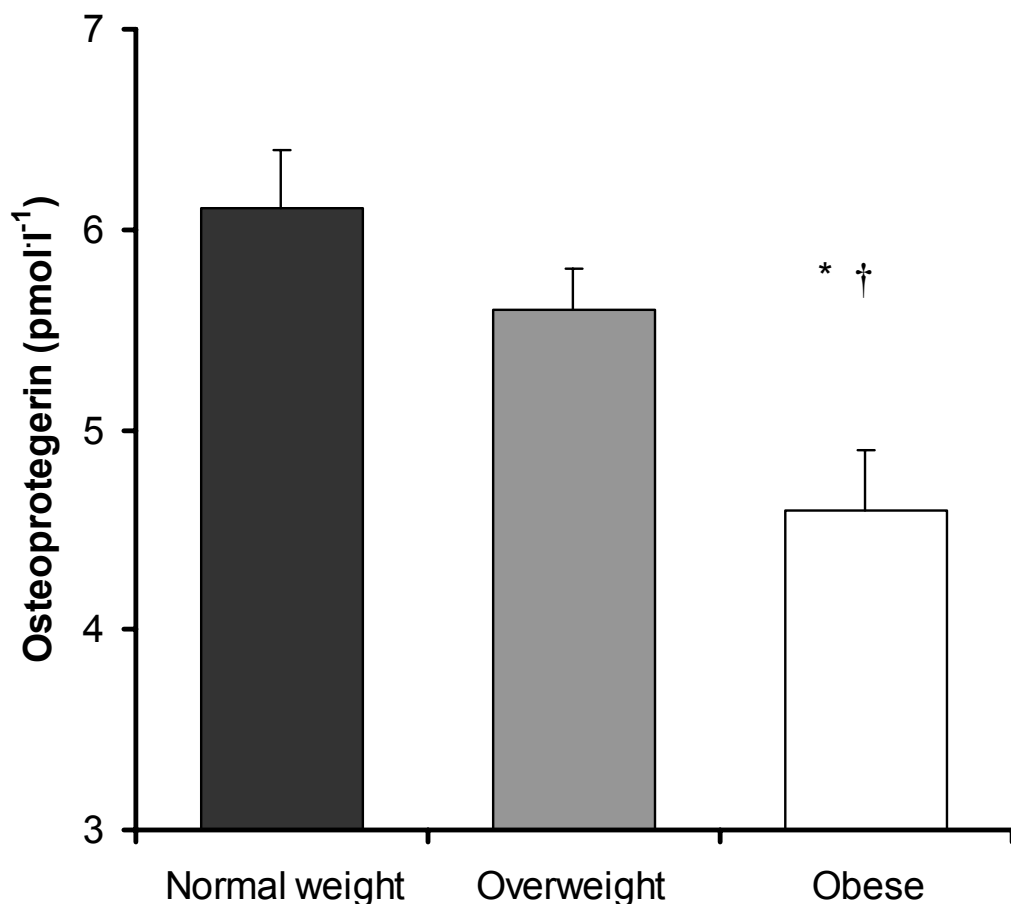
Figure 3.2 Osteprotegerin for Normal weight, Overweight and Obese subjects. Values are mean ± SEM.*p <0.05 vs. Normal weight, †p < 0.05 vs. Overweight.

3.3.4 Correlation Analysis

OPG showed a significant inverse correlation with BMI and waist circumference (Figure 3.3) and a positive relationship with VO_{2max}. There were also significant relationships between OPG and several other metabolic indices including a significant inverse correlation with fasting glucose,

fasting insulin, AUC glucose, AUC insulin, HOMA-IR and was positively correlated with OGIS and adiponectin (Table 3.4). The correlation between OPG and adiponectin persisted after additional adjustment for BMI. Controlling for age and gender, TRAIL was significantly related to fat mass ($r = 0.255$, $p < 0.05$) and waist circumference ($r = 0.207$, $p < 0.05$), these relationships were maintained after additional adjustment for BMI. ($r = 0.373$, $p < 0.001$), ($r = 0.257$, $p < 0.05$).
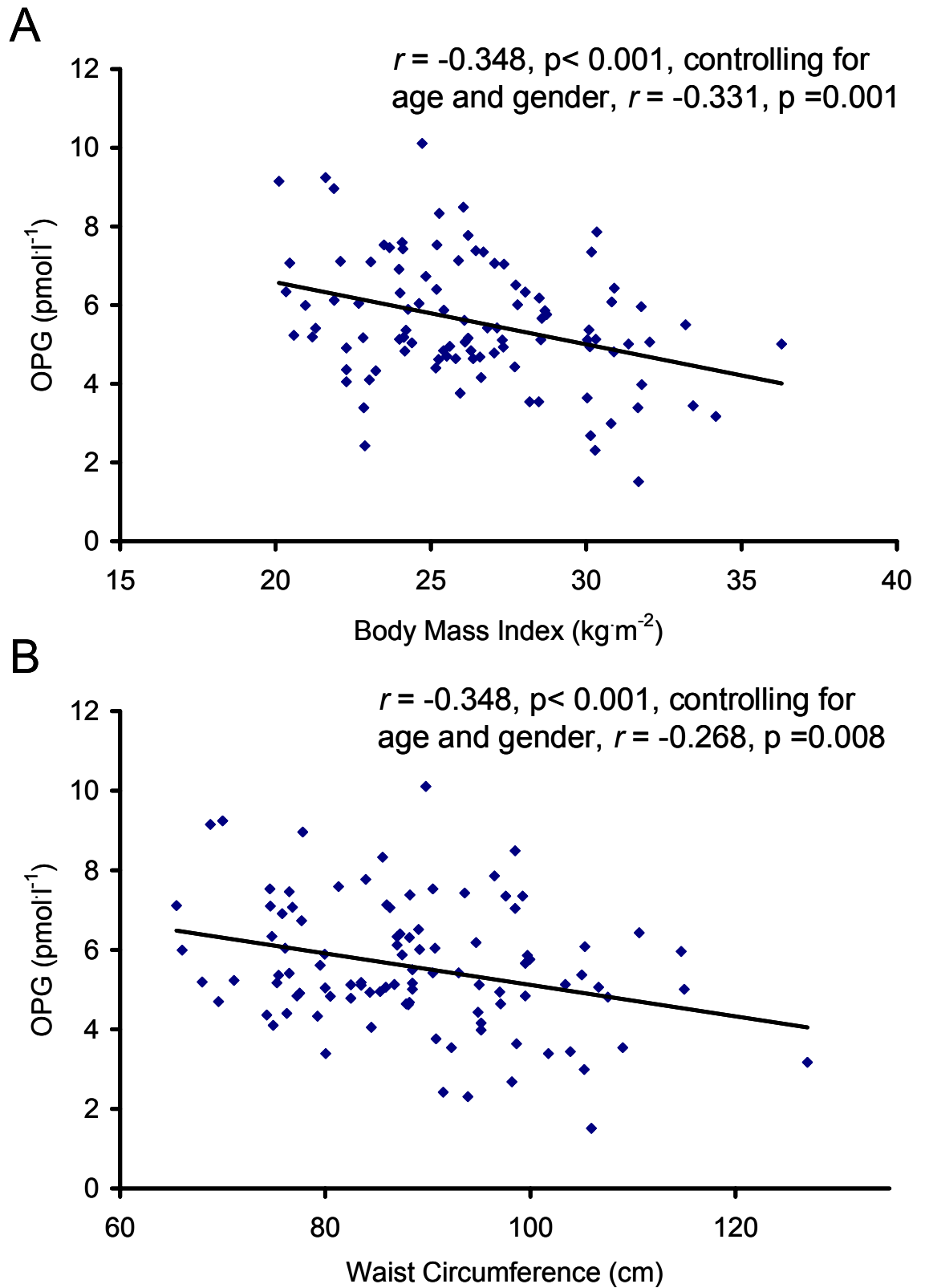
Figure 3.3 Relationship between A) OPG and BMI, B) OPG and waist circumference.

Table 3.4 Age and Gender Adjusted Correlations Between OPG and Anthropometric and Metabolic Indices.

	r	p
BMI (kg·m^{-2})	-0.331	***
Waist Circumference (cm)	-0.268	**
VO$_{2\ max}$ (ml·kg·min^{-1})	0.237	*
Fasting Glucose (mmol·l$^{-1)}$)	-0.248	*
Fasting Insulin (pmol·l^{-1})	-0.202	**
AUC Glucose (mmol·l·min)	-0.279	**
AUC Insulin (pmol·l·min)	-0.271	**
HOMA-IR	-0.222	*
OGIS (ml·min·m^{-2})	0.221	*
Adiponectin (µg·ml^{-1})	0.391	***

BMI (Body mass index), VO$_{2\ max}$, (maximal oxygen consumption), AUC Glucose (area under the glucose curve), AUC Insulin (area under the insulin curve), HOMA-IR (Matthews *et al.*, 1985), OGIS (Mari *et al.*, 2001). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.4 Summary

The main findings of Experiment I are that obese subjects who are normal glucose tolerant and free from cardiovascular disease, have lower circulating osteoprotegerin when compared with normal weight and overweight individuals. In this cohort OPG is positively correlated with adiponectin and insulin sensitivity.

Chapter IV The relationship between OPG, TRAIL, sRANKL and markers of inflammation in Type 2 Diabetes and Vascular Disease.

4.1 Introduction

Rationale

Type 2 diabetes mellitus is associated with an accelerated pathogenesis of atherosclerosis and a more than threefold increased risk of cardiovascular disease (Kannel & McGee, 1979). Arterial calcification is a prominent feature of atherosclerosis and common in patients with type 2 diabetes (Chen & Moe, 2003). It is an independent risk factor for cardiovascular mortality in both newly diagnosed (Niskanen *et al.*, 1994) and established type 2 diabetes (Lehto *et al.*, 1996). Arterial calcification of the tunica media was first identified almost a century ago, but our understanding that this may be an active, rather than a passive, carefully-regulated process has further developed in recent times with the identification of a possible role for the OPG/RANKL/TRAIL pathway in this process (Doherty *et al.*, 2004). Studies of serum RANKL have been inconclusive, with both increased (Kiechl *et al.*, 2007) and reduced (Schoppet *et al.*, 2003) risk of CVD disease being reported with elevated levels of RANKL concentration. Only one paper has measured RANKL levels in individuals with type 2 diabetes, finding no difference from healthy individuals (Secchiero *et al.*, 2006). TRAIL also appears to affect the vasculature and may contribute to plaque instability (Sato *et al.*, 2006), though others have shown that administration of TRAIL to atherogenic Apo E-/- mice induced plaque regression and stabilisation of residual plaques (Secchiero *et al.*, 2006). Whether serum OPG, RANKL and TRAIL are higher in patients with type 2 diabetes compared to non-diabetic individuals is still under investigation. Studies to date suggest higher serum OPG levels in type 2 diabetes but these studies have often mixed groups of diabetic and non diabetic patients, examined OPG in patients with diabetes related microvascular complications, and have poorly defined control groups with no attempt to control for underlying metabolic bone disease, which could affect serum OPG and RANKL levels. It is well-accepted that inflammation plays an important role in the pathogenesis of diabetes and IL-6 and hsCRP are frequently used to gain a measure of the degree of underlying inflammation (Wellen & Hotamisligil, 2005). Whether OPG/RANKL/TRAIL could be a reflection of low-grade vascular inflammation in individuals with diabetes is not known.

Aims

The aim of this experiment was to measure serum OPG/RANKL/TRAIL in a cohort of well controlled type 2 diabetic patients with no evidence of underlying metabolic bone disease and compare them to a healthy age and BMI control group. We also determined if any differences could be attributed to the presence of underlying vascular disease or inflammation.

Hypothesis

We hypothesised that OPG, along with other traditional inflammatory markers would be higher in type 2 diabetic patients. We also tested the hypothesis that OPG would be a sensitive marker of inflammation that would distinguish between diabetics and normoglycemic controls irrespective of prior history of vascular disease in these patients.

4.2 Materials and Methods

4.2.1 Experimental Design Overview

One Hundred and ten subjects volunteered to participate in this study. Fifty eight normoglycemic, healthy subjects free from CVD were recruited from Dublin City University and sixty two patients with type 2 diabetes were recruited from the diabetes clinic in Beaumont Hospital. A plain language statement was given to those expressing an interest in the study, after which a briefing meeting was scheduled to allow for questions to be asked about the study and written informed consent was provided by individuals wishing to participate. Subjects reported to the laboratory in the morning following an overnight fast at which point fasting blood samples were collected. Subjects were interviewed by a physician and had anthropometric measurements taken. Oral glucose tolerance tests (OGTT) to ensure normal glucose tolerance and exercise stress tests (see previous chapter for method) were performed on all of the healthy controls to rule out undiagnosed hyperglycaemia or CVD Full clinical history and physical examination were performed on all study subjects. The study was approved by the Dublin City University Research Ethics and Beaumont Hospital Ethics Committee and conformed to the Declaration of Helsinki. Finally, each participant completed a health history questionnaire underwent a medical screening examination.

4.2.2 Assessment of Bone Mineral Density

Bone mineral density (BMD) was measured using the GE Lunar Prodigy 2 DEXA scanner (GE Medical Systems, UK). Participants were positioned as per manufacturer instructions and bone mineral density was reported as grams of bone mineral content (BMC) per projected area (g⋅cm^{-2}) The mean of the lumber spine (L1 – L4) and the total BMD at the femur were used to classify patients according to WHO criteria (Brown & Josse, 2002). A sub-group of 66 participants (53% and 57% of those with and without diabetes respectively) underwent DEXA scanning. It was originally intended to conduct a DEXA scan on all participants in this experiment, however due to serious flooding in August 2008 the DEXA scanner was rendered unusable. A new DEXA scanner was acquired, but after initial quality control measures it was found that there were significant irregularities in the results being produced. It was felt at this time that the results measured on the two separate machines were not comparable and therefore only those scans taken on the original machine were used for analysis.

4.2.3 Statistical Procedures

SPSS 15.0 for Windows (SPSS Inc., USA) was used for statistical analysis. Data are reported as means ± SEM. Non-normally distributed variables including (fasting plasma glucose, IL-6, and hsCRP) were log-transformed for the purpose of analysis. Differences between groups were assessed using the unpaired Student t-test. The degree of relationship was calculated using Pearson's product moment (r). All data presented are adjusted for age and gender. Multiple linear regression analysis was performed with OPG as the dependent variable and age, gender, BMI, waist circumference, blood pressure, fasting glucose, total and LDL cholesterol, TRAIL, hsCRP, IL-6 as the independent variables. A $p<0.05$ was taken as indicative of statistical significance. Statistical analysis was carried out using SPSS statistical package (version 15.0; SPSS Inc., Chicago IL, USA).

4.2.4 Subject Characteristics

Demographics for the type 2 diabetic patients and healthy controls are described in Table 4.1.

Patients were matched for age, gender and BMI.

Table 4.1 Subject Characteristics

	Normal	Type 2 Diabetes
N	58	62
Age (years)	55.6 ± 1.2	58.31.2
Gender (M:F)	28:30	40:22
BMI (kg/m^2)	29.5 ± 0.4	30.0 ± 0.5
Waist Circumference (cm)	101.3 ± 1.4	105.0 ± 1.3 ~

BMI (Body mass index), Values are mean ± SEM. ~ p = 0.06

4.3 Results

Subject Metabolic and Cardiovascular Characteristicsare presented in Table 4.2. Waist circumference, systolic blood pressure, medication use (anti-hypertensive, statin, ACE inhibitor/ARB, and aspirin use), fasting plasma glucose and triglycerides were all significantly higher in diabetics, while total HDL and LDL cholesterol were lower. Characteristics of the disease state, medication and complications of the type 2 diabetics are presented in Table 4.3.

Table 4.2 Subject Metabolic and Cardiovascular Characteristics.

	Normal	Type 2 Diabetes
N	58	62
Waist Circumference (cm)	101.3 ± 1.4	105.0 ± 1.3 ~
Systolic BP (mmHg)	131.14 ± 2.53	142.3 ± 2.26 ***
Diastolic BP (mmHg)	81.8 ± 1.4	80.26 ± 1.26
Current smokers	4.7%	9.8%
Anti-hypertensive use	13.2%	86.7% ***
ACE/ARB use	8.6%	67.2% ***
Statin use	15.1%	82.0% ***
Aspirin use	5.7%	78.7% ***
Fasting glucose (mmol·l^{-1})	5.2 ± 0.2	7.9 ± 0.2 ***
Total Cholesterol (mmol·l^{-1})	5.4 ± 0.1	4.1 ± 0.1 ***
LDL Cholesterol (mmol·l^{-1})	3.4 ± 0.1	2.0 ± 0.1 ***
HDL Cholesterol (mmol·l^{-1})	1.4 ± 0.04	1.2 ± 0.04 ***
Triglycerides (mmol·l^{-1})	1.4 ± 0.1	2.0 ± 0.1 ***
TNF-α (pg/ml)	1.5 ± 0.3	1.4 ± 0.3

BP (blood pressure), LDL (Low density lipoprotein), HDL (Hofbauer *et al.*, 2002), TNF-α (Tumour necrosis factor alpha) Values are mean ± SEM, *$p < 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* Type 2 diabetes

Table 4.3 Characteristics of the Disease State in Patients with Type 2 Diabetes.

	Median (range) or n (%)
Duration of diabetes (years)	7 (1-20)
HbA1c (%)	7 (5.1-10)
Insulin treated	12 (19.67%)
Metformin treated	37 (60.66%)
Sulphonylurea treated	21 (34.43%)
TZD treated	3 (5.92%)
Diet alone	8 (13.12%)
Microvascular complications	15 (24.19%)
Macrovascular complications	20 (32.26%)

HbA1c (haemoglobin A1c), TZD (Thiazolidinediones)

4.3.3 Effect of Glycaemic Status on Inflammatory Markers

OPG (5.7 ± 0.2 *vs.* 4.9 ± 0.2 pmol$^{.}$l^{-1}; $p < 0.05$), IL-6 (3.0 ± 0.4 *vs.* 1.8 ± 0.4 pg$^{.}$ml^{-1}, $p < 0.05$) and hsCRP (2.2 ± 0.3 *vs.* 1.6 ± 0.3 mg$^{.}$L^{-1}, $p < 0.05$) were significantly higher in type 2 diabetic patients and adiponectin was significantly lower (4.8 ± 0.5 *vs.* 7.2 ± 0.5 µg$^{.}$ml^{-1}; $p < 0.05$) compared to the healthy non-diabetic controls and there were no differences in serum TRAIL or RANKL between those with or without diabetes (Figure 4.1).
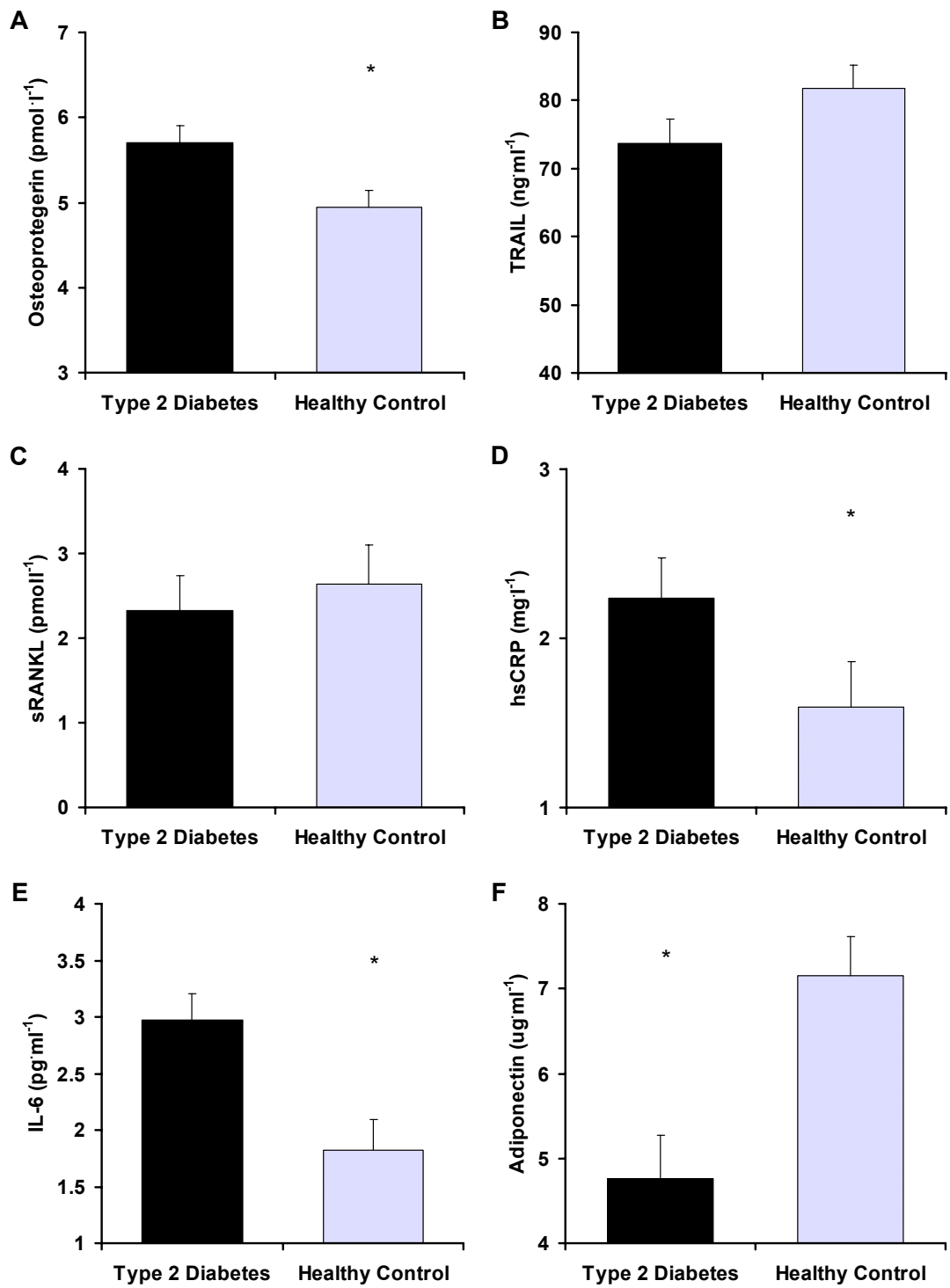
Figure 4.1 Circuling concentrations of (A) Osteoprotegerin, (B) TRAIL, (C) sRANKL (D) hsCRP, (E) IL-6 and (F) Adiponectin in Type 2 diabetics and in age and BMI matched normoglycemic healthy controls. Data are presented as mean ± SEM. *p < 0.05 vs. Type 2 diabetes.

4.3.4 Gender Breakdown

In the entire group, OPG (5.77 ± 0.26 *vs.* 5.05 ± 0.18 pmol·l⁻¹, *p* < 0.05) and adiponectin (7.80 ± 0.66 *vs.* 4.23 ± 0.37 µg·ml⁻¹, *p* < 0.0001) were higher in women than men. There were no significant gender differences for TRAIL, hsCRP, sRANKL or IL-6. OPG was correlated with age (r = 0.247, p < 0.05).

4.3.5 Correlation Analysis

TRAIL was correlated with LDL (r = 0.303, p < 0.01) and waist circumference (r = 0.202, p < 0.05). In the healthy control group, controlling for gender there was a correlation between OPG and age (r = 0.364, p < 0.01). When both age and gender were controlled for, OPG was correlated with waist circumference (r = -0.262, p < 0.05), adiponectin (r = 0.366, p < 0.01), total cholesterol (r = 0.380, p < 0.01), LDL (r = -0.336, p < 0.05), fasting plasma glucose (r = -0.363, p < 0.05) and showed a strong tendency towards a significant inverse correlation with TRAIL (r = -0.294, p = 0.053), Left Hip BMD (r = -0.320, p = 0.079) and right hip BMD (r = -0.326, p = 0.074). None of these relationships with OPG were evident in the type 2 diabetic cohort. TRAIL was however correlated with LDL (r = 0.325, p < 0.05)

4.3.6 Subset Analysis on the Effect of Vascular Disease on Inflammatory Markers

To investigate whether the elevated OPG, IL-6 and hsCRP observed in the diabetes group was due to the higher prevalence of vascular disease within this group, we compared mean values of these proteins, after exclusion of type 2 diabetes patients with either micro- or macro-vascular disease (Table 4.4). OPG was still significantly higher in those with diabetes (5.68 ± 0.25 *vs.* 4.93 ± 0.2 pmol·l⁻¹, p < 0.05) than normal controls, while the significant difference previously seen with IL-6 (2.29 ± 0.26 *vs.* 1.95 ± 0.23 pg·ml⁻¹, p = 0.24) and hsCRP (1.93 ± 0.27 *vs.* 1.59 ± 0.24 mg·L⁻¹, p = 0.37) were now lost. In this group OPG correlated with IL-6 after correction for age and gender (r = 0.24, p < 0.05), but this association was lost after correction for glycaemic status. There was no correlation between RANKL or TRAIL and IL-6, or hsCRP in either group.

Table 4.4 OPG, RANKL, TRAIL, IL-6 and hsCRP in Type 2 diabetics and Healthy Controls in subjects free from vascular disease.

	Type 2 Diabetes	Normal
N	38	58
Age (years)	56.0 ± 1.6	55.6 ± 1.2
Gender (M:F)	23:15	28:30
BMI (kg·m^{-2})	30.61 ± 0.6	29.48 ± 0.42
OPG (pmol·l^{-1})	5.68 ± 0.25	4.93 ± 0.20*
TRAIL (ng·ml^{-1})	76.74 ± 4.33	82.19 ± 3.77
RANKL (pmol·l^{-1})	2.25 ± 0.47	2.60 ± 0.43
IL-6 (pg·ml^{-1})	2.29 ± 0.26	1.95 ± 0.23
hsCRP (mg·l^{-1})	1.93 ± 0.27	1.59 ± 0.24
TNF-α (pg·ml^{-1})	1.42 ± 0.28	1.43 ± 0.26

BMI (Body mass index), TRAIL (TNF-related apoptosis inducing ligand), sRANKL (soluble receptor activator of NF-κβ ligand), Interleukin 6 (IL-6), hsCRP (high sensitivity c-reactive protein), TNF-α (Tumour necrosis factor alpha). Values are mean ± SEM. * $p < 0.05$ *vs.* Type 2 Diabetes

4.3.7 Bone Mineral Density and markers of inflammation

In the subset of 66 participants (53% and 57% of those with and without diabetes respectively) that underwent DEXA scanning, there was no significant difference between OPG, adiponectin, sRANKL and TRAIL between those who were osteopaenic and those who had BMD in the normal range.

4.4 Summary

The main findings of Experiment II are that OPG but not RANKL or TRAIL is significantly increased in type 2 diabetes. IL-6 and hsCRP is higher in individuals with diabetes and adiponectin is lower, but unlike OPG, they are no longer different when subjects with vascular disease are excluded.

Chapter V The effect of Obesity, Glycaemic Status and an acute glucose load on circulating concentrations of OPG

5.1 Introduction

Rationale

Type 2 diabetes, impaired glucose tolerance and obesity are characterized by fasting and postprandial hyperinsulinaemia (Reaven *et al.*, 1993a), (Cavaghan & Polonsky, 2005) and insulin resistance. Unravelling the specific metabolic effects of elevated circulating insulin from failing insulin action remains challenging. However, several large, longitudinal studies have described a link between hyperinsulinaemia and the development of cardiovascular disease. (Pyorala *et al.*, 1985), (Welborn & Wearne, 1979), (Eschwege *et al.*, 1985). As well as its traditional glucose lowering role, insulin is a vasoactive peptide capable of exerting significant hemodynamic effects (Cersosimo & DeFronzo, 2006) including increased sympathetic activity, renal sodium retention, and vascular smooth muscle cell proliferation. (Goalstone *et al.*, 1998), (Kawasaki *et al.*, 2000). Indeed the activity of endothelial nitric oxide synthase, a potent vasodilator is increased several fold by insulin responsive cytokines such as IL-1β, IL-6, TNF-α, interferon-γ and adenosine (Landry & Oliver, 2001). The Framingham Offspring Study (Meigs *et al.*, 2000) showed that there was a consistent relationship between hyperinsulinaemia and the procoagulant state, which was evaluated by measuring PAI-1, tissue plasminogen activator, von Willebrand factor, fibrinogen, plasma viscosity, and factor VII antigen (Uwaifo & Ratner, 2003). Nevertheless, other studies have found that that arterial infusion of insulin leads to an up regulation of both endothelin, a vasoconstrictor, and the vasodilator nitric oxide (Cardillo *et al.*, 1999). Recent studies have suggested an important role for insulin in the inhibition of OPG expression and secretion. Olesen *et al.* (2005) demonstrated *in vitro* that human vascular smooth muscle cells incubated with insulin exhibit markedly reduced OPG production when compared to controls (Olesen *et al.*, 2005). One *in vivo* study which examined the effect of 6 months of insulin therapy in young type 1 diabetics on OPG levels and endothelial function via flow-mediated endothelium-dependent arterial dilation found that OPG decreased significantly in these patients and that this decrease was strongly correlated with the change in flow-mediated endothelium-dependant arterial dilation (Xiang *et al.*, 2007). Another very recent paper has also demonstrated an acute effect of insulin administration in reducing OPG secretion to the circulation in lean, type 2 diabetic and obese subjects during a euglycaemic-hyperinsulinaemic clamp. It was found that serum OPG was markedly reduced in all three groups and that the lean control group showed a significantly greater decrease than the type 2 diabetic and obese

subjects (Jorgensen *et al.,* 2009). Interaction between insulin and the OPG / RANKL / RANK / TRAIL axis may be one mechanism by which elevated fasting and postprandial hyperinsulinaemia can independently affect the development of cardiovascular disease.

Aims

The purpose of this study was to examine changes in circulating OPG levels with varying degrees of glucose tolerance and to investigate the influence of adiposity and inflammatory processes on OPG concentrations.

Hypothesis

We hypothesized that the deteriorating inflammatory state coupled with the sharp rise in hyperinsulinaemia with decreasing glucose tolerance would uncouple the relationship between OPG and insulin sensitivity that we observed in our previous experiments. We also propose that acute hyperinsulinaemia associated with an oral glucose load may act to suppress OPG secretion and that this regulation would be differentially regulated dependant of glycaemic status.

5.2 Materials and Methods

5.2.1 Experimental Design Overview

Sixty one male subjects were recruited to participate in this study. Twenty patients with type 2 diabetes and twenty male patients with either impaired glucose tolerance or impaired fasting glucose were recruited from the diabetes clinic in Beaumont Hospital. An additional twenty one healthy obese male subjects free from CVD were recruited from Dublin City University. All three groups were age and BMI matched. In addition, data from twenty one lean age matched subjects who were part of the cohort used in the Experiment 1 were also included in the data set (Figures 5.1 and 5.4) for comparative purposes. Ethical approval was obtained from the Beaumont hospital and Dublin City University Research Ethics Committee. Volunteers were excluded if they had evidence of malignancy, renal impairment (serum creatinine >120 μmol·l^{-1}), type 1 diabetes, pregnancy, any disorder of calcium metabolism (i.e. hyper- or hypo-calcaemia),

previous diagnosis of osteoporosis or use of medications affecting bone metabolism (i.e. calcium, vitamin D, bisphosphonates, oestrogen preparations, strontium, parathyroid hormone), recent (within previous 6 months) history of a macrovascular event (defined as an acute coronary syndrome, transient ischaemic attack, stroke, lower limb ischaemic event or any vascular interventional procedure), and osteoporosis on DEXA scan. Subjects reported to the laboratory in the morning following an overnight fast at which point fasting blood samples were collected. Oral glucose tolerance tests (OGTT) to assess glycaemic status were carried out on all subjects. Subjects were interviewed by a physician and had anthropometric measurements taken. A full clinical history and physical examination were performed by a medical doctor on all study subjects.

5.2.2 Assessment of Glycaemic Status

Subjects were assigned to a category of either normoglycaemia (NGT Obese), impaired glucose tolerance/impaired fasting glucose (IFG / IGT) or type 2 diabetes based on previous clinical history and WHO guidelines for an OGTT (Reinauer *et al.*, 2002). In brief; subjects were classified with impaired fasting glucose if they had fasting blood glucose between 6.1 and 6.9 mmol·l^{-1}. They were considered to have impaired glucose tolerance if their fasting plasma glucose was ≤ 7.0 mmol·l^{-1} and their 2 hr plasma glucose was between 7.8 and 11.0 mmol·l^{-1}. They were adjudged to be diabetic if either their fasting glucose was ≥ 7.0 mmol·l^{-1} and/or their 2 hr plasma glucose was ≥ 11.0 mmol·l^{-1}. Normal glucose tolerance (NGT) was considered to be a fasting plasma glucose < 6.1 mmol·l^{-1} and a 2 hr plasma glucose less than 7.8 mmol·l^{-1} in conjunction with a medical examination and interview which did not show any prior history of glycaemic dysfunction. All blood sampling and biochemical analysis were carried out as described previously in section 3.2.6 and 3.2.7.

5.2.3 Statistical Procedures

SPSS 15.0 for Windows (SPSS Inc., USA) was used for statistical analysis. Data are reported as means ± SEM. Normally distributed variables were explored using simple bivariate or partial regression. Non-normally distributed variables were log-transformed. A one-way analysis of covariance (ANCOVA) was used to examine differences between glycaemic categories with

age as a covariate. Bonferroni's post hoc test was applied to determine differences among means. Statistical significance was set at $p < 0.05$.

5.2.4 Subject Characteristics

Age and BMI for all subjects are presented in Table 5.1.There was no significant difference between groups for age or BMI.

Table 5.1 Subject Characteristics

	Type 2 Diabetes	IGT / IFG	NGT Obese
	(20)	(20)	(21)
Age (years)	56.2 ± 2.1	56.8 ± 2.3	53.8 ± 2.2
BMI ($kg^.m^{-2}$)	30.5 ± 0.6	30.5 ± 0.8	30.2 ± 0.5

BMI (Body mass index)

5.3 Results

5.3.1 Markers of Insulin Sensitivity

Indicators of metabolic function and insulin sensitivity are presented in Table 5.2 for the three subject groups. In order to assess the potential influence of adiposity and relative insulin resistance on circulating OPG levels in clinically normal glucose tolerant subjects, twenty one lean age matched males from experiment 1 were included later for further analysis (Figure 5.1)

Table 5.2 Subject Characteristics and indicators of insulin sensitivity.

	Type 2 Diabetes	IGT / IFG	NGT Obese
	(20)	(20)	(21)
Fasting Glucose (mmol·l$^{-1)}$)	7.4 ± 0.2	6.2 ± 0.1 *	5.2 ± 0.1 * †
Fasting Insulin (pmol·l^{-1})	118.8 ± 15.6	127.7 ± 12.6	39.9 ± 4.3 * †
2 hr Glucose (mmol·l^{-1})	12.6 ± 0.7	8.6 ± 0.3 *	5.1 ± 0.3 * †
2 hr Insulin (pmol·l^{-1})	493.4 ± 74.5	526.8 ± 55.2	39.6 ± 24.6 * †
HOMA-IR	6.4 ± 0.8	5.8 ± 0.6 *	1.6 ± 0.2 * †
OGIS (ml·min·m^{-2})	289 ± 10	332 ± 10	424 ± 9 * †
AUC Glucose (mmol·l·min)	1593 ± 62	1218 ± 30 *	816 ± 32 * †
AUC Insulin (pmol·l·min)	64475 ± 9176	60693 ± 6032	29677 ± 3492 * †

NGT (Normal Glucose Tolerance), HOMA-IR (Matthews *et al.*, 1985), OGIS (Mari *et al.*, 2001), AUC Glucose (area under the glucose curve), AUC Insulin (area under the insulin curve). Values are mean ± SEM. * $p < 0.05$ *vs.* Type 2 Diabetes, † $p < 0.05$ vs. IGT / IFG.

Characteristics of the disease state and medication of the type 2 diabetics are presented in Table 5.3

Table 5.3 Characteristics of the Disease State in Patients with Type 2 Diabetes.

	Median (range) or n (%)
Duration of diabetes (years)	5 (1-13)
Insulin treated	3 (15%)
Metformin treated	16 (80%)
Sulphonylurea treated	5 (25%)
TZD treated	1(5%)
Diet alone	2 (10%)

TZD (Thiazolidinediones)
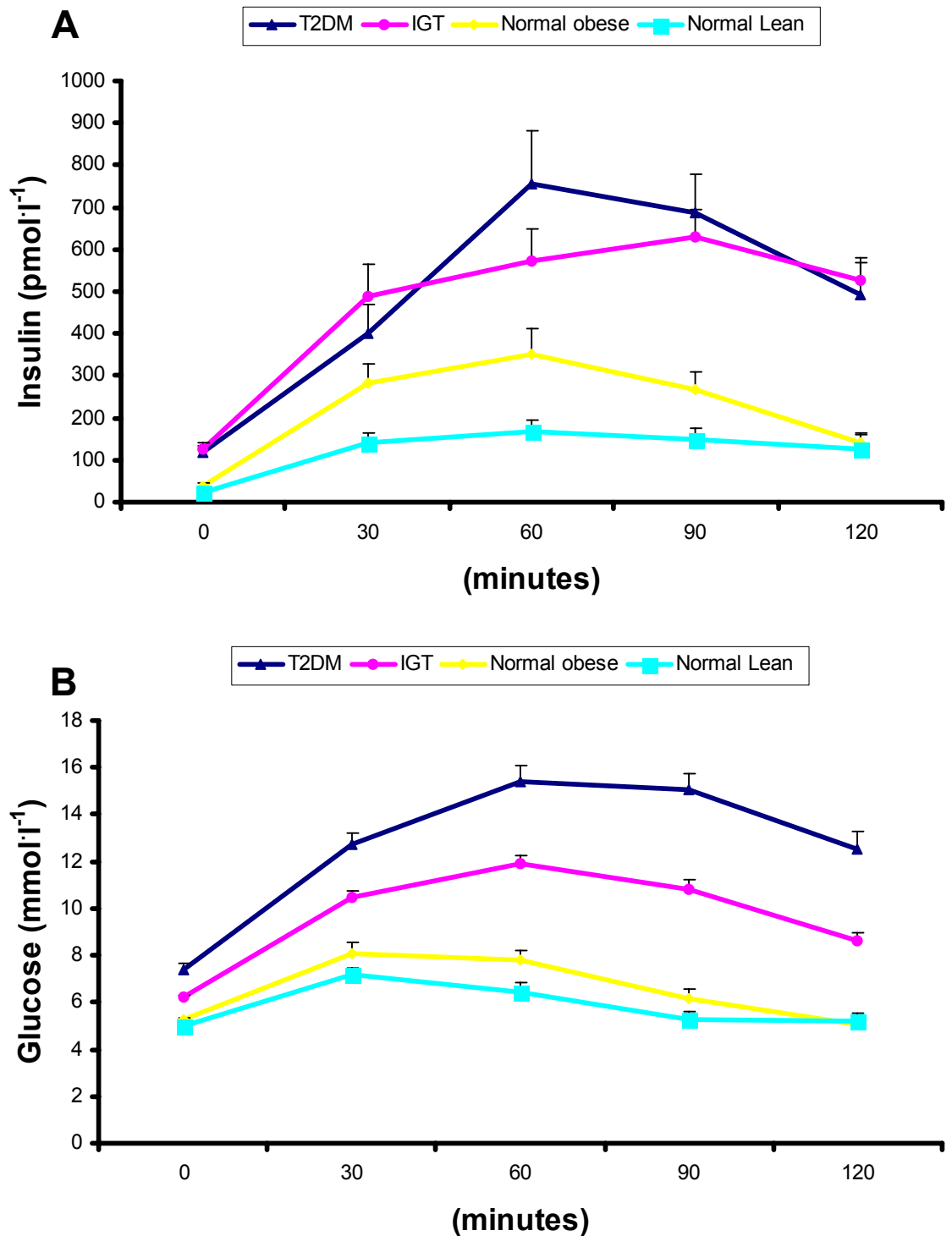
Figure 5.1 Insulin (A) and Glucose (B) kinetics in response to a 75 g Oral Glucose Tolerance Test in Type 2 diabetics ▲ , those with Impaired Glucose Tolerance or Impaired Fasting Glucose (Pomplun *et al.*, 2007) ♦ , age and BMI matched normoglycemic controls ● and a lean age matched control group ■.

5.3.2 Insulin, Glucose, OPG and hsCRP Kinetics in Response to the OGTT

The type 2 diabetics and the IGT/IFG group had significantly higher plasma insulin concentrations at all time points than both the age and BMI matched group or the lean age

matched group $p < 0.01$. There was no significant difference in plasma insulin levels at any time point between the type 2 diabetics and IGT/IFG group or between the two normoglycemic groups. Plasma glucose was significantly higher in the type 2 diabetic group at all time points ($p < 0.01$) and IGT/IFG plasma glucose levels were significantly higher at all time points that the two normoglycaemic groups ($p < 0.01$). There was no significant difference in plasma glucose levels between the obese and lean normoglycaemic groups (Figure 5.1). However there were significant differences in fasting glucose, fasting insulin, HOMA-IR, OGIS as well other indicators of insulin sensitivity and glycaemic control between the groups (Table 5.1). There was no significant change in hsCRP in response to the OGTT in any of the glycaemic conditions. OPG was significantly reduced in response to the oral glucose load in the IFG / IGT but was unchanged in type 2 diabetics and normoglycemic obese subjects (Figure 5.2).

5.3.3 hsCRP and Insulin Sensitivity

OGIS (424 ± 9 *vs.* 332 ± 10, $p < 0.05$ and *vs.* 289 ± 10 ml$^.$min$^.$m^{-2}, $p < 0.05$) and adiponectin (4.3 ± 0.3 *vs.* 3.1 ± 0.3, $p < 0.05$ and *vs.* 2.9 ± 0.2 µg$^.$ml^{-1}, $p < 0.05$) were significantly higher in healthy age and BMI controls than in either IGT / IFG subjects or type 2 diabetics. hsCRP was significantly lower in the healthy matched control group (1.2 ± 0.2 *vs.* 4.6 ± 0.9 mg$^.$L^{-1} $p < 0.05$) and the IGT / IFG group (2.4 ± 0.7 *vs.* 4.6 ± 0.9 mg$^.$L^{-1}, $p < 0.05$). There was no significant difference in hsCRP levels between the healthy matched control group and the IGT / IFG subjects. OPG (4.7 ± 0.3 *vs.* 6.0 ± 0.5, $p < 0.05$ and *vs.* 6.3 ± 0.3 pmol$^.$l^{-1}, $p < 0.05$) was significantly lower in healthy controls than in either IGT / IFG subjects or type 2 diabetics. There was no significant difference in OPG levels between the type 2 diabetics and IGT/IFG groups (Figure 5.1). In order to assess the independent effect of obesity on circulating OPG levels, a fourth group of age matched lean controls from Experiment 1 were included for analysis. OPG levels in this group of age matched normoglycemic lean males were then compared to the other groups. The lean control group had significantly higher levels of OPG than the obese normoglycemic group, however there was no significant difference between this group (6.0 ± 0.3 pmol.$^{l-1}$) and either the type 2 diabetics (6.3 ± 0.3 pmol$^.$l^{-1}) or the IGT / IFG groups (6.0 ± 0.5 pmol$^.$l^{-1}) (Figure 5.2). This finding which may be explained by the relative fasting and postprandial hyperinsulinaemia, that although not of clinical significance may still be sufficient to suppress OPG production and secretion and in the absence of a significant inflammatory state

to give impetus to increased OPG production as indicated by what would be considered normal hsCRP levels.
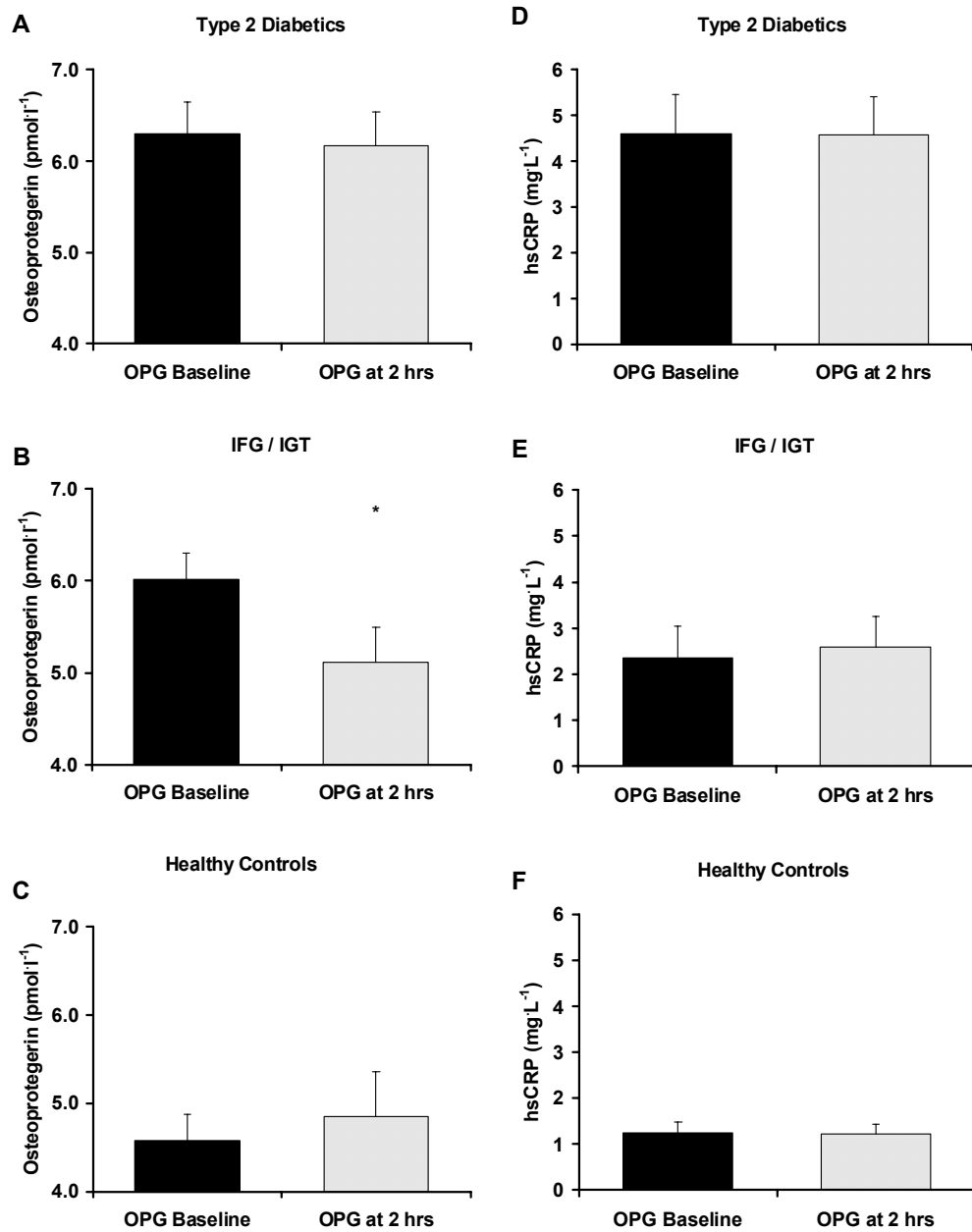
Figure 5.2 Circulating concentrations of Osteoprotegerin (A), (B), (C) and hsCRP (D), (E), (F) in Type 2 diabetics, pre-diabetic (IFG/IGT) and normoglycemic obese controls at baseline and 2hrs after a 75 g oral glucose load (OGTT).
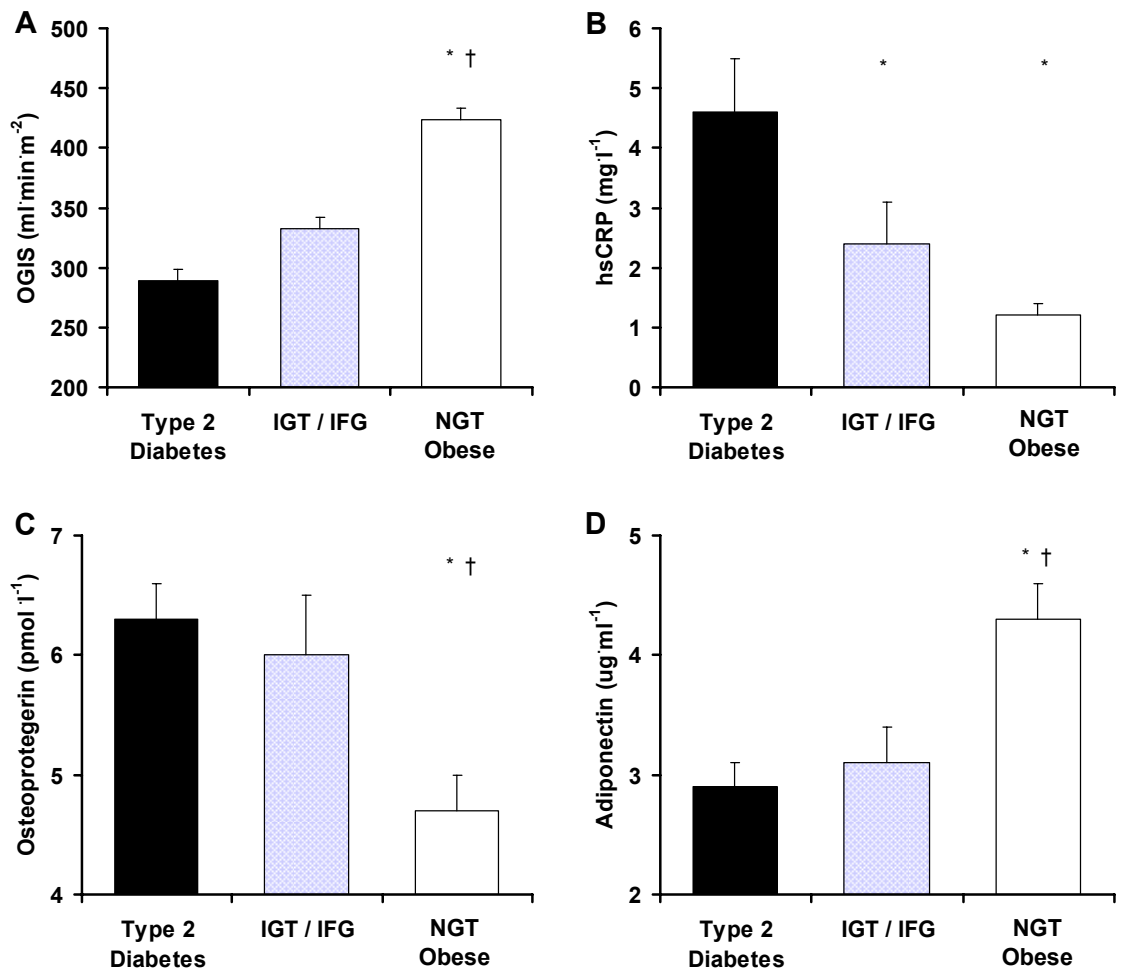
Figure 5.3 Differences in markers of insulin sensitivity and systemic inflammation in Type 2 diabetics, those with Impaired Glucose Tolerance (Pomplun *et al.*, 2007) or Impaired Fasting Glucose (IFG) and age and BMI matched normoglycemic controls (A) Oral Glucose Insulin Sensitivity (OGIS), (B) high sensitivity C-reactive Proteins (hsCRP), (C) Osteprotegerin (D) Adiponectin. *$p < 0.05$ vs. Type 2 diabetes, † $p < 0.05$ vs. IGT / IFG.
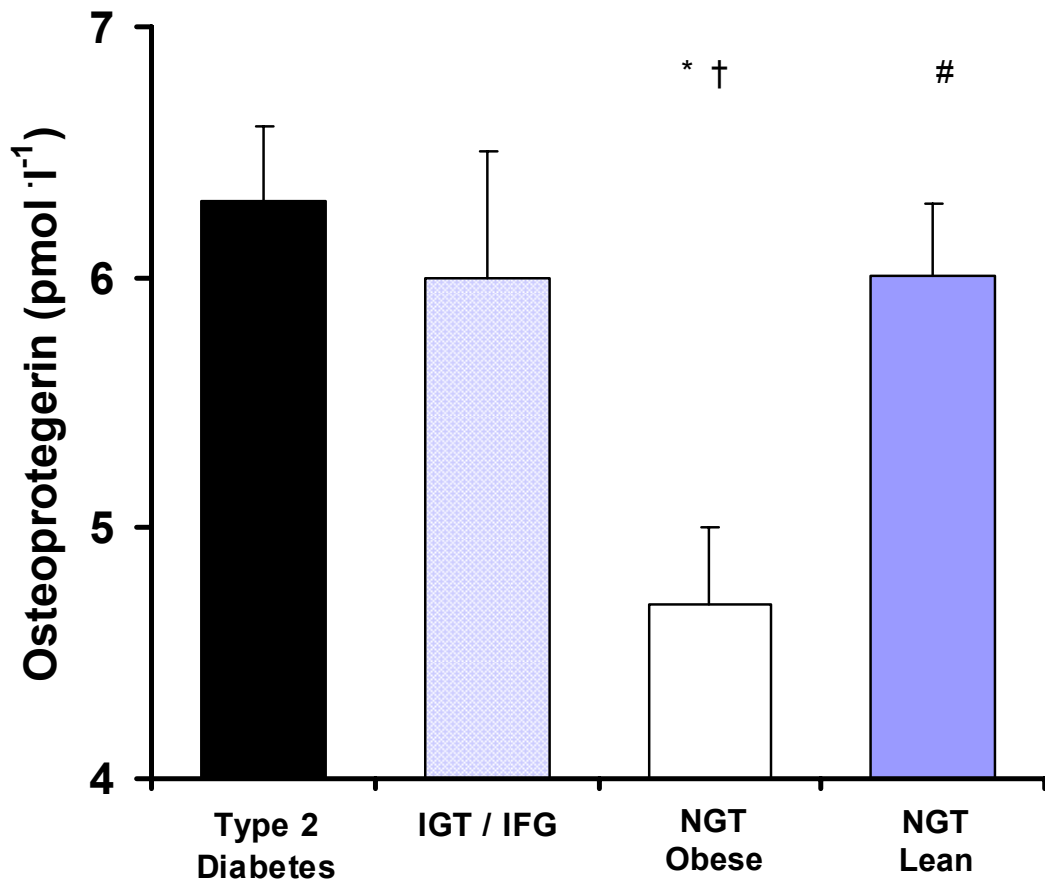
Figure 5.4 Osteprotegerin in Type 2 diabetics, those with Impaired Glucose Tolerance or Impaired Fasting Glucose (IGT/ IFG), age and BMI matched normoglycemic subjects (NGT Obese) and an age matched normoglycemic lean control group (Nissen & Sharp, 2003).

5.4 Summary

The main findings of Experiment III are that there is no difference in OPG between prediabetic and type 2 diabetic cohorts, but both have higher levels than matched obese controls. Interestingly, OPG in lean insulin-sensitive subjects is comparable to that of the pre-diabetic and type 2 diabetic patients but significantly higher than their matched lean counterparts.

Chapter VI General Discussion

The goal of this thesis was to investigate changes in Osteprotegerin concentrations in various stages of metabolic dysfunction, including obesity, insulin resistance and cardiovascular disease. The principle findings of the experimental studies presented here were as follows.

Experiment I

    I.   Obese subjects who have normal glucose tolerance and are free from cardiovascular disease have lower circulating levels of OPG than their lean age matched counterparts.

    II.   Osteoprotegerin is inversely correlated with insulin sensitivity, adiponectin and indicators of total body and visceral adiposity and positively correlated with aerobic fitness.

    III.   TRAIL is positively correlated with both fat mass and waist circumference, independent of age, gender and BMI.

Experiment II

    IV.   Osteprotegerin and IL-6 are significantly higher as and adiponectin significantly lower in type 2 diabetics than in age and gender matched normoglycemic controls, while there is no difference in TNF-α, TRAIL or sRANKL concentrations.

    V.   Osteoprotegerin is higher in type 2 diabetics after excluding patients with previously diagnosed vascular disease, a distinction which could not be made using traditional inflammatory markers such as IL-6, hsCRP or TNF-α.

Experiment III

    VI.   There is no difference in OPG concentrations between those with prediabetes and overt type 2 diabetes, however both conditions appear to have significantly higher levels of OPG than age and BMI matched obese normoglycemic controls.

    VII.   Interestingly, lean normoglycemic subjects have OPG concentrations which are similar to that of both pre-diabetic and type 2 diabetic patients but significantly higher than their matched obese counterparts.

The most significant finding from this series of experiments is the differential regulation of circulating OPG in obese and diabetic patients as presented in Figure 5.4. Circulating OPG has been reported to be significantly higher in patients with type 2 diabetes (Yaturu *et al.*, 2008), (Secchiero *et al.*, 2006), (Olesen *et al.*, 2005), (Rasmussen *et al.*, 2006), to correlate with the presence of coronary artery disease (Jono *et al.*, 2002), (Schoppet *et al.*, 2003), and to be an independent predictor of cardiovascular mortality (Browner *et al.*, 2001), (Kiechl *et al.*, 2004), (Ueland *et al.*, 2004), (Omland *et al.*, 2008). Indeed it has also been shown that OPG can independently predict silent coronary artery disease in type 2 diabetic patients (Avignon *et al.*, 2005). However, in the majority of studies the control groups are often age and BMI matched. Therefore, the assumption that circulating OPG progressively increases with increasing weight and insulin resistance may not be correct. There have been few published papers that have examined the relationship between adiposity, insulin sensitivity and OPG in a normal population free from overt cardio-metabolic disease. The findings from this thesis demonstrate an uncoupling of an insulin or insulin resistant mediated decrease in circulating OPG and suggest that elevated OPG in diabetic patients may be the result of inflammatory processes.

In Experiment I we tested the hypothesis that in a healthy cohort, in the absence of a significant inflammatory promoter, that OPG may be differentially regulated in obesity and, that OPG may be chronically related to fasting and postprandial insulin excursions as assessed by an oral glucose tolerance test. Our findings from Experiment I indicate that obese subjects with normal glucose tolerance who are free from cardiovascular disease as confirmed by an exercise stress test and medical examination have lower circulating OPG compared with matched normal weight and overweight individuals. We also observed a positive relationship between OPG and adiponectin which was mirrored by concomitant inverse relationships with glucose stimulated insulin secretion in addition to similar significant negative relationships with both fasting insulin and glucose. These findings would at first seem to be at odds with previous studies that have found a positive relationship between circulating OPG levels and metabolic dysfunction (Anand *et al.*, 2006), (Avignon *et al.*, 2005), (Knudsen *et al.*, 2003), (Terekeci *et al.*, 2009), (Browner *et al.*, 2001), (Jono *et al.*, 2002), (Kiechl *et al.*, 2004), (Schoppet *et al.*, 2003), (Ziegler *et al.*, 2005). A number of growth factors and inflammatory cytokines which are key players in the pathogenesis of atherosclerosis and coronary artery disease have also been implicated in the regulation of OPG in the vascular wall. Vascular endothelial cell-expression of OPG can be

induced by the addition of the inflammatory cytokines; TNF-α, IL-1α and IL-1β, (Secchiero *et al.*, 2006), (Ben-Tal *et al.*, 2007). In vascular smooth muscle cells, a number of cytokines have been shown to augment OPG *expression in vitro*, including TNF-α, IL-1β, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and angiotensin II (Collin-Osdoby *et al.*, 2001), (Olesen *et al.*, 2005), (Ben-Tal *et al.*, 2007), (Zhang *et al.*, 2002). A recent study by Olesen *et al.*, (2005) also suggested an important role for insulin itself in the inhibition of OPG expression and secretion and demonstrated *in vitro* that human vascular smooth muscle cells incubated with insulin exhibit markedly reduced OPG production when compared to controls (Olesen *et al.*, 2005). One *in vivo* study examined the effect of 6 months of insulin therapy in young type 1 diabetics on OPG levels and endothelial function and found that OPG decreased significantly (Xiang *et al.*, 2007). Another very recent paper has also observed a negative effect of acute insulin administration on OPG secretion to the circulation of lean, type 2 diabetic and obese subjects during a hyperinsulinaemic-euglycaemic clamp (Jorgensen *et al.*, 2009). By excluding subjects with metabolic or cardiovascular disease and therefore those with a significant underlying inflammatory process, we have demonstrated for the first time that subtle, sub-clinical, changes in fasting ambient insulin or insulin sensitivity coincide with a reduction in circulating OPG concentrations. Previously the role of obesity in the regulation of circulating osteoprotegerin has not been clear. Some studies report a decrease in OPG in obese subjects compared to lean controls (Ugur-Altun *et al.*, 2005), (Holecki *et al.*, 2007) but other studies have not found a relationship between OPG and BMI (Gannage-Yared *et al.*, 2006), (Gannage-Yared *et al.*, 2008), (Jorgensen *et al.*, 2009). Weight gain is accompanied by a number of metabolic alterations including a decrease in insulin sensitivity. Ugur-Altan *et al.*, (2005) divided a group of obese healthy subjects into tertiles based on insulin resistance as assessed using the HOMA-IR method and compared these groups to lean control subjects. When OPG was corrected for BMI it was significantly lower in all obese tertiles compared to lean controls. They also found that OPG was significantly lower in the least insulin sensitive obese tertile compared to the most sensitive. In Experiment I, insulin sensitivity was significantly lower in obese compared with overweight and normal weight groups and there was a positive relationship between OPG and insulin sensitivity for all subjects (Ugur-Altun *et al.*, 2005). We found an inverse relationship between fasting OPG and the area under the curve for glucose and insulin during the OGTT in support of recent studies suggesting that elevated insulin may be an important effector exerting downward pressure on circulating OPG concentrations. When Jorgensen *et al.*, (2009) reported

a decrease in OPG in response to hyperinsulinaemia, the magnitude of OPG-lowering effects of insulin was decreased by approximately 50 % in the obese and type 2 diabetic groups compared to lean controls (Jorgensen *et al.*, 2009). A decrease in OPG has also been reported in lean and morbidly obese subjects following an oral glucose tolerance test (Hofso *et al.*, 2009) and in lean males following a hyperglycaemic clamp (Knudsen *et al.*, 2007). In this study, Knudsen *et al.*, (2007) found that the decrease in OPG was related to the change in serum insulin and not glucose during the hyperglycaemic clamp. Therefore, subtle increases in fasting insulin secretion, as observed in Experiment I, may be adequate to decrease chronic OPG production in an obese normoglycemic cohort. If insulin sensitivity was a major regulator of serum OPG, exercise training or weight loss might be expected to induce a change in its circulating concentrations. We found a positive correlation between OPG and aerobic fitness which might suggest that exercise training may increase serum OPG in healthy subjects. However, other studies using a dietary restriction-induced weight loss intervention reported a further decrease in OPG (Holecki *et al.*, 2007) or no change as a result of gastric banding (Gannage-Yared *et al.*, 2008). No studies that we are aware of, have, as of yet examined the impact of exercise training on levels of circulating OPG.

The positive relationship between OPG and adiponectin was robust and maintained after additional correction for BMI. Adiponectin is an adipocyte-specific endocrine protein with anti-inflammatory and insulin sensitising actions. Circulating adiponectin is lower in obese subjects compared to lean controls and is also decreased with cardiovascular disease and type 2 diabetes. It is not yet known if adiponectin secretion and OPG appearance are directly related physiological processes *in vivo* or if the positive correlation between OPG and adiponectin reported here, and in other studies (Gannage-Yared *et al.*, 2006), (Gannage-Yared *et al.*, 2008), is evident only in healthy cohorts. Interestingly receptors for adiponectin are present in both osteoblasts and osteoclasts, suggesting that adiponectin influences bone metabolism in an autocrine / paracrine as well as an endocrine manner (Berner *et al.*, 2004), (Shinoda *et al.*, 2006). There is now also evidence of a similar role for OPG in adipose tissue. An *et al.*, (2007) demonstrated that OPG and RANKL are expressed in differentiating 3T3L1 adipocytes and that OPG mRNA expression in this cell model could be attenuated in response to insulin and increased in response to TNF-α, much the same as in the VSMC model (Olesen *et al.*, 2005). Interestingly, the treatment of these cells with the insulin-sensitizer, rosiglitazone, led to a dose-

dependant decrease in OPG mRNA (An *et al.*, 2007). Recombinant adiponectin increases bone resorption *in vitro* by reducing osteoblast formation through RANKL secretion and the inhibition of OPG production (Luo *et al.*, 2006). The majority of published human studies on the subject also indicate that adiponectin is a negative regulator of BMD in both men and women (Misra *et al.*, 2007), (Peng *et al.*, 2008), (Lenchik *et al.*, 2003), (Jurimae & Jurimae, 2007), (Richards *et al.*, 2007). This negative effect may be mediated by the promotion of bone resorption (Peng *et al.*, 2008). However, some studies have shown a positive effect of adiponectin on BMD (Tamura *et al.*, 2007) or no effect (Oh *et al.*, 2004). The relationship observed in Experiment I between OPG and adiponectin may be indicative of consistently elevated BMDs in obese relative to lean populations.

Circulating TRAIL and RANKL are ligands for the soluble OPG receptor. We found a positive relationship between TRAIL, fat mass and waist circumference but did not observe a difference across BMI categories. TRAIL may exert an effect at the level of the vascular wall and *in vitro* evidence suggests that it can promote apoptosis in vascular smooth muscle cells, leading to increased plaque instability (Sato *et al.*, 2006). On the other hand, OPG appears to promote endothelial cell survival (Cross *et al.*, 2006), (Malyankar *et al.*, 2000), possibly by inhibition of TRAIL-induced apoptosis (Pritzker *et al.*, 2004). We did not find any significant changes or relationships for the other OPG ligand, sRANKL. This may be due to the fact that our subject cohort were healthy as other studies have reported elevated RANKL to be associated with increased (Kiechl *et al.*, 2007) and decreased (Schoppet *et al.*, 2003) cardiovascular disease risk. This inconsistency may also result from different assay methodologies. Several studies have used commercially available assays that measure unbound and uncomplexed forms of both RANKL or OPG (Xiang *et al.*, 2006), (Knudsen *et al.*, 2003), (Rasmussen *et al.*, 2006), (Jorgensen *et al.*, 2009). The RANKL assay used in these experiments measured soluble RANKL (sRANKL) i.e. bound to OPG in addition to the free component. The OPG assay used in this body of work measured both total OPG, including OPG bound to RANKL and TRAIL and has been used to measure OPG in many cohorts (Gannage-Yared *et al.*, 2006), (Gannage-Yared *et al.*, 2008), (Anand *et al.*, 2006), (Schoppet *et al.*, 2003). It is problematic to compare the OPG concentrations from studies that have used different commercially available assays because of the difficulty in ascribing an exact molecular weight to the OPG-isoforms measured in different assays, thus making a conversion from non-SI to SI units problematic. Therefore,

previous studies that have exclusively measured uncomplexed OPG may have unintentionally excluded a large portion of the biologically active circulating OPG that has either bound to TRAIL or RANKL or indeed has undergone some other non-specific binding. Therefore changes in circulating free OPG observed in some previous studies in response to either insulin therapy or acute insulin infusion are as likely to reflect changes in the concentrations of known ligands, RANKL or TRAIL and thus their increased binding to OPG.

Type 2 diabetes mellitus is associated with accelerated atherosclerosis and a threefold increased risk of cardiovascular disease (Kannel & McGee, 1979). Arterial calcification, a prominent feature of atherosclerosis, is prevalent and extensive in patients with diabetes (Chen & Moe, 2003) and is an independent predictor of cardiovascular mortality in both newly diagnosed (Niskanen et al., 1994) and established type 2 diabetes (Lehto et al., 1996). OPG-knockout mice development osteoporosis and marked vascular calcification of the aorta and renal arteries (Bucay et al., 1998). Administration of recombinant OPG reverses arterial calcification (Min et al., 2000), (Price et al., 2001) and suggests that OPG plays an active role in the prevention of vascular calcification. The purpose of Experiment II was to measure serum OPG/RANKL/TRAIL in a cohort of well controlled type 2 diabetic patients with no evidence of underlying metabolic bone disease, comparing them to an equivalent age and BMI matched, normoglycemic, and healthy cohort and to determine whether any differences in OPG relate to the presence of underlying vascular disease or inflammation. We found that OPG, IL-6 and hsCRP (but not RANKL or TRAIL) were higher in patients with diabetes than in controls. OPG correlated with age and fasting glucose in healthy controls, but not in those with diabetes. After exclusion of diabetic subjects who had a previous history of vascular disease, OPG was still higher in those with diabetes, but IL-6 and hsCRP were no longer significantly different. Our findings from Experiment II indicate that OPG is significantly higher in diabetic patients, regardless of the presence or absence of diabetes related complications, when compared to an age and BMI matched control group with normal glucose tolerance. We did not find a significant difference between diabetics and healthy subjects for TNF-α irrespective of prior history of vascular dysfunction. Despite the fact that TNF-α is considered to be a potent promoter of OPG production, this result is perhaps not surprising as although increased TNF-α expression in obesity is evident in adipose tissue, muscle, and macrophages. Several studies, indicate that circulating TNF-α concentrations are not elevated in obese rodents and humans (Xu et al.,

2002), (Cawthorn & Sethi, 2008). This finding has suggested that adipose tissue does not release TNF-α systemically in great quantities, which might imply that TNF-α exerts its effects locally rather than in an endocrine manner. Analysis and interpretation of the circulating concentrations of TNF-α in both obesity and type 2 diabetes have been somewhat complicated by the fact that even when using ELISA based assays from the same manufacturer some studies have reported values based on the use of assays that have measured high sensitivity TNF-α (Ng *et al.*, 1999), (Plomgaard *et al.*, 2007) and others have reported values using standard TNF-α assays (MacEneaney *et al.*, 2009). It is likely that given the nature of the small changes in TNF-α observed in human studies that a high sensitivity assay such as that employed here would be more appropriate to assess circulating levels in healthy populations. For these reasons, after we found that there were no differences in TNF-α levels between the diabetic and normoglycaemic groups, we decided it would not be prudent to measure TNF-α prospectively in Experiment III or retrospectively in Experiment 1.

No difference was observed in sRANKL or TRAIL between the two groups, suggesting that their role as a biomarker, specifically of metabolic dysfunction may be limited. To the best of our knowledge, this is the first study to investigate the triumvirate of OPG/RANKL/TRAIL in such detail in patients with type 2 diabetes. Our findings for OPG in this cohort are in agreement with those of Xiang *et al.*, (2006) and Kim *et al.*, (2005) who found higher serum OPG in patients with diet controlled diabetes and no history of vascular disease (Xiang *et al.*, 2006), (Kim *et al.*, 2005). Both of these studies contained patients with newly-diagnosed diabetes, and it is not known the effect, if any, of glucose toxicity at diagnosis on serum OPG levels. Previous studies examining the relationship between OPG, RANKL, TRAIL and diabetes have had conflicting results. Some of the earlier studies investigating the role of OPG as a marker of cardiovascular disease did not include a homogeneous group of type 2 diabetics and no effort was made to exclude patients with underlying metabolic bone disease or who were on medications which could have interfered with bone metabolism (Schoppet *et al.*, 2003), (Browner *et al.*, 2001), (Kiechl *et al.*, 2004), (Omland *et al.*, 2008). This may be why an increase in circulating OPG has been consistently linked with the onset, progression and severity of cardiovascular disease (Jono *et al.*, 2002), (Schoppet *et al.*, 2003). OPG is an independent risk factor for incident cardiovascular disease (Kiechl *et al.*, 2004), (Ueland *et al.*, 2004), heart failure (Omland *et al.*, 2008), (Ueland *et al.*, 2004), all cause (Browner *et al.*, 2001) and vascular mortality (Kiechl *et*

al., 2004), (Omland *et al.*, 2008), (Ueland *et al.*, 2009). When the severity of vascular disease is assessed by coronary angiography, circulating OPG increases proportionally with the number of diseased vessels (Jono *et al.*, 2002), (Schoppet *et al.*, 2003). In patients with type 2 diabetes OPG is increased in some (Browner *et al.*, 2001), (Xiang *et al.*, 2006) but not all (Jorgensen *et al.*, 2009) studies. The reason for this may be related to the presence or absence of micro and macrovascular complications in the studied groups. In studies that used a non-diabetic control group, circulating OPG was similar between controls and diabetic patients without vascular complications (Knudsen *et al.*, 2003), (Terekeci *et al.*, 2009), while other studies have shown significantly higher OPG in diabetes patients with asymptomatic silent coronary artery disease (Avignon *et al.*, 2005), (Avignon *et al.*, 2007) or microvascular complications including microalbuminuria (Xiang *et al.*, 2006), retinopathy (Knudsen *et al.*, 2003) and neuropathy (Terekeci *et al.*, 2009). It is not known why circulating OPG is increased with type 2 diabetes and why this response is in contrast to obesity-related changes. It may be related to the increased presence of pro-inflammatory cytokines as the *in vitro* incubation of human vascular smooth muscle cells (Olesen *et al.*, 2005) and human microvascular endothelial cells (Secchiero *et al.*, 2006), (Collin-Osdoby *et al.*, 2001) with TNF-α , but not glucose, increases OPG production. This would also support the suggestion that OPG protects against the development of vascular damage as OPG-deficient mice have increased arterial calcification (Bennett *et al.*, 2006) that can be reversed following OPG replacement (Price *et al.*, 2001). In Experiment II when the diabetic and normoglycemic groups were combined and analyzed together, OPG correlated positively with age and adiponectin and was inversely related to waist circumference, total cholesterol and fasting plasma glucose. However, none of these relationships were found when the diabetic group were analyzed in isolation. The finding of an inverse relationship between adiponectin and OPG in the grouped data is reminiscent of our finding in Experiment I. It appears that diabetes, possibly as a result of the inflammatory state or ambient hyperinsulinaemia, may 'uncouple' the positive relationship between OPG and insulin sensitivity observed in healthy groups.

The role of insulin in this process is important but difficult to definitively prove in dynamic human experiments. The vasoactive role of insulin is mediated by the regulation of a number of important inflammatory cytokines that promote increase endothelial nitric oxide synthase activity (Landry & Oliver, 2001). Insulin may have an even wider role in the metabolic regulation of non

traditional tissues such as bone and the vascular endothelium. The relationship between glycaemic status and bone metabolism is complex and has been poorly understood. Despite higher BMD in diabetes, the risk of fractures in patients with type 2 diabetes is significantly increased (Carnevale *et al.*, 2004). Indeed recent observations suggest that bone resorption is acutely reduced in the postprandial period (Clowes *et al.*, 2002) and after an oral glucose load (Bjarnason *et al.*, 2002). Clowes *et al.*, (2002) demonstrated that a hyperinsulinaemic-hypoglycaemic clamp led to a significant reduction in markers of bone turnover. The purpose of Experiment III was to investigate if and how OPG changes with progressive insulin resistance. In addition, we sought to examine if an acute differential effect of an oral glucose load and the resultant transient hyperinsulinaemia on circulating OPG could be identified. Our findings indicate that there was no significant change in hsCRP, a traditional marker of inflammation in response to a glucose load in the total cohort or in any of the specific groups. When the total group was analyzed in Experiment III we did not find a difference between OPG at baseline and 120-min after the glucose challenge. Nevertheless OPG was positively correlated with AUC insulin and HOMA-IR which conflicts with our findings from Experiment 1. However, when glycaemic status was statistically controlled for using a partial correlation analysis, these relationships were lost.

There was no significant change in OPG levels in either the normoglycemic-obese category or in the type 2 diabetics. Interestingly, circulating OPG levels did decrease 2 hrs after glucose consumption in the IFG / IGT group. At baseline OPG was lower in the normoglycemic obese group than the other categories and there were no significant differences between the lean, type 2 diabetic, and IFG/IGT groups. We found that glucose intolerance was associated with lower adiponectin levels and higher hsCRP levels. The finding of increased adiponectin in obese-normoglycaemic relative to pre-diabetic and type 2 diabetics is supported by Hofso *et al.*, (2009) who also showed that adiponectin was lower in lean and morbidly obese normoglycemic subjects than pre-diabetic or new onset diabetes patients matched for age and BMI. Hofso *et al.*, (2009) also saw that lean subjects had significantly lower CRP levels than their obese and pre-diabetic counterparts and that diabetics had significantly elevated CRP levels compared to the other groups, a finding which is consistent with our observations in Experiment III. Interestingly using an assay which measures unbound OPG, the authors did not find any significant difference between groups for circulating fasting OPG which again may indicate an

inherent problem for reports that have studied OPG dynamics in that there are two widely used assays which measure very different forms of the glycoprotein yet attempt to draw comparable conclusions.

Taken together the results of this thesis would cast doubt on the generalized assumption of a progressive increase in circulating OPG with metabolic and cardiovascular disease. We suggest that the relationships observed in previous studies are specific and cogent only for the metabolic phenotype under investigation. Here we postulate one possible explanation for the often contradictory reports in the literature is that as an individual progresses from being lean and insulin sensitive to obese and relatively but NOT clinically insulin resistant, that fasting and postprandial hyperinsulinaemia suppress OPG production and secretion, as indicated by our findings in Experiment I and supported by others (Ugur-Altun *et al.*, 2005). As the individual progresses to develop type 2 diabetes the appearance of an array of inflammatory cytokines which have been demonstrated to promote OPG secretion from several tissue types becomes the dominant regulator of OPG appearance. We therefore suggest that in place of the previously proposed observation of a linear relationship between OPG and disease progression that a V shaped function is more likely (Figure 6.1), with decreasing OPG as a result of hyperinsulinaemia in obesity and elevated OPG occurring in response to the onset and the worsening state of chronic inflammation, a characteristic of progressive dysglycaemia, and as evidenced by our findings in Experiment III.
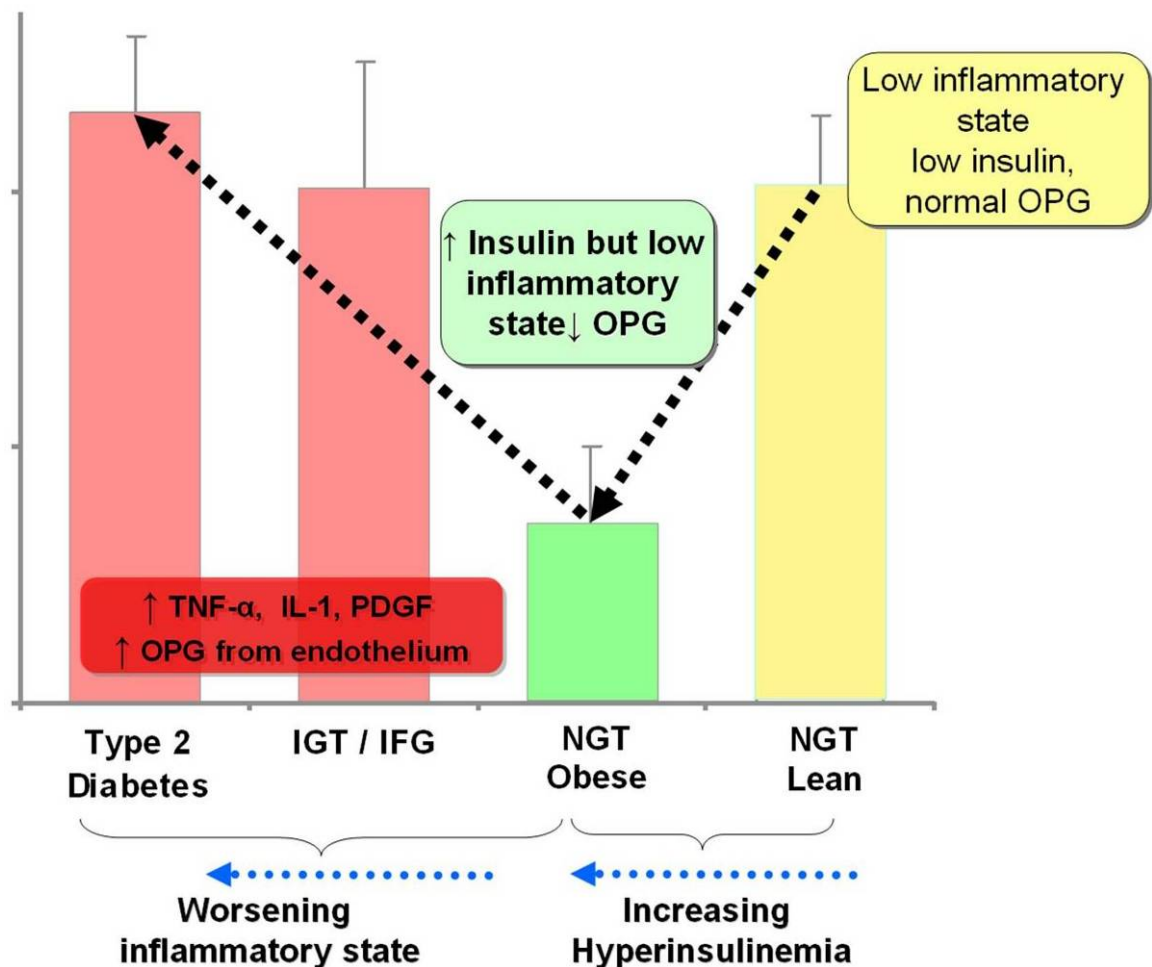
Figure 6.1 Postulated model for the V-shaped regulation of OPG in response to hyperinsulinaemia and the inflammatory process. The progression from lean and insulin sensitive to obese and hyperinsulinemic with a significant inflammatory process leads to an insulin-mediated suppression of OPG production. The inflammatory process associated with the development of type 2 diabetes leads to an increase in OPG secretion.

Increasingly it has been recognized that adipose tissue is an active metabolic tissue releasing cytokines such as adiponectin, TNF-α, IL-6, leptin and many others that have pleiotropic endocrine actions in the circulation, which can affect insulin sensitivity positively or negatively and contribute to vascular dysfunction. The vascular endothelium, like adipose tissue, can also produce and release glycoproteins yet little is known about the biological interaction of such factors. Although there have been significant advances in our understanding of these signalling mechanisms at the molecular and cellular level, we have not developed an integrated understanding of biological processes.

It appears that insulin reliably suppresses OPG production under a wide variety of *in vitro* and *in vivo* conditions (An *et al.*, 2007) and is also upregulated by a number of inflammatory mediators. Recombinant adiponectin reduces osteoblast formation via a mechanism whereby RANKL

mRNA production is increased and OPG decreased in cultured human osteoblasts (Luo *et al.*, 2006). There is also considerable epidemiological evidence that shows a consistent inverse relationship between adiponectin and BMD (Misra *et al.*, 2007), (Peng *et al.*, 2008), (Lenchik *et al.*, 2003), (Jurimae & Jurimae, 2007), (Richards *et al.*, 2007). When this is considered in conjunction with the fact that TNF-α appears to potently suppress adiponectin mRNA production in adipocytes (Ruan *et al.*, 2002), one can begin to see that OPG may be an excellent example of a protein who's regulation sits at the nexus of what were once considered to be disparate and insular metabolic tissues. Despite substantial advances in our comprehension of the integrative nature of metabolic homeostasis and dysfunction in health in disease in the last 20 years, we are still only beginning to understand the true extent of this integration. The findings in this thesis suggest that OPG production and secretion is subject to complex regulation by mediators produced from an array of tissue types, and that its perturbations in dysglycaemia, cardiovascular disease, arterial calcification as well as osteogenic disorders give evidence for a complex adipose-vascular-osteo-inusulinar axis.

There are a number of limitations to the present series of experiments and generally to the field of *in vivo* research into OPG. As has previously been discussed OPG secretion can be attributed to a number of cellular sources and tissues. All of the literature to date describing OPG in human models of disease has measured circulating OPG, which may be too crude a measure. Currently there is no method to categorically attribute circulating levels to OPG to a particular cellular source or indeed pathology, a criticism which is equally valid for the data presented here. As mentioned earlier in this manuscript there is a discrepancy in the type of assays used in such studies, where some report unbound OPG and others including the work presented here report total OPG, making meta-analysis of the full body of literature on the subject difficult. It would be useful if future studies that examined the effect of either therapeutic intervention or *in vitro* manipulation on OPG production were to measure both total and unbound OPG in addition to TRAIL and RANKL to assess the true dynamic nature of this family of molecules in response to a variety of stimuli. In conclusion, the results from this thesis suggest that an obesity-related decrease in insulin sensitivity or an increase in insulin secretion coincides with reduced circulating OPG in normoglycemic individuals. Furthermore, in this population OPG is positively correlated with insulin sensitivity and adiponectin. We also present evidence that these relationships are not maintained, possibly due to the systemic inflammation

found in pre-diabetes and type 2 diabetes, whereupon OPG is elevated relative to obese males with moderate hyperinsulinaemia but is not significantly different from lean males with low fasting insulin and negligible systemic inflammation. To What effect this fall in serum OPG, in association with increasing adiposity observed here, may have on vascular function in healthy subjects and why and how OPG increases in diabetic and non-diabetic patients with documented vascular disease appears to be complex and requires further study.

Chapter VII References

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Chaper VIII Appendices

DCU

Preparation for your Oral Glucose Tolerance Test

1. **You will be asked to attend room XB30 (Metabolic Research Unit) situated in the basement of the Science Block**

2. **You should ensure that your diet in the 3 days prior to your visit is unrestricted and rich in carbohydrates**

3. **You should not engage in any strenuous physical activity in the 24 hours prior to your visit to the lab**

4. **You should ensure that you have fasted for 12 hrs prior to your visit to the lab, consuming only water in this time and during the test.**

5. **You should ensure that you wear loose fitting comfortable clothes for the test**

6. **You should not take any medication on the morning of or during the test**

7. **You should refrain from smoking on the morning of or during the test**

Other notes:
We have set up an email station that you can use for work purposes during your Oral Glucose Tolerance Test. There are also a number of live network points in the room which you are free to make use of should you require them.

Check List

Consent signed	☐
OGTT	☐
Medical Screening	☐
Resting Blood Pressure	☐
Skin folds	☐
Circumferences	☐
Spirometry	☐
Resting ECG	☐
Aerobic fitness test	☐

Start Time: _____ Blood Pressure ___ / ___ D.O.B. ___ /____ /_____
      Sex: M / F

Room Temp _____ Pressure _____ mmHg Height:_____m Weight: _____kg

Pre test check list

Consent signed: ☐ 12 hr fast: ☐ Dietary advice: ☐ No smoking: ☐ No medication: ☐

Resting Blood Samples

     Purple x 2: ☐   Green x 1: ☐   Blue x 2: ☐   Red x 3: ☐

Check	Time	Time	Comments	Sample 1	Sample 2	mean	Insulin
☐		-10					☐
☐		0	75g CHO				☐
☐		30					☐
☐		60					☐
☐		90					☐
☐		120					☐
☐		180					☐

Finish Time: _____

Circumferences

	1	2	3	Ave.		1	2	3	Ave.
Bicep					**Hips**				
Chest					**Thigh**				
Waist					**Calf**				

Skin Folds

	Measurement 1	Measurement 2	Measurement 3	Ave.
Triceps				
Pectoralis				
Subscapular				
Abdominal				
Midaxillary				
Suprailiac				
Thigh				

Signature of Tester: _____

Aerobic Fitness Assessment

Temp: _____°C   Barometric Pressure: _____mmHg

Date of Birth**: ____ /____ /_____**   Sex   M / F

Height: ____ m   Weight: _____ kg

RBP: Manual   ¹___ / ___   ²___ / ___   ³___ / ___   ⁴___ / ___

RBP: Automated   ¹___ / ___   ²___ / ___   ³___ / ___   ⁴___ / ___

Consent signed: ☐   Medical history: ☐   Resting ECG: ☐

Protocol used   _____

Stage	RPE	HR	BP
Warm up			
1			
2			
3			
4			
5			
6			
7			
8			
10			
11			
12			
13			
14			
15			
16			

Appendix C Preparticpation Screening

Department: School of Health and Human Performance, Dublin City University

Principal investigators
Dr. Donal O'Gorman   (01 7008060),   donal.ogorman@dcu.ie
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Pre-participation screening form

First name   **Surname**

_____   _____

Telephone Home   **Telephone Work**   **Telephone Mobile**

_____   _____   _____

Email

_____

Estimate your height   Estimate your weight

_____ft _____in   _____st _____lbs

Have you ever been told by a doctor that you have diabetes?   Yes☐   No☐

Have you ever been told by a doctor you have a heart condition?   Yes☐   No☐

Appendix D Physician's Medical Screening Form

DCU

DATE ☐☐ ☐☐ ☐☐☐☐

| *CONTACT DETAILS)* |

Last name: _____   **First name**: _____

Date of birth:_____   **Age** _____

Address:
_____

_____

Mobile   _____ Work:_____

Home:_____

Email Address: _____

Next of kin

Name   _____   **Contact tel:** _____

Relationship to you_____
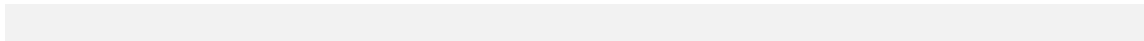
MEDICAL HISTORY (PHYSICIAN ADMINISTERED)

1. Do you suffer from any of the following (tick box)?   **Yes**   **No**

a)   High blood pressure (hypertension)   □   □

b)   Angina   □   □

     i.e   chest pain, neck pain, jaw pain, arm pain
or undue breathless on exertion
(such as walking fast or walking up a hill)

c)   Heart disease of any sort   □   □

     e.g.   heart attack
blocked blood vessels to the heart
abnormal heart rhythm

d)   Peripheral vascular disease   □   □

     e.g.   intermittent claudication (calf pain on walking)
stroke

e)   Elevated blood cholesterol or triglycerides   □   □

f)   Diabetes   □   □

2. Have you ever had any of the following (tick box)?   **Yes**   **No**

a)   A heart attack   □   □

b)   Heart surgery   □   □

c)   An angiogram   □   □

d)   Insertion of a stent   □   □

e)   Treatment of an irregular heart beat   □   □

f)   A blackout (loss of consciousness)   □   □

**3. Please list any other medical conditions you suffer from
at present or have suffered from in the past**

1. _____

2. _____

3. _____

4. _____

4. List any medications which you are now taking

_____

_____

_____

5. Your family history

Do any of your first degree relatives (parents, brothers, sisters) suffer from any of the following (tick box if yes)?

		Yes	No
a)	heart disease	☐	☐
b)	high blood pressure	☐	☐
c)	diabetes	☐	☐

Has any first degree relative of yours died from heart disease? Yes ☐

No   ☐

6. Alcohol / Cigarettes

Do you consume alcohol regularly?   Yes ☐   No ☐

If yes, how many units per week? _____

Do you smoke?   Yes ☐   No ☐

If yes, how many cigarettes a day? _____

7. Your Exercise Pattern

Do you take part in regular exercise of physical activity ? Yes ☐   No ☐

If yes, give details (how often per week, duration per session)

_____

_____

PHYSICAL EXAMINATION

Blood Pressure ___ / ____   **Pulse**____

GENERAL APPEARANCE

Subject looks: Healthy____   Not healthy___   Very ill____

SUMMARY FINDINGS

	Nothing Abnormal Found	**Details if Abnormal**
HEAD AND NECK	☐	_____
		_____
CHEST AND LUNGS	☐	_____
		_____
HEART	☐	_____
		_____
ABDOMEN	☐	_____
		_____
EXTREMITIES	☐	_____
		_____
NEUROMUSCULAR	☐	_____
		_____

RESTING ECG

Descriptive Analysis:

Rate: _____bpm

Rhythm: _____

Arrhythmias _____

_____

_____

Clinical Impression:

_____

_____

_____

_____

According to the medical history and physical exam, does subject qualify for this research study?

Yes ☐

No ☐

Comments:

_____

_____

_____ _____

Physician's signature (Date)

David Ashley

The Role of Serum Osteoprotegerin as a Biomarker of Metabolic Dysfunction in Obesity and Type 2 Diabetes

Thesis overview, April 2010

Introduction

Increasing prevalence of Obesity & Type 2 Diabetes

Obesity

Prevalence (%)

Diabetes

Prevalence (%)

Prevalence of diabetes in adult population (20-79 years) (%)

7.8%
8.6%
10.1%

2003² 2010¹ 2030¹

1) The International Diabetes Federation, Diabetes Atlas Fourth Edition (2009).
2) The International Diabetes Federation, Diabetes Atlas Second Edition (2006).
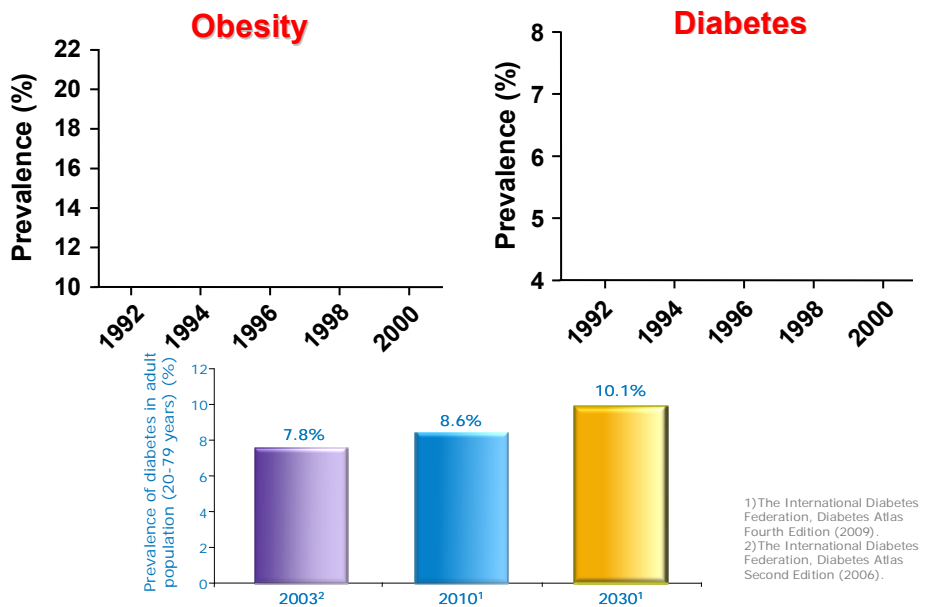
Introduction
Pathophysiological features of Type 2 Diabetes

Impaired insulin secretion

Increased hepatic glucose production

Decreased incretin effect

Increased lipolysis

HYPERGLYCAEMIA

Decreased glucose uptake

Increased FA Production

↓ insulin secretion

↑ insulin resistance
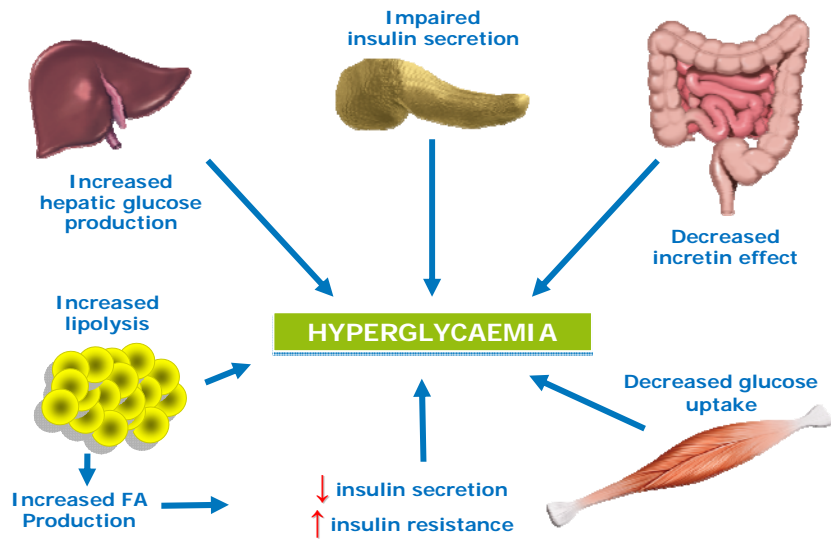
Adapted from: Stumvoll M, et al. Lancet. 2005;365:1333-46.

Pro-hyperglycaemic	Anti-hyperglycaemic
Resistin	Leptin
TNF- α	Adiponectin
IL-6	Visfatin
RBP-4	Omentin

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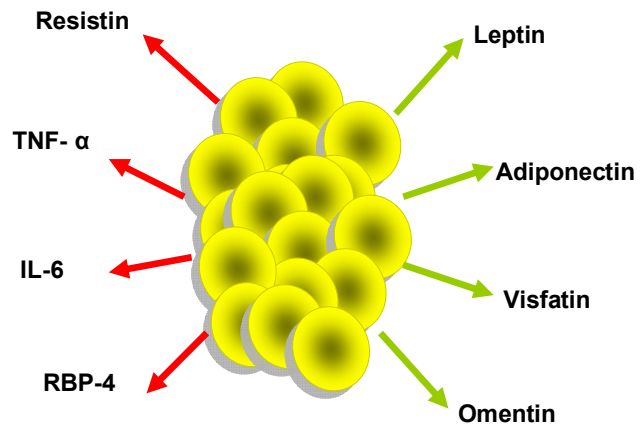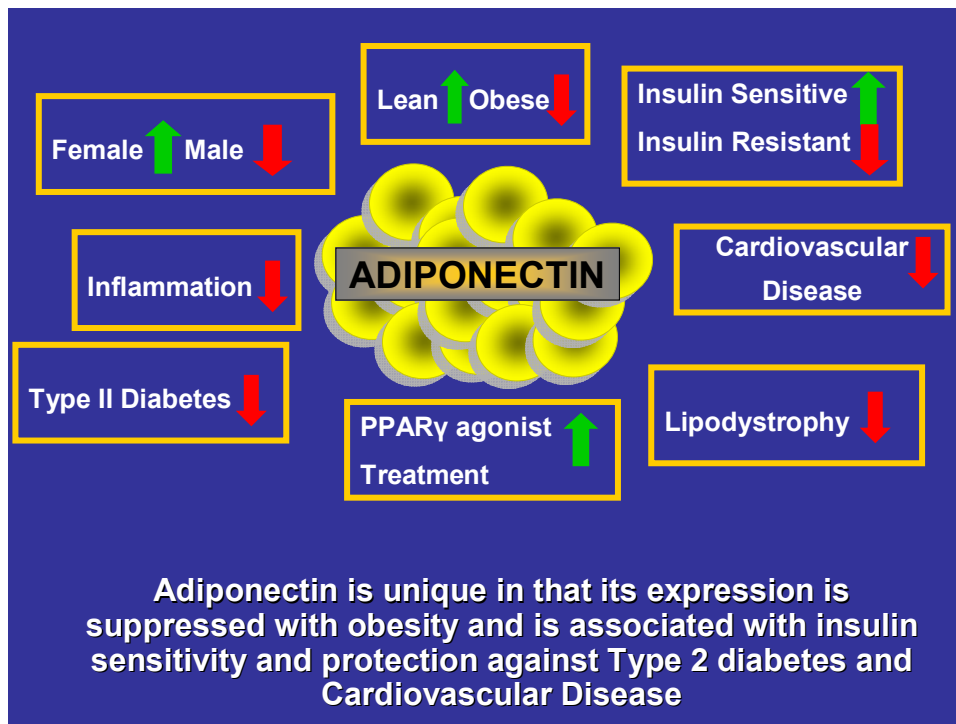
Introduction
Adipose Tissue, Cardiovascular Disease and Bone

- Adipocytokines are involved in the regulation of glucose and lipid metabolism.
- They exert anti- and pro-inflammatory effects and are involved in blood pressure control, haemostasis and bone mass turnover
- Evidence suggests that CVD and osteoporosis often coexist
- Several proteins such as osteocalcin, osteopontin and bone morphogenic protein, which were once thought to be bone-specific in their biological action, have been identified in atherosclerotic lesions.
- Such observations have given rise to the suggestion of the existence of an "Osteo-adipose-vascular" network
- One such protein that has garnered considerable interest in recent years is the novel glycoprotein osteoprotegerin (OPG)
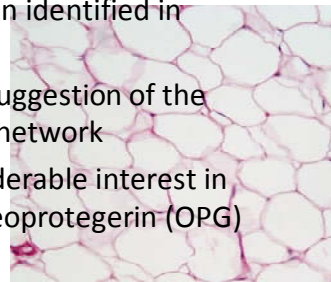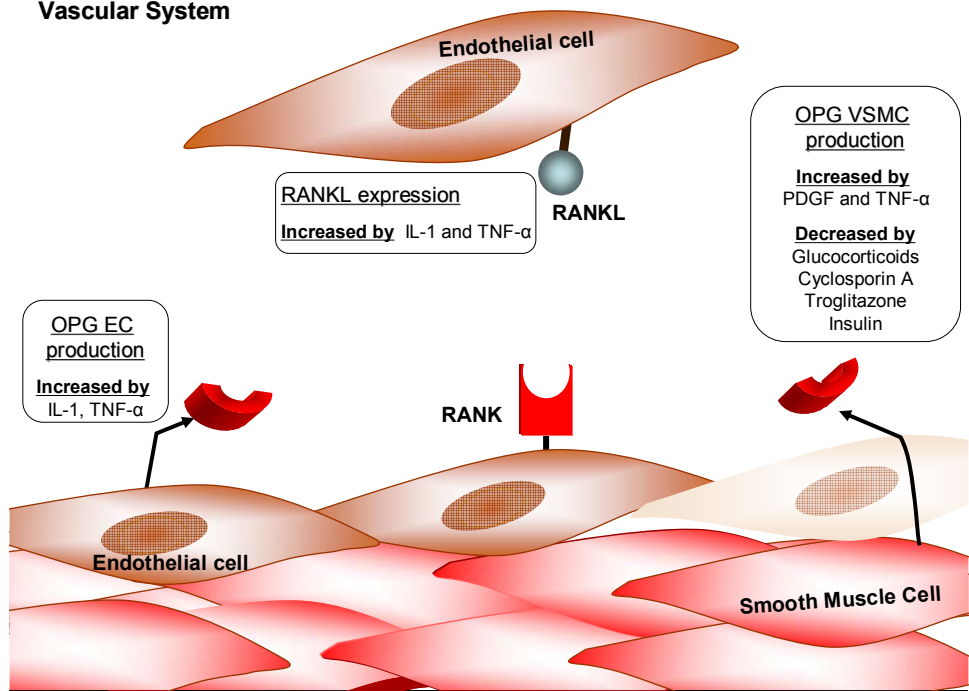
Introduction
Osteoprotegerin (OPG)

OPG knockout

- OPG knockout mice develop severe osteoporosis in addition to vascular calcification

- This suggests a protective role for OPG in the vascular system,

- There is emerging evidence for its involvement in the vascular system, with its expression observed in vascular tissues

- In vitro studies have demonstrated the ability of pro-inflammatory cytokines to upregulate OPG levels in both endothelial and vascular smooth muscle cells

- Suggesting a role for OPG in vascular disease, since inflammatory factors are thought to be key to the progression of CAD and atherosclerosis

Stromal Cell or mature Osteoblast

OPG

RANK

RANKL

Osteoclast precursor

TRAF 6 — NF-κB

NF-κB

C-Fos

NFATc1

Nucleus

Osteoclastogenic genes

Vascular System

Endothelial cell

RANKL

RANKL expression

Increased by IL-1 and TNF-α

OPG VSMC production

Increased by
PDGF and TNF-α

Decreased by
Glucocorticoids
Cyclosporin A
Troglitazone
Insulin

OPG EC production

Increased by
IL-1, TNF-α

RANK

Endothelial cell

Smooth Muscle Cell
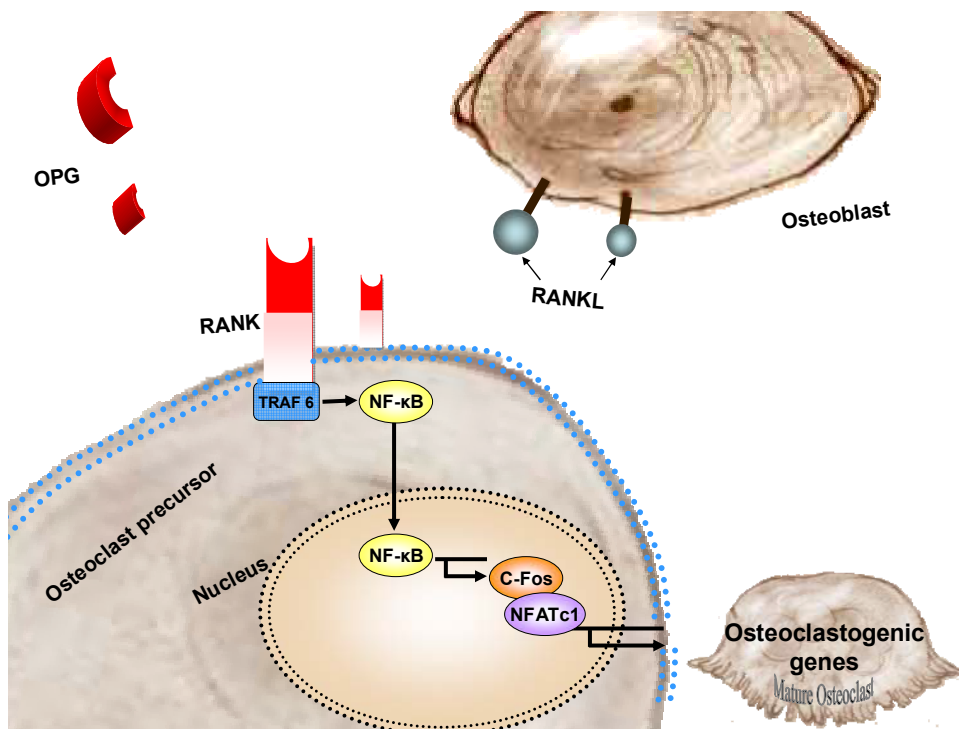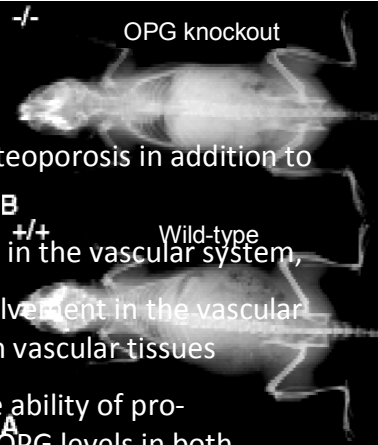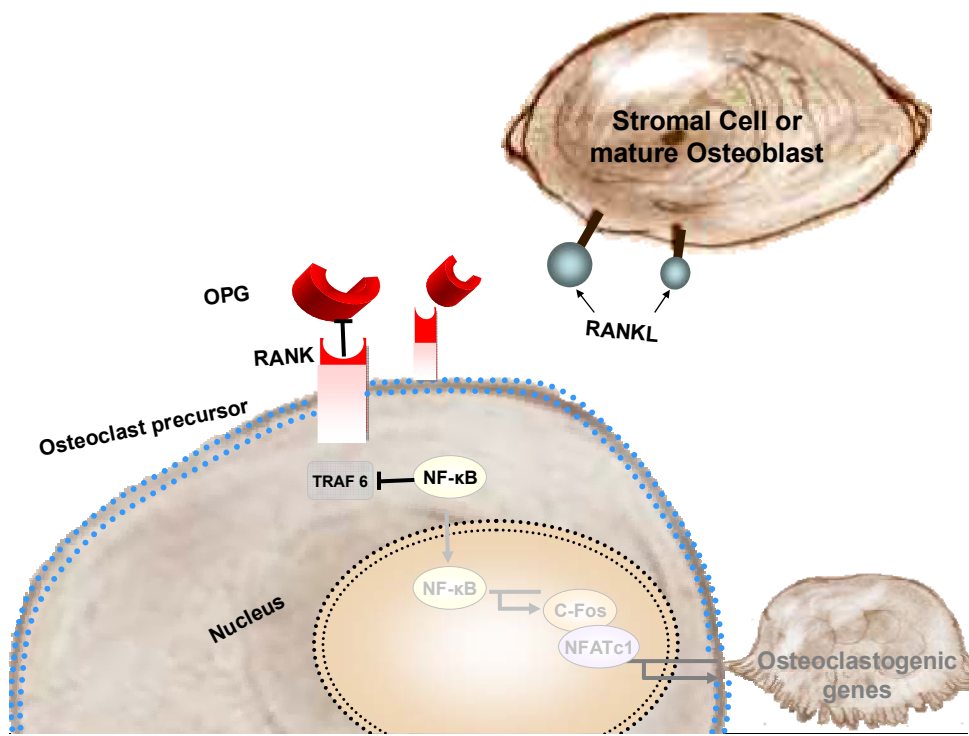
Thesis Aims & objectives

To investigate the role of OPG in obesity and metabolic dysfunction and to further elucidate and explore the relationship between the OPG / RANK / TRAIL axis and established markers of inflammation and insulin sensitivity

Examine the impact of diabetes and vascular disease on their circulating concentrations while probing how these novel markers relate to other traditional inflammatory markers and adipocytokines.

Study the influence of glycaemic status and adiposity together on serum levels of OPG and to interrogate if a worsening glycaemic status can influence its relationship with adiponectin and systemic inflammation.

Experiment I

An investigation of serum OPG, TRAIL and sRANKL levels and their relationship with indicators of adiposity and insulin sensitivity in a healthy, representative Irish cohort.

Introduction/rationale

- Circulating OPG is significantly higher in patients with type 2 diabetes and is higher in the tunica media of type 2 diabetics than matched normal controls

- It has also been shown that circulating concentrations of OPG can independently predict silent CAD in type 2 diabetic patients

- Despite the higher circulating and tissue concentrations of OPG in patients with CVD there has been little research on high risk obese subjects

- Gannage-Yared et al. (2006) examined the relationship between OPG and components of the metabolic syndrome in 151 healthy ageing men.

- They found that OPG was inversely correlated with fasting plasma glucose and insulin sensitivity and positively correlated with adiponectin.

- Most of the research to date which has indicated that OPG is associated with a negative coronary outlook has been conducted in patients with underlying CVD

- Few published papers have examined the relationship between insulin sensitivity, adiposity and OPG in a healthy population free from CVD
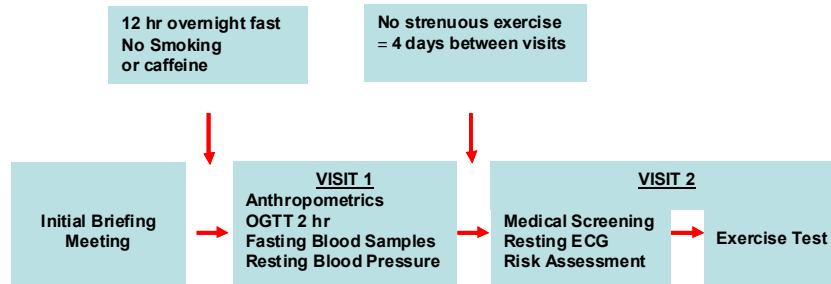
Introduction/rationale

Aims
The purpose of this study was to determine if BMI and insulin sensitivity influence the concentrations of serum OPG and TRAIL in subjects who do not have cardiovascular or metabolic disease.

Hypothesis
That in a healthy cohort, circulating OPG would be lower in obese subjects and inversely related to insulin resistance.

Experimental Design

- 136 subjects

- 36 excluded because of undiagnosed hypertension, impaired glucose tolerance or abnormal ECG

12 hr overnight fast No Smoking or caffeine	No strenuous exercise = 4 days between visits

| Initial Briefing
Meeting | → | **VISIT 1**
Anthropometrics
OGTT 2 hr
Fasting Blood Samples
Resting Blood Pressure | → | **VISIT 2**
Medical Screening
Resting ECG
Risk Assessment | → | **Exercise Test** |

Methods

- Glucose   Dual channel YSI 2300
- Insulin   AutoDELFIA® Perkin Elmer FIA
- Adiponectin,
  IL-6 and TRAIL   ELISA, RnD
- CRP   Immunonephelometry (Randox)
- Lipids   Spectrophotometric analysis (Randox)
- OPG   ELISA, Biomedica
- sRANKL   ELISA, Biomedica

Results

Subject Characteristics

Table 3.1 Selected Anthropometric and Cardiovascular Characteristics of Subjects

	Normal weight	Overweight	Obese
	(36)	(41)	(23)
Gender (male/female)	19/17	19/22	11/12
Age (years)	44.4 ± 1.5	46.7 ± 2.0	47.2 ± 2.8
BMI (kg·m^{-2})	22.8 ± 0.2	26.7 ± 0.2 *	31.4 ± 0.3 * †
Waist Circumference (cm)	78.5 ± 1.2	89.8 ± 1.2 *	102.2 ± 2.0 * †
Waist to Hip Ratio	0.82 ± 0.01	0.87 ± 0.01 *	0.93 ± 0.02 * †
Body fat (%)	19.6 ± 1.15	27 ± 1.16 *	31.9 ± 1.5 * †
VO$_{2\,max}$ (ml·kg·min^{-1})	41.5 ± 1.9	37.5 ± 1.5	29.6 ± 1.7 * †
Systolic BP (mmHg)	117.1 ± 2.2	120.4 ± 1.6	130.3 ± 2.2 * †
Diastolic BP (mmHg)	74.0 ± 1.5	76.5 ± 1.4	80.6 ± 2.1 *

Results

A

B

Table 3.2 Metabolic Markers and Indicators of Insulin Sensitivity.

	Normal weight	Overweight	Obese
Fasting Glucose (mmol·l^{-1})	4.8 ± 0.1	5.0 ± 0.1	5.3 ± 0.1 * †
Fasting Insulin (pmol·l^{-1})	26.4 ± 3.5	38.2 ± 4.2 *	51.3 ± 5.6 *
Triglycerides (mmol·l^{-1})	0.97 ± 0.05	1.32 ± 0.10 *	1.55 ± 0.15 *
HOMA-IR	0.83 ± 0.12	1.25 ± 0.14 *	1.8 ± 0.2 * †
OGIS (ml·min·m^{-2})	533 ± 11	512 ± 9	451 ± 11 * †
AUC Glucose (mmol·l·min)	671 ± 18	738 ± 19 *	831 ± 36 * †
AUC Insulin (pmol·l·min)	20487 ± 2444	29585 ± 4285	42336 ± 4979 * †
hs-CRP (mg·L^{-1})	0.92 ± 0.18	0.92 ± 0.08	1.16 ± 0.15
Adiponectin (µg·ml^{-1})	9.9 ± 0.9	6.6 ± 0.5 *	4.8 ± 0.5 *
TRAIL (pg·ml^{-1})	72.2 ± 5.4	81.6 ± 3.9	82.4 ± 6.9
sRANKL (pg·ml^{-1})	3.4 ± 0.6	3.0 ± 0.4	2.9 ± 0.7

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TRAIL

- TRAIL was significantly related to

- Fat mass (r = 0.255, p < 0.05)

- Waist circumference (r = 0.207, p < 0.05)
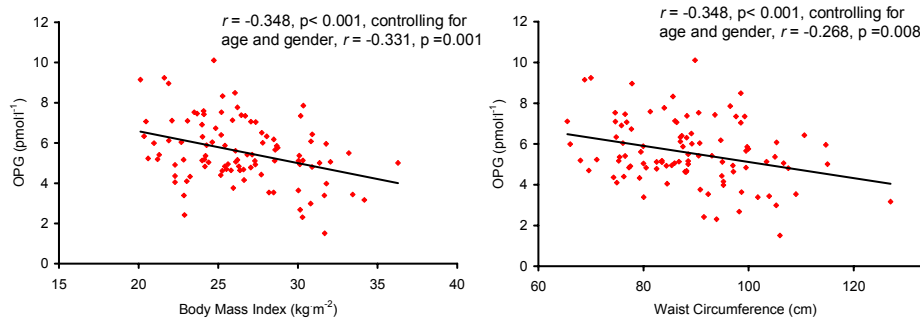
Osteoprotegerin and Obesity
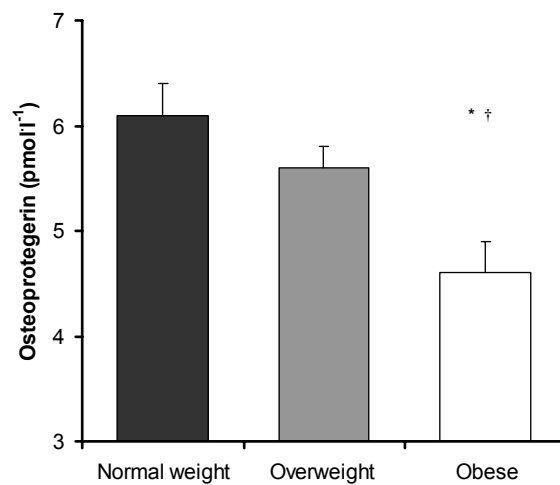
Results

Osteoprotegerin- Correlation Analysis

Table 3.3 Age and Gender Adjusted Correlations Between OPG and Anthropometric and Metabolic Indices.

	r	p
BMI (kg·m^{-2})	-0.331	***
Waist Circumference (cm)	-0.268	**
VO$_{2\,max}$ (ml·kg·min^{-1})	0.237	*
Fasting Glucose (mmol·l^{-1})	-0.248	*
Fasting Insulin (ρmol·l^{-1})	-0.202	**
AUC Glucose (mmol·l·min)	-0.279	**
AUC Insulin (pmol·l·min)	-0.271	**
HOMA-IR	-0.222	*
OGIS (ml·min·m^{-2})	0.221	*
Adiponectin (µg·ml^{-1})	0.391	***

Results

Osteoprotegerin and Adiposity

r = -0.348, p< 0.001, controlling for age and gender, r = -0.331, p =0.001

r = -0.348, p< 0.001, controlling for age and gender, r = -0.268, p =0.008

Summary

The main findings of Experiment I are that obese subjects who are normal glucose tolerant and free from CVD, have lower circulating OPG when compared with normal weight and overweight individuals. In this cohort OPG is positively correlated with adiponectin and insulin sensitivity.
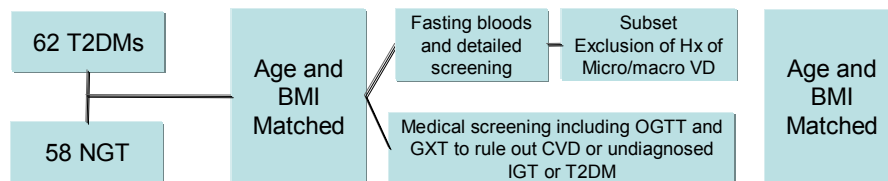
Experiment II

The relationship between OPG, TRAIL, sRANKL and markers of inflammation in Type 2 Diabetes and Vascular Disease.

Experimental design

- 110 subjects volunteered to participate in this study.

- 58 normoglycemic, healthy subjects free from CVD were recruited from DCU

- 62 patients with type 2 diabetes were recruited from the diabetes clinic in Beaumont Hospital.

- OGTT to ensure normal glucose tolerance and exercise stress tests were performed on all of the healthy controls to rule out undiagnosed hyperglycaemia or CVD

62 T2DMs		Age and BMI Matched	Fasting bloods and detailed screening	Subset Exclusion of Hx of Micro/macro VD	Age and BMI Matched
58 NGT			Medical screening including OGTT and GXT to rule out CVD or undiagnosed IGT or T2DM		

Introduction/rationale

- Arterial calcification is a prominent feature of atherosclerosis and common in patients with type 2 diabetes.

- Our understanding of this process has further developed in recent times with the identification of a possible role for the OPG/RANKL/TRAIL axis in the process

- Studies of serum RANKL have been inconclusive, with both increased and reduced risk of CVD disease being reported with elevated concentrations

- Only one paper has measured RANKL levels in individuals with type 2 diabetes, finding no difference from healthy individual (Secchiero *et al.*, 2006).

- Studies to date suggest higher serum OPG levels in type 2 diabetes

- However many of these studies have had poorly defined control groups

- IL-6 and hsCRP are frequently used to gain a measure of the degree of underlying inflammation

- Whether OPG, RANKL or TRAIL could reflect low-grade vascular inflammation in individuals with diabetes is not yet known

Results

Subject Characteristics

Table 4.1 Subject Metabolic and Cardiovascular Characteristics.

	Normal	Type 2 Diabetes
N	58	62
Age (years)	55.6 ± 1.2	58.31.2
Gender (M:F)	28:30	40:22
BMI (kg/m^2)	29.5 ± 0.4	30.0 ± 0.5
Waist Circumference (cm)	101.3 ± 1.4	105.0 ± 1.3 ~
Systolic BP (mmHg)	131.14 ± 2.53	142.3 ± 2.26 ***
Diastolic BP (mmHg)	81.8 ± 1.4	80.26 ± 1.26
Current smokers	4.7%	9.8%
Anti-hypertensive use	13.2%	86.7% ***
ACE/ARB use	8.6%	67.2% ***
Statin use	15.1%	82.0% ***
Aspirin use	5.7%	78.7% ***
Fasting glucose (mmol l^{-1})	5.2 ± 0.2	7.9 ± 0.2 ***
Total Cholesterol (mmol l^{-1})	5.4 ± 0.1	4.1 ± 0.1 ***
LDL Cholesterol (mmol l^{-1})	3.4 ± 0.1	2.0 ± 0.1 ***
HDL Cholesterol (mmol l^{-1})	1.4 ± 0.04	1.2 ± 0.04 ***
Triglycerides (mmol l^{-1})	1.4 ± 0.1	2.0 ± 0.1 ***
TNF-α (pg/ml)	1.5 ± 0.3	1.4 ± 0.3

Experimental design

Correlation Analysis

- TRAIL was correlated with LDL (r = 0.303, p < 0.01) and waist circumference (r = 0.202, p < 0.05). .
- In the healthy control group, controlling for gender there was a correlation between OPG and age (r = 0.364, p < 0.01)
- When both age and gender were controlled for, OPG was correlated with
  – waist circumference (r = -0.262, p < 0.05),
  – adiponectin (r = 0.366, p < 0.01),
  – total cholesterol (r = 0.380, p < 0.01),
  – LDL (r = -0.336, p < 0.05),
  – fasting plasma glucose (r = -0.363, p < 0.05)
  – TRAIL *strong trend (r = -0.294, p = 0.053)*
- None of these relationships with OPG were evident in the type 2 diabetic cohort

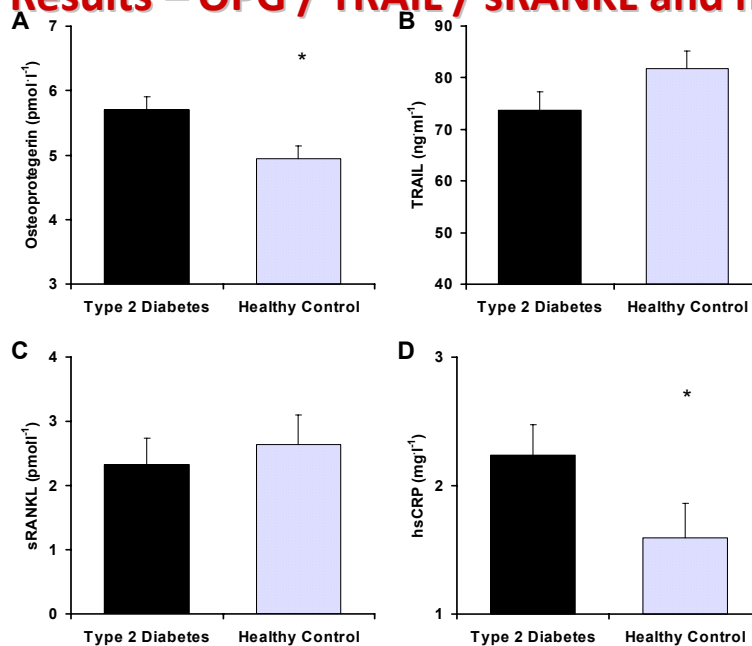# Results

Metabolic data

Table 4.2 Characteristics of the Disease State in Patients with Type 2 Diabetes.

	Median (range) or n (%)
Duration of diabetes (years)	7 (1-20)
HbA1c (%)	7 (5.1-10)
Insulin treated	12 (19.67%)
Metformin treated	37 (60.66%)
Sulphonylurea treated	21 (34.43%)
TZD treated	3 (5.92%)
Diet alone	8 (13.12%)
Microvascular complications	15 (24.19%)
Macrovascular complications	20 (32.26%)

Results – OPG / TRAIL / sRANKL and hsCRP

Results – Adipocytokines

Vascular Disease and inflammatory markers

Subset Analysis on the Effect of Vascular Disease on Inflammatory Markers

To investigate whether the elevated OPG, IL-6 and hsCRP observed in the diabetes group was due to the higher prevalence of vascular disease within this group, we compared mean values of these proteins, after exclusion of type 2 diabetes patients with either documented micro- or macro-vascular disease.

- OPG was still significantly higher in type 2 diabetics than normal controls
- The significant difference previously seen with IL-6 and hsCRP was no longer present
- In this group OPG correlated with IL-6 after correction for age and gender ($r = 0.24$, $p < 0.05$), but this association was lost after correction for glycaemic status.
- There was no correlation between RANKL or TRAIL and IL-6, or hsCRP in either group.

Summary

The main findings of Experiment II are that OPG but not RANKL or TRAIL is significantly increased in type 2 diabetes. IL-6 and hsCRP is higher in individuals with diabetes and adiponectin is lower, but unlike OPG, they are no longer different when subjects with vascular disease are excluded.

Experiment III

The effect of Obesity, Glycaemic Status and an acute glucose load on circulating concentrations of OPG

Introduction/rationale

Aims

To examine changes in OPG levels with varying degrees of glucose tolerance and to investigate the influence of adiposity and inflammatory processes on OPG concentrations.

Hypothesis

The deteriorating inflammatory state coupled with the sharp rise in hyperinsulinaemia with decreasing glucose tolerance uncouples the relationship between OPG and insulin sensitivity that was observed in Experiment I and II

Introduction/rationale

- As well as its role in maintaining normoglycemia, insulin is a vasoactive peptide capable of exerting pleiotropic hemodynamic effects

- Recent studies have suggested an important role for insulin in the inhibition of OPG expression and secretion.

- Olesen et al. (2005) showed that vascular smooth muscle cells incubated with insulin exhibit markedly reduced OPG production compared to controls

- Xiang et al. (2007) found that OPG was decreased in response to 6 months of insulin therapy in young type 1 diabetics and that this decrease was strongly correlated with changes in endothelial function.

- Jorgensen et al. (2009) examined the effect of insulin infusion (hyperinsulinaemic clamp) on OPG production in lean, type 2 diabetic and obese subjects

- OPG was markedly reduced in all groups and but the lean control group showed a significantly greater decrease than the type 2 diabetic and obese subjects.

- Interaction between insulin and the OPG / RANKL / RANK / TRAIL axis may be one mechanism by which elevated fasting and postprandial hyperinsulinaemia can independently affect the development of cardiovascular disease.

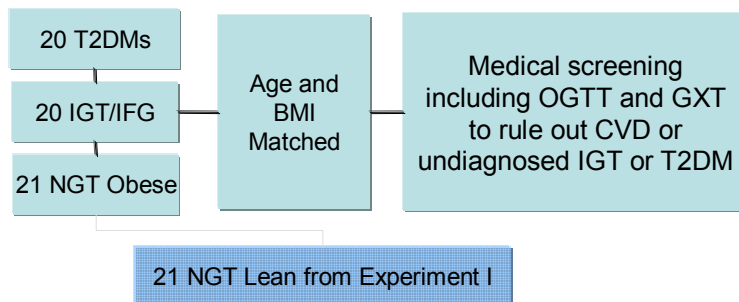## Experiment III –Glycaemic Status, glucose loading and OPG
Results

Subject Characteristics

Table 5.1 Subject Characteristics and indicators of insulin sensitivity.

	Type 2 Diabetes	IGT / IFG	NGT Obese
	(20)	(20)	(21)
Age (years)	56.2 ± 2.1	56.8 ± 2.3	53.8 ± 2.2
BMI (kg·m^{-2})	30.5 ± 0.6	30.5 ± 0.8	30.2 ± 0.5
Fasting Glucose (mmol·l$^{-1)}$)	7.4 ± 0.2	6.2 ± 0.1 *	5.2 ± 0.1 * †
Fasting Insulin (pmol·l^{-1})	118.8 ± 15.6	127.7 ± 12.6	39.9 ± 4.3 * †
2 hr Glucose (mmol·l^{-1})	12.6 ± 0.7	8.6 ± 0.3 *	5.1 ± 0.3 * †
2 hr Insulin (pmol·l^{-1})	493.4 ± 74.5	526.8 ± 55.2	39.6 ± 24.6 * †
HOMA-IR	6.4 ± 0.8	5.8 ± 0.6 *	1.6 ± 0.2 * †
OGIS (ml·min·m^{-2})	289 ± 10	332 ± 10	424 ± 9 * †
AUC Glucose (mmol·l·min)	1593 ± 62	1218 ± 30 *	816 ± 32 * †
AUC Insulin (pmol·l·min)	64475 ± 9176	60693 ± 6032	29677 ± 3492 * †

Experiment II – OPG inflammatory markers and type 2 diabetes
Experimental design

- 61 male subjects were recruited to participate in this study
- 20 patients with type 2 diabetes
- 20 patients with either IGT/IFG
- 21 healthy obese male subjects free from CVD were recruited from DCU
- All three groups were age and BMI matched.
- Data from 21 lean age matched subjects from experiment I were also included in the later analysis
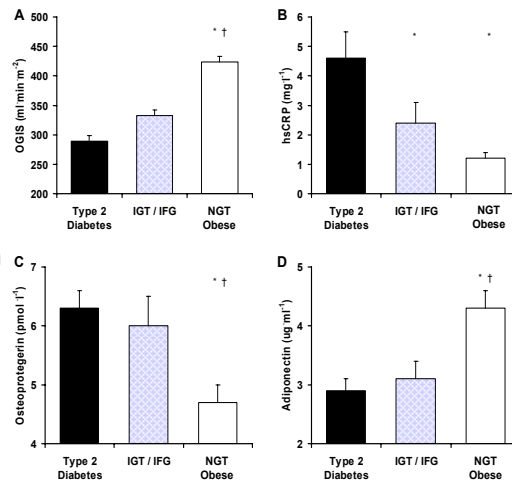
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┌──────────────┐
│  20 T2DMs    │
└──────────────┘
┌──────────────┐   ┌───────────┐   ┌─────────────────────────┐
│  20 IGT/IFG  │───│ Age and   │───│ Medical screening       │
└──────────────┘   │ BMI       │   │ including OGTT and GXT   │
┌──────────────┐   │ Matched   │   │ to rule out CVD or      │
│ 21 NGT Obese │   └───────────┘   │ undiagnosed IGT or T2DM │
└──────────────┘                   └─────────────────────────┘

        ┌──────────────────────────────────┐
        │  21 NGT Lean from Experiment I    │
        └──────────────────────────────────┘
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Results – OPG

OGIS and adiponectin were significantly higher in healthy age and BMI matched controls than in either IGT / IFG subjects or type 2 diabetics

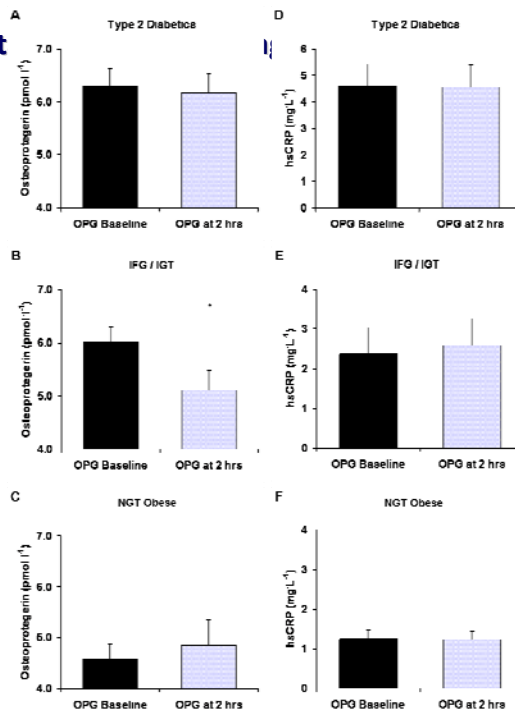hsCRP was significantly lower in the healthy matched control group and the IGT / IFG group
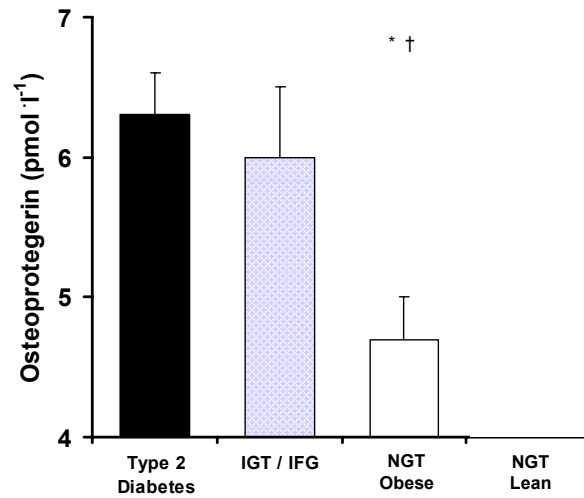
OPG was significantly lower in the NGT Obese group

Results – OPG

• OPG was significantly reduced in response to the oral glucose load in the IFG / IGT group but was unchanged in type 2 diabetics and normoglycemic obese subjects

• There was no significant change in hsCRP in response to the OGTT in any of the glycaemic conditions.

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Results – OPG

Summary

The main findings of Experiment III are that there is no difference in OPG between prediabetic and type 2 diabetic cohorts, but both have higher levels than matched obese controls. Interestingly, OPG in lean insulin-sensitive subjects is comparable to that of the pre-diabetic and type 2 diabetic patients but significantly higher than their matched lean counterparts.

General Discussion and Conclusion
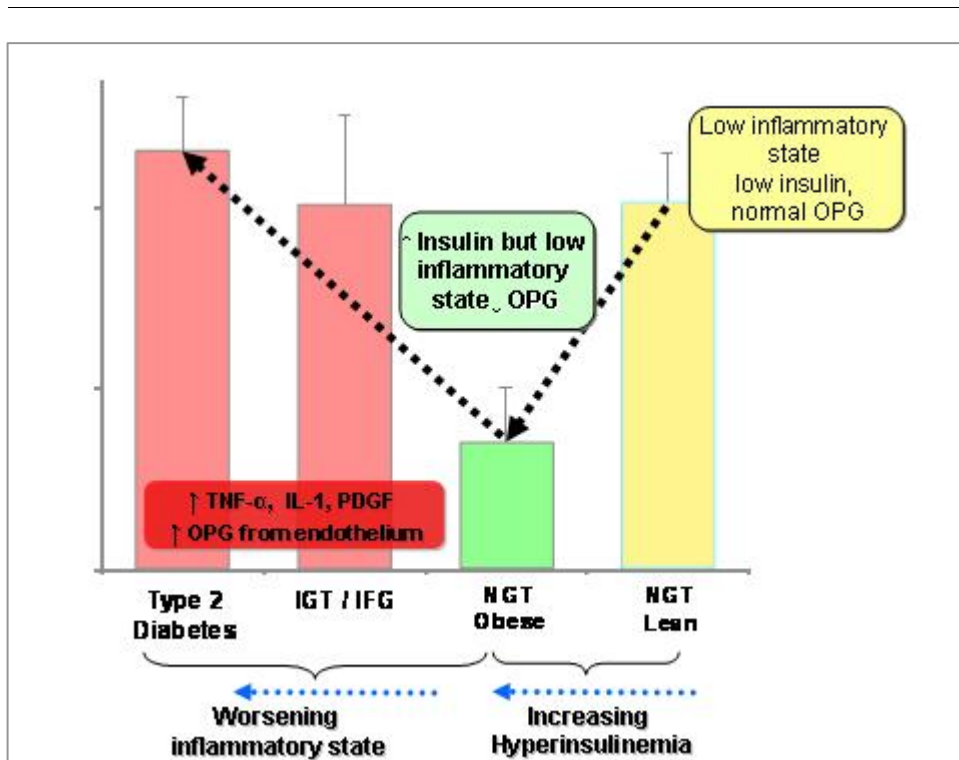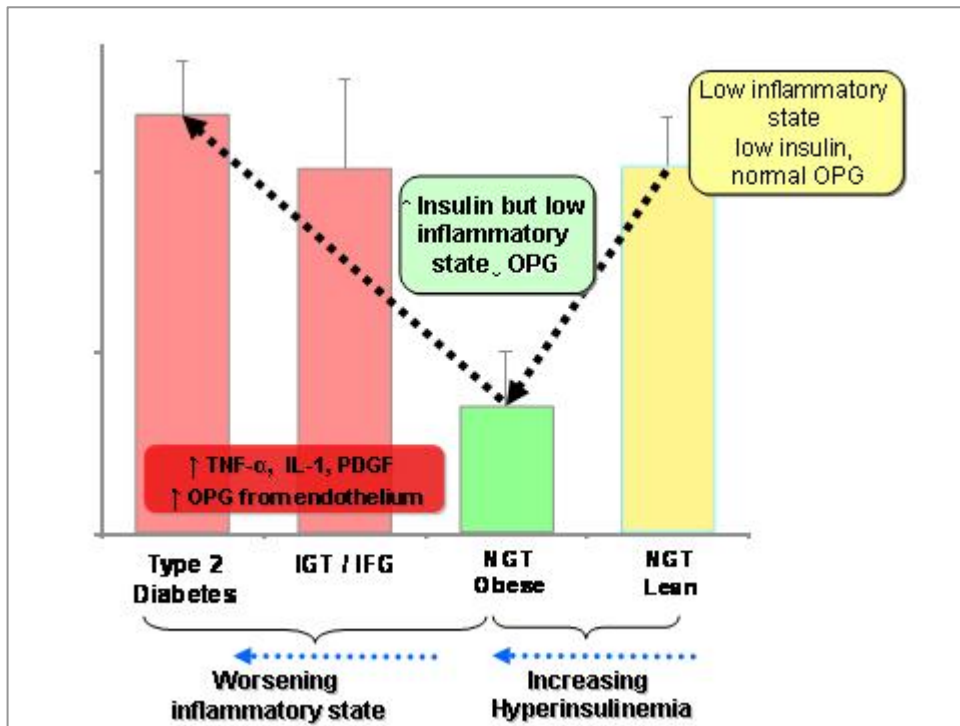
General discussion
Future directions

Future studies that examine the effect of either therapeutic intervention or *in vitro* manipulation on OPG production should measure both total and unbound OPG in addition to TRAIL and RANKL an order to assess the true dynamic nature of this family of molecules in response to a variety of stimuli.

Thank You

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