Thesis

Lipids, Oxidative stress and Endothelial Function.

Dr Richard A Anderson
Declaration

I declare that this thesis has not been presented to the University of London or any other University in support of an application for any degree.
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Abstract

Subject and aims of work presented in this thesis are that the ingestion of a fatty meal induces a state of transient endothelial dysfunction through a mechanism caused by increased oxidative stress. In type 2 diabetic subjects where there is generally an exaggerated response to a fatty meal, the effect on the vascular endothelium and generation of oxidative stress may be enhanced.

I examined the effect of attenuating post-prandial lipaemia on endothelial function and oxidative stress in type 2 diabetes. I then studied the effect of antioxidant therapy (Vitamin C) alone on endothelial function and oxidative stress after a fatty meal and then in combination with Insulin lispro and chronic vitamin C therapy on post-prandial lipaemia, oxidative stress and endothelial function in patients with type 2 diabetes.

Using the technique of *Ex vivo* EPR spin trapping, I attempted to study the mechanism of lipoprotein peroxidation caused by post-prandial oxidative stress in type 2 diabetics and finally I examined the effect of a fatty meal on platelet nitrate responsiveness in type 2 diabetes.
Principal aims and objectives

Chapter 1
General background
Factors that determine the response to a fatty meal
The effect of a fatty meal on other lipoproteins
The fatty meal and its link with atherosclerosis
The endothelium and endothelial dysfunction
The effect of a fatty meal on the endothelium and in particular its effect in type 2 diabetics.
The effect of a fatty meal on platelet function.

Chapter 2
An overview on methodology used.

Chapter 3
The issue addressed in this chapter is to establish whether the ingestion of a fatty meal in healthy individuals has an impact on endothelial function and markers of oxidative stress and to compare these findings with subjects who have impaired fat handling and possibly depressed antioxidant defences (T2DM subjects).

Chapter 4
To examine the effect of attenuating post-prandial lipaemia on endothelial function and oxidative stress in type 2 diabetes.

Chapter 5
The effect of antioxidant therapy (Vitamin C) on endothelial function and oxidative stress after a fatty meal.
Chapter 6
The effects of Insulin lispro and chronic vitamin C therapy on post-prandial lipaemia, oxidative stress and endothelial function in patients with type 2 diabetes mellitus.

Chapter 7
Ex vivo EPR Spin Trapping to Study the Mechanism of Lipoprotein Peroxidation Caused by Post-Prandial Oxidative Stress in Type 2 Diabetics.

Chapter 8
The effect of a fatty meal on platelet nitrate responsiveness in type 2 diabetes

Conclusions and future directions

TABLE OF CONTENTS
DECLARATION.............................................................................................................
TABLE OF CONTENTS............................................................................................
LIST OF FIGURES....................................................................................................
LIST OF TABLES......................................................................................................
ACKNOWLEDGEMENTS..........................................................................................
SUBJECT AND AIMS OF WORK PRESENTED IN THIS THESIS..............................

CHAPTER 1
INTRODUCTION
(a) POST PRANDIAL LIPAEMIA
(b) VASCULAR DAMAGE
(c) OXIDATIVE STRESS
(d) PROTHROMBOSIS
CHAPTER 2

METHODS

(a) ENDOTHELIAL FUNCTION (FLOW-MEDIATED DILATATION)
(b) ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY
(c) EPR SUBFRACTIONATION
(d) LIPID PEROXIDATION
(e) LIPID SUBFRACTIONATION
(f) PLATELET AGGREGATION

RESULTS

CHAPTER 3
POST PRANDIAL LIPAEMIA IN HEALTHY INDIVIDUALS COMPARED TO TYPE 2 DIABETES (T2DM).

CHAPTER 4
ATTENUATION OF PPL BY CIPROFIBRATE THERAPY AND ITS EFFECT ON ENDOTHELIAL FUNCTION/OXIDATIVE STRESS IN T2DM.

CHAPTER 5
ATTENUATION OF OXIDATIVE STRESS BY ANTIOXIDANT THERAPY AND ITS EFFECT ENDOTHELIAL FUNCTION/OXIDATIVE STRESS ON T2DM.

CHAPTER 6
THE EFFECT OF INSULIN THERAPY ON PPL/ENDOTHELIAL FUNCTION AND OXIDATIVE STRESS IN T2DM.

CHAPTER 7
INSIGHTS INTO THE MECHANISM OF OXIDATIVE STRESS CAUSED BY PPL IN T2DM.

CHAPTER 8
PPL AND ITS EFFECT ON PLATELET NITRATE RESPONSIVENESS IN T2DM.
CONCLUSIONS
DISCUSSION
FUTURE DIRECTIONS
REFERENCES
PUBLICATIONS
PRESENTATIONS
**Abbreviations**

CHD – Coronary heart disease

PP – Post prandial

PPL – Post prandial lipaemia

LDL – Low density lipoprotein

HDL – High density lipoprotein

VLDL – Very low density lipoprotein

TG – Triglycerides

LPL – Lipoprotein lipase

FFA – Free fatty acids

CM (CMR) – chylomicrons (remnants)

T2DM – Type 2 diabetes

CETP - Cholesterol ester transfer protein

IDL – Intermediate density lipoprotein

LRP - LDL receptor-related protein

EDHF - Endothelial derived hyperpolarizing factor

NO – Nitric oxide

ADP/ATP – Adenosine di/triphosphate

NOS – Nitric oxide synthase

SNP - Sodium nitroprusside

VCAM-1 - Vascular cell adhesion molecule-1

ICAM-1 - Intercellular adhesion molecule-1

t-PA - Tissue-type plasminogen activator

EDD - Endothelium-dependent dilatation
FMD – Flow-mediated dilatation

ACE – Angiotensin converting enzyme

ADMA – Asymmetric dimethylarginine

LOOH – Lipid hydroperoxides

FR (LDFR) – (Lipid derived) free radicals.

AUC – Area under the curve

OCP – Oral contraceptive pill

TBARS – Thiobarbituric acid reacting substances

EPR – Electron paramagnetic resonance spectroscopy

HbA1c – Glycosylated Haemoglobin

IGT – Impaired glucose tolerance

ED – Endothelial dysfunction

BMI – Body mass index

OS – Oxidative stress

EID – Endothelial independent vasodilatation

WHO – World health organisation

HOPE – Heart outcomes prevention outcomes study

L-NMMA – L-Nitromonomethylarginine

UKPDS – UK prospective diabetes study
List of Tables

Chapter 3

Table 1. Glucose and lipid parameters in T2DM and control subjects, fasting, 4 and 8 hours after consumption of a fatty meal.

Table 2. Distribution of cholesterol and TG amongst major lipoprotein subclasses in both groups in the fasting state.

Table 3. Cholesterol and TG distribution in the major subclasses during PPL in both groups.

Table 4. Post-prandial AUC for TG content (µmol/ml/8 hrs) of lipoproteins in both groups, following a fatty meal.

Table 5. Correlation of fasting FMD with various parameters in the T2DM group.

Table 6. Correlation of PPL and metabolic parameters with the reduction in FMD in the T2DM group.

Chapter 4

Table 1. Patient characteristics.

Table 2. Fasting biochemistry in both groups at baseline and after treatment.

Table 3: Fasting cholesterol and TG distribution among major lipoproteins in both groups at baseline and after treatment.

Table 4. Post-prandial AUC for TG content (µmol/ml/8 hrs) of major lipoproteins in both groups.

Chapter 5

Table 1: Patient characteristics at baseline with either vitamin C or placebo

Table 2: Cholesterol and TG content of the major lipoprotein subclasses in both groups at baseline.

Table 3: Post-prandial AUC for TG and cholesterol content of major lipoproteins in both groups at baseline.

Table 4: Correlation of fasting and post-prandial deterioration in endothelial function.
Table 5: Factors correlating most significantly with the improvement in fasting and post-prandial FMD following vitamin C therapy.

Chapter 6

Table 1. Patient characteristics at baseline and after treatment with Insulin in combination with either vitamin C or placebo.

Table 2: Cholesterol and TG content of the major lipoprotein subclasses in both groups at baseline and after insulin.

Table 3: Post-prandial AUC for TG and cholesterol content of major lipoproteins in both groups at baseline and after insulin.

Table 4. Fasting baseline and post-treatment vascular parameters in both groups.

Table 5. Post-prandial vascular parameters in both groups at baseline and after treatment.

Table 6: Fasting and post-prandial TBARS and EPR measurements in both groups at baseline and after treatment.

Table 7: Baseline correlation of fasting and post-prandial deterioration in endothelial function.

Table 8: Factors correlating most significantly with the improvement in fasting and post-prandial FMD following insulin and vitamin C therapy.

Chapter 7

Table 1. Lipid subfractions EPR fasting and post-prandially.

Table 2. Plasma measures lipid derived free radicals and lipid peroxidation products.

Chapter 8

Table 1. Patients/normal subjects’ characteristics.

Table 2. Metabolic data.

Table 3. Correlation of fasting platelet NO responses to SNP and GTN with various parameters in the T2DM group.
List of Figures

Chapter 1

Figure 1. Fat absorption

**Figure 2.** Schematic diagram of Lipoprotein interactions with lipoprotein lipase at the endothelial cell surface.

Figure 3: Effects of NO via guanylate cyclase activation

Figure 4. Lipid hydroperoxide metabolism.

Chapter 2

Photo 1. Wall tracking equipment.

Figure 1. Chemical structure of PBN.

Figure 2. Trapping of R· by PBN, PBN-LO- and PBN-L-

Chapter 3

Figure 1. Post prandial rises in LDFR and TBARS in healthy controls and T2DM subjects.

Figure 2. Post prandial changes in FMD in healthy controls and T2DM subjects.

Chapter 4.

Figure 1. Post-prandial lipaemia in Ciprofibrate group at baseline and after 3 months treatment.

Figure 2. Post-prandial lipaemia in placebo group at baseline and after 3 months treatment.

Figure 3. Flow mediated endothelium dependent vasodilatation (FMD) in placebo and Ciprofibrate groups at baseline and after treatment.

Figure 4. GTN induced endothelium independent brachial artery dilatation at baseline and after 3 months in both groups.

Figure 5. Fasting and post-prandial oxidative stress in placebo and Ciprofibrate groups at baseline and after treatment.

Chapter 5

Figure 1 Changes in EPR in each group

Figure 2. Markers of lipid peroxidation
Figure 3. Changes in endothelial function post-prandially

Chapter 6

Figure 1. Fasting and post-prandial TBARS at baseline and post intervention.

Figure 2. Fasting and post-prandial EPR at baseline and post intervention.

Figure 3. Fasting and post-prandial %FMD changes at baseline and post intervention.

Figure 4. TG content of the major lipoprotein subclasses in both groups at baseline and post insulin therapy.

Figure 5. Post-prandial AUC for TG content of major lipoproteins in both groups at baseline and after insulin.

Chapter 7.

Figure 1. Trapping of R· by PBN

Figure 2. Lipid hydroperoxide metabolism.

Chapter 8

Figure 1. Fasting ADP induced platelet aggregation in T2DM and controls.

Figure 2. Fasting SNP and GTN responses in controls and T2DM subjects

Figure 3. Fasting markers of oxidative stress in controls and T2DM subjects.

Figure 4. Fasting and postprandial SNP platelet responses in T2DM.

Figure 5. Fasting and postprandial markers of oxidative stress.

Figure 6. Fasting and post-prandial endothelial function (FMD) in T2DM subjects.
Oral Presentations

European Society of Cardiology, Barcelona, 1999

1. Ciprofibrate blunts post-prandial lipaemia and attenuates the associated endothelial dysfunction and oxidative stress in NIDDM.


American College of Cardiology, New Orleans, 1999

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How are fatty meals atherothrombotic?
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Chapter I

Introduction

Endothelial dysfunction is a complex disorder resulting, in common with many diseases, from the interaction of genetic predisposition and environmental factors. Although our genetic background may set the stage, individual factors, such as diet, smoking, and other elements of lifestyle, are likely to determine the initiation and progression of endothelial dysfunction into CHD. In particular, endothelial dysfunction is thought to be an important factor in the development of atherosclerosis, hypertension and heart failure. Indeed recent studies have shown a close association between impaired endothelial function and progression of cardiovascular disease.

Epidemiological studies have demonstrated a link between eating habits, health and disease. The modern lifestyle with a tendency for high fat diets combined with a reduction in physical activity is detrimental to health. The trend towards increasing urbanization has been directly associated with an increase in dietary fat, and a shift from plant to animal proteins (1). Thirty years ago, the Seven Countries Study (2) demonstrated that a high saturated fat intake strongly predicted coronary heart disease mortality. A link reinforced by the more recent Ireland-Boston diet and Honolulu Heart studies (3,4). A chronic high saturated fat intake results in only modest increases in total cholesterol, raising the possibility that other mechanisms may be involved in the pathological process. Furthermore, with the increasing prevalence of fatty meals linked with popular “fast foods” the role of fatty meal ingestion on the atherogenic diathesis needs re-evaluating. William Heberden (5) made the first observation of a post-prandial (PP) effect on the circulation of blood over two hundred years ago. Its role in exacerbating anginal symptoms is well described (6). In this thesis I aim to illustrate the role that fatty meal ingestion can have via its post-prandial effects on lipoprotein interactions with the vascular endothelium.
Post Prandial Lipaemia

The major part of a lifetime is spent in the post-prandial state. The duration of this period depends on the composition of the meal, but varies between 3 hours for a high glucose meal and 8 hours in a high fat meal. Studies performed in the fasting state are thus ‘post-absorptive’ when TG metabolism and transport have reached equilibrium, this therefore monitors the ‘endogenous pathway’. Whereas studies in the post-prandial phase monitor the ‘exogenous pathway’, i.e., the body’s ability to handle ingested fat.

It is already well known that atherogenesis can be driven by abnormalities in the endogenous pathway, e.g., LDL receptor abnormalities expressing as homo and heterozygous familial hypercholesterolaemia. However, it was only in 1979 that Zilversmit hypothesised that abnormalities in the exogenous pathway of lipid handling could be atherogenic (7). Clinically we do not account for this notion as evidenced by the reliance on the fasting serum cholesterol and TG levels to guide both cardiovascular risk profiles and therapy.

What is PPL?

It is the state of absorption during which TG metabolic capacity is under challenge. Put simply, it is the rise in plasma TG (and by implication TG-rich lipoproteins) following a fatty meal.

Mechanism of fat absorption

Absorption of dietary fat occurs in the duodenum and proximal jejunum. The initiating step involves hydrolysis of dietary triacylglycerols in the stomach by lingual and gastric lipases. The products of this, are carried in a lipid emulsion into the small intestine where they mix with bile and pancreatic juice. The latter is the source of several hydrolytic enzymes, one of which, cholesterol esterase hydrolyzes triacylglycerols, phospholipids and the small proportion of dietary cholesterol that is esterified (8).

The second major intraluminal event involves incorporation of unesterified cholesterol, which has extremely low aqueous solubility, into mixed micelles containing bile acids. Micelles
act as shuttles, delivering fatty acids and monoglycerides to the intestinal microvilli, where they dissociate and diffuse inside the enterocyte. Subsequently, intestinal cells resynthesize TG molecules and incorporate them to chylomicrons that are secreted to the intestinal lymph and thence into the circulation.

Figure 1

Mechanism of absorption.
Factors that determine PPL

The magnitude and duration of the post-prandial hypertriglyceridaemia is determined by a number of factors.

Table 1

<table>
<thead>
<tr>
<th>Factors influencing post-prandial hypertriglyceridaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
</tr>
<tr>
<td>Lipoprotein lipase (hepatic or endothelium-bound)</td>
</tr>
<tr>
<td>Age</td>
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<tr>
<td>Sex</td>
</tr>
<tr>
<td>Obesity</td>
</tr>
<tr>
<td>Exercise</td>
</tr>
<tr>
<td>Apolipoproteins</td>
</tr>
<tr>
<td>LDL receptor protein and activity</td>
</tr>
</tbody>
</table>

(a) Lipoprotein Lipase

The enzyme lipoprotein lipase (LPL) is the key enzyme for hydrolysis of TG from dietary fat (chylomicrons and VLDL) to FFA’s and monoglycerides (9). Through the action of LPL, chylomicrons and VLDL are depleted of their TG cores to form CM remnants and LDL, respectively. LPL is synthesized mainly in adipose tissue and muscle, and functions at the vascular endothelium where it is activated by apoC-II, an apolipoprotein present at the surface of chylomicrons, VLDL and HDL. Therefore LPL holds a key role in controlling lipoprotein/FFA interactions at the endothelial interface.
Figure 2. Schematic diagram of Lipoprotein interactions with lipoprotein lipase (LPL) at the endothelial cell surface releasing free fatty acids, (remnants) CMr/VLDLr and IDL.
Lipoprotein lipase is bound to the vascular endothelium attached to heparan-sulphate anchors, it has been shown to have multiple interactions with lipoproteins and components of the blood vessel wall (10). It is the rate-limiting enzyme for hydrolysis of TG in circulating lipoproteins. The enzyme is synthesized in a variety of tissues, including macrophages (11) and in particular the vascular endothelium. Hydrolysis of TG by LPL primarily occurs in the microvasculature. Lipolysis products can be transported through the circulation to medium-large conductance vessels which are the primary targets of atherogenesis and can be influenced by lipolysis products generated in the microcirculation. If there is a defect in muscle and adipose LPL (eg T2DM), the vessel will be exposed to greater numbers of chylomicrons. Since the action of LPL is limited by substrate concentration, more lipolysis then will be performed by arterial wall LPL.

LPL lipolytically-generated FFA are important sources of cellular energy and are substrates for lipid biosynthesis (12), but these FFA have also been implicated as a causal factor for injury to arterial wall cells and foam cell formation (7). Indeed elevated circulating free fatty acid levels impair endothelium-dependent vasodilatation (13). They can also in excess, impede endothelial cells to produce prostacyclin and cGMP, resulting in an increase in oxidative stress at the endothelial cell surface, impeding directly and indirectly platelet aggregation (14). The clinical relevance of LPL activity has been shown recently, with a significant correlation to angina pectoris severity (15).

(b) Hepatic Lipase

Hepatic lipase is the second major enzyme in the vascular bed responsible for TG and phospholipid hydrolysis (16). Hepatic lipase unlike LPL does not require apoC-II for activation, it therefore preferentially acts on HDL$_2$ particles. Its role is manifest, if decreased LPL activity leads to pronounced PPL, which through cholesterol ester transfer protein (CETP) increases TG content of HDL$_2$ which allows its conversion to HDL$_3$ by hepatic lipase resulting in low HDL$_2$.  

23
(c) Apolipoproteins

ApoC-I and ApoC-II interfere with removal of TG-rich lipoproteins (CM, CMR, IDL and VLDL) via the LDL receptor-related protein (17). ApoE-polymorphisms are also an important determinant for receptor mediated clearance of remnant particles. The most obvious manifestation of this is in ApoE-2 carriers manifesting with type III hyperlipidaemia which is characterised by high circulating VLDL and premature atherosclerosis.

(d) LDL receptor-related (LRP) and LDL receptor

Chylomicron remnants and IDL bind in addition to LRP, the LDL receptor via apoE (18). Chylomicron remnant removal in the circulation is approximately 30% by a LDL receptor-dependent mechanism (19).

(e) Insulin

Insulin in addition to lowering glucose, plays a pivotal role in post-prandial lipid metabolism, by modulating the activity of the key lipolytic enzymes, lipoprotein lipase and hepatic lipase. It thus suppresses lipolysis at the adipocyte so restricting the flux of non esterified free fatty acids to the liver as well as enhancing LDL receptor expression (20).

(f) Age, Sex and Exercise

There is a tendency of increasing age being accompanied by greater PP responses (21). Women have lower TG responses to an oral fat load test with different retinyl ester and HDL responses when compared to men although visceral adiposity is a major contributing factor in this gender difference and results in the exaggerated PP TG-rich lipoprotein response noted in men (22). There is markedly greater PPL in obese subjects with area under the curve for plasma TG being at least twice that of lean individuals. This is in part due to chronic elevations in circulating FFA increasing VLDL lipoproteins (23).
Exercise increases the rate of removal of circulating TG in endurance-training men compared to sedentary men (24). It appears that differences are due to increases in LPL activity, particularly in muscle, and an increase in TG uptake in muscle. When exercise is discontinued for 3 weeks, the magnitude of PPL returns to sedentary pre-existing levels.

**The effect of PPL on other lipoproteins**

PPL has marked effects on other circulating lipoproteins in the circulation. After ingestion of a fat load, or in the presence of high TG (Post-prandially) there is a net transfer of TG from VLDL and chylomicrons to LDL and HDL to the lower density particles. There is a simultaneous back-transfer of cholesteryl ester from LDL and HDL to the lower-density particles. Consequently there is cholesterol enrichment of VLDL and remnants and thus they are more atherogenic. Relative TG enrichment of LDL and HDL allows CETP to work through this to establish equilibrium and result in small dense LDL particles and cholesterol poor small HDL. The end result is more easily oxidizable LDL and lesser antioxidant capacity of HDL, therefore combined they are both made more atherogenic. Coupled with cholesterol enriched VLDL this completes an atherogenic diathesis (25). The action of CETP in the PP state is necessary for the negative association of PP TG levels and HDL-C (26).

**PPL and atherosclerosis**

During this hypertriglycerideamic phase the vascular endothelium is exposed to potentially harmful lipoproteins (27) (VLDL, IDL, LDL and chylomicron [CM] remnants), the so-called circulating atherogenic lipoproteins. The concentrations of these circulating atherogenic lipoproteins are higher in subjects with coronary artery disease (CAD) than normal healthy subjects (28) and predictors of recurrent events in patients with CAD (29). The remnant levels of these lipoproteins have also been correlated with the severity of coronary atherosclerosis in cases of sudden death (30).
Elevated fasting levels of TG and remnant lipoproteins may also be implicated in the pathogenesis of CAD. Subjects with elevated fasting triglycerides but normal LDL-cholesterol levels exhibit endothelial dysfunction and elevated fasting remnant lipoprotein levels are associated with impaired coronary vasomotor function (31). TG-rich lipoprotein remnant particles have been independently associated with the presence, severity and progression of atherosclerosis (32), and high fasting remnant lipoprotein levels predict future coronary events in subjects with CAD (33).

However it seems that post-prandial TG levels may provide an independent marker of risk for the development of atherosclerosis. Patients with CAD (28,34), and offspring of patients with premature CAD (35) exhibit exaggerated TG responses to standard fat containing meals. Elevated plasma TG levels after an oral high fat load predict carotid artery intimal thickness (36), carotid atherosclerosis and exercise-induced ischaemic ECG changes (37,38). Furthermore intervention trials have shown that reduction of TG-rich lipoprotein in plasma leads to a slower rate of progression of coronary atherosclerosis (39) and coronary events (40), data consistent with the involvement of TG metabolism in atherogenesis. This raises the possibility of an enhanced atherogenic risk attributable to repeated episodes of post-prandial lipaemia and the subsequent exposure to circulating TG-rich atherogenic lipoproteins. How may acute elevation of TG-rich lipoproteins exert a deleterious vascular effect after a fatty meal?
Vascular damage

Normal Endothelial Function

The endothelium lies between the lumen and the vascular smooth muscle. Although one cell layer thick, it is able to sense changes in haemodynamic forces, or blood-borne signals, by membrane receptor mechanisms and respond to physical and chemical stimuli by synthesis or release of a variety of vasoactive and thromboregulatory molecules and growth factors. The endothelium therefore plays a key role in vascular homeostasis through the release of a variety of autocrine and paracrine substances. In addition to vasodilatation, a healthy endothelium is antiatherogenic because of effects that include inhibition of platelet aggregation and adhesion (41), smooth muscle cell proliferation (42) and leukocyte adhesion (43). Therefore the endothelium is in the frontline in any assault causing vascular damage, and causes disruption in its prime functions, which are expanded upon below.

Regulation of vascular tone

The endothelium controls underlying smooth muscle tone in response to certain pharmacologic and physiologic stimuli (44). This involves a number of lumen membrane receptors and complex intracellular pathways and the synthesis and release of a variety of relaxing and constricting substances including: Nitric oxide, endothelium derived hyperpolarizing factor (EDHF), prostacyclin and endothelins. In addition to making their own vasoactive mediators, endothelial cells may transduce signals from, or even inactivate, circulating vasodilators and constrictors, such as thrombin, bradykinin, ADP and ATP (45).

Nitric Oxide (NO) is synthesized from L-Arginine by the enzyme, nitric oxide synthase (NOS) (46). The reaction is stereospecific, and L-arginine is converted to NO and L-citrulline. NOS has at least 3 isoforms the main isoform discussed will be eNOS (constitutive or endothelial), the other two being neuronal (n) NOS and inducible (i) NOS. NO maintains low arterial tone at rest, in both the systemic and pulmonary circulation (47). Nitric oxide release is
stimulated by increased flow (leading to increased shear stress on the endothelium) (48) and by bradykinin, thrombin, acetylcholine and a variety of other circulating agents that activate specific endothelial cell membrane receptors (49).

The vasodilatory activity of NO is due to interaction with the iron atom of heme in the cytosolic enzyme soluble guanylate cyclase, causing its activation and thereby increasing intracellular cyclic guanosine monophosphate (cGMP) levels from GTP (50). In smooth muscle cells, this results in a reduction of intracellular calcium and thereby relaxation (51). NO released by the coronary microvessels may also influence myocardial contractility (52). The same pathway is involved in the mechanism of action of exogenous nitrovasodilators, such as sodium nitroprusside (SNP) and nitroglycerin on smooth muscle and platelet activity.
Leukocyte adhesion

The adherence of circulating monocytes to an intact intimal surface is an early event in atherogenesis. Adhesion of monocytes occurs through rolling and tethering of monocytes to endothelial cells, mediated by adhesion molecules. Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression are tonically inhibited by NO both in vitro and in vivo (53, 54). Therefore endothelium-derived NO plays a key role in the regulation of the interaction between the endothelium and circulating monocytes.
Thromboreistance

The endothelium has anticoagulant, antiplatelet and fibrinolytic properties. Endothelial cells are the major site for anticoagulant reactions involving thrombin (55). Platelet adhesion to endothelial cells is markedly inhibited by endothelium-derived NO and prostacyclin (56), furthermore not only is NO a potent inhibitor of platelet adhesion but also of aggregation (57). The same stimuli that activate platelets, such as thrombin, ADP and ATP, also act to release prostacyclin from the endothelium, which allows the endothelium to limit the extent of platelet plug formation (58). The reactions between platelets and endothelium regulate platelet function, coagulation cascades and local vascular tone (59).

Endothelial cells can also secrete tissue-type plasminogen activator (t-PA) (60). t-PA release is stimulated in vivo by noradrenaline, vasopressin or stasis within the vessel lumen. Thrombin may also stimulate t-PA release, providing a further endothelium-mediated safeguard against uncontrolled coagulation. Therefore if there is endothelial dysfunction and/or activation then the balance shifts towards a procoagulant state.

What is endothelial dysfunction?

Endothelial dysfunction is now recognised as a key early event in atherogenesis (61). Simply it is the failure of the endothelium to respond to physical and chemical physiological stimuli with resultant failure of vascular relaxation. When endothelium-dependent vasorelaxation is impaired, there is likely to be either decreased production of and/or increased inactivation of nitric oxide.

Since this is the principal vasodilator substance released by the endothelium, concentrating on just endothelial vasodilatation is however misleading. ‘Endothelial dysfunction’ may refer to impairment of other endothelial functions such as anticoagulation and anti-inflammatory properties (62,63). Dysfunction of endothelial cells is however, a systemic process and the major initiating event in atherosclerosis.
The mechanisms underlying altered endothelium-dependent vascular relaxation in various disease states is almost certainly multifactorial and varies according to the pathological condition, its duration and the vascular bed studied. Deficiency of either the substrate for the enzyme NOS or one of its cofactors will attenuate NO-mediated vasodilatation. Alterations in endothelial cell signalling may also impair appropriate activation of the NOS in response to neurohumoral or mechanical stimuli. In advanced atherosclerosis expression of NOS in the endothelium declines, almost certainly decreasing endothelium-dependent vascular relaxation. There is also evidence that in certain diseases, such as hypercholesterolaemia, diabetes, hypertension and heart failure that NO bioavailability is reduced because of inactivation by excessive production of superoxide anions in the vascular wall. Experimental evidence has demonstrated that endothelium-derived nitric oxide may be an important endogenous antiatherogenic molecule and that reduction in nitric oxide activity facilitates the progression of atherosclerosis (64).

Specific instances of endothelial dysfunction in relationship to specific situations will be addressed in the relevant chapters.

In summary, the loss of the protective actions of nitric oxide, i.e. endothelial dysfunction, may result in a number of abnormalities. These may enhance progression of atherosclerosis including: increased release of vasoconstrictor substances; expression of surface adhesion molecules which facilitate monocyte recruitment and egress into the intima; production of growth factors which promote vascular smooth muscle cell proliferation and migration; and enhanced thrombogenicity mediated by increased platelet activation, plasminogen activator inhibitor-1, and expression of tissue factor.
Oxidative stress

Concept of oxidative stress

A free radical is any species that contains an unpaired electron which is capable of independent existence. They are produced continuously in cells either as by-products of metabolism or, by electron leakage from mitochondrial respiration. Important free radicals in humans, include superoxide (O$_2^\cdot$) and nitric oxide (NO). Although O$_2^\cdot$ and NO are chemically relatively unreactive species, they can undergo further reactions to form highly reactive species such as hydrogen peroxide H$_2$O$_2$, the hydroxyl radical (OH) and peroxynitrite (ONOO') which are damaging in biological systems.

\[
\begin{align*}
O_2^\cdot + NO & \rightarrow ONOO' \\
O_2^\cdot + O_2^- & \rightarrow H_2O_2, O_2 \\
ONOO' & \rightarrow OH'
\end{align*}
\]

Cells have developed a comprehensive set of antioxidant defence mechanisms to prevent free radical formation and to limit their damaging effects. These include the enzymes, superoxide dismutase (SOD), glutathione peroxidase (GSH peroxidase) and catalase. There are further antioxidant defences including Vitamin C and E. A section highlighted in the methods section.

Oxidative stress results when the oxidising species predominate over the antioxidant defences to promote pathological processes. In vivo, after bursts of OH' or O$_2^\cdot$ ions there is a coincident burst of conjugated dienes, lipid hydroperoxides, malondialdehyde and lipid derived free radicals.

Lipids are particularly vulnerable to oxidative attack, which results in the production of lipid peroxides. Lipid hydroperoxides (LOOH) are formed as the by-product of a number of
biological processes. In the absence of metals, such as iron or copper ions, decomposition of lipid peroxides can occur to form reactive alkoxy (LO') and peroxyl (LOO') radicals which can induce and propagate further lipid peroxides in the process of lipid peroxidation.

\[
\begin{align*}
\text{LH} & \xrightarrow{R'} \text{L}^- \\
\text{RH} & \xrightarrow{O_2} \text{LOO}^- \\
\text{LOOH} & \xrightarrow{\text{Fe}^{2+}} \text{LO}^- + \cdot \text{OH}
\end{align*}
\]

Figure 4: Lipid hydroperoxide metabolism

Not only can alkoxy and peroxyl radicals propagate lipid peroxidation but they are also capable of inducing damage to other biomolecules and can react with nitric oxide (65) and thereby down regulate its effects on the vasculature. NO has been shown to react (2 X 10^9 M^-1 s^-1) with lipid hydroperoxyl and/or lipid alkoxy radicals (LO'), resulting in LOONO or LONO formation respectively, which in turn leads to chain termination (66) Therefore experimental detection of these radicals provides important biological information on the interaction between lipids, free radicals and NO.

In this thesis, I have used the technique known as paramagnetic resonance spectroscopy in determining ‘oxidative stress’ and will expand on the methodology and principles behind this technique in the methodology section.
**Post-prandial lipaemia and effects on vascular endothelium**

Impaired endothelium-dependent dilatation (EDD) is a feature of heart failure and atherosclerosis, which may be due to reduced production, and/or increased inactivation of NO (by reaction with oxygen free radicals or cyclooxygenase) and the presence of major risk factors including chronically elevated cholesterol, homocysteine, blood pressure and diabetes. Atherosclerotic risk might therefore be expected to be increased if fatty meals induce transient endothelial dysfunction, particularly if occurring two to three times a day on a repeated basis.

Human vascular endothelial function may be evaluated ultrasonically as brachial artery flow-dependent dilatation, a process mediated by shear stress-induced nitric oxide (NO) release (67). A technique that will be expanded upon in the methods section. Flow-dependent dilatation is diminished after a fatty meal in healthy subjects, starting after two hours with a nadir at three and four hours. The change is independent of total or LDL-cholesterol levels (68). The phenomenon does not occur with low fat meals, implying a causal role for TG and TG-rich lipoproteins. This appears consistent with in vitro and in vivo studies, which show that remnant lipoprotein particles are associated with impaired endothelium-dependent coronary vasodilatation (69)(70). Furthermore, recent evidence has shown that remnant lipoproteins induce proatherothrombogenic changes in endothelial cells via increases in adhesion molecule and tissue factor expression partly via a redox sensitive mechanism (and therefore blunted by HDL-C) (71).

Normal individuals with high fat intakes and disease states with abnormal fat handling (PPL) would therefore be expected to have either/both repetitive and prolonged endothelial dysfunction, giving a cyclical stimulus to atherothrombosis.

Post-prandial endothelial dysfunction clearly may be limited by interventions modifying the production or inactivation of NO. Angiotensin converting enzyme (ACE) inhibition by Quinapril prevents post-prandial endothelial dysfunction in healthy individuals. Angiotensin II receptor antagonists provide lesser benefit, the difference supporting a protective role for bradykinin-induced nitric oxide (NO) synthesis by endothelial cells following ACE inhibition.
Endothelial dysfunction associated with PPL is also prevented by prior folate supplementation, an intervention that under different circumstances increases NO production by increasing the availability of tetrahydrobiopterin, an essential co-factor for NO synthase activity. It may also improve endothelial redox status and in general folic acid has been shown to possess direct free radical scavenging effects in vitro (73). Recent evidence has suggested that PP elevations of asymmetric dimethylarginine (ADMA) may also play a role in the PP deterioration of endothelial dysfunction in type 2 diabetics blunting NO production at an endothelial level (74).

There is increasing evidence that fatty meal ingestion leads to oxidative stress, a condition in which NO inactivation may be increased. During oxidative stress, the superoxide anion can rapidly bind NO, attenuating flow-dependent dilatation either directly or via the toxic cellular consequences of the resultant peroxynitrite formation. Thus increased NO activity might explain the partial prevention of PPL related endothelial dysfunction by the antioxidant vitamins E and C. Red wine also decreases fatty meal-induced changes in lipid hydroperoxides through its antioxidant (polyphenol) effect and might be expected to improve endothelial function although so far evidence is conflicting, possibly due to differences in fat content in the meals studied (75,76). Williams et al showed that cooked rather than uncooked fats cause post-prandial endothelial dysfunction and postulated that ingested products of lipid peroxidation induce endothelial dysfunction (77). This environment of increased oxidative stress may play a role in endothelial cell surface disruption, as shown in vitro recently (78) and result in increased platelet/endothelial cell adhesion in the PP phase.

Not all meals lead to endothelial dysfunction. Although most studies of post-prandial phenomena utilize a standard butter fat meal, Vogel et al (79) have investigated the impact of different meal types on endothelial function. Olive oil based meals have similar effects to butter fat products inducing PP endothelial dysfunction. Canola oil enriched with \( \alpha \)-linolenic acid (\( \omega-3 \)), a component of the diet in the Lyon heart study (80) did not cause significant PP endothelial
dysfunction. Fish oils eaten in the form of salmon resulted in a lesser absolute rise in plasma TG and also did not result in PP endothelial dysfunction (81). These findings may help to explain the low incidence of CAD in Eskimos whose high fat intake is mainly based on fish oil and offer a theoretical basis for at least in part some of the benefit seen in the Lyon heart study (80).

**Post-prandial endothelium and type 2 diabetes**

Patients with type 2 (T2DM) diabetes exhibit exaggerated and prolonged PPL and also premature atherosclerosis (82). Fatty meal-induced decreases in endothelial function and increases in oxidative stress are greater in type 2 diabetics than in healthy controls (83). Several adverse processes are co-operating in these patients. Oxidative stress may be accentuated by low circulating levels of high density lipoprotein (HDL) since HDL normally shuttles lipid hydroperoxides to the liver for inactivation (84) and thus may be expected to reduce levels of lipid-derived free radicals that remain in the vicinity of the endothelium. Diabetic patients also exhibit fasting; increased TG enrichment of VLDL and LDL particles, the abnormality most closely associated with PPL-related endothelial dysfunction. It appears therefore that patients with type 2 diabetes, who are generally predisposed to abnormal fat handling, demonstrate a constellation of post-prandial adverse events; superimposed on a background of increased free fatty acid fluxes, decreased antioxidant capacity and endothelial dysfunction in the fasting state. It therefore seems highly likely that excessive PPL contributes to the risk of macro-vascular disease in type 2 diabetes.
Prothrombosis: Post-prandial Hypercoagulability?

Clotting Factors

As well as affecting major changes on vasomotor endothelial dysfunction, PPL would also be expected to have other effects mediated by dysfunctional endothelium, like effects on the haemostatic system.

Indeed, PPL has been shown to be associated with transient activation of factors VII and XII (85). Coagulation factor VII circulates in plasma mainly as an inactive zymogen; approximately 1% of it circulates as activated factor VII (FVIIa). Activated factor VII is a potent procoagulant, initiating the thrombotic response to atheromatous plaque rupture by complexing with exposed tissue factor to activate the coagulation cascade (86). Activated factor VII levels correlate positively with dietary fat intake and show a measurable decrease within 24 hours of a reduction in total fat consumption (87). In adults taking a diet rich in long-chain saturated fatty acids (88), activated factor VII increases after a meal containing more than 90g fat (89). Factor VII activation correlates quantitatively with the degree of PP triglyceridaemia and seems to be related to free fatty acid production during lipolysis of TG-rich lipoproteins by lipoprotein lipase on the endothelial cell surface. It appears that large PP TG-rich lipoproteins are required in vivo (90) and that long chain saturated fatty acids provide the surface that activates the coagulation system at the endothelial bound lipoprotein lipase/lipoprotein interface (91). The post-prandial rise in activated factor VII can be attenuated however. There is limited evidence that acutely, a meal containing n-3 polyunsaturated fatty acids does not increase activated factor VII (92) or induce endothelial dysfunction (81), suggesting that the two outcomes may be linked. Mechanistic considerations that may in part account for the cardioprotective effect of fish oils.
Fibrinolytic resistance

Not only does PPL induce a prothrombotic tendency but a fatty meal can also decrease the fibrinolytic capacity in the blood. Tissue-type plasminogen activator (t-PA) is the principal fibrinolytic mechanism that operates in the coronary vascular bed (93). Therefore any increase in the natural inhibitor of this plasminogen activator inhibitor-1 (PAI-1), will intimately influence coronary thrombosis in vivo.

Increases in long-term plasma TG levels chronically are associated with increased PAI-1 activity due to activation of the promoter gene by VLDL (94). This undermines the principal fibrinolytic protection in the coronary vasculature in conditions like type 2 diabetes. There is also evidence that PAI-1 activity and PAI-1 antigen is increased acutely after a fat load by 76 and 64% respectively. This infers relative fibrinolytic resistance in the immediate aftermath of a fatty meal (95). Two and four hours after a fatty meal in CAD patients there is also a significant rise in plasma fibrinogen again reinforcing the procoagulant milieu (96).

Platelets

The importance of platelet activation in atherosclerotic vascular disease is supported by both epidemiological and randomized control trial data (97). In cross sectional analysis of the Caerphilly study data, there was an association between increased platelet aggregation and cardiovascular disease (98). The importance of the role of platelets in atherosclerosis is currently emphasized by the number of antiplatelet agents currently under investigation in clinical trials. Potentially altering endothelial function can indirectly alter platelet function.

A number of recent investigations have demonstrated that factors associated with endothelial dysfunction also may induce diminution of tissue responsiveness to NO, both at the level of the blood vessel wall (99-101) and platelet (102,103). This has led to the concept that endothelial dysfunction represents one component of a multi-tissue disorder of vascular homeostasis, in
which smooth muscle and platelet dysfunction are equally important as abnormal endothelial physiology (104-6). There is increasing evidence of a correlation between the extent of endothelial and smooth muscle dysfunction in both coronary (105) and peripheral (104) vascular beds, raising the possibility of common pathogenetic mechanisms. Furthermore, resistance to anti-aggregatory effects of NO donors in platelets appears to be engendered by reversible inactivation of soluble guanylate cyclase together with "scavenging" of NO by $O_2^-$ (11), mechanisms potentially applicable to smooth muscle dysfunction.

The role of PPL and platelet physiology will be addressed in chapter 8.
**Aims**

**Principal aims and objectives**

**Chapter 1**

Outline factors that determine the response to a fatty meal

The effect of a fatty meal on other lipoproteins

The fatty meal and its link with atherosclerosis

The endothelium and endothelial dysfunction

The effect of a fatty meal on the endothelium and in particular its effect in type 2 diabetics.

**The effect of a fatty meal on platelet function.**

**Chapter 2**

An overview on methodology used.

**Chapter 3**

To establish whether the ingestion of a fatty meal in healthy individuals has an impact on endothelial function and markers of oxidative stress and to compare these findings with subjects who have impaired fat handling and possibly depressed antioxidant defences (T2DM subjects).

**Chapter 4**

To examine the effect of attenuating post-prandial lipaemia on endothelial function and oxidative stress in type 2 diabetes.

**Chapter 5**

To examine the effect of antioxidant therapy (Vitamin C) on endothelial function and oxidative stress after a fatty meal.

**Chapter 6**

To examine the effects of a short lived Insulin, Lispro and chronic vitamin C therapy on post-prandial lipaemia, oxidative stress and endothelial function in patients with type 2 diabetes mellitus.
Chapter 7
To use *Ex vivo* EPR Spin trapping to Study the Mechanism of Lipoprotein Peroxidation Caused by Post-Prandial Oxidative Stress in Type 2 Diabetics.

Chapter 8
To study the effect of a fatty meal on platelet nitrate responsiveness in type 2 diabetes

*The hypothesis to be tested in this MD thesis:*

The ingestion of a fatty meal induces a state of transient endothelial dysfunction through a mechanism caused by increased oxidative stress. In type 2 diabetic subjects where there is generally an exaggerated response to a fatty meal, the effect on the vascular endothelium and generation of oxidative stress is enhanced. Modulation of lipid metabolism and antioxidant defences can exert beneficial effects on the vascular endothelium.
Chapter 2

Methods

Post-Prandial Lipaemia – The Standard Fatty Meal.

Central to the study of post-prandial lipid metabolism is the consumption of a standard fatty meal. This was undertaken as described by Patsch (26), and was well tolerated by all subjects. The test meal consisted of 325g of whipping cream, 20g of strawberry-flavoured Nesquick, 15g of granulated sugar and 12g of dry skimmed milk. Of the 1400 calories 3% were derived from protein (9.5g), 14% from carbohydrates (48g) and 83% from fat (130g). The total cholesterol content was 480 mg, with 60 % of the total fat content in the form of saturated fat (~80g). The ingredients were well mixed and the quantity of meal adjusted to provide 65 g of fat per m² of body surface area (26), using a standard normogram. Blood samples were taken at baseline and at 2,4,6 and 8 hours following the meal to enable analysis of plasma lipid and glucose profiles during post-prandial lipaemia.

Assessment of endothelial function

General

Tests to assess the endothelial vasomotor function in humans became available and were either invasive in nature and therefore neither applicable to large study groups nor ideal for repeated studies. In 1992/3 groups began using a non-invasive ultrasound technique for assessment of endothelial function (107,100). In my studies during my MD period, a high resolution ultrasonic wall tracking system developed at our centre was used. There are other methods for measuring endothelial function and are briefly outlined below.
Intracoronary studies

Infusion of acetylcholine (up to $10^{-6}$M) into the coronary circulation has been used to assess conduit vessel vasomotion. This has served as the gold standard over the last decade for endothelial function testing. Recently coronary blood flow assessed using intracoronary doppler wires (108,109) has been used for assessing resistance vessel function in the coronary circulation.

Positron Emission Tomography (PET)

Quantitative assessment of myocardial blood flow and metabolic activity can be made by PET scanning (110). Basal and hyperaemic flow can be obtained to calculate coronary flow reserve (109), this is in part an assessment of endothelial function.

Impedance plethysmography

Hokanson et al (111) described electrically calibrated plethysmography for direct measurement of limb blood flow. It involves intraarterial infusions of acetylcholine to assess endothelial function. There are some concerns regarding day-to-day variability.

Venous studies

Recently described novel investigations into the venous endothelium have been published but are out of the remit of this thesis (112). In view of the facilities available and the ease of repeated studies over intervention phases, the ultrasound technique was used for all studies.

Brachial artery Ultrasound Wall tracking.

Flow-mediated dilatation as observed in response to reactive hyperaemia relies on the ability of the intact endothelium to release nitric oxide (NO). This has been confirmed in conduit arteries such as the brachial (113) and radial arteries (114). In both arteries the specific NO inhibitor L-NMMA abolished at least 70% of the observed vasodilatory response to reactive hyperaemia. In contrast, aspirin (cyclooxygenase inhibition) did not affect flow-mediated dilatation (113,115). Non nitric oxide mediators such as EDHF may also contribute to FMD.
Do studies in the brachial artery have relevance to the coronary circulation?

It is a widely held belief that the brachial artery does not develop any discernible atherosclerosis. A recent post-mortem study gives structural evidence of concurrent correlating atherosclerotic changes in the brachial, carotid and left anterior descending coronary artery (116). Furthermore the severity of the atherosclerotic lesions in the brachial artery was also significantly correlated with both coronary and carotid lesion severity. Suggesting that the brachial artery is a reasonable surrogate marker for studying coronary atherosclerosis.

Do structural correlates indicate functional relevance?

Functional coronary-brachial artery correlations.

Coronary vasomotor responses to acetylcholine infusions have been compared with brachial artery vasodilatory responses to reactive hyperaemia and a close correlation between responses in the two arteries was found (117). In a group of patients referred for coronary angiography, the positive predictive value of abnormal brachial dilatation in predicting coronary endothelial dysfunction was 95%. Furthermore FMD of the brachial artery correlates inversely with the extent of coronary atherosclerosis (117,118). It is unknown whether a similar correlation exists in young healthy subjects in whom abnormal brachial artery vascular reactivity has been observed, a point that is of relevance to the control group in study 1.

There are number of factors that influence the assessment of brachial endothelial function. Vessel size is of critical importance for the vasodilatory response. Controlling for this factor in these studies are of obvious importance. Hormonal variations are also vitally important in women. Endothelium dependent vasodilatation in the brachial artery varies during the menstrual cycle (119). FMD increases significantly during the follicular and luteal phase when serum oestradiol levels are high, although baseline vessel size remains constant. Control for menstrual cycle was made in all studies and subjects as all women in the studies were premenopausal.
Physical activity also modifies the measurements of FMD. Exercise training can improve FMD in healthy individuals (120) therefore in the intervention studies, subjects were asked to and subsequently followed their normal exercise patterns between studies.

Practicalities

Studies were performed on subjects following >10 minutes supine rest with their arm held outstretched on a pneumatic cushion in a temperature controlled room (21-23C). The brachial artery was identified using the ultrasound transducer. Anatomical landmarks (usually arterial bifurcations) were identified to allow repeat studies on the same section of artery. A stand off device containing ultrasound coupling gel was placed beneath the transducer to prevent compression of the anterior wall of the artery. The transducer was held in a clamp and a 2-dimensional B-mode image of the brachial artery was obtained. The M-mode cursor was then positioned perpendicular to the vessel and the horizontal distance between the cursor and the anatomical landmark noted. With the ultrasound machine in M-mode, the radio frequency (RF) signals from the M-mode output were digitised and relayed to the high resolution Valdirec Wall-Track System™ (WTS), resolution +/-3μm. The sampling frequency of the RF signals was 1KHz and the total recording time was 10 seconds. On completion of data acquisition, the first RF signal was displayed on the computer screen. The operator marked the positions of the anterior and posterior vessel walls using sample volume markers. The WTS tracked the vessel wall movements using the stored RF signals, to produce displacement waveforms of the anterior and posterior artery walls, which enabled end-systolic intraluminal diameters to be determined and thus the maximum change in diameter or distension for each beat. Blood pressure was recorded non-invasively using photoplethysmography (Finapres™) with a cuff on the middle finger of the arm being studied.

See Picture 1
Picture 1

The picture on the top left shows 'wall tracking' equipment with the arm held outstretched and the ultrasound probe in the appropriate position.

The next picture shows the M-mode picture (top right) taken with a post dilatation picture on the bottom left.

Finally on the bottom right is the RF signal computer image revealing the markers that clearly define the innermost arterial trace.
Reproducibility

The mean coefficient of variation for baseline arterial diameter measurements in 20 subjects performed 3 weeks apart were 6.8%.

Protocol

Measurements were made at baseline, during increased blood flow secondary to reactive hyperaemia in the hand and after sublingual GTN (400μg). Hyperaemia was produced by releasing a paediatric sphygmomanometer wrist cuff inflated to systolic pressure plus 50mmHg for 5 minutes. Blood pressure was measured continuously and blood flow was recorded for 15 seconds before and until 90 seconds after cuff release. Internal brachial artery diameter was measured 60 seconds after cuff release. Further measurements were made after 15 minutes to confirm vessel recovery. Repeat measurements of brachial artery diameter and blood flow were taken 3 minutes after GTN. Flow and GTN-mediated vasodilatation data are presented as the percentage diameter change in the brachial artery at respective time points and are used as surrogates for endothelium dependent and independent vasodilatation.

Recent guidelines published from the ‘International Brachial Reactivity Task Force’ have concentrated on upper arm cuff occlusion as the method of preference in inducing flow-mediated vasodilatation. Studies however have shown that using a lower arm cuff for occlusion the reactive hyperaemia seen is almost solely NO mediated whereas following upper cuff occlusion only approximately 35% of dilatation is NO mediated (121).

Limitations

There are weaknesses in every technique of assessing endothelial vasomotor function. It can be difficult to acquire optimal images of the brachial artery in some individuals. The position of the transducer must be kept constant during the various phases of the test. Visual analysis is difficult and time consuming. It is important to aware of the wide ranges of flow-mediated
dilatation even in normal subjects (116). In the studies outlined in this thesis, this technique has been used for studying early arterial changes in healthy controls and type 2 diabetics to enable study of transient changes in vasomotor aspects of endothelial function.

1. Oxidative stress measurements

Measurement of oxidative stress.

Due to the short half-life of most reactive free radicals, oxidative stress reactions are usually inferred from the reaction products produced from their interactions with biological substrates. Lipid peroxidation products are the most commonly measured as indices of oxidative damage in vivo. These include exhaled alkanes, isoprostanes and aldehydic products such as 4-hydroxyalkenals and malondialdehyde (MDA) (122). The most widely used index of lipid peroxidation is MDA formation assayed with the thiobarbituric acid (TBA) assay. However, besides MDA, several other compounds including bilirubin are also reactive towards TBA. Additionally, the TBA assay is carried out in strongly acidic conditions with elevated temperatures which may produce MDA as an artifact (123). Moreover, it is often difficult to determine the tissue of origin of the MDA and related products and their temporal relationship. Thus, establishing the involvement of free radicals in the pathogenesis of disease is extremely difficult, partly due to the short lifetimes of these species but also due to the lack of sufficiently sensitive technology to detect free radicals directly in biological systems.

EPR spectroscopy and spin trapping.

Electron paramagnetic resonance (EPR) spectroscopy is the most powerful and direct method for the detection and identification of free radicals. It overcomes many of the problems inherent in other indirect methods of measuring lipid peroxidation and oxidative stress and allows the detection and identification of free radicals produced in vivo. Many free radicals, including
superoxide and lipid free radicals, react too quickly to be observed directly in the laboratory under normal conditions. To detect these species, a chemical trap has to be used and the resultant radical-adduct is stable enough to be characterised by EPR spectroscopy under physiological conditions (124). These 'spin-traps' are too toxic to be used in patients, however they are ideal for detecting secondary free radicals arising from degradation of plasma lipid hydroperoxides in the blood as it is taken (125,126).

Measurement of venous free radicals

It has been well established that the formation of lipid radicals follows oxidative damage in vivo (127). In this study we used the technique of ex vivo spin trapping to investigate the formation of free radicals in venous blood (128,129). Reactive free radicals decay too quickly to be observed directly by EPR spectroscopy under normal laboratory conditions. However, they may be 'trapped' by some chemicals ('spin traps') to form long-lived stable radical adducts which can be observed and identified by EPR spectroscopy. While such spin traps are too toxic to be injected into humans, they can be conveniently used to trap free radicals in blood samples ex-vivo. This technique has recently been used and validated to identify lipid-derived free radicals generated post-exercise in humans (130) and in the coronary sinus of patients undergoing elective cardioplegia (131) and coronary angioplasty (132). Reactive oxygen species induce lipid peroxidation producing secondary lipid radicals, which are spin-trapped and detectable in blood samples ex vivo (131). Lipid derived free radicals were measured ex vivo in venous blood, after fasting and 4 hours post-prandially. 2.0-2.5mls of venous blood was taken directly into foil covered (to prevent photolytic degradation of the spin trap) vacuum-sealed EDTA glass tubes, containing 1ml of the spin trap, α-phenyl N-tert-butyl nitrone (PBN) (0.125mols/l). Following centrifugation at 2000rpm for 5 minutes the PBN adduct was extracted twice from the plasma supernatant with toluene, dried under nitrogen gas and reconstituted in degassed chloroform. EPR spectra were recorded on a Varian E104 spectrometer operating at 9.1GHz at 10mW power, 1
Gauss modulation, 0.25s time constant and 100G scan range. EPR spectral parameters were obtained from data acquisition and processing using in-house EPR computational software. EPR spectral peak heights were taken as a good correlation of spin-adduct concentration after confirmation of peak to peak line width conformity and double integration on selected samples. The separation between sets of peaks in the EPR spectrum (coupling constant) was used to identify the lipid radical species trapped. These were identified as lipid (L') and alkoxyl (LO') free radicals. Analysis of the EPR spectra from spin-trapped radicals derived from venous blood samples suggested that the radicals trapped were alkoxyl radicals (coupling constants $a_N = 13.9$ Gauss, $a_B = 2.2$ Gauss) and lipid radicals ($a_N = 14.1$ Gauss; $a_B = 4.0$ Gauss). These assignments, which agree with previous studies (130), suggest that these radicals are derived from decomposition of lipid hydroperoxides in the extracellular compartment. Detection of secondarily formed lipid free radicals strongly supports the presence of peroxidative damage.
Figure 1

Chemical structure of PBN

Trapping of R· by PBN

\[
\text{H}^-, \text{C}_0
\]

R· can be PBN-LO' and PBN-L'.

Figure 2

\[
\text{ROOH} - e^- \rightarrow \text{ROO}^- + \text{H}^+ (2)
\]

\[
\text{ROOH} + e^- \rightarrow \text{RO}^- + \text{HO}^- (3)
\]

H^+

R1

Both these molecules can undergo B-scission to form CH₃· radicals.

The PBN-LOO' is unlikely to be detected as so unstable.
Potential precursor for LO-PBN adduct may be lipid hydroperoxides.

EPR signal intensity directly proportional to FR adduct content.

**Summary of EPR as a basis of oxidative stress measurement.**

When PBN (the spin trap) is added to a system and an EPR spectrum results, this is *prima facie* (128) evidence that a transient free radical was present. The identity of the radical can be made by analysis of the splitting pattern (see EPR spectra) and electron-nuclear hyperfine coupling constants of the EPR spectrum. The separation between sets of peaks in the EPR spectrum (coupling constant) was used to identify the lipid radical species trapped. These were identified as lipid (L·) and alkoxy (LO·) free radicals. The EPR spectral peak heights were taken as a good correlation of spin-adduct concentration after confirmation of peak to peak line width conformity and double integration.

An example of a typical is spectra is shown below.
Spin labelling of Lipoproteins

This methodology is dealt in more detail in the relevant chapter.

We utilised density gradient ultracentrifugation to separate the different classes of lipoproteins (i.e., chylomicrons, VLDL, LDL, HDL) following ex vivo spin labelling and use the EPR assay to detect the presence of radical adducts in each lipoprotein class in the post-prandial phase.

For total plasma lipid peroxidation-derived free radical (LPDFR) measurements, 3ml venous blood was taken directly into a sealed glass tube, containing 1 ml of PBN (0.125 mol l⁻¹) in saline. Following centrifugation (1000 g, 5 minutes) 2 ml of plasma was removed, and was extracted with toluene, dried under nitrogen gas and reconstituted in deoxygenated chloroform. EPR spectra were recorded on a Bruker EMX X-band spectrometer.

To determine LPDFR in different lipoprotein subclasses, venous blood was drawn into PBN-containing vacutainers, and plasma prepared as above. 2 ml plasma was then carefully deoxygenated by carefully blowing under a stream of oxygen-free nitrogen for 10 minutes, layered on the top of 20 ml of sodium chloride solution of relative density 1.005 (that had been deoxygenated with oxygen-free nitrogen) and then centrifuged at 25000 g for 14 hours at 10 °C. The chylomicron/VLDL containing fractions at the top of the tube, and the HDL/LDL containing fractions at the bottom were removed, and extracted with toluene and measured by EPR as for whole plasma above, with results presented as total arbitrary units per total lipoprotein fraction.

Thiobarbituric acid-reactive substances (TBARS)

To enable, more than one measure of 'oxidative stress' in our studies, we also used the more traditional measurement of lipid peroxidation markers (TBARS). Lipid peroxidation is a well established mechanism of cellular injury in both animals and humans. This process leads to the destruction of membrane lipids and production of lipid peroxides and their by-products such as aldehydes. Malonaldehyde (MDA) and 4-hydroxyalkenals, such as 4-hydroxy-2(E)-nonenal...
(4-HNE), are end products derived from the breakdown of polyunsaturated fatty acids and related esters. Measurements of such aldehyde’s provides a convenient index of lipid peroxidation (133). The LPO-586 assay is based on the reaction of a chromogenic reagent with MDA and 4-HNE at 45°C. One molecule of either MDA or 4-HNE reacts with 2 molecules of the reagent (10.3mM N-methyl-2-phenylindole, in acetonitrile) to yield a stable chromophore with maximal absorbance at 586nm. We used this assay for stored (at −70°C) plasma level of TBARS, an indicator of lipid peroxides in plasma (134) using the above spectrophotometric assay (LPO-586) (Oxis). The results are expressed as μmol/L and give values for malonaldehyde (MDA) and 4-hydroxyalkenals (4-HNE) combined. The reproducibility of this assay using the above protocol over the period analysing our samples is SEM <5%.

**Limitations of the TBARS assay.**

Lipid peroxidation products are the most commonly measured as indices of oxidative damage in vivo. These include exhaled alkanes, isoprostanes and aldehydic products such as 4-hydroxyalkenals and malondialdehyde (MDA) (126). This is an indirect estimate of free radical-evoked cell damage. The most widely used index of lipid peroxidation is MDA formation assayed with the thiobarbituric acid (TBA) assay. However, besides MDA, several other compounds including bilirubin are also reactive towards TBA. Additionally, the TBA assay is carried out in strongly acidic conditions with elevated temperatures which may produce MDA as an artifact (122). Moreover, it is often difficult to determine the tissue of origin of the MDA and related products and their temporal relationship.

**Biochemical measurements.**

Cholesterol and TG concentrations (μmol/ml) in each gradient fraction were measured spectrophotometrically using standard diagnostic reagent kits (135,136). Amounts of cholesterol and TG (μmoles) in each gradient fraction were calculated and the cholesterol and TG content of
HDL, LDL and VLDL computed by summation of the appropriate fractions (as determined by agarose gel electrophoresis). (137,138). Plasma glucose was measured by a hexokinase-based technique and HbA1c via enzyme immunoassay (138).

**Lipoprotein separation.**

Chylomicron free plasma was analysed to assess the compositional variation in the three major lipoprotein subclasses during PPL in T2DM and control subjects in chapter 3 and pre and post fibrate therapy in chapter 4.

Whole blood was collected into EDTA tubes and centrifuged at 20,000 g for 20 minutes. 2.4ml of this chylomicron free plasma was placed into a tube containing 0.8ml of iodixanol (Liposep™). The iodixanol/plasma mixture was filled with Hepes buffered saline in order to facilitate VLDL and LDL separation. After centrifugation, a standard photometric technique was used to measure the cholesterol and TG concentration in each gradient fraction. By plotting the TG and cholesterol content of each fraction against fraction number, the fractions corresponding to HDL, LDL and VLDL could be identified. This fraction-banding pattern was further confirmed by agarose gel electrophoresis. The total TG and cholesterol concentration in each of the lipoprotein classes was calculated by summing the concentrations of TG and cholesterol in those fractions corresponding to each particular lipoprotein class. This provided a measure of TG and cholesterol within each lipoprotein expressed as µmolar concentrations per ml. The total concentrations of HDL and LDL may be calculated using this methodology (137).

**Platelets**

The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to the vessel wall injury, following platelet activation. The platelet surface has various receptors and there are diverse stimuli which activate platelets, with equally diverse platelet responses to these stimuli, mediated by the binding of various stimulants to specific platelet receptors.
It is possible to characterize platelet responses into the ‘reversible’ platelet responses, which include adhesion, shape change and reversible aggregation and the ‘irreversible’ platelet responses that comprise release reaction and secondary irreversible aggregation (139). With regards this thesis, the sole marker of platelet function is that of aggregation ex vivo to adenosine diphosphate (ADP).

**Platelet Methodology**

**Blood sampling and preparation of platelets**

Precautions were taken to minimise the potential for activation of the clotting system or platelets during blood sampling. Venous blood was collected from the antecubital fossa in plastic tubes containing 1:10 volume of acid citrate anticoagulant; acidified citrate was utilised in order to minimise deterioration of platelet function during experiments (140,141).

**Platelet Aggregation Studies**

Aggregation in whole blood was examined utilising a whole blood impedance aggregometer (ChronoLog Corporation). Tests were performed at 37°C and stirring speed of 900rpm. Samples of blood were diluted twofold with normal saline (final volume 1ml) and prewarmed for 5 minutes at 37°C. Aggregation was induced with adenosine 5'-diphosphate (ADP)(1μM) and responses monitored continually for 7 min, and responses were recorded for electrical impedance in Ohms. Inhibition of aggregation was tested with exogenous NO donors, SNP and GTN (final concentration of 10 and 100μmol/L respectively) were added to samples 1 min before ADP. The duration of incubations were estimated as those optimal in preliminary experiments. In control tests, physiological saline was added in appropriate volumes. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of SNP and GTN studied.
Limitations

There are weaknesses in the methodology of this section of my thesis. The use of one concentration of ADP although based on previous experimental data could potentially limit the NO donor/platelet responsiveness data. Full dose response curves may have illuminated the actual dose that a significant difference in NO donor resistance was demonstrated. Furthermore no PP platelet responses were performed in healthy controls which may have delineated the question of platelet responsiveness postprandially. Platelet aggregometry techniques do not necessarily correlate with other tests of platelet activation and may have serious limitations in extrapolating to in vivo measures. There is uncertainty whether the ex vivo conditions in the platelet aggregometer truly reflect in vivo aggregation.
Chapter 3

Post-prandial lipaemia, Endothelial Function and Oxidative Stress in healthy individuals compared to type 2 diabetic subjects.

Does post-prandial lipaemia induce endothelial dysfunction and oxidative stress?

To establish whether the ingestion of a fatty meal in healthy individuals had an impact on endothelial function and markers of oxidative stress. Furthermore to compare these findings with subjects who had impaired fat handling and possibly depressed antioxidant defences (T2DM subjects).

Introduction

PPL represents the state of absorption during which the TG metabolic capacity is under challenge. The magnitude of PPL may be defined as the extent and duration of the rise in plasma TG after a standard fatty meal, peaking at 4 hours and in T2DM lasting up to 12 hours. After a fatty meal, healthy subjects demonstrate endothelial dysfunction for up to 4 hours, which can be attenuated by the antioxidant vitamin C (68) suggesting a possible free radical mediated mechanism. Endothelial dysfunction was not observed however with low fat meals (81) suggesting a role for TG and potentially TG-rich lipoproteins in this phenomenon.

In the fasting state, subjects with elevated triglycerides but normal LDL-cholesterol levels exhibit endothelial dysfunction (141) and elevated fasting remnant lipoprotein levels are associated with impaired coronary vasomotor function (31). A significant relationship between PPL and coronary artery disease (CAD) has been reported in association with both normal and abnormal carbohydrate metabolism (33). TG-rich lipoprotein remnant particles have been independently associated with the presence, severity and progression of atherosclerosis (142) with higher levels of remnant lipoprotein particles in the fasting state, being shown to predict future coronary events in subjects with CAD (29).
T2DM is associated with a markedly increased risk of macrovascular disease, particularly affecting coronary artery disease (CAD). The UKPDS 33 demonstrated that intensive glycaemic control has no significant impact on macrovascular disease incidence (143). The Paris prospective study demonstrated that plasma triglyceride (TG) level was the most important predictor of coronary death in T2DM (144). This infers that abnormal lipid handing in T2DM may be more likely to impart the large macrovascular risk associated with T2DM. In T2DM, there is a consistent, prolonged and exaggerated post-prandial (PP) excursion in plasma TG with subsequent TG enrichment of lipoprotein particles (145), resulting in an ‘atherogenic’ lipid profile (including abnormal PPL) and fasting endothelial dysfunction. The object of this study was therefore to test the hypothesis that TG-rich lipoproteins produced during PPL induce transient endothelial dysfunction and oxidative stress in healthy individuals and compare this effect to T2DM subjects; where there is decreased antioxidant defences and an excess of these TG-rich lipoproteins both in the fasting and post-prandial state.

Study subjects.

12 consecutive patients with T2DM (mean age 47.3 years; range 35-53; 7 men and 5 women) (diagnosed on WHO criteria) whose glycaemic control was moderate (HbA1c 6.5-10%, mean 8.5%) were enrolled in this study and compared with 12 healthy volunteers (mean age 43 years; range 30-63; 5 men and 7 women). All subjects were non-smokers, normotensive, and had no history or clinical evidence of overt vascular disease; had fasting cholesterol levels less than 6.5mmol/L, and were not taking aspirin, lipid lowering or hormone replacement therapy, or supplemental vitamins. All women in both groups were studied at approximately the same time in their menstrual cycles. The diabetic group had a significantly higher body mass index (32.2 ± 4.1 vs. 27.5 ± 3.2 p<0.05). All T2DM subjects were either diet controlled or were on oral hypoglycaemic agents. Those subjects taking oral agents omitted their medication the morning of
Written informed consent was obtained from all subjects, and the local research ethics committee approved the study.

Study Protocol.

Studies began at 9am after a 12-hour overnight fast. Blood was drawn for serum total, LDL and HDL cholesterol, triglycerides, lipid subfractionation, glycosylated haemoglobin, glucose and venous FR measurements, and brachial artery endothelial function was measured. Each subject was then given a standard fatty tolerance test meal (28), in the form of homogenised milk shake containing 80 grams of saturated fat, with a total calorific value of 1480 Kcal. Lipid profiles and plasma glucose were repeated at 2 hourly intervals over the following 8 hours. Lipoprotein subfractions were assessed at baseline, 4 and 8 hours post prandially, with lipid derived FR’s and endothelial function tested at baseline and during the peak lipaemic phase, namely 4 hours following meal ingestion. (68)

Endothelial function

Measured as described in the methods section.

Oxidative stress measurements

Measured as described in the methods section;

(a) Electron paramagnetic resonance spectroscopy.

(b) Thiobarbituric acid reacting substances.

Biochemical Measurements

Measured as described in the methods section.

Lipoprotein subfractions

Measured as described in methods section.
Statistics

Statistical analysis was performed on Minitab (version 12). Conventional methods were used for calculating mean, standard deviation and checks for normal distribution. Group differences in continuous variables were determined by two-tailed t-test. Statistical significance for differences in continuous variables between groups was tested by one way analysis of variance. As a measure of plasma glucose and the total amounts of lipid and lipoprotein present in plasma during the 8-hour post-prandial period, areas under the curve (AUC) were calculated for plasma concentrations without subtraction of baseline values. AUC was calculated using Simpson’s integration rule (145). This measure of AUC, without subtraction of baseline values was used since the principal aim of this study was to investigate the effect of post-prandial lipaemia on measures of endothelial function and oxidative stress. Multiple-regression analysis was used to study the independent relationship between variables with logarithmic transformation of skewed data, statistical significance was inferred when p < 0.05.
Results

Post-prandial lipaemia

There were no significant quantitative changes in total, LDL or HDL cholesterol or plasma glucose during the PP period in either group. Although, as expected plasma glucose remained consistently higher in the T2DM group compared to controls (Table 1). Table 1 also illustrates the changes in plasma TG over 8 hours; plasma TG increased significantly in response to the meal, with peak levels attained after four hours and in the T2DM group remaining elevated after 8 hours. The total AUC-TG (mmol/l/8hrs) was significantly higher in the T2DM group than in controls (191.8 ± 108 [T2DM] vs. 64.25 ± 10.75 [controls], p < 0.001), fasting plasma TG levels correlating with total AUC in both groups (r = 0.883, p < 0.001 [T2DM], r = 0.71, p = 0.003 [controls]).

Table 3 illustrates the compositional changes in HDL, LDL and VLDL as a result of PPL. There was significant TG enrichment of all lipoproteins in the T2DM subjects at both 4 and 8 hours following the fatty meal. In the healthy subjects, there was TG-enrichment of VLDL. PPL in the T2DM group resulted in the production of TG rich lipoproteins, with AUC for TG content of HDL, LDL and VLDL being significantly greater in the T2DM group than controls (Table 4). As expected TG enrichment of HDL-C and VLDL-C correlated with AUC TG, r=0.55, 0.52; p=0.002 and p=0.03 respectively.

Cholesterol distribution amongst the lipoproteins remained unchanged at all post-prandial time points in both groups. The cholesterol content of VLDL in the T2DM subjects remained significantly elevated compared to controls. Whereas, the cholesterol content of HDL remained significantly lower in T2DM subjects than the control subjects (Table 2).
Endothelial Function.

Fasting FMD in healthy controls, demonstrated no significant correlation with any measured metabolic parameter. There was a significant decrement in FMD post-prandially in controls (Graph 2). In the diabetic subjects there was lower fasting FMD, which deteriorated significantly post-prandially. Baseline brachial artery diameters were 3.49 ± 0.23mm and 3.38 ± 0.42mm in the control and T2DM groups respectively. In the T2DM group, fasting FMD, demonstrated a significant correlation with a variety of parameters including total, HDL and LDL-cholesterol, plasma TG, chronic glycaemia and TBARS (Table 5), no correlation was noted with compositional analyses of lipoprotein content.

Post-prandial endothelial function

In healthy controls, the reduction in FMD associated with PPL correlated inversely with fasting HDL-C levels (r = - 0.84, p = 0.001). There was no correlation with any other measured variable including the magnitude of post-prandial hypertriglyceridaemia. In the T2DM group, the reduction in FMD associated with PPL was related to several parameters. (Table 6). The magnitude of post-prandial hypertriglyceridaemia (AUC TG), fasting plasma TG levels and the TG enrichment of both VLDL and LDL demonstrated the strongest correlation with reduction in FMD in the T2DM group. Total plasma HDL-C however correlated inversely with the reduction in FMD during PPL in both groups. Post-prandially, there were no significant changes in endothelium-independent vasodilatation in either group (Graph 2).

Oxidative stress

Lipid-derived free radicals increased post-prandially in both controls (2.4 ± 0.1 to 3.3 ± 0.2, p<0.05) and T2DM (2.25 ± 0.4 to 4.5 ± 1.3 units p<0.05), the increase being greater in T2DM (p<0.05) (Graph 1). Analysis of the EPR spectra from spin-trapped radicals derived from venous blood samples suggested that the radicals trapped were alkoxyl radicals (coupling constants aN = 13.9 Gauss, aβH = 2.2
Gauss) and carbonyl radicals (aN= 14.1 Gauss; a\beta H = 4.0 Gauss). These assignments, which agree with previous studies (20) suggest that these radicals are derived from decomposition of lipid hydroperoxides in the extracellular compartment. Detection of secondarily formed lipid free radicals strongly supports the presence of peroxidative damage. Fasting markers of lipid peroxidation were significantly greater in T2DM subjects compared to controls whilst fasting lipid derived free radicals were similar in both groups. (Graph 1) In T2DM, fasting TBARS positively correlated with HbA1c, VLDL-C (r=0.535, p<0.05) and LDL-C (r=0.529, p=0.05). There was also a negative correlation with fasting FMD (r=-0.72, p<0.05). There was no relationship between fasting TBARS and any measured variable in control subjects.

**Correlation between PPL and oxidative stress.**

PPL in both groups resulted in augmented oxidative stress, the rise in free radicals correlated significantly with the extent of VLDL TG enrichment [AUC for VLDL-TG content (r = 0.59, p = 0.02), (T2DM), (r = 0.55, p = 0.03) (Controls)]. Both groups also exhibited significant increases in TBARS post-prandially, indicating increased oxidative stress post-prandially. There were no correlations between the post-prandial rise in oxidative stress and the magnitude of PPL as defined by AUC for plasma TG in both groups. Due to the complex nature of the interaction between PP lipid metabolism and its effect on FMD in T2DM, multiple regression analysis was applied to assess the associations between FMD and measured variables. In a stepwise model, the change in FMD during PPL was assessed as the dependent variable against all measured factors. The change in oxidative stress, HbA1c, AUC for plasma TG and the AUC for plasma glucose were found to be significant determinants (p = 0.002) of the observed reduction in FMD, accounting for 64.2% of observed variation in the change in FMD. Using fasting FMD as the dependent variable, age, fasting TG, HbA1c and fasting LDL-C levels (p < 0.0001), were seen to be the most significant determinants, accounting for 75.7% of variation.
Table 1

Glucose and lipid parameters in T2DM and control subjects, fasting, 4 and 8 hours after consumption of a fatty meal.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4 hours</th>
<th>8 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma TG</strong></td>
<td>Controls</td>
<td>1.33 ± 0.5</td>
<td>2.2 ± 0.7*</td>
</tr>
<tr>
<td></td>
<td>T2DM</td>
<td>3.5 ± 2.1†</td>
<td>9.1 ± 8.2*†</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>Controls</td>
<td>5.7 ± 0.75</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>T2DM</td>
<td>5.9 ± 1.1</td>
<td>6.1 ± 1.1</td>
</tr>
<tr>
<td><strong>LDL-C</strong></td>
<td>Controls</td>
<td>3.2 ± 0.25</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>T2DM</td>
<td>3.7 ± 0.8</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td><strong>HDL-C</strong></td>
<td>Controls</td>
<td>1.39 ± 0.3</td>
<td>1.38 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>T2DM</td>
<td>1.04 ± 0.22†</td>
<td>0.99 ± 0.25†</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>Controls</td>
<td>4.7 ± 0.4</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>T2DM</td>
<td>11.9 ± 3.3†</td>
<td>11.2 ± 3.4†</td>
</tr>
</tbody>
</table>

(* denotes P<0.05 vs baseline). (†denotes P<0.05 vs Controls at the same time points). All measurements in mmol/l.
Table 2
Distribution of cholesterol and TG amongst major lipoprotein subclasses in both groups in the fasting state. Data expressed as mean ± SD, lipoprotein cholesterol and TG content represented in μmol/ml.

<table>
<thead>
<tr>
<th></th>
<th>Controls – TG content</th>
<th>T2DM -TG content</th>
<th>Controls – Cholesterol</th>
<th>T2DM – Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.7 ± 0.14</td>
<td>2.68 ± 1.12*</td>
<td>1.06 ± 1.25</td>
<td>2.06 ± 1.21*</td>
</tr>
<tr>
<td></td>
<td>0.31 ± 0.11</td>
<td>0.97 ± 0.44*</td>
<td>4.5 ± 1.7</td>
<td>4.31 ± 1.16</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.11</td>
<td>0.57 ± 0.48*</td>
<td>1.85 ± 0.9**</td>
<td>1.14 ± 0.9</td>
</tr>
</tbody>
</table>

* Denotes P<0.05 T2DM vs Controls at baseline, ** denotes p<0.05 controls vs T2DM at baseline.
Table 3

Cholesterol and TG distribution in the major subclasses during PPL in both groups. Data is expressed as mean ± SD and lipoprotein cholesterol and TG content is represented as µmol/ml.

<table>
<thead>
<tr>
<th></th>
<th>VLDL 4hrs</th>
<th>VLDL 8hrs</th>
<th>LDL - 4hrs</th>
<th>LDL - 8hrs</th>
<th>HDL - 4hrs</th>
<th>HDL - 8hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls TG</td>
<td>0.86 ± 0.56</td>
<td>0.72 ± 0.59</td>
<td>0.34 ± 0.14</td>
<td>0.31 ± 0.17</td>
<td>0.14 ± 0.07</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>T2DM TG</td>
<td>4.73 ± 2.26*</td>
<td>3.32 ± 1.65*</td>
<td>1.04 ± 0.78*</td>
<td>0.92 ± 0.66*</td>
<td>0.68 ± 0.65*</td>
<td>0.66 ± 0.61*</td>
</tr>
<tr>
<td>Controls Cholesterol</td>
<td>1.11 ± 1.02</td>
<td>0.99 ± 1.03</td>
<td>4.1 ± 1.68</td>
<td>4.32 ± 1.63</td>
<td>1.72 ± 0.81**</td>
<td>1.71 ± 0.76**</td>
</tr>
<tr>
<td>T2DM Chol</td>
<td>2.39 ± 1.08*</td>
<td>2.54 ± 1.11*</td>
<td>4.78 ± 1.81</td>
<td>4.51 ± 1.76</td>
<td>1.15 ± 0.51</td>
<td>1.21 ± 0.52</td>
</tr>
</tbody>
</table>

* Denotes P<0.05 T2DM vs Controls, ** Denotes P<0.05 Controls vs T2DM.

Table 4:

Post-prandial AUC for TG content (µmol/ml/8 hrs) of lipoproteins in both groups, following a fatty meal. Data is expressed as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>AUC VLDL-TG</th>
<th>AUC LDL-TG</th>
<th>AUC HDL-TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2DM</td>
<td>1.79 ± 0.22*</td>
<td>1.22 ± 0.35*</td>
<td>1.01 ± 0.43*</td>
</tr>
<tr>
<td>Controls</td>
<td>1.13 ± 0.41</td>
<td>0.56 ± 0.27</td>
<td>0.81 ± 0.28</td>
</tr>
</tbody>
</table>

* Denotes P < 0.05 T2DM > Controls, when analysed using 2 sample Mann-Whitney test.
Table 5. Correlation of fasting FMD with various parameters in the T2DM group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>-0.026</td>
<td>0.912</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>-0.375</td>
<td>0.094</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.53</td>
<td>0.014*</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>-0.55</td>
<td>0.005*</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>-0.52</td>
<td>0.016*</td>
</tr>
<tr>
<td>Insulin (lu/l)</td>
<td>-0.23</td>
<td>0.118</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>-0.58</td>
<td>0.006*</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.47</td>
<td>0.034*</td>
</tr>
<tr>
<td>EPR peak heights (Units)</td>
<td>-0.32</td>
<td>0.16</td>
</tr>
<tr>
<td>TBARS (μmol/l)</td>
<td>-0.72</td>
<td>0.008*</td>
</tr>
</tbody>
</table>
Figure 1

Post prandial rises in LDFR and TBARS in healthy controls and T2DM subjects.

(* denotes P<0.05 vs baseline). Error bars indicate ± SD.
Figure 2

Post prandial changes in vascular responses in healthy controls and T2DM subjects.

(* denotes $P<0.05$ vs baseline). (+ denotes $P<0.05$ vs Controls). Error bars indicate ± SD.
Table 6.
Correlation of PPL and metabolic parameters with the reduction in FMD in the T2DM group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TG (mmol/l)</td>
<td>0.52</td>
<td>0.015*</td>
</tr>
<tr>
<td>AUC plasma TG</td>
<td>0.51</td>
<td>0.03*</td>
</tr>
<tr>
<td>Fasting Insulin (IU/l)</td>
<td>0.060</td>
<td>0.79</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.33</td>
<td>0.14</td>
</tr>
<tr>
<td>AUC HDL-C (mmol/l/8hr)</td>
<td>-0.49</td>
<td>0.033*</td>
</tr>
<tr>
<td>AUC LDL-C (mmol/l/8hr)</td>
<td>0.37</td>
<td>0.099</td>
</tr>
<tr>
<td>AUC Total Cholesterol (mmol/l/8hr)</td>
<td>0.22</td>
<td>0.33</td>
</tr>
<tr>
<td>Increase in oxidative stress</td>
<td>0.26</td>
<td>0.22</td>
</tr>
<tr>
<td>AUC HDL-TG content (µmol/ml/8hr)</td>
<td>-0.33</td>
<td>0.11</td>
</tr>
<tr>
<td>AUC LDL-TG content (µmol/ml/8hr)</td>
<td>0.49</td>
<td>0.03*</td>
</tr>
<tr>
<td>AUC VLDL-TG content (µmol/ml/8hr)</td>
<td>0.58</td>
<td>0.02*</td>
</tr>
</tbody>
</table>
Discussion.

Previous reports of endothelial dysfunction in association with PPL in healthy individuals have suggested that TG-rich lipoproteins and oxidative stress may be involved (68). The present study demonstrates that in healthy controls post-prandial lipaemia induces endothelial dysfunction, which is inversely correlated with fasting HDL-C, but not with the magnitude of post-prandial hypertriglyceridaemia. Therefore HDL-C appears protective against PP endothelial dysfunction. Secondly venous lipid-derived free radicals increase in association with endothelial dysfunction, which correlates with VLDL TG enrichment, implying that TG-rich VLDL induces oxidative stress and is associated with endothelial dysfunction after a fatty meal.

The study also shows fasting endothelial dysfunction in T2DM subjects, a finding that correlates with glucose, plasma TG, total and LDL-Cholesterol and inversely with HDL-C. This is consistent with previous data indicating impaired agonist-induced vasodilatation correlating with plasma TG levels and HDL/total cholesterol levels (146). Furthermore the deterioration in FMD in the T2DM group most strongly correlates with TG enrichment of both VLDL and LDL (the potential atherogenic molecules); In addition PP oxidative stress correlated strongly with TG-enrichment of VLDL. These observations suggest that following a fatty meal in patients with T2DM, the atherogenic insult appears to be TG-rich VLDL and LDL and involves endothelial dysfunction and oxidative stress. The elevations of alkoxyl and carbonyl free radicals post-prandially can cause endothelial dysfunction by a number of mechanisms. They can inhibit cellular processes and react rapidly with nitric oxide (NO) to terminate chain propagation in vivo (147) and can also subsequently form reactive nitrogen species (148) resulting in blunted flow-mediated vasodilatation. This transient increase in oxidative stress is superimposed on existing elevations in lipid peroxidation, which relates to fasting endothelial function. The augmented oxidative stress seen in diabetics may be the result of greater free radical production than controls or alternatively decreased antioxidant defenses. In subjects with impaired glucose tolerance (IGT) and T2DM, there is a deficit in antioxidant capacity, involving particularly low levels of vitamin C and urate (149). Depressed vitamin E levels have also been found in diabetic patients with increased lipid
peroxides (150) and when associated with hypertriglyceridaemia (151). Our TBAR data concurs with these findings. In vitro data would seem to indicate that PP lipid changes (via TG enriched VLDL) increase the oxidative milieu present in the arterial wall. Monocytes and polymorphonuclear cells release more superoxide anions when exposed to plasma from hypertriglyceridaemic patients (152,153), a phenomenon also positively correlated with plasma TG and negatively with HDL. Furthermore leucocyte activation secondary to hypertriglyceridaemia may contribute to the increased oxidative stress seen in T2DM (154).

The source of the increase in oxidative stress observed in this study is unclear. Two measures of oxidative stress were used i.e. EPR spectroscopy measurements and TBARS. In the fasting state, direct measurement of carbonyl and alkoxyl radicals were similar to normal controls in the fasting state, indicating that 'active' lipid peroxidation was similar at baseline. However TBARS provide evidence of previous lipid peroxidation with stable end product accumulation and were significantly greater in fasting T2DM subjects than those of controls, consistent with previous data (150,151). The PP rise in carbonyl and alkoxyl radicals indicates a transient rise in reactive species at the 4 hour time point, accompanying ED. This suggests an acute oxidative milieu post-prandially, a finding substantiated by the rise in TBARS. This data supports in vitro data indicating that oxidative damage caused by lipid components in remnant lipoproteins, especially peroxidized phospholipids, deteriorates cell surface membranes and may be partly responsible for remnant induced impairment of endothelial-dependent relaxation (71).

The data suggest that oxidative stress results from TG enrichment of lipoproteins, as indicated by the correlation with TG enrichment of VLDL. Oxidative stress is probably causally related to the post-prandial impairment of EF since antioxidants attenuate this transient impairment of EF in normal subjects in previous studies and both measures of oxidative stress were elevated post-prandially in both groups (68). TG enriched VLDL was a significant determinant of the deterioration in PP endothelial dysfunction; supporting observations by Lewis and colleagues in fasting subjects (141) but is at variance with the observation of normal EF in patients with profound hypertriglyceridaemia, (type V hyperlipidaemia)
These findings including those of the present study suggest that functioning lipoprotein lipase is required for the endothelial damage by these lipoproteins.

AUC for plasma glucose and basal metabolic control (HbA1c) were also significant determinants of PP endothelial dysfunction. These observations are supported by data demonstrating endothelial dysfunction in acute hyperglycaemia (156). Poor overall metabolic control may play a role in PP endothelial dysfunction by several potential mechanisms. Increased supply of atherogenic free fatty acids (FFA’s) including production of reactive aldehydes, enhanced production of advanced glycation end products and glycation of lipoproteins.

PPL affects lipoprotein composition by TG enrichment. LDL-cholesterol enriched with TG results in smaller dense LDL-C particles and large VLDL-C particles. These have a prolonged endothelial residence time and thus increased propensity to both absorption by macrophages and endothelial cell injury (27), a view supported by the present data. VLDL particles enriched with TG, are toxic to endothelial cells in vitro. Recent data shows that VLDL particle exposure from hypertriglyceridemic patients (equivalent to post-prandial levels) results in NF-kB activation in endothelial cells after 6 hours, implying that TG-rich lipoproteins and oxidative stress are intimately linked (157). In addition lipid enriched HDL particles have an impaired endothelial protective capacity in hypertriglyceridaemia, in particular diminished antioxidant potential, particularly the ability to shuttle reactive hydroperoxides from the endothelium to the liver for excretion (158). HDL inhibits endothelial cell injury mediated by TG-rich lipoproteins remnants (159) a factor which may explain our observation of reduced FMD and fasting HDL-C levels in both groups.

TG-rich lipoproteins can also influence EF indirectly by changing the distribution of LDL particles to a smaller, denser LDL population. Plasma TG being the dominant factor in reducing LDL size (160), a factor that may be important chronically. An acute increase in FFA fluxes in the PP phase can contribute to the endothelial dysfunction and oxidative stress seen. This could be mediated by either facilitation of excess cholesterol-rich lipoproteins entering the intima (161,162) or by decreasing the endothelial
protective properties of albumin (163) as VLDL causes endothelial cell toxicity when FFA/albumin ratio is elevated (163). FFA’s have also been shown to impair cultured endothelial cell responsiveness to NO and prostacyclin (14). Furthermore they have been shown to increase markers of lipid peroxidation (163) and therefore may underlie the present observations of greater deterioration in endothelial dysfunction and elevated TBARS seen in the diabetic subjects. It may also explain the non-significant fall in endothelial-independent vasodilatation post-prandially in both groups.

This study shows that free radicals increase post-prandially in normal individuals. HDL appears to protect against the transient impairment of endothelial function by TG-rich VLDL lipoproteins during PPL. In T2DM subjects, where fasting ED is present, PPL results in further decrements in endothelial function, which appears to be related TG-rich VLDL, LDL and a greater increase in oxidative stress. These phenomena may be related to compounding effects of hyperglycaemia and dyslipidaemia. Therapeutic interventions aimed at decreasing TG-VLDL and oxidative stress post-prandially may therefore attenuate endothelial dysfunction and atherogenesis in both healthy subjects and appears particularly important in T2DM.
Conclusions

In healthy individuals:

1. PPL is associated with endothelial dysfunction and increased oxidative stress.
2. TG enriched VLDL correlated with the degree of oxidative stress seen.
3. HDL-C is inversely correlated with the increase in oxidative stress.

In T2DM subjects:

1. A fatty meal results in greater PPL than healthy controls.
2. PPL induces greater oxidative stress in association with endothelial dysfunction.
3. TG-VLDL and TG-LDL are correlated with the PP deterioration in endothelial dysfunction.
4. TG-VLDL is correlated with the PP rise in oxidative stress.
5. There is fasting endothelial dysfunction.
Chapter 4
To examine the effect of attenuating post-prandial lipaemia on endothelial function and oxidative stress in subjects with type 2 diabetes (T2DM).

Introduction

Cardiovascular disease is the most common complication of T2DM (164). Many of the established risk factors for atherosclerosis are characteristic of T2DM. The so-called metabolic syndrome (165) includes a dyslipidaemia characterised by hypertriglyceridaemia, reduced HDL-Cholesterol (HDL-C) and abnormal post-prandial lipaemia (PPL) (166-168,144). Recent evidence suggests that glycaemia is not the major determinant of CHD in T2DM (143). Fasting hypertriglyceridaemia, appears to be an independent predictor of CHD in type 2 diabetes in both cross sectional (144) as well as prospective studies (168). Furthermore, fasting hypertriglyceridaemia (with elevated TG rich VLDL) in non-diabetic subjects with normal total and LDL cholesterol levels is associated with endothelial dysfunction (141).

PPL represents the state of absorption during which the TG metabolic capacity is under challenge. Since many of the factors involved in post-prandial lipid metabolism are insulin sensitive (166) there is a clear potential for abnormalities of PPL to arise in T2DM. PPL in T2DM consists of a prolonged and exaggerated excursion in plasma TG with subsequent lipoprotein TG enrichment (169). A significant relationship between PPL and both severity and progression of coronary atherosclerosis has been described in subjects with and without T2DM (28,141). After a fatty meal, non-diabetic subjects demonstrate transient ED, which can be attenuated by the antioxidant vitamins C and E (170). No dysfunction is noted following a low fat meal. Thus lipoprotein TG enrichment during PPL results in an ‘atherogenic’ lipoprotein profile, putatively causing enhanced oxidative stress and endothelial dysfunction (ED).

From chapter 3, the previous study demonstrates that in healthy controls post-prandial lipaemia induces endothelial dysfunction, and this phenomenon is inversely correlated with
fasting HDL-C, but not with the magnitude of post-prandial hypertriglyceridaemia. Therefore HDL-C may have a protective role against PP endothelial dysfunction. Secondly venous lipid-derived free radicals (a marker of oxidative stress) increase in association with endothelial dysfunction, which correlates with VLDL TG enrichment, implying that TG-rich VLDL may induce oxidative stress and is associated with endothelial dysfunction after a fatty meal in healthy individuals.

In T2DM, it has been shown that there is fasting endothelial dysfunction in T2DM subjects, a finding that correlates with glucose, plasma TG, total and LDL-Cholesterol and inversely with HDL-C. This is consistent with previous data indicating impaired agonist-induced vasodilatation correlating with plasma TG levels and HDL/total cholesterol levels (146). Secondly the deterioration in FMD in the T2DM group most strongly correlates with TG enrichment of both VLDL and LDL (the potential atherogenic molecules); Thirdly PP oxidative stress correlated strongly with TG-enrichment of VLDL. These observations suggest that following a fatty meal in subjects with T2DM, the atherogenic insult may be TG-rich VLDL and LDL, involving both endothelial dysfunction and oxidative stress.

Numerous studies have demonstrated the presence of endothelial dysfunction (ED) in T2DM, such that ED is a recognised association of T2DM (14). However these studies were conducted in the fasting state and may actually underestimate the true extent of ED in T2DM. From the previous findings in chapter 3, it was hypothesised that fibrate therapy may impact on endothelial function; oxidative stress and PPL in T2DM. To this end it was decided to study the effects of a fibric acid derivative (Ciprofibrate), a widely used class of lipid regulating agents, which exerts a variety of effects on lipid and lipoprotein metabolism (171). In particular, ciprofibrate attenuates PPL (172), decreases TG enrichment of lipoproteins and in particular reduced TG rich VLDL and LDL.
Subjects

20 T2DM patients (mean age 47.5 years, age range 35-53, 11M, 9F – diagnosis based on WHO criteria) - mean HbA1c 8.5% were recruited. All subjects were non-smokers, normotensive with fasting total cholesterol < 6.5 mmol/l. Each subject had a normal resting electrocardiogram and no personal or family history of premature vascular disease. A family history of premature vascular disease was defined as the absence of symptomatic macrovascular disease in a first degree relative prior to the age of 65. All subjects were not taking aspirin, lipid lowering agents, or supplemental vitamin therapy. Female subjects were pre-menopausal and were studied at approximately the same time in their menstrual cycles (Follicular phase). Written consent was obtained from all subjects, with approval from the local ethics committee.

Study protocol

Studies were commenced following a 12-hour overnight fast. After 30 minutes of supine rest venous blood was drawn for measurement of total, LDL and HDL cholesterol, plasma triglyceride, insulin, glucose and glycosylated haemoglobin (HbA1C), 5 ml of venous blood was also drawn to enable measurement of venous free radicals. Each subject was given a fatty meal (17) containing 80 grams of saturated fat, lipid profiles and plasma glucose were repeated 2 hourly over the following 8 hours. Endothelial function and oxidative stress were assessed fasting and during peak lipaemia. Each patient was randomised to three months treatment with either Ciprofibrate 100mg once daily or matched placebo, after which the above investigations were repeated.
Methods

Endothelial function
Measured as described in the methods section.

Oxidative stress measurements
Measured as described in the methods section;
(a) Electron paramagnetic resonance spectroscopy.
(b) Thiobarbituric acid reacting substances.

Biochemical Measurements
Measured as described in the methods section.

Lipoprotein subfractions
Measured as described in methods section.

Statistical analysis

Statistical analysis was performed on Minitab (Version 12). Conventional methods were used for calculating mean, standard deviation and checks for normal distribution. Group differences in continuous variables were determined by a two-tailed t-test. Statistical significance for differences in continuous variables between groups was tested by one way analysis of variance. As a measure of plasma glucose and the total amounts of lipid and lipoprotein present in plasma during the 8-hour post-prandial period, areas under the curve (AUC) were calculated for plasma concentrations without subtraction of baseline values. This measure of AUC was used since the principle aim of this study was to investigate the effect of PPL on fasting measures of endothelial function and oxidative stress. Multiple-regression analysis was used to study the independent relationship between variables with logarithmic transformation of skewed data, statistical significance was inferred when $p < 0.05$. 
Results

Patient characteristics

There were no significant differences in blood pressure, or BMI between groups at baseline or after treatment (Table 1).

Biochemistry

There were no differences in total, LDL cholesterol, insulin, glucose and HbA1C between groups at baseline and after treatment. There was a significant reduction in fasting plasma TG following ciprofibrate, with an associated rise in HDL-C. Table 3 illustrates the fasting VLDL, LDL and HDL compositional changes in both placebo and ciprofibrate groups as a result of three months treatment (Table 2).

Post-prandial lipaemia:

AUC for post-prandial plasma TG (mmol/l/8hrs) was similar in both groups at baseline (199.8 ± 98 [Ciprofibrate], 188.6 ± 110 [placebo]). There was a significant reduction in post-prandial hypertriglyceridaemia following Ciprofibrate (Fig 1) resulting in reduced plasma TG AUC (199.8 ± 98 [baseline], 78.9 ± 21.6 [3-months]; p < 0.05). No changes were noted following placebo (Fig 2).

AUC for the TG content of HDL, LDL and VLDL was initially similar in both groups (Table 3). After 3 months of Ciprofibrate, post-prandial lipoprotein TG enrichment was significantly reduced (Table 4). There was no significant increase in glucose between fasting and 4-hours. Post-prandial glycaemia (AUC glucose) was unchanged by treatment.
**Vascular data:**

At baseline, fasting and post-prandial FMD was similar in both groups, with a significant reduction in post-prandial FMD (Fig 3). Fasting FMD significantly improved in the ciprofibrate group $3.8 \pm 1.8\%$ vs. $4.8 \pm 1.1\%$, $p< 0.05$. Similarly, post-prandial FMD also improved significantly $1.8 \pm 1.3\%$ vs. $3.4 \pm 1.1\%$, $p < 0.05$. There were no changes following placebo (Fig 3). GTN mediated vasodilatation was similar in both groups, with no significant changes following treatment (Fig 4). Fasting and post-prandial resting arterial diameter, resting and hyperaemic blood flow were similar in both groups, before and after treatment.

**Free radical data:**

At baseline both groups exhibited similar measures of fasting and post-prandial oxidative stress (Fasting – $2.33 \pm 1.1$ [ciprofibrate group], $2.4 \pm 1.8$ [placebo group]; post-prandial - $3.85 \pm 2.5$ [ciprofibrate], $3.7 \pm 2.9$ [placebo]) (Fig 5). After treatment, fasting oxidative stress was similar in both groups with no change from baseline ($2.27 \pm 1.3$ [ciprofibrate], $2.36 \pm 1.5$ [placebo]). After treatment the post-prandial excursion in oxidative stress was attenuated by ciprofibrate, ($2.57 \pm 1.9$ vs. $3.89 \pm 2.5$, $p < 0.05$).

**Correlation between post-prandial lipaemia, endothelial function and oxidative stress**

Fasting FMD correlated inversely with LDL-C ($r = -0.55$, $p = 0.03$ [ciprofibrate], $r = -0.52$, $p = 0.02$ [placebo]) and TG content of VLDL ($r = -0.51$, $p = 0.03$ [ciprofibrate], $r = -0.50$, $p = 0.04$). HDL-C correlated positively with FMD ($r = 0.52$, $p = 0.03$ [ciprofibrate], $r = 0.55$, $p = 0.02$ [placebo]). The post-prandial reduction in FMD correlated with post-prandial TG enrichment of VLDL ($r = 0.52$, $p = 0.04$ [ciprofibrate], $r = 0.54$, $p = 0.03$ [placebo]) and LDL ($r = 0.55$, $p = 0.03$ [ciprofibrate], $r = 0.52$, $p = 0.04$ [placebo]) and inversely with 4-hour HDL-C ($r = -
Baseline fasting oxidative stress demonstrated no significant correlations. The post-prandial increase in oxidative stress correlated with post-prandial TG enrichment of VLDL (AUC for VLDL TG content) \((r = 0.51, p = 0.04 \text{[ciprofibrate]}, r = 0.53, p = 0.03 \text{[placebo]})\). After treatment there were no significant correlations (at the 95% confidence level) between the fasting and post-prandial improvement in endothelial function and any changes in the measured metabolic parameters as a result of ciprofibrate therapy.
Table 1:
Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Ciprofibrate (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Ciprofibrate 3-months</th>
<th>Placebo 3-months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Systolic BP (mm/hg)</td>
<td>139.4 ± 23.6</td>
<td>144.1 ± 31.7</td>
<td>141.2 ± 25.8</td>
<td>146.9 ± 22.5</td>
</tr>
<tr>
<td>Resting Diastolic BP (mm/hg)</td>
<td>75.5 ± 9.8</td>
<td>72.7 ± 6.3</td>
<td>73.9 ± 7.7</td>
<td>75.1 ± 6.8</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>32.7 ± 4.2</td>
<td>31.3 ± 6.7</td>
<td>32.9 ± 4.9</td>
<td>31 ± 6.3</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49.2 ± 3.7</td>
<td>48.7 ± 4.5</td>
<td>48.2 ± 2.7</td>
<td>49.6 ± 6.5</td>
</tr>
<tr>
<td>Sex</td>
<td>6M / 4F</td>
<td>5M / 5F</td>
<td>5M / 4F</td>
<td>4M / 4F</td>
</tr>
</tbody>
</table>

Table 2:
Fasting biochemistry in both groups at baseline and after treatment (Mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Ciprofibrate (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Ciprofibrate 3-months</th>
<th>Placebo 3-months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>11.1 ± 3.3</td>
<td>10.1 ± 4.1</td>
<td>10.5 ± 3.4</td>
<td>11.2 ± 4.6</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.9 ± 1.1</td>
<td>5.6 ± 1.4</td>
<td>5.6 ± 1.3</td>
<td>5.7 ± 1.2</td>
</tr>
<tr>
<td>LDL - C (mmol/l)</td>
<td>3.57 ± 0.6</td>
<td>3.46 ± 0.9</td>
<td>3.51 ± 0.5</td>
<td>3.42 ± 0.5</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.03 ± 0.2</td>
<td>1.09 ± 0.2</td>
<td>1.26 ± 0.1*</td>
<td>0.98 ± 0.3</td>
</tr>
<tr>
<td>Insulin (IU/l)</td>
<td>32.1 ± 17</td>
<td>31.9 ± 10.8</td>
<td>28.9 ± 10.1</td>
<td>32.4 ± 14.7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.3 ± 1.3</td>
<td>8.01 ± 1.5</td>
<td>7.9 ± 1.5</td>
<td>8.15 ± 1.6</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>2.8 ± 2.1</td>
<td>2.8 ± 1.7</td>
<td>1.5 ± 0.8*</td>
<td>3.1 ± 2.7</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 compared to baseline.
Table 3:
Fasting cholesterol and TG distribution among major lipoproteins in both groups at baseline and after treatment. Cholesterol and TG concentrations are expressed as μmol/ml (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Ciprofibrate (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Ciprofibrate (Post-treatment)</th>
<th>Placebo (post-treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL TG content</td>
<td>2.54 ± 0.98</td>
<td>2.61 ± 1.14</td>
<td>1.46 ± 0.55*</td>
<td>2.53 ± 1.06</td>
</tr>
<tr>
<td>VLDL Cholesterol</td>
<td>2.02 ± 1.29</td>
<td>2.07 ± 1.19</td>
<td>1.08 ± 0.65*</td>
<td>2.09 ± 1.05</td>
</tr>
<tr>
<td>LDL TG content</td>
<td>0.99 ± 0.39</td>
<td>0.94 ± 0.51</td>
<td>0.40 ± 0.24*</td>
<td>0.92 ± 0.36</td>
</tr>
<tr>
<td>LDL Cholesterol</td>
<td>4.28 ± 1.20</td>
<td>4.39 ± 1.21</td>
<td>4.31 ± 1.27</td>
<td>4.43 ± 1.29</td>
</tr>
<tr>
<td>HDL TG content</td>
<td>0.59 ± 0.49</td>
<td>0.55 ± 0.41</td>
<td>0.13 ± 0.11*</td>
<td>0.51 ± 0.44</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>1.09 ± 0.7</td>
<td>1.16 ± 0.9</td>
<td>1.79 ± 0.22**</td>
<td>1.18 ± 0.65</td>
</tr>
</tbody>
</table>

* Denotes P < 0.05, baseline > post-ciprofibrate TG and cholesterol content.
** Denotes P < 0.05, post treatment > baseline cholesterol content.
Table 4.
Post-prandial AUC for TG content ($\mu$ mol/ml/8 hrs) of major lipoproteins in both groups (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>AUC VLDL-TG content</th>
<th>AUC LDL-TG content</th>
<th>AUC HDL-TG content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofibrate group (Baseline)</td>
<td>1.66 ± 0.35</td>
<td>1.19 ± 0.45</td>
<td>1.07 ± 0.31</td>
</tr>
<tr>
<td>Placebo group (Baseline)</td>
<td>1.72 ± 0.41</td>
<td>1.25 ± 0.30</td>
<td>1.03 ± 0.48</td>
</tr>
<tr>
<td>Ciprofibrate group (3-mths)</td>
<td>1.19 ± 0.21*</td>
<td>0.76 ± 0.48*</td>
<td>0.88 ± 0.21*</td>
</tr>
<tr>
<td>Placebo group (3-mths)</td>
<td>1.79 ± 0.57</td>
<td>1.21 ± 0.22</td>
<td>1.07 ± 0.39</td>
</tr>
</tbody>
</table>

* Denotes P < 0.05, For TG content reduction in ciprofibrate group after 3 months
Fig 1. Post-prandial lipaemia in Ciprofibrate group at baseline and after 3 months treatment

* Denotes $P > 0.05$, for Pre-treatment $>$ post-treatment
Fig 2: Post-prandial lipaemia in placebo group at baseline and after 3 months treatment
**Fig 3**: Flow mediated endothelium dependent vasodilatation (FMD) in placebo and Ciprofibrate groups at baseline and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>4-Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td><img src="baseline_fasting.png" alt="Bar chart" /></td>
<td><img src="baseline_4-hours.png" alt="Bar chart" /></td>
</tr>
<tr>
<td>3-Months</td>
<td><img src="3-months_fasting.png" alt="Bar chart" /></td>
<td><img src="3-months_4-hours.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

Ciprofibrate group

Placebo group

* P < 0.05 (Fasting post-treatment vs Baseline); ** P < 0.05 (Post-prandial post-treatment vs baseline)
Fig 4: GTN induced endothelium independent brachial artery dilatation at baseline and after 3 months in both groups.
**Fig 5:** Fasting and post-prandial oxidative stress in placebo and Ciprofibrate groups at baseline and after treatment

* Denotes $p < 0.05$, post-treatment < baseline in Ciprofibrate group.
Discussion

This study demonstrates an association between exaggerated post-prandial lipaemia with the production of atherogenic lipoproteins enhanced oxidative stress and augmented endothelial dysfunction in T2DM, which is attenuated by ciprofibrate.

Fasting endothelial function correlated inversely with LDL-C levels and TG content of VLDL, and positively with HDL-C levels. The post-prandial deterioration in endothelial function correlated inversely with HDL-C and positively with, the post-prandial TG enrichment of VLDL and LDL. Post-prandial TG enrichment of VLDL was the only parameter to correlate with post-prandial oxidative stress. There was also a trend toward reduced post-prandial GTN responsiveness, which has been previously described in other studies examining the effects on of transient hypertriglyceridaemia on endothelial function (173). This effect may partly due to increased free fatty acid levels, which may down regulate smooth muscle responsiveness to NO (13).

Associations between endothelial function and both LDL-C and HDL-C in T2DM have been previously described (174,175). This is however the first study to demonstrate a direct association between TG rich lipoproteins and endothelial dysfunction, both fasting and post-prandial in T2DM. Furthermore, this study demonstrates an association between TG rich VLDL and enhanced oxidative stress in T2DM.

Lipoprotein analysis, from the Monitored Atherosclerosis Regression Study (MARS) (176) demonstrate the importance of TG rich VLDL and IDL as predictors of atherosclerotic disease progression. TG rich VLDL particles preferentially undergo endocytosis by receptors on macrophages to form foam cells (177). Furthermore lipolytic products of TG rich VLDL are toxic to endothelial cells and macrophages (177,178). TG rich Apo-B containing particles have also been isolated in excess from atherosclerotic plaques (179). Increased free fatty acid fluxes particularly during PPL may potentate the effects of TG rich VLDL on endothelial function, since VLDL causes enhanced endothelial toxicity when FFA / albumin ratios are elevated (180).
TG enrichment of VLDL also affects LDL metabolism (181), with increased synthesis of atherogenic small dense particles. Such LDL particles are more toxic to endothelial cells, readily undergo oxidation and exhibit enhanced pro-oxidant potential (182).

During PPL there was TG enrichment of LDL and HDL particles. Hydrolysis of TG in these TG rich LDL particles contributes further to the production of smaller, denser particles. TG rich, cholesterol deplete HDL particles, partly produced as a result of enhanced neutral lipid exchange with TG rich VLDL, demonstrate decreased endothelium protective properties, including reduced antioxidant properties (183).

Not only are these particles produced in excess during PPL in type 2 diabetes, there is also reduced particle catabolism as a result of impaired LPL function (82) and defective particle clearance as a result of increased particle competition for receptor mediated endocytosis (185). Functional LPL is required to produce such an atherogenic lipoprotein profile, and thus mediate the effects of hypertriglyceridaemia on endothelial function, since in hypertriglyceridaemic patients with LPL deficiency, endothelial function is preserved (173).

T2DM is associated with enhanced oxidative stress (185), which represents a state of disequilibrium between free radical production and antioxidant defences. This study supports an association between transient hypertriglyceridaemia with the production of TG rich VLDL particles during PPL in T2DM with increased oxidative stress. The precise mechanisms accounting for this observation remain speculative and may involve a variety of complex changes in lipoprotein metabolism (173).

Enhanced oxidative stress has a variety of important effects in atherogenesis, including lipoprotein oxidation - in particular LDL oxidation (186), this may be of particular relevance in T2DM, in which such particles predominate. Oxidised LDL has both pro-oxidant properties and enhanced endothelial toxicity (187). Enhanced oxidative stress may also directly induce endothelial dysfunction by decreasing synthesis and release of NO by endothelial cells and by inactivating NO in the sub-endothelial space (183). Furthermore, the alkoxyl radicals detected by
the EPR methodology employed in this study have been shown to directly interact with NO (149). Thus the observation of deteriorating endothelial function associated with PPL in T2DM may result from a combination of dyslipidaemia and oxidative stress.

Following ciprofibrate, fasting and post-prandial endothelial function significantly improved, coupled with a reduction in post-prandial oxidative stress. Fasting plasma TG and post-prandial AUC for TG were also reduced, whilst HDL-C levels were increased. There were also modest but non-significant reductions in total, LDL cholesterol, plasma insulin and HbA1c. Furthermore, there was TG depletion of all lipoproteins, with cholesterol enrichment of HDL and cholesterol depletion of VLDL.

The non-significant reduction in fasting insulin and HbA1c may reflect improved insulin sensitivity and as this positively relates to endothelial NO synthesis (186), this could potentially account for some of the observed change in endothelial function. However improved fasting endothelial function correlates most strongly with reduced fasting plasma TG, and TG depletion of VLDL and HDL (r = 0.54, 0.46 and 0.48 respectively). Moreover the improvement in post-prandial endothelial function correlates most strongly with attenuation of post-prandial hypertriglyceridaemia and lipoprotein TG enrichment. Additionally, the changes in post-prandial oxidative stress correlate most strongly with attenuation of VLDL TG enrichment. These correlations were not however significant at the 95% confidence level, which may be due to the relatively small sample size and small absolute differences, rather than a true lack of biological significance.

Fibrates cause decreased production and enhanced catabolism of TG rich VLDL (172). There is subsequently reduced neutral lipid exchange resulting in TG depletion of LDL and HDL with cholesterol enrichment of increased concentrations of HDL. This results in production of larger, less dense particles with reduced atherogenic potential, which may be of even greater significance during PPL.
The mechanisms by which ciprofibrate improves endothelial function and oxidative stress during PPL remain speculative. Attenuating the magnitude and duration of the endothelium’s exposure to atherogenic lipoproteins may be of benefit, with reduced TG rich VLDL particles and increased HDL-C levels being of particular importance. Although ciprofibrate improved endothelial function both in the fasting state and post-prandially, it did not however blunt the decrement in endothelial function post-prandially. The mechanism for this is not entirely clear but highlights the atherogenicity of PPL even when modulated (to non-diabetic levels). A theory supported by the effect of PPL on endothelial function in healthy individuals.

In summary, we confirmed the earlier findings that PPL in T2DM results in endothelial dysfunction and enhanced oxidative stress. Ciprofibrate therapy, by attenuating PPL and modifying the atherogenic lipoprotein profile, leads to significant improvement in fasting and post-prandial endothelial function, and attenuates post-prandial oxidative stress.

**Clinical Implications**

This study highlights the importance of PPL on endothelial function, in this case particularly in T2DM. Can intervention with fibric acid derivatives therefore impact upon secondary prevention of coronary heart disease in T2DM by mechanisms including improving both fasting and post-prandial endothelial function.

Recent results from the Veterans Affairs HDL Intervention study (VA-HIT) study and Bezaﬁbrate Infarction Prevention study (BIP) (187) are conflicting in their results. In the VA-HIT study (188), there was an overall 22% reduction in major CHD, a quarter of the 2531 participants were diabetics. The subgroup analysis revealed that diabetics accrued similar benefits to non-diabetics with ﬁbrate therapy. In the BIP study there was a 9.4% reduction (p=0.26) in non-fatal events, with only 10% being diabetics. In fact, the confidence limits for both studies overlap and
therefore the differences seen may be due to chance and overall there was an approximate 16% reduction in CHD risk combining both data sets (189).

Where fibrate therapy would be expected to be particularly beneficial are in those subjects with features of the ‘metabolic syndrome’. The metabolic syndrome includes features such as high TG’s, low HDL-C, small dense LDL-C and abnormal PPL; coupled with this is a preponderance of insulin resistance, glucose intolerance/diabetes, hypertension and obesity. Over 80% of the VA-HIT subjects had 2 or more features of the syndrome and 50% with 3 or more. This may explain the relative greater benefit seen in the VA-HIT study compared to the BIP study.

More definitive answers from this area will be available later this year from the Diabetic Atherosclerosis Intervention Study (DAIS) (190) looking at angiographic regression in T2DM. Further primary and secondary prevention results will be available in 2005 from the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study until then the exact role for fibrate therapy as secondary prevention is unclear.
Chapter 5.

Prolonged deterioration of endothelial dysfunction in response to post-prandial lipaemia is attenuated by vitamin C in type 2 diabetes.

Introduction

A fatty meal, results in alimentary or post-prandial lipaemia (PPL) which causes transient perturbations in circulating lipoprotein particles. It is that period during absorption, when capacity for triglyceride (TG) metabolism is exceeded and is characterised by increased plasma levels of TG and subsequent lipoprotein enrichment by TG. This results in TG-enriched VLDL, a propensity for small dense LDL, and low HDL levels (7). Endothelial function is diminished after a fatty meal in healthy subjects, starting after two hours with a nadir at three and four hours, the change being independent of total/LDL-cholesterol levels (68). The phenomenon does not occur with low fat meals, implying a causal role for TG and TG-rich lipoproteins. Remnant lipoprotein particles are associated with impaired endothelium-dependent coronary vasodilatation (69,70), furthermore, remnant lipoproteins also induce proatherothrombogenic changes in endothelial cells via increases in adhesion molecule and tissue factor expression partly via a redox sensitive mechanism (and therefore blunted by HDL-C) (71). Findings consistent with chapter 3 showing HDL-C levels related to the degree of PP endothelial dysfunction after a fatty meal (83).

Post-prandial endothelial dysfunction may be limited by interventions modifying the production or inactivation of NO. Angiotensin converting enzyme (ACE) inhibition prevents post-prandial ED in healthy individuals. Angiotensin II receptor antagonists provide lesser benefit, the difference supporting a protective role for bradykinin-induced nitric oxide (NO) synthesis by endothelial cells following ACE inhibition (72). ED associated with PPL is also prevented by prior folate supplementation, an intervention that increases NO production by increasing the availability of tetrahydrobiopterin, an essential co-factor for NO synthase activity. It may also improve endothelial redox status and possess direct scavenging effects in vitro (73).
PP elevations of asymmetric dimethylarginine (ADMA) may also play a role in the PP deterioration of ED in T2DM blunting NO production at an endothelial level (74).

There is increasing evidence that fatty meal ingestion leads to oxidative stress, where NO inactivation may be increased. We have shown that PPL in healthy individuals results in ED with significant elevations in both lipid derived free radicals and products of lipid peroxidation in the plasma (chapter 3), (83). An observation that is related to the TG enrichment of VLDL and is inversely related to protective HDL levels. Indeed PPL related endothelial dysfunction is attenuated by the antioxidant vitamins E and C in healthy individuals (170). Furthermore the redox-sensitive transcription factor NF-kB is activated PP in monocytes coinciding with peaks in TG and chylomicrons, implying an increase in oxidative stress, reversible with the presence of antioxidants from red wine (quercetin and alpha-tocopherol) in healthy volunteers (191).

Patients with T2DM exhibit exaggerated and prolonged PPL (82). Fatty meal-induced decreases in endothelial function and increased oxidative stress are greater in T2DM than in healthy controls (83) at 4 hours. Diabetic patients also exhibit fasting increased TG enrichment of VLDL and LDL particles, low HDL-C levels, the abnormalities most closely associated with PPL-related ED. These features would therefore be expected to present throughout an 8 hour time period.

We therefore sought to establish; (1) the duration of both endothelial dysfunction and increased oxidative stress after a fatty meal in T2DM and (2) examine whether the oral antioxidant therapy vitamin C (1g bd) prior to and concomitant with a fatty meal could improve endothelial function and blunt the rise in oxidative stress without manipulating lipid metabolism.
Methods

Subjects

20 T2DM subjects (mean age 53.5 ± 8.8 years; range 38-68 years; 14 men and 6 women) (diagnosed on WHO criteria) whose glycaemic control was moderate (HbA1c 6.5-10%, mean 8.4%) were enrolled in the study. All subjects were non-smokers, normotensive, and had no history or clinical evidence of overt vascular disease; had fasting cholesterol levels less than 6.5mmol/L, and were not taking aspirin, lipid lowering or hormone replacement therapy, nor supplemental vitamins. All women in both groups were studied at the same time in their menstrual cycles (follicular phase). All subjects were either diet controlled or were on oral hypoglycaemic agents. Those subjects taking oral agents omitted their medication on the morning of the study. Written informed consent was obtained from all subjects, and the local research ethics committee approved the study.

Study Protocol.

As previously described in the methods section.

Subjects were randomized in a double blind fashion to a 3 day period of either placebo or vitamin C (1g bd). With the study protocol repeated after 1g VC ingestion with a repeat study fat meal.

Measurement of endothelial function.

As previously described in the methods section.

Lipid assay and biochemical measurements.

As previously described in the methods section.

Measurements of oxidative stress

(a) Venous lipid-derived free radicals.

As previously described in the methods section.

(b) Thiobarbituric acid-reactive substances (TBARS)

As previously described in the methods section.
Data analysis.

Statistical analysis was performed on Minitab (version 12). Conventional methods were used for calculating mean, standard deviation and checks for normal distribution. Group differences in continuous variables were determined by two-tailed t-test. Statistical significance for differences in continuous variables between groups was tested by one way analysis of variance. As a measure of plasma glucose and the total amounts of lipid and lipoprotein present in plasma during the 8-hour post-prandial period, areas under the curve (AUC) were calculated for plasma concentrations without subtraction of baseline values. AUC was calculated using Simpson's integration rule (147). This measure of AUC, without subtraction of baseline values was used since the principal aim of this study was to investigate the effect of post-prandial lipaemia on measures of endothelial function and oxidative stress. Multiple-regression analysis was used to study the independent relationship between variables with logarithmic transformation of skewed data, statistical significance was inferred when p < 0.05.

Results

Patient characteristics and Biochemistry

There was no significant difference between groups at baseline, with total, LDL cholesterol, glucose (fasting and post-prandial), HbA1c, blood pressure in particular remaining unchanged (Table 1). Cholesterol and TG content of the major lipoprotein subclasses (µmol/ml) in both groups at baseline were similar and did not change with placebo nor vitamin C (Table 2).
Post-prandial lipaemia.

AUC for post-prandial plasma TG (mmol/l/8hrs) was similar in both groups (56.9 ± 8 [vit C], 52.6 ± 11 [placebo]), post-prandial AUC for TG and cholesterol content (μ mol/ml/8 hrs) of major lipoproteins in both groups at baseline were also similar and again did not change after intervention (Table 3). The intervention of VC/placebo did not alter any of the lipid variables.

Markers of oxidative stress

There was a significant rise in oxidative stress measured by EPR spectroscopy in both groups at baseline at the 4 hour time point. (VC group) 3.01 ± 0.62 to 4.34 ± 0.72 (p<0.005) and (P) 2.8 ± 0.5 to 4.37 ± 0.78 (p<0.05). This rise was completely abolished by concurrent VC treatment 3.1 ± 0.4 down to 2.9 ± 0.6 but not placebo 3.2 ± 0.4 to 3.96 ± 0.5. The 8 hour measures of oxidative stress tailed off further with VC but not placebo (Figure 1), it did not however fall significantly from the 4 hour timepoint.

This pattern was maintained in oxidative stress measured by markers of lipid peroxidation (Figure 2). These measures of oxidative stress showed a significant increase at the 4hr timepoint from 5.6 ± 1.6 to 8.3 ± 1.6. VC treatment significantly blunted the rise in TBARS from 6.6 ± 2.6 to 7.5 ± 2.9 compared to placebo. The 8hr timepoint showed a variable response. At baseline in both groups there is a drop from a peak at 4hrs. (VC – group) 8.3 ± 1.6 to 5.8 ±1.9; (placebo group) 6.7 ± 0.9 to 6.6 ± 0.9, (p=ns).

Changes in endothelial function

In response to a fatty meal there was a consistent significant deterioration in FMD at 4hrs both at baseline in the two groups and after placebo therapy (Fig 3.). At 8hrs FMD approached baseline in a variable fashion but generally though not significantly remained below the fasting
level. There was however marked improvement in FMD after VC therapy at all time points (Figure 3.) (P<0.05).

Although VC therapy did not completely attenuate PP endothelial function, there was only a 15% relative reduction in PP endothelial function compared to 33% in the baseline study. Therefore there was relative blunting of PP endothelial dysfunction.

**Correlation between PPL, endothelial function and oxidative stress.**

Tables 4 and 5 illustrate the associations between PPL FMD and oxidative stress at baseline and after treatment. At baseline, fasting FMD correlated inversely with LDL-C and positively with HDL-C concentrations. The deterioration in post-prandial FMD correlated with the magnitude of post-prandial hypertriglyceridaemia and with TG enrichment of VLDL. Whilst fasting oxidative stress measurements demonstrated no significant correlations, the post-prandial increases in EPR (at 4 and 8 hours) correlated with post-prandial hypertriglyceridaemia and TG enrichment of VLDL (table 5).

Following treatment, the improvements in fasting FMD correlated most significantly with reduced oxidative stress (EPR) (vitamin C group only). The improvements in post-prandial FMD (at 4 and 8 hours) correlated with reduced free radical levels (EPR) in the vitamin C group.
Discussion

PPL in T2DM subjects in our study exhibit prolonged endothelial dysfunction up to 8 hours after fatty meal ingestion. The antioxidant vitamin C improves endothelial function at 0, 4 and 8 hours and attenuates PP induced oxidative stress at 4 hours only. Vitamin C blunted but did not abolish the deterioration of endothelial function PP; concomitantly it completely abolished the increase in oxidative stress. Significant determinants of PP endothelial dysfunction was TG enrichment of VLDL and LDL particles therefore although VC therapy improves baseline and PP endothelial function, there still remained some decrement in function.

This study reinforces the observations that at baseline, fasting FMD correlated inversely with LDL-C and positively with HDL-C concentrations (83). The deterioration in post-prandial FMD correlated with the magnitude of post-prandial hypertriglyceridaemia and with TG enrichment of VLDL. Whilst fasting oxidative stress demonstrated no significant correlations, the post-prandial increases in oxidative stress correlated with post-prandial hypertriglyceridaemia and TG enrichment of VLDL.

Following treatment, the improvements in fasting and post prandial endothelial function correlated most significantly with vitamin C reduction in oxidative stress. There was however still a decrement in PP endothelial function despite VC therapy indicating that lipoprotein interactions with the endothelium continue to blunt some endothelial function. Lipoprotein lipase (LPL) is the key enzyme for hydrolysis of TG from dietary fat (chylomicrons and VLDL) to free fatty acids (FFA) and monoglycerides (192). LPL is bound to the vascular endothelium and acts on chylomicrons and VLDL to deplete them of TG to form CM remnants and LDL, respectively. Therefore LPL holds a key role in controlling lipoprotein/FFA interactions at the endothelial interface. FFA have also been implicated as a causal factor for injury to arterial wall cells and foam cell formation. PP endothelial dysfunction and arterial injury may result from a direct effect of circulating lipoprotein lipolysis causing elevated circulating free fatty acid levels released at the endothelial cell surface (13), impairing endothelium-dependent vasodilatation via a decrease
in production of prostacyclin and cGMP (14). This PP endothelial dysfunction may be overcome by using different fat products. Canola oil enriched with α-linolenic acid (α-3), a component of the diet in the Lyon heart study (80) did not cause significant PP endothelial dysfunction (81). Fish oils eaten in the form of salmon resulted in a lesser absolute rise in plasma TG and also did not result in PP endothelial dysfunction (81). Therefore the source of fat ingested that is incorporated within the lipoproteins metabolised by LPL may be crucial in how toxic the resultant FFA are to the endothelium.

It has been shown that in healthy humans acute elevations of glucose induces endothelial dysfunction PP, a phenomenon blunted by VC (193). In that study, no rise in PP glucose concentrations after a fatty meal was observed. In the present study PP elevations in plasma glucose are unlikely to be the major determinant of endothelial dysfunction.

In chapter 4, it was shown that modification of TG metabolism can protect endothelial function. Ciprofibrate improves both fasting and post-prandial endothelial function in subjects with T2DM, with favourable blunting of PP induced oxidative stress (83). Despite these impressive results there was still a small deterioration in endothelial function at the 4 hour timepoint. These findings nevertheless imply that fibrates might have an important role to play in the prevention of coronary events in T2DM. Prospective studies are awaited but recent trial data show that non-diabetic CAD mortality is decreased in subjects with low HDL cholesterol taking fibrate therapy (192).

In both the previous study with ciprofibrate and the present study with VC, despite attenuating either lipid metabolism (with ciprofibrate) or oxidative stress (with VC) singly there remains a decrement in PP endothelial function. This may indicate that both processes acting together may play a role in PP endothelial dysfunction and that both strategies need to be employed with fatty meal ingestion to completely abolish endothelial dysfunction PP in T2DM.
### Table 1:

Patient characteristics at baseline with either vitamin C or placebo

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 10, 4F</td>
<td>N = 10, 2F</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.7 ± 6.9</td>
<td>53.6 ± 7.9</td>
</tr>
<tr>
<td>BMI (Kg/m)</td>
<td>29.2 ± 4.8</td>
<td>28.6 ± 5.5</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>5.4 ± 0.7</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.3 ± 0.3</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.92 ± 0.1</td>
<td>0.93 ± 0.09</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Systolic BP (mm/hg)</td>
<td>141 ± 11</td>
<td>143 ± 13</td>
</tr>
<tr>
<td>Diastolic BP (mm/hg)</td>
<td>85 ± 10</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>Fasting Insulin (mu/l)</td>
<td>30.6 ± 10.8</td>
<td>28.3 ± 12.9</td>
</tr>
<tr>
<td>Fasting Glc (mmol/l)</td>
<td>8.4 ± 0.7</td>
<td>9.1 ± 1.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.5 ± 0.8</td>
<td>8.4 ± 0.7</td>
</tr>
<tr>
<td>AUC Glc (mmol/l/8hours)</td>
<td>62.9 ± 11.6</td>
<td>64.8 ± 17.8</td>
</tr>
</tbody>
</table>
Table 2:

Cholesterol and TG content of the major lipoprotein subclasses (μmol/ml) in both groups at baseline (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Vitamin C (Post VC)</th>
<th>Placebo (Baseline)</th>
<th>Placebo (Post Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL - TG</td>
<td>3.79 ± 1.91</td>
<td>3.71 ± 2.3</td>
<td>3.89 ± 2.28</td>
<td>3.7 ± 1.8</td>
</tr>
<tr>
<td>LDL - TG</td>
<td>2.11 ± 0.98</td>
<td>2.09 ± 0.81</td>
<td>2.05 ± 0.72</td>
<td>1.98 ± 0.9</td>
</tr>
<tr>
<td>HDL - TG</td>
<td>1.12 ± 0.31</td>
<td>1.09 ± 0.37</td>
<td>1.25 ± 0.44</td>
<td>1.12 ± 0.5</td>
</tr>
<tr>
<td>VLDL - Chol</td>
<td>2.81 ± 1.13</td>
<td>2.55 ± 1.01</td>
<td>2.77 ± 1.26</td>
<td>2.93 ± 0.98</td>
</tr>
<tr>
<td>LDL - Chol</td>
<td>4.15 ± 1.84</td>
<td>4.3 ± 1.9</td>
<td>4.13 ± 1.89</td>
<td>4.4 ± 2.1</td>
</tr>
<tr>
<td>HDL - Chol</td>
<td>0.93 ± 0.11</td>
<td>0.88 ± 0.2</td>
<td>0.97 ± 0.21</td>
<td>1.02 ± 0.18</td>
</tr>
</tbody>
</table>
Table 3: Post-prandial AUC for TG and cholesterol content (µ mol/ml/8 hrs) of major lipoproteins in both groups at baseline (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Vitamin C (Post VC)</th>
<th>Placebo (Baseline)</th>
<th>Placebo (Post Placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylo - TG</td>
<td>32.5 ± 12.4</td>
<td>30.4 ± 10.2</td>
<td>34.3 ± 15.8</td>
<td>31 ± 11.3</td>
</tr>
<tr>
<td>VLDL - TG</td>
<td>23.8 ± 9.4</td>
<td>21.8 ± 10.9</td>
<td>25.7 ± 12.2</td>
<td>22.8 ± 9.8</td>
</tr>
<tr>
<td>LDL - TG</td>
<td>11.2 ± 5.7</td>
<td>10.4 ± 7.3</td>
<td>12.6 ± 7.1</td>
<td>11 ± 6.1</td>
</tr>
<tr>
<td>HDL - TG</td>
<td>4.5 ± 1.9</td>
<td>5 ± 3.1</td>
<td>3.9 ± 2.7</td>
<td>4.7 ± 3.7</td>
</tr>
<tr>
<td>Chylo - Chol</td>
<td>4.8 ± 2.1</td>
<td>5.9 ± 3.6</td>
<td>5.2 ± 3.2</td>
<td>5.7 ± 2</td>
</tr>
<tr>
<td>VLDL - Chol</td>
<td>5.87 ± 3.3</td>
<td>6.02 ± 2.8</td>
<td>6.22 ± 4.1</td>
<td>5.8 ± 5.3</td>
</tr>
<tr>
<td>LDL - Chol</td>
<td>7.5 ± 3.5</td>
<td>8.2 ± 3.1</td>
<td>8.2 ± 2.9</td>
<td>8.5 ± 4.1</td>
</tr>
<tr>
<td>HDL - Chol</td>
<td>2.5 ± 1.1</td>
<td>2.2 ± 2</td>
<td>3.1 ± 1.4</td>
<td>2.7 ± 1.8</td>
</tr>
</tbody>
</table>
Table 4:
Correlation of fasting and post-prandial endothelial function (FMD) with metabolic parameters.

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Correlation co-efficient (r value)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C (mmol/l)</td>
<td>-0.61 (fasting FMD)</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.6 (fasting FMD)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial TG (AUC -TG) (mmol/l/8-hours)</td>
<td>0.49 (Change in FMD at 4–hours)</td>
<td>0.027</td>
</tr>
<tr>
<td>Post-prandial TG (AUC -TG) (mmol/l/8-hours)</td>
<td>0.47 (Change in FMD at 8–hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (mmol/ml/8-hours)</td>
<td>0.45 (Change in FMD at 4–hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (mmol/ml/8-hours)</td>
<td>0.4 (Change in FMD at 8–hours)</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Table 5:
Factors correlating with the improvement in fasting and post-prandial FMD following vitamin C therapy.

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Correlation Coefficient (r value)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced fasting free radical levels (EPR- arbitrary units)</td>
<td>0.55 (improvement in fasting FMD)</td>
<td>0.027</td>
</tr>
<tr>
<td>Reduced 4–hour free radical levels (EPR – arbitrary units)</td>
<td>0.46 (improvement in 4–hour FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Reduced 8–hour free radical levels (EPR – arbitrary units)</td>
<td>0.42 (improvement in 8–hour FMD)</td>
<td>0.038</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.46 (Baseline increase in EPR at 4 hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (AUC VLDL-TG)</td>
<td>0.36 (Baseline increase in EPR at 4 hours)</td>
<td>0.045</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (AUC VLDL-TG)</td>
<td>0.4 (Baseline increase in EPR at 8 hours)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.34 (Baseline increase in EPR at 8 hours)</td>
<td>0.05</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.4 (Baseline increase in TBARS at 4 hours)</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Figure 1 Changes in EPR in each group

![Bar chart showing changes in EPR in each group with annotations for statistical significance.]

* Denotes p < 0.05 cf. 0 hours
§ Denotes p < 0.05 cf. Baseline

Figure 2. Markers of lipid peroxidation

![Bar chart showing markers of lipid peroxidation with annotations for statistical significance.]

§ Denotes p < 0.05 cf. Baseline
* Denotes p < 0.05 cf. 0 hours

y-Axis indicates µmol/L
Figure 3. Changes in endothelial function post-prandially.

* Denotes p < 0.05 cf. Baseline
§ Denotes p < 0.05 cf. 0 hours
Chapter 6

Chronic Vitamin C and insulin therapy.

The effects of Insulin lispro and chronic vitamin C therapy on post-prandial lipaemia, oxidative stress and endothelial function in patients with type 2 diabetes mellitus.

Introduction

Cardiovascular disease is a common complication of T2DM (165). Several risk factors for atherosclerosis co-segregate in T2DM, (166) including the typical dyslipidaemia characterised by reduced HDL-cholesterol (HDL-C), hypertriglyceridaemia and abnormal post-prandial lipaemia (PPL) (167). PPL represents a state in which triglyceride (TG) metabolism is compromised. Since many of the factors involved in post-prandial lipid metabolism are insulin sensitive (166) there is clear potential for abnormalities of PPL to arise in T2DM. These include prolonged and exaggerated excursions in plasma TG and TG rich lipoproteins (TGRL) (167), which associate with atherosclerotic disease severity and progression in subjects with and without diabetes (168).

Endothelial dysfunction (ED), with reduced bioavailable nitric oxide (NO), is a pivotal event in atherogenesis (193) and is a consistent finding in T2DM. The underlying mechanisms involve hyperglycaemia (194), increases in free fatty acid concentrations (175), lipid abnormalities (187) and the production of TGRL associated with enhanced oxidative stress particularly during PPL (187,83).

Transient ED has been described in association with PPL in diabetic and non-diabetic subjects in chapter 3 (83), correlating with enhanced OS and TGRL levels, in particular TG rich VLDL (195). Furthermore, modifying PPL and reducing TGRL levels by fibrate therapy improves endothelial function and reduces OS associated with PPL in patients with T2DM as shown in chapter 4 (195).
T2DM is associated with reduced endogenous levels of vitamin C (196), which may be an independent cardiovascular risk factor (197). The beneficial effects of vitamin C therapy on endothelial function in T2DM have been consistently demonstrated (198), with recent evidence suggesting that vitamin C administration may also attenuate transient endothelial dysfunction associated with both hyperglycaemia and PPL in healthy subjects (199,170).

Insulin therapy in T2DM is conventionally initiated on glycaemic criteria. Insulin retards the progress of microvascular complications in T2DM (143) and may reduce macrovascular risk (143). Insulin improves endothelial-dependent and independent vasodilator function in T2DM (200) and has various effects on lipid metabolism, including suppression of hepatic free fatty acid release, reduced plasma TG and attenuated synthesis of atherogenic lipoproteins (201,202).

With these issues in mind we investigated the effect of insulin therapy combined with either vitamin C or placebo on PPL and the associated changes in endothelial function and oxidative stress.

Methods

Subjects

Twenty T2DM subjects (mean age 53.2 years; age range 39 – 59 years; 17 men - mean HbA1c 8.4 %) were studied (Table 1). All were non-smokers and receiving standard oral hypoglycaemic agents. Patients with vascular disease, taking antihypertensives, lipid lowering agents, aspirin or supplemental vitamins were excluded from the study. Females were pre-menopausal and studied in the follicular phase. Written consent was obtained from all subjects with local ethics committee approval.

Study Design

Studies commenced following a 12-hour overnight fast with omission of hypoglycaemic agents on the study day. After 30 minutes of supine rest venous blood was drawn for measurement of total, LDL and HDL cholesterol, plasma triglyceride, insulin, glucose and
glycosylated haemoglobin (HbA1c). 5 ml of venous blood was drawn to enable measurement of oxidative stress. Each subject was given a fatty meal (32) containing 80 grams of saturated fat, lipid profiles and plasma glucose measurements were repeated 2 hourly over the following 8 hours. Endothelial function and oxidative stress were assessed fasting and at 4 and 8 hours. Oral hypoglycaemics were discontinued and replaced by pre-meal bolus insulin lispro (0.2 U/Kg). Subjects were closely supervised by a dedicated specialist nurse, with individual dose adjustments made to match pre-study glucose levels based on a four week period of home blood glucose monitoring carried out by each subject prior to entry into the study. Subjects also received either oral vitamin C 1 gram daily or matching placebo and after six weeks the studies were repeated with omission of all treatment on the study day.

**Endothelial function assessment**

This was assessed as previously described.

**Biochemical measurements**

These were assessed as previously described.

**Lipoprotein separation**

These were assessed as previously described.

**Measurement of oxidative stress**

1. **Measurement of venous free radicals.**

As previously described except that EPR spectra were recorded on a Bruker EMX X-band spectrometer and therefore absolute EPR values are different from those measured on a Varian spectrometer in Chapters 3-5.

2. **Thiobarbituric acid-reactive substances (TBARS)**

As previously described.
Statistical analysis

Conventional methods were used for calculating mean, standard deviation and checks for normal distribution. Group differences in variables were determined by two-tailed t-test. Statistical significance for differences between groups was tested by one way analysis of variance. As a measure of plasma glucose and the total amounts of lipid and lipoprotein present during PPL, areas under curve (AUC) were calculated for plasma concentrations without subtraction of baseline values. Multiple-regression analysis was used to study the relationship between variables with logarithmic transformation of skewed data.

Results

Patient characteristics (Table 1): Blood pressure, age and BMI remained unchanged.

Biochemistry (Table 1): Total, LDL cholesterol, glucose (fasting and post-prandial) and HbA1c also remained unchanged. There were non-significant reductions in fasting insulin, with significant reduction in fasting TG and rise in HDL-C (maintained during PPL). Table 2 and figure 4 illustrate the fasting VLDL, LDL and HDL compositional changes, with marked reduction in lipoprotein TG content.

Post-prandial lipaemia:

AUC for post-prandial plasma TG (mmol/l/8hrs) was similar in both groups (56.9 ± 8 [Vit C], 52.6 ± 11 [placebo]), with significant reductions following insulin (Fig 1) (AUC-TG - 56.9 ± 8 to 40.1 ± 10.3 [Vit C group], 52.6 ± 11 to 39.1± 12.5 [placebo group]; p < 0.05) associated with reduced lipoprotein TG content (Table 3 and figure 4 and 5).

Plasma insulin levels rose during PPL, with small reductions following treatment 66.7 ± 18.5 [4-hour], 49.4 ± 13.9 [8-hours] – baseline Vitamin C to 61.4 ± 23.6 [4-hour], 42.8 ± 21.7 [8-
hours] – post-treatment. 63.1 ± 17.8 [4-hours], 43.6 ± 17.2 [8-hours] – baseline placebo to 59.8 ± 21.4 [4-hours], 40.9 ± 24.2 [8-hours] – post-treatment - placebo).

Vascular Data:

Baseline fasting and post-prandial FMD was similar in both groups, with a significant reduction in post-prandial FMD at 4 hours, maintained at 8 hours (Fig 2). Fasting FMD improved in both groups following insulin, with augmented improvement in the vitamin C group (Fig 2) (1.1 ± 1.2 to 4.2 ± 1.1%, p < 0.001 [placebo] and 0.9 ± 1.1 to 6.1 ± 1.3%, p < 0.001 [vitamin C], FMD Vit C group >. FMD placebo group, p < 0.05).

These changes were maintained at 4 and 8 hours (Fig 2) (0.3 ± 1.2 to 3.1 ± 0.9%, P < 0.01 [placebo 4-hours], 0.7 ± 1.1 to 3.76 ± 1.1%, p < 0.001 [placebo 8-hours] and 0.7 ± 1.5 to 4.9 ± 2.1%, p < 0.01 [vit C 4-hours], 0.8 ± 0.9 to 5.8 ± 0.6%, p < 0.01 [Vit C 8-hours]).

Fasting and post-prandial GTN responses increased in both groups following insulin (Tables 4 and 5), while all other vascular parameters remained unchanged.

Oxidative stress data

(a) EPR data

Both groups exhibited similar baseline levels. After treatment fasting and post-prandial levels fell with greater reduction in the vitamin C group (Table 6).

(b) TBARS data

Both groups initially demonstrated similar TBARS measurements (Table 6), following insulin fasting and post-prandial levels fell with greater reductions in the vitamin C group.
Correlation between PPL, endothelial function and oxidative stress

Tables 7 and 8 illustrate the associations between PPL FMD and OS at baseline and after treatment. At baseline, fasting FMD correlated inversely with LDL-C and positively with HDL-C concentrations. The deterioration in post-prandial FMD correlated with the magnitude of post-prandial hypertriglyceridaemia and with TG enrichment of VLDL. Whilst fasting OS demonstrated no significant correlations, the post-prandial increases in EPR correlated with post-prandial hypertriglyceridaemia and TG enrichment of VLDL, while the changes in TBARS correlated with post-prandial hypertriglyceridaemia (Table 8).

Following treatment, the improvements in fasting FMD correlated most significantly with reduced OS (EPR) (vitamin C group only), increase in HDL-C concentrations and reduction in VLDL TG content. The improvements in post-prandial FMD correlated with reduced free radical levels (EPR) in the vitamin C group and was associated with various lipid parameters in both groups. The most significant associations occurred with the reduction in post-prandial hypertriglyceridaemia, increase in HDL-C concentrations and reduction in TG content of VLDL. The reduction in fasting and post-prandial OS in both groups demonstrated only an association with increased HDL-C concentrations (fasting $r = 0.49$, 4-hours $r = 0.42$, 8-hours $r = 0.44$; $p < 0.05$ [vit C group]; fasting $r = 0.46$, 4-hours $r = 0.43$, 8-hours $r = 0.41$; $p < 0.05$ [placebo group]).

Discussion

Following 6 weeks of insulin therapy, fasting and post-prandial endothelial function (FMD) improved and OS reduced. Endothelium-independent responses also increased while the FMD and OS changes were augmented by concomitant vitamin C therapy.

Plasma TG fell, PPL was attenuated, and TGRL levels reduced with HDL-C increased, while there was no change in average glucose levels. These changes were the consequence of
chronic insulin therapy, since the subjects had not received any insulin for at least 12 hours prior to being studied.

Insulin lispro is identical in structure to human insulin, except that the amino acids at positions 28 and 29 of the B chain are reversed, resulting in reduced tendency to self-associate (203). When injected subcutaneously, insulin lispro is absorbed more rapidly than soluble insulin, producing earlier peak activity (120 minutes post-injection, lasting approximately 5-6 hours) and shorter duration of action (203). Thus, compared with conventional subcutaneous human insulin, insulin lispro when injected prior to a meal produces metabolic effects, which more closely mimic those of endogenous insulin release. Insulin lispro was therefore used in an attempt to provide a more physiological means of supplementing the impaired post-prandial early phase insulin release in T2DM (203). An initial dose of 0.2 U/Kg was used to reduce the possibility of hypoglycaemia, with individual dose adjustments made to match pre-study glucose levels as closely as possible.

This study reaffirms the association between endothelial function with both LDL and HDL-C in T2DM (83). Whilst supporting an association between TGRL with endothelial dysfunction in T2DM (83), this study confirms a role for TG rich VLDL particles in the pathogenesis of enhanced OS in T2DM.

TG rich VLDL particles preferentially undergo endocytosis by macrophages forming foam cells (177), while lipolytic products of TG rich VLDL are toxic to endothelial cells and macrophages (178,179). Increased free fatty acid (FFA) fluxes particularly during post-prandial lipaemia may potentiate the toxic effects of TG rich VLDL on endothelial cells (183). Increased levels of TG rich VLDL may also promote ED by promoting increased synthesis of toxic small dense LDL particles (183).

TG depletion of VLDL following insulin correlated with improvements in endothelial function. Thus by reducing levels of TG rich VLDL, improved endothelial function following insulin may be partly due to attenuation of the atherogenic effects of these particles.
TG depletion of VLDL as a result of exogenous insulin administration is described in T2DM (202). This may be due to reduced hepatic synthesis of these particles as a result of reduced FFA levels, which function as a major substrate for the production of TG rich VLDL (203). Irrespective of glucose and FFA levels insulin suppresses the synthesis of apolipoprotein B in TG rich particles, resulting in relatively greater reduction in hepatic synthesis of TG rich VLDL (202).

Insulin is also responsible for other lipid changes that could enhance endothelial function, including reduced FFA levels. This may prove beneficial since FFA are considered to be directly toxic to the endothelium and potentiate the effects of TG rich VLDL. These effects may be particularly relevant during PPL, which is associated with fluxes of increased FFA (165).

Insulin also reduces TG and increases HDL-C (201), confirmed in the present study in the fasted and post-prandial states. The reduction in TG may be partly due to the effects of insulin on reducing hepatic production of TGRL (183), whilst the increase in HDL-C may be a secondary consequence of reduced TG and TGRL (183). These effects may also represent improved insulin sensitivity, supported by the observed small reductions in insulin levels in this study. Insulin therapy has been shown to increase insulin sensitivity in T2DM (202). The present observations were however statistically non-significant and may simply reflect sample size.

The TG and HDL-C changes correlated with improvements in endothelial function. This may be due to the reduced production of atherogenic TGRL and small dense LDL particles associated with hypertriglyceridaemia (183) and increased endothelium protective effect of increased HDL concentration (183).

Insulin also reduced fasting and post-prandial LDL and HDL TG content. Excess hydrolysis of TG within TG rich LDL particles results in increased production of smaller more dense and thus more atherogenic particles, with increased pro-oxidant properties. Thus reducing TG rich LDL may influence OS and endothelial function through reduced levels of small, dense LDL. TG rich HDL particles demonstrate decreased endothelium protective and antioxidant
properties (181). Thus increased levels of TG deplete, but relatively cholesterol enriched particles may contribute to the enhanced antioxidant and endothelium protective potential of the increased HDL-C concentrations.

Reduced TGRL following insulin may not only reflect reduced synthesis but also enhanced catabolism. The catabolism of TGRL is mediated by lipoprotein lipase (LPL), which may have reduced lipolytic activity in T2DM (144). Although acute administration of exogenous insulin may up-regulate LPL enzymatic activity (203), there is no evidence that chronic administration of exogenous insulin has similar effects.

Insulin not only improved endothelial function but also caused reductions in OS, which were augmented in the vitamin C group. This study reaffirms an association between hypertriglyceridaemia and TGRL in particular TG rich VLDL with enhanced OS in T2DM.

Enhanced OS has a variety of important effects including LDL oxidation (182), particularly relevant in T2DM where the smaller denser particles demonstrate an increased susceptibility to oxidation. Enhanced OS may also directly induce endothelial dysfunction by decreasing synthesis and release of NO by endothelial cells and by inactivating NO in the subendothelial space (64). Furthermore, the alkoxyl radicals detected by EPR have been shown to directly interact with NO (66,183). The improvements in endothelial function following insulin may reflect underlying reduced OS. Insulin may have decreased OS measures in general by a complex combination of effects. By decreasing overall oxidative stress insults (by lowering TGRL and modifying other lipid particles) and improving antioxidant protection (increasing HDL, possibly improving eNOS activity). This may account for the augmented improvements in the vitamin C group, where the reductions in OS were greater.

Vitamin C is a low-molecular weight antioxidant that scavenges free radicals, the levels of which are reduced in T2DM (198). Treatment with vitamin C may supplement endogenous levels, thus reducing OS and so improving endothelial function. This study supports previous observations of beneficial effects of vitamin C on endothelial function in T2DM (199). Moreover
It demonstrates that chronic vitamin C therapy may attenuate endothelial dysfunction associated with PPL in T2DM. It also demonstrates that the effects of vitamin C on endothelial function in T2DM may be augmented by concomitant chronic insulin therapy. The magnitude of change in OS with chronic vitamin C therapy may be greater than that seen in chapter 5 because of replenishment of antioxidant stores over the time course of the experiment rather than the acute ingestion seen in chapter 5.

Insulin can improve endothelial function by beneficial lipid changes as described. It may also directly improve endothelial function, by increasing endothelial NO synthesis as a result of increased eNOS gene expression (204). Such an effect may be augmented by any improvement in insulin sensitivity since insulin sensitivity positively correlates with endothelial NO synthesis (186).

Insulin also increased endothelium-independent (vascular smooth muscle dependent) vasodilatation. Various mechanisms may account for these observations including increased bioavailability of exogenous as well as endogenous NO due to increased synthesis (186), and reduced inactivation due to decreased OS, TGRL and small dense LDL. Vascular smooth muscle cells in the context of insulin resistance may exhibit nitrate resistance (206), thus any potential improvement in insulin sensitivity may be reflected in vascular smooth muscle cells as increased nitrate responsiveness.

In summary, this study confirms that insulin therapy in T2DM results in reduced OS and improved fasting and post-prandial vascular reactivity, with augmented effects on endothelial function and OS produced by additional vitamin C. The underlying mechanisms may include increased HDL-C concentrations; modified TG metabolism with attenuated PPL and reduced levels of TGRL with supplemental effects of vitamin C on antioxidant defences.

This study thus supports potentially important vascular effects of chronic insulin therapy, in particular modifying endothelial function, decreasing oxidative stress and attenuating PPL. The effects appear to be independent of changes in glycaemic control. The vascular effects of insulin
therapy in T2DM appear to be augmented by supplemental vitamin C. Larger long-term randomised controlled trials are however required to fully evaluate the effects of insulin and vitamin C therapy vascular risk in patients with T2DM.
Figure 1. Fasting and post-prandial TBARS (in μmol/L) at baseline and post intervention.

Denotes p < 0.05 Baseline > Post-treatment, § Denotes p < 0.05 Placebo > Vitamin C + Insulin.
All data is ± SEM.

Figure 2. Fasting and post-prandial EPR (in A/units) at baseline and post intervention.
* Denotes $p < 0.05$ Baseline $>$ Post-treatment, § Denotes $p < 0.05$ Placebo $>$ Vitamin C + Insulin

Figure 3. Fasting and post-prandial FMD changes at baseline and post intervention.

* Denotes $p < 0.05$ Baseline $<$ Post-treatment
§ Denotes $p < 0.05$ Placebo $<$ Vitamin C + Insulin
Figure 4. TG content of the major lipoprotein subclasses in both groups at baseline and post insulin therapy.

Dark blue and light blue pattern are placebo and placebo/insulin groups respectively, whilst the vitamin C and vitamin C/insulin groups are in single shades.

* P< 0.05 Baseline < Post insulin
Figure 5. Post-prandial AUC for TG content (µ mol/ml/8 hrs) of major lipoproteins in both groups at baseline and after insulin (mean ± SD).

Denotes p < 0.05 Baseline > Post Insulin when analysed using 2 sample Mann-Whitney test.
Table 1: Patient characteristics at baseline and after treatment with Insulin in combination with either vitamin C or placebo

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Vitamin C (Post – treatment)</th>
<th>Placebo (Post – treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 10, 2F</td>
<td>N = 10, 1F</td>
<td>N = 10, 2F</td>
<td>N = 10, 1F</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.7 ± 6.9</td>
<td>53.6 ± 7.9</td>
<td>52.7 ± 6.9</td>
<td>53.6 ± 7.9</td>
</tr>
<tr>
<td>BMI (Kg/m)</td>
<td>29.2 ± 4.8</td>
<td>28.6 ± 5.5</td>
<td>29.3 ± 4.9</td>
<td>28.7 ± 5.2</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>5.4 ± 0.7</td>
<td>5.5 ± 0.6</td>
<td>5.4 ± 0.7</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.3 ± 0.3</td>
<td>3.4 ± 0.5</td>
<td>3.3 ± 0.3</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.92 ± 0.1</td>
<td>0.93 ± 0.09</td>
<td>1.21 ± 0.1*</td>
<td>1.19 ± 0.14*</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.4</td>
<td>1.4 ± 0.2*</td>
<td>1.3 ± 0.4 *</td>
</tr>
<tr>
<td>Systolic BP (mm/hg)</td>
<td>151 ± 11</td>
<td>149 ± 13</td>
<td>148 ± 13</td>
<td>148 ± 14</td>
</tr>
<tr>
<td>Diastolic BP (mm/hg)</td>
<td>85 ± 10</td>
<td>84 ± 9</td>
<td>84 ± 9</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>Fasting Insulin (mu /l)</td>
<td>30.6 ± 10.8</td>
<td>28.3 ± 12.9</td>
<td>25.9 ± 12.8</td>
<td>23.7 ± 15.6</td>
</tr>
<tr>
<td>Fasting Glc (mmol/l)</td>
<td>8.4 ± 0.7</td>
<td>9.1 ± 1.3</td>
<td>8.1 ± 1</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.5 ± 0.8</td>
<td>8.4 ± 0.7</td>
<td>8.4 ± 0.9</td>
<td>8.5 ± 0.9</td>
</tr>
<tr>
<td>AUC Glc (mmol/l / 8hours)</td>
<td>62.9 ± 11.6</td>
<td>64.8 ± 17.8</td>
<td>59.8 ± 14.7</td>
<td>60.6 ± 15.1</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 Post treatment vs. Baseline
Table 2: Cholesterol and TG content of the major lipoprotein subclasses (μmol/ml) in both groups at baseline and after insulin (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Vitamin C (Post-Ins)</th>
<th>Placebo (Post-Ins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL - TG</td>
<td>3.79 ± 1.91</td>
<td>3.89 ± 2.28</td>
<td>2.83 ± 1.27*</td>
<td>2.91 ± 1.34*</td>
</tr>
<tr>
<td>LDL - TG</td>
<td>2.11 ± 0.98</td>
<td>2.05 ± 0.72</td>
<td>1.51 ± 0.42*</td>
<td>1.48 ± 0.37*</td>
</tr>
<tr>
<td>HDL - TG</td>
<td>1.12 ± 0.31</td>
<td>1.25 ± 0.44</td>
<td>0.79 ± 0.22*</td>
<td>0.81 ± 0.26*</td>
</tr>
<tr>
<td>VLDL - Chol</td>
<td>2.81 ± 1.13</td>
<td>2.77 ± 1.26</td>
<td>2.86 ± 1.19</td>
<td>2.79 ± 1.33</td>
</tr>
<tr>
<td>LDL - Chol</td>
<td>4.15 ± 1.84</td>
<td>4.13 ± 1.89</td>
<td>4.09 ± 2.10</td>
<td>4.11 ± 1.91</td>
</tr>
<tr>
<td>HDL - Chol</td>
<td>0.93 ± 0.11</td>
<td>0.97 ± 0.21</td>
<td>0.89 ± 0.2</td>
<td>0.95 ± 0.17</td>
</tr>
</tbody>
</table>

* P<0.05 Baseline vs. Post-Insulin

Table 3: Post-prandial AUC for TG and cholesterol content (μ mol/ml/8 hrs) of major lipoproteins in both groups at baseline and after insulin (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Vitamin C (Post – Ins)</th>
<th>Placebo (Post – Ins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylo - TG</td>
<td>32.5 ± 12.4</td>
<td>34.3 ± 15.8</td>
<td>22.6 ± 10.4*</td>
<td>23.7 ± 14.8*</td>
</tr>
<tr>
<td>VLDL - TG</td>
<td>23.8 ± 9.4</td>
<td>25.7 ± 12.2</td>
<td>18.5 ± 6.8*</td>
<td>19.7 ± 7.4*</td>
</tr>
<tr>
<td>LDL - TG</td>
<td>11.2 ± 5.7</td>
<td>12.6 ± 7.1</td>
<td>7.3 ± 4.2 *</td>
<td>8.5 ± 5.2 *</td>
</tr>
<tr>
<td>HDL - TG</td>
<td>4.5 ± 1.9</td>
<td>3.9 ± 2.7</td>
<td>2.4 ± 1.3*</td>
<td>1.9 ± 1.9*</td>
</tr>
<tr>
<td>Chylo - Chol</td>
<td>4.8 ± 2.1</td>
<td>5.2 ± 3.2</td>
<td>5.4 ± 3.1</td>
<td>5.6 ± 3.9</td>
</tr>
<tr>
<td>VLDL – Chol</td>
<td>5.87 ± 3.3</td>
<td>6.22 ± 4.1</td>
<td>6.1 ± 4.6</td>
<td>5.95 ± 3.9</td>
</tr>
<tr>
<td>LDL – Chol</td>
<td>7.5 ± 3.5</td>
<td>8.2 ± 2.9</td>
<td>7.9 ± 5.1</td>
<td>8.4 ± 3.6</td>
</tr>
<tr>
<td>HDL – Chol</td>
<td>2.5 ± 1.1</td>
<td>3.1 ± 1.4</td>
<td>2.9 ± 1.8</td>
<td>3.3 ± 1.5</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 Baseline > Post Insulin when analysed using 2 sample Mann-Whitney test.
### Table 4: Fasting baseline and post-treatment vascular parameters in both groups

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Vitamin C (Post-Ins)</th>
<th>Placebo (Post-Ins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting arterial diameter (mm)</td>
<td>3.76 ± 0.19</td>
<td>3.82 ± 0.2</td>
<td>3.80 ± 0.14</td>
<td>3.84 ± 0.28</td>
</tr>
<tr>
<td>Resting blood flow (ml/min)</td>
<td>241 ± 28</td>
<td>236 ± 39</td>
<td>252 ± 47</td>
<td>247 ± 54</td>
</tr>
<tr>
<td>GTN induced dilatation (% change from rest)</td>
<td>9.8 ± 3.4</td>
<td>9.6 ± 4.2</td>
<td>11.5 ± 3.1*</td>
<td>11.8 ± 3.8*</td>
</tr>
<tr>
<td>Hyperaemic blood flow (% increase)</td>
<td>408 ± 16</td>
<td>414 ± 27</td>
<td>416 ± 19</td>
<td>427 ± 30</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 Post-Insulin > baseline.

### Table 5: Post-prandial vascular parameters in both groups at baseline and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>VC 4hr Baseline</th>
<th>VC 8hr Baseline</th>
<th>Placebo 4hr Baseline</th>
<th>Placebo 8hr Baseline</th>
<th>VC – 4hr Post-Ins</th>
<th>VC – 8hr Post-Ins</th>
<th>Placebo 4hr Post-Ins</th>
<th>Placebo 8hr Post-Ins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting arterial diameter (mm)</td>
<td>3.79±0.1</td>
<td>3.82±0.2</td>
<td>3.84±0.22</td>
<td>3.81±0.19</td>
<td>3.83±0.18</td>
<td>3.81±0.1</td>
<td>3.78±0.2</td>
<td>3.81±0.2</td>
</tr>
<tr>
<td>Resting blood flow (ml/min)</td>
<td>250 ± 28</td>
<td>229 ± 41</td>
<td>244 ± 39</td>
<td>219 ± 31</td>
<td>246 ± 39</td>
<td>233 ± 19</td>
<td>220 ± 23</td>
<td>231 ± 29</td>
</tr>
<tr>
<td>GTN induced dilatation (% change from rest)</td>
<td>9.5 ± 3.4</td>
<td>9.2 ± 4.2</td>
<td>9.7 ± 4.9</td>
<td>9.1 ± 3.9</td>
<td>11.9±3.6*</td>
<td>11.3±4.3*</td>
<td>11.8±4.1</td>
<td>11.2±3.9</td>
</tr>
<tr>
<td>Hyperaemic blood flow (% increase)</td>
<td>411 ± 36</td>
<td>437 ± 52</td>
<td>436 ± 29</td>
<td>422 ± 30</td>
<td>422 ± 35</td>
<td>440 ± 61</td>
<td>433 ± 38</td>
<td>409 ± 49</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 Post-Insulin vs. Baseline
Table 6: Fasting and post-prandial TBARS (μmol/l) and EPR (arbitrary units) measurements in both groups (units, mean ± SD) at baseline and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C (Baseline)</th>
<th>Placebo (Baseline)</th>
<th>Vitamin C (Post-treatment)</th>
<th>Placebo (Post-treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting TBARS</td>
<td>5.67 ± 2.6</td>
<td>5.71 ± 2.42</td>
<td>4.91 ± 2.23*</td>
<td>5.22 ± 2.15*§</td>
</tr>
<tr>
<td>4-Hours TBARS</td>
<td>8.3 ± 2.5</td>
<td>7.8 ± 3.08</td>
<td>5.33 ± 2.12*</td>
<td>6.31 ± 1.93*§</td>
</tr>
<tr>
<td>8-Hours TBARS</td>
<td>6.2 ± 2.4</td>
<td>6.6 ± 2.9</td>
<td>4.97 ± 1.83*</td>
<td>5.66 ± 2.11*§</td>
</tr>
<tr>
<td>Fasting EPR</td>
<td>8.2 ± 2.8</td>
<td>9.1 ± 3.5</td>
<td>2.4 ± 1.3*</td>
<td>6.8 ± 2.1*§</td>
</tr>
<tr>
<td>4-Hours EPR</td>
<td>13.1 ± 4.7</td>
<td>12.5 ± 5.9</td>
<td>4.2 ± 1.9*</td>
<td>8.9 ± 3.1*§</td>
</tr>
<tr>
<td>8-Hours EPR</td>
<td>11.4 ± 3.6</td>
<td>10.8 ± 4.1</td>
<td>2.8 ± 0.9*</td>
<td>7.2 ± 2.3*§</td>
</tr>
</tbody>
</table>

* Denotes p < 0.05 Baseline > Post-treatment
§ Denotes p < 0.05 Placebo > Vitamin C
Table 7: Baseline correlation of fasting and post-prandial deterioration in endothelial function (FMD)

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Correlation co-efficient ( r value)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C (mmol/l)</td>
<td>-0.61 (fasting FMD)</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.55 (fasting FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial TG (AUC –TG) (mmol/l/8-hours)</td>
<td>0.48 (Change in FMD at 4 hours)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial TG (AUC –TG) (mmol/l/8-hours)</td>
<td>0.43 (Change in FMD at 8 hours)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (mmol/ml/8-hours)</td>
<td>0.44 (Change in FMD at 4 hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (mmol/ml/8-hours)</td>
<td>0.39 (Change in FMD at 8 hours)</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Table 8: Factors correlating most significantly with the improvement in fasting and post-prandial FMD following insulin and vitamin C therapy.

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Correlation Coefficient (r value)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced fasting free radical levels (EPR - arbitrary units)</td>
<td>0.51 (improvement in fasting FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Increase HDL-C concentrations</td>
<td>0.44 (improvement in fasting FMD)</td>
<td>0.04</td>
</tr>
<tr>
<td>Reduced 4 - hour free radical levels (EPR – arbitrary units)</td>
<td>0.46 (improvement in 4 – hour FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Reduced 8 - hour free radical levels (EPR – arbitrary units)</td>
<td>0.40 (improvement in 8 – hour FMD)</td>
<td>0.04</td>
</tr>
<tr>
<td>Reduced post-prandial TG (AUC –TG) (mmol/l/8hours)</td>
<td>0.42 (improvement in 4 – hour FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Reduced post-prandial TG (AUC –TG) (mmol/l/8hours)</td>
<td>0.38 (improvement in 8 – hour FMD)</td>
<td>0.04</td>
</tr>
<tr>
<td>Increased HDL- C (4-hours)</td>
<td>0.41 (improvement in 4 – hour FMD)</td>
<td>0.03</td>
</tr>
<tr>
<td>Increased HDL- C (8-hours)</td>
<td>0.38 (improvement in 8 – hour FMD)</td>
<td>0.05</td>
</tr>
<tr>
<td>Reduced Post-prandial VLDL TG content (mmol/l/8hours)</td>
<td>0.35 (improvement in 4 – hour FMD)</td>
<td>0.08</td>
</tr>
<tr>
<td>Reduced Post-prandial VLDL TG content (mmol/l/8hours)</td>
<td>0.33 (improvement in 8 – hour FMD)</td>
<td>0.1</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.46 (Baseline increase in EPR at 4 hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (AUC VLDL-TG)</td>
<td>0.38 (Baseline increase in EPR at 4 hours)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.39 (Baseline increase in TBARS at4hours)</td>
<td>0.04</td>
</tr>
<tr>
<td>Post-prandial VLDL TG content (AUC VLDL-TG)</td>
<td>0.43 (Baseline increase in EPR at 8 hours)</td>
<td>0.03</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.44 (Baseline increase in EPR at 8 hours)</td>
<td>0.02</td>
</tr>
<tr>
<td>Post-prandial hypertriglyceridaemia (AUC TG)</td>
<td>0.40 (Baseline increase in TBARS at8hours)</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Chapter 7
Subfractionation Chapter

Clinical Ex vivo EPR Spin Trapping to Study the Mechanism of Lipoprotein Peroxidation Caused by Post-Prandial Oxidative Stress in Type 2 Diabetics

Introduction

The ingestion of fat in the diet is associated with significant increases in plasma triglycerides and TG-rich lipoproteins. The ensuing alimentary lipaemia is associated with elevations in markers of oxidative stress and decreases in bioavailable NO (83,68). It is unclear whether the source of this increased oxidative stress, is via dietary ingestion of lipid peroxides or through induction of an oxidative process in the vasculature causing endothelial dysfunction that impairs vascular dilatation with production of peroxidised and oxidised lipoproteins.

Previous reports (83,68) have suggested that some lipid hydroperoxides present in food may escape reduction or degradation during absorption across the intestinal barrier and that these peroxides may induce endothelial dysfunction, the earliest manifestation of atherosclerosis (208). This phenomenon may be a contributing factor to the correlation found between PPL and increased risk of cardiovascular disease. However there is growing evidence that PP lipoproteins can induce oxidative stress and decrease endothelial function, therefore bioavailable NO. In vitro data would seem to indicate that PP remnant lipoproteins increase oxidative damage caused by lipid components (particularly peroxidized phospholipids), by damaging cell surface membranes and being at least partly responsible for decreases in endothelium-dependent relaxation (71). Monocytes and polymorphonuclear cells release more superoxide anions when exposed to plasma from hypertriglyceridaemic (equivalent to post-prandial levels) patients (152,153), a phenomenon also positively correlated with plasma TG and negatively with HDL.

Within the bloodstream itself, the free radical peroxidation of lipoprotein lipids has received much study, with the extent of lipid peroxidation being an indication of the overall balance of imposed oxidative stress versus the protective effect of antioxidants (209). Oxidised
lipoproteins are implicated in the atherosclerotic process, and endothelial dysfunction generally, and lipid peroxidation within the bloodstream is therefore an important parameter (210,211). In most reports, lipid peroxidation is studied by assaying for the stable products produced when lipids are oxidised, such as aldehydes (thiobarbituric acid reactive substances TBARs), conjugated dienes or isoprostanes (211-3). However because these are relatively stable species, whose rates of biological clearance are usually unknown, they can unsuitable for many time-course studies where it is important to know when free radical generation and damage occurred. In addition, the actual identity of the damage product, and the reactions that caused its formation can be difficult to determine definitively (e.g. TBAR’s) although in many cases it would in fact be desirable to determine if and what free radical intermediates were involved.

EPR spectroscopy is a sensitive and specific technique for detecting and identifying free radical species, but because of their reactivity, these are usually short-lived in biological systems and so their steady-state concentrations are below the limit of detection. To overcome this difficulty the technique of spin trapping was developed, which involves adding an exogenous compound (the spin trap) to the system under study; the spin trap reacts with reactive free radical species to form more stable radical adducts that can be studied by EPR (214-6). This is shown for addition of a radical (R-) to the spin trap N-t-butyl-α-phenylnitrone (PBN) in reaction 1. By identifying the radical adducts, it is possible to determine the nature of the original reactive free radicals, and from changes in the EPR signal intensity one can study either kinetics or the effects of added agents on radical generation. EPR spin trapping has been of great value in the study of the importance and reactions of free radicals in biological systems from the in vitro to in vivo (217,129,130).
Importantly in comparison to TBAR measurements, spin trapping will only detect transient radical species formed during ongoing lipid peroxidation, and so the detection of radical adducts enables better time resolution of when lipid peroxidation occurs. We have therefore used the technique of spin trapping, with EPR detection of the resultant radical adducts, for studying the ongoing lipid peroxidation in clinically-derived blood samples, and use the term lipid peroxidation-derived free radicals (LPDFR) to describe what is actually measured. Since it is not currently possible to administer spin traps directly to human subjects, we have used a rapid sampling and mixing approach in which venous blood is directly drawn into a vacutainer containing spin trap (α-phenyl-tert-butynitrone, PBN) followed by solvent extraction of the radical adducts and EPR spectroscopy (131,129,128).

The system studied here has been the PP oxidative stress induced by a fatty meal in T2DM in whom the ingestion of a high fat content meal is associated with significant and prolonged increases in plasma triglycerides and TG-rich lipoproteins, and lipid peroxidation (215,216). We had previously shown that post-prandial lipaemia in T2DM subjects results in increases in lipid peroxidation measured as both lipid peroxidation derived free radicals (LPDFR, measured as PBN radical adducts) and plasma TBARs (Fig 1) greater than those seen in normal
controls, and also significant decreases in endothelial function (83). However, it is unclear whether the cause of these increases in oxidative stress in this system is due to:

a) dietary ingestion of lipid hydroperoxides that then provide 'seeding' hydroperoxides to initiate lipid peroxidation predominantly within the chylomicrons/VLDL (that initially carry dietary lipids following gastrointestinal absorption);

b) through inducing a state of generalised oxidative stress within the vasculature (such as by activating neutrophils, and/or endothelial NAD(P)H oxidases) thereby causing a general peroxidation of all lipoprotein classes present, or;

c) simply as a result of increased availability of substrates for lipid peroxidation.

It has been suggested that some lipid hydroperoxides present in food may escape reduction or degradation during absorption across the intestinal barrier (219) with these hydroperoxides then able to undergo either oxidative or reductive heterolysis to produce alkoxyl and peroxyl radicals respectively (Reactions 2 and 3) (220). However, due to the acid environment of the stomach, it might reasonably be expected that acid-catalysed Hock cleavage of dietary hydroperoxide would dominate (Reaction 4), resulting in the uptake of carbonyl-modified lipid species that can be detected as TBARs (218). Experimental evidence obtained from feeding rats purified lipid hydroperoxides indicated that these hydroperoxides are degraded into aldehydes (i.e. TBARs) in the stomach, with these aldehydes then undergoing absorption (221,222). Other authors have instead emphasized the importance of the reduction of dietary hydroperoxides during transport in the gut (225).

$$\text{ROOH} - e^- \rightarrow \text{ROO}^- + \text{H}^+ \quad (2)$$

$$\text{ROOH} + e^- \rightarrow \text{RO}^- + \text{HO}^- \quad (3)$$
Whatever the potential mechanisms of hydroperoxide metabolism may be, it would be highly desirable to determine whether the lipid oxidation observed in post-prandial diabetics results from absorption and metabolism of dietary hydroperoxides, or induction of oxidative stress through another mechanism (such as stimulation of neutrophils). Since dietary-derived lipids (and their hydroperoxides if present) will be transported in chylomicrons/VLDL after a meal, if the PBN-radical adducts observed in previous experiments (83) result directly from a ‘seeding’ effect of absorbed hydroperoxides, then the majority of the lipid-derived PBN adducts will be generated in the chylomicrons. If however, a generalized induction of oxidative stress in the bloodstream is induced, lipid-derived PBN adducts would be formed throughout different classes of lipoproteins (HDL, LDL, etc.). Thus in this study, we have used the experimental system as delineated in the methodology section, but have further used ultracentrifugation to separate lipoprotein classes after the ex vivo spin trapping process and determined the relative amounts of PBN-radical adducts in both the chylomicron/VLDL fractions and the LDL/HDL fractions in response to a fatty meal in a cohort of T2DM. In a limited number of subjects, the effects of co-administration of ascorbate (1 g) concurrent with the fatty meal have been examined.
Materials and Methods

Subjects

9 T2DM subjects (mean age 56 ± 3.4 years; 7 men and 2 women, diagnosed on WHO criteria) whose glycaemic control was moderate (HbA1c 6.5-10%, mean 8.8%) were enrolled in the study. All subjects were non-smokers, normotensive, and had no history or clinical evidence of overt vascular disease; had fasting cholesterol levels less than 6.5mmol/L, and were not taking aspirin, lipid lowering, hormone replacement therapy, or supplemental vitamins. All subjects were either diet controlled or were on oral hypoglycaemic agents. Written informed consent was obtained from all subjects, and the local research ethics committee approved the study.

Post-Prandial Lipaemia – The Standard Fatty Meal.

Central to this study of post-prandial lipaemia is the consumption of a standard fatty meal. The test meal is as described in the methods section. Blood samples were taken at baseline and at 4 hours following the meal to enable analysis of plasma lipids, lipoproteins and lipid derived free radicals during post-prandial lipaemia. The 4 hour time point was chosen as most ingested fat is in the chylomicron and free NEFA phases rather than into LDL and HDL subfractions (223) and coincides with the nadir of bioavailable NO in the vasculature (79,83,223).

Protocol

Studies began at 09:00 after a 12 hour overnight fast. Blood was drawn for triglycerides, TBAR and LPDFR measurements. Each subject was then given a standard fat tolerance test meal (as above). Blood was again drawn for measurements during the peak lipaemic phase, 4 hours following meal ingestion (83). Plasma TBAR and triglycerides were determined as indicated previously in the methods section.
Subfractionation EPR

For total plasma lipid peroxidation-derived free radical (LPDFR) measurements, 3 ml venous blood was taken directly into a sealed glass tube (vacutainer), containing 1 ml of PBN (0.125 mol l⁻¹) in saline. Following centrifugation (1000 g, 5 minutes) 2 ml of plasma was removed, and was extracted with toluene, dried under nitrogen gas and reconstituted in deoxygenated chloroform. EPR spectra were recorded on a Bruker EMX X-band spectrometer using the following parameters: Modulation amplitude 0.2 mT, sweep width 6 mT, microwave power 10 mW, X axis resolution 1024 points with conversion time 0.655 sec, time constant 0.655 sec. To determine LPDFR in different lipoprotein subclasses, venous blood was drawn into PBN-containing vacutainers, and plasma prepared as above. 2 ml plasma was then carefully deoxygenated to prevent further lipid peroxidation by carefully blowing under a stream of oxygen-free nitrogen for 15 minutes, layered on the top of 20 ml of sodium chloride solution of relative density 1.005 (that had been deoxygenated with oxygen-free nitrogen) and then centrifuged at 25000 g under vacuum for 14 hours at 10 °C. The chylomicron/VLDL containing fractions at the top of the tube, and the HDL/LDL containing fractions at the bottom were removed, and extracted with toluene and measured by EPR as for whole plasma above, but modulation 0.25 mT, microwave power 20 mW and with 20 scans, and results presented as total arbitrary units per total lipoprotein fraction. Flow mediated dilatation of the brachial artery, was used as a measure of endothelial function, this is a well validated non-invasive measure of bioavailable nitric oxide (NO⁻) activity in the vascular bed (79).

Results and Discussion

The results of the whole plasma LPDFR, TBAR and triglyceride determinations are shown in table 1. It can be seen that whilst plasma TG and TBARs both increased significantly at the 4 hours time point indicating PPL and OS, there was not a significant increase in LPDFR. However, in subjects given ascorbate concurrent with the fatty meal, a large decrease in both
LPDFR and TBARs at 4 hours was observed, indicating that oxidative stress had been reduced by this treatment, as has previously been reported (223). Thus, the failure to observe PP increases in LPDFR in addition to TBARs is felt simply to result from the smaller study size than that used previously. As witnessed in table 2, there was a significant decrease in endothelial function (FMD) in the post-prandial state, in accord with previous studies (83).

The results of free radical measurements in the isolated VLDL/chylomicron and HDL/LDL subfractions are shown in Table 2, and it can be seen that although there was considerable variation between individuals, there were detectable levels of LPDFR (i.e. ongoing lipid peroxidation) in both lipoprotein fractions, and that these were both increased, at the 4 hour PP time point. The considerable variation in LPDFR is similar to that observed in the measurement of PP lipid hydroperoxides by Ursini et al. where the percentage increase ranged from 13 to 674 % compared to same subject in the fasted state (216). The percent increase in LPDFR was broadly in accord with the percentage increase in TBARs for the same subject. The effect of concurrent ascorbate administration was to substantially decrease the levels of LPDFR in both lipoprotein fractions. These results are in contrast to those of Delmas Beauvieux et al., in which EPR spin trapping with the trap diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) was used in T2DM subjects but did not yield detectable EPR spectra (224), although TBARs were increased compared to normal controls. This is most likely related to the different hydrophobcities of DEPMPO and PBN (octanol/water partition coefficient – 0.1 and 12) favouring the partition of PBN into lipoprotein particles to detect lipid peroxidation.

LPDFR measurements indicate that in these T2DM subjects, there is detectable ongoing lipid peroxidation in both chylomicron/VLDL and HDL/LDL fractions, even after an overnight fast that might be expected to clear the former. However, the PP state is much more persistent in T2DM (typically persisting for 12 hours as opposed to 4 hours in controls) (83) and so it would be expected that significant levels of these lipoprotein (and hence LPDFR in these particles) might persist in these subjects. It is also known that HDL carries the bulk of lipid hydroperoxides
in normal control subjects during the fasting state (225) and so ongoing lipid peroxidation (producing LPDFR) would also be expected in these particles. The depletion of antioxidant capacity in T2DM will also tend to promote lipid peroxidation in all lipoprotein classes (226).

In the post-prandial state, increases in LPDFR were observed in both the chylomicron/VLDL and HDL/LDL fractions, which generally correlated with TBAR’s in each subject, and which were substantially decreased by coadministration of ascorbate. The increases in LPDFR were not limited to the chylomicron/VLDL fractions, and so it is unlikely that the post-prandial increases in lipid peroxidation simply result from the incorporation of dietary hydroperoxides into these particles, followed by peroxidation induced by these species. Rather, it would indicate that there is a generalized increase in oxidative stress within the bloodstream in the PP state in these subjects (supported by the decrease in FMD), resulting in initiation of lipid peroxidation within all classes of lipoproteins: this initiation results in ongoing lipid peroxidation (detected at LPDFR) and hence stable markers of lipid peroxidation such as TBARs. The significant decreases in LPDFR and TBARs caused by the concurrent administration of ascorbate with the fatty meal could result from scavenging of aqueous phase oxidants before they can initiate lipid peroxidation in the lipoproteins and/or the regeneration of lipid-soluble antioxidants within the lipoprotein particles (226). This study therefore provides evidence that there is a generalized increase in bloodstream oxidative stress occurs post-prandially in T2DM, and that this results in increases in lipid peroxidation.
Table 1. Below are the results for the separate lipid subfractions both fasting and post-prandially in individual subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>CM/VLDL 0 hours</th>
<th>LDL/HDL 0 hours</th>
<th>CM/VLDL 4 hours</th>
<th>LDL/HDL 4 hours</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>39.4</td>
<td>27.5</td>
<td>35.8</td>
<td>17.5</td>
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<tr>
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<td>3</td>
<td>8.2</td>
<td>19.5</td>
<td>17.6</td>
<td>16.3</td>
</tr>
<tr>
<td>4</td>
<td>37.3</td>
<td>34.6</td>
<td>72</td>
<td>61.3</td>
</tr>
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<td>14.1</td>
<td>11.1</td>
<td>14.9</td>
<td>12.5</td>
</tr>
<tr>
<td>6</td>
<td>5.8</td>
<td>3.2</td>
<td>9.8</td>
<td>7.4</td>
</tr>
<tr>
<td>7</td>
<td>21.4</td>
<td>15.1</td>
<td>24.03</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>15.4</td>
<td>26.1</td>
<td>10</td>
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</tr>
<tr>
<td>10</td>
<td>21</td>
<td>18</td>
<td>11.4</td>
<td>13.9</td>
</tr>
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</table>

Table 2. Below are the results for the plasma measures lipid derived free radicals and lipid peroxidation products in individual subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>EPR-0 hours</th>
<th>EPR-4 hours</th>
<th>Tbars-0 hours</th>
<th>Tbars-4 hours</th>
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<td>4990</td>
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<td></td>
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<td>3</td>
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<td>4875</td>
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<td>4.7</td>
</tr>
<tr>
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<td>5000</td>
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<td>10.6</td>
<td>6.4</td>
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<td>3000</td>
<td>6.9</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>10155</td>
<td>2000</td>
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<tr>
<td>10</td>
<td>9847</td>
<td>2500</td>
<td>3.2</td>
<td>2.86</td>
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Chapter 8

The effect of a fatty meal on platelet nitrate responsiveness in type 2 diabetes.

Introduction

Type 2 diabetes (T2DM) is characterized by a high incidence of cardiovascular disease (227,228) with a fourfold increase in the incidence of coronary artery disease compared to non-diabetics. Macrovascular disease accounts for 80% of deaths in T2DM (229). Indeed diabetics without previous myocardial infarction have a cardiovascular risk equivalent to non-diabetic subjects who have had a prior history of myocardial infarction (230). Furthermore, diabetics who suffer acute coronary events have poorer outcomes than non-diabetics suffering similar events (231,232). There are numerous vascular, haemostatic and fibrinolytic abnormalities in T2DM, which may provide a basis for the increased susceptibility of T2DM subjects to atherothrombotic disease (233).

The precise mechanism responsible for these abnormalities are uncertain, but is likely to include diminution of the endothelial NO barrier to platelet aggregation/cellular infiltration (193). Endothelial dysfunction is typically present in T2DM (100) and is believed to contribute importantly to the increased vascular risk in some other disorders; such as hypercholesterolaemia (234) hypertension (235) and ischaemic heart disease (62) where platelet responsiveness to NO is also reduced. Vascular responsiveness to exogenous nitrates in T2DM has been observed to be attenuated in the brachial (236) and in the coronary arteries (241).

Platelet hyperaggregability is also likely to play a part in the increased susceptibility of T2DM subjects to atherothrombotic disease. The increased risk of acute thrombosis in T2DM may be in part due to altered platelet function. There is an increase in platelet adhesion and aggregation to ADP (237,238). Furthermore there is an increase in platelet-dependent thrombin generation (239) and impaired platelet mediated vasodilatation (238,240). Abnormal platelet function appears partly mediated via increased blood glucose (239,240). At a
cellular level there are alterations in calcium signalling, either due to excess superoxide anion production or to attenuated feedback control by nitric oxide due to decreased production (241).

Platelet hyporesponsiveness to the antiaggregatory effects of NO donors may also be present in T2DM. Limited data suggests anomalies in guanylate cyclase activity (242) and reduced platelet sensitivity to GTN in obese T2DM subjects only but not lean T2DM subjects (236). As regards platelet/NO interactions, the phenomenon of ‘nitrate resistance’, has been fully characterised recently by Chirkov et al (242) in patients with stable angina pectoris, and shown to be mediated by a combination of mechanisms. NO ‘scavenging’ by O₂⁻ production and (presumably a redox-dependent) inactivation of platelet soluble guanylate cyclase. The latter was identified as a component mechanism in diabetics by Chirkov et al in 1990 (242).

We therefore examined platelet responsiveness to NO, in T2DM and controls and related our findings to measurements of endothelial function and vascular NO-induced vasodilatation in T2DM subjects. The objectives of the current study were: (1) to compare platelet responsiveness to NO in fasting T2DM with those from normal subjects (2) To determine whether the postprandial state affected platelet sensitivity to NO in T2DM subjects. (3) to evaluate extent of correlation between platelet responsiveness to NO in diabetics and measures of oxidative stress primarily but also endothelial function and vascular hypo-responsiveness to NO. Other factors to be examined as possible modulators of platelet responses included age, body mass index (BMI), lipids, acute and chronic glycaemia.
Methods

Subjects

Studies were performed in the following groups:

(a) Normal subjects n=15, 5 women aged 30 to 74, (mean 42.8 ± 12.2 years) taking no medications affecting platelet aggregation or antioxidants.

(b) T2DM subjects diagnosed on WHO criteria, n=12, 4 women aged 38 to 68 years (mean age 53.5 ± 8.8 years) whose glycaemic control was moderate (HbA1c 6.5-10%, mean 8.1%) were enrolled in the study.

All subjects were non-smokers, normotensive, and had no history or clinical evidence of overt vascular disease; had fasting cholesterol levels less than 6.5mmol/L, and were not taking aspirin, lipid lowering or hormone replacement therapy, nor supplemental vitamins. The diabetic group had a significantly higher body mass index (31 ± 3.6 vs. 26.8 ± 3.1, p<0.05). All T2DM subjects were either diet controlled or were on oral hypoglycaemic agents. Those subjects taking oral agents omitted their medication on the morning of the study. Written informed consent was obtained from all subjects, and the local research ethics committee approved the study.

Study Protocol.

Studies began at 9am after a 12-hour overnight fast. Blood was drawn for serum total, LDL and HDL cholesterol, triglycerides (TG), glycosylated haemoglobin (HbA1c), insulin, glucose and venous FR measurements, and brachial artery endothelial function was measured. Each subject was then given a standard fat tolerance test meal (32), as previously described. Lipid profiles and plasma glucose were repeated 4 hours post prandially, with lipid derived FR’s and endothelial function tested at baseline and during the peak lipaemic phase, ie 4 hours following meal ingestion. (32)
Blood sampling and preparation of platelets

Measurements of oxidative stress and platelet reactivity were assessed both in the fasting and post-prandial state as indicated. Precautions were taken to minimise the potential for activation of the clotting system or platelets during blood sampling. Venous blood was collected from the antecubital fossa in plastic tubes containing 1:10 volume of acid citrate anticoagulant; acidified citrate was utilised in order to minimise deterioration of platelet function during experiments (243).

Platelet Aggregation Studies

Aggregation was examined in whole blood utilising a whole blood impedance aggregometer (ChronoLog Corporation) as described in chapter 2. Tests were performed at 37°C and stirring speed of 900rpm. Samples of blood were diluted twofold with normal saline (final volume 1ml) and prewarmed for 5 minutes at 37°C. Aggregation was induced with adenosine 5'-diphosphate (ADP) (1μM) and responses measured as changes in electrical impedance (Ohms) continually for 7 minutes. Inhibition of aggregation was assessed with the exogenous NO donor, SNP (final concentration 10 μmol/L) and GTN (final concentration 100μmol/L) which were added to samples 1 min before ADP. The duration of incubations were estimated as those optimal in preliminary experiments. In control tests, physiological saline was added in appropriate volumes. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of SNP and GTN.

Measurements of oxidative stress

(a) Venous lipid-derived free radicals.

Measured as outlined in the methods section.

(b) Thiobarbituric acid-reactive substances (TBARS)

Measured as outlined in the methods section.
**Measurement of endothelial function.**

Measured as outlined in the methods section.

**Lipid assays and biochemical measurements.**

Measured as outlined in the methods section.

**Data analysis.**

The principal hypothesis was tested via comparison of SNP and GTN responses for fasting normal subjects and those with T2DM in the fasting and postprandial state. Differences between normal subjects and patients with diabetes, where data were normally distributed, were examined via non-paired t-test; otherwise comparison utilized a Mann-Whitney test. Pre and postprandial comparisons were subject to paired t-test analysis.

Evaluation of possible correlates of platelet responsiveness to SNP and GTN in diabetic subjects was performed via a univariate analysis, we used the following parameters as possible correlates: age, BMI, lipids, glucose, HBA1c, (acute and chronic glycaemia), oxidative stress as measured via TBARS and EPR spectroscopy, Markers of endothelial function and vascular GTN responses. In view of possible interdependence of TBARS and EPR spectroscopy data, the model was also evaluated with one of these parameters excluded in turn. Results are expressed as mean ± SEM unless otherwise stated.
**Results**

**Patients/normal subjects’ characteristics**

Gender distribution was similar in patients and controls but patients were slightly older than controls (p<0.05). Fasting glucose, glycosylated haemoglobin, BMI and number of subjects taking oral medications were also greater in the T2DM group, p<0.05 (See Table 1).

**Fasting platelet reactivity**

Platelet reactivity to 1μM ADP differed significantly between diabetic and normal subjects (Figure 1) in the fasting state 13.85 ± 1 vs 10.76 ± 1.1 (p<0.05). There was no significant inter-gender difference in ADP responses. Inhibition of platelet aggregation by SNP and GTN was 73.1 ± 5.9% and 50.3 ± 7.7% in healthy controls respectively compared to 15.4 ± 7% and 19.5 ± 8.2% in fasting diabetics (p<0.001, p<0.02) respectively (Figure 2). Individual responses to SNP and GTN were normally distributed in healthy subjects, but in diabetic subjects the distribution was skewed, with the bulk of diabetic subjects showing markedly reduced SNP and GTN responses. SNP and GTN responses were significantly correlated with each other (p=0.001).

**Measurements of oxidative stress**

Two indices of plasma oxidative stress were used in this study, lipid derived free radicals directly sampled from venous blood, which gives a snapshot of oxidative stress, and longer lived markers of lipid peroxidation, (TBARS) as composites of background oxidative stress. Analysis of the EPR spectra from spin-trapped radicals derived from venous blood samples suggested that the radicals trapped were alkoxyl radicals (coupling constants aN = 13.9 Gauss, aβH = 2.2 Gauss) and carbonyl radicals (aN= 14.1 Gauss; aβH = 4.0 Gauss). These assignments, which agree with our previous studies (29-31), suggest that these radicals are derived from decomposition of lipid hydroperoxides in the extracellular compartment. Detection of secondarily formed lipid free radicals strongly supports the presence of continuing peroxidative damage in vivo.
Fasting measures of oxidative stress

(EPR Spectroscopy and lipid peroxidation products)

Results are summarized in figure 3. Concentrations of secondary free radicals were significantly elevated (p<0.001) in fasted diabetics (3605 ± 559) vs controls (880 ± 96). Concentrations of TBARS were also elevated in fasted diabetic subjects (6.54 ± 0.83) compared to controls (3 ± 0.34) (p<0.002).

Post-prandial effects

Fasting and post-prandial changes platelet reactivity in T2DM.

In the T2DM subjects fasting and post-prandial platelet reactivity to ADP was similar (13.85 ± 1 and 14.86 ± 1.1 respectively. There was no significant difference between SNP and GTN responses in the fasting (15.4 ± 7% and 19.5 ± 8.2%) vs post-prandial states (20 ± 7.1% and 7.7 ± 3%) respectively (Figure 4).

Fasting and post-prandial changes in oxidative stress in T2DM.

There were significant increases in both lipid derived free radicals from 3605 ± 559 to 4313 ± 580 post-prandially (p<0.02) (Figure 5) and markers of lipid peroxidation from 6.54 ± 0.83 to 8.8 ± 0.6μmol/L postprandially (p<0.05).

Fasting and post-prandial vascular data.

Endothelial function measured as percentage flow-mediated dilatation, deteriorated PP from 0.65 ±0.3% to −0.68 ±0.3% (p<0.005) (Figure 6) but vascular NO responses measured as percentage brachial artery dilatation in response to GTN remained unchanged 12.2 ±1.2% and 13.1 ±0.6% PP (t=-0.65, p=0.49).
Determinants of SNP and GTN responses and vascular parameters: multiple regression in the fasting state.

On univariate analysis, there tended to be an inverse relationship between SNP and GTN responses in the diabetic subjects and plasma levels of lipid derived free radicals ($r = -0.543$, $p=0.06$ [SNP]) and ($r = -0.515$, $p=0.08$ [GTN]). No such correlations were present with TBARS. No such correlation with either marker of oxidative stress was present in healthy controls. Fasting insulin levels were significantly correlated with platelet ADP responses ($r=0.642$, $p=0.02$).

Endothelial function and vascular GTN responses were independent variables except for a tendency to deteriorate with age ($r=-0.527$, $p=0.07$) and ($r=-0.534$, $p=0.07$) respectively. Glucose was positively correlated with age ($r=-0.709$, $p=0.01$). No other metabolic parameter correlated with platelet or vascular responses. Glucose also had a tendency to correlate with HbA1c ($r=0.544$, $p=0.06$). In particular both BMI and serum cholesterol were not related to SNP responses in any way.

Determinants of SNP and GTN responses and vascular parameters: multiple regression in the post-prandial state.

Apart from a tendency for PP SNP responses to be related to endothelium independent vasodilatation ($r=0.512$, $p=0.08$) there were no other correlates of platelet and vascular function. There were some metabolic parameters that correlated with plasma glucose; Age ($r=-0.576$, $p=0.05$) and cholesterol ($r=0.668$, $p=0.02$). There was also a tendency for PP TG levels to be correlated with PP glucose ($r=0.548$, $p=0.06$).
Discussion

The major findings in this study were marked abnormalities of platelet function in T2DM. There was both increased platelet aggregation to ADP and decreased platelet NO responsiveness in T2DM. The magnitude of this impaired NO responsiveness was even greater than that previously observed in subjects with angina pectoris (242). Platelet NO responsiveness was not critically interdependent on body mass index unlike a previous study (236) which seemed to indicate that obesity per se was responsible for this phenomenon. Our study also indicates that platelet NO responsiveness is associated with increased oxidative stress but unlike our observations concerning endothelial function in this and previous studies (83,195) does not appear critically sensitive to incremental oxidative stress.

There is increased ADP induced platelet aggregation in T2DM in this study, a finding consistent with other studies (236,237,240). Only two previous studies have reported abnormal NO responsiveness in platelets from T2DM subjects. Trofossi et al (236) reported that there was abnormal NO responsiveness to GTN in platelets but this finding was attributed to obesity rather than T2DM per se, as lean T2DM subjects in that study exhibited normal NO platelet responsiveness. A further study found impaired NO responsiveness in platelets in diabetes but this was only shown in a combined cohort of both insulin dependent and T2DM subjects (242). Therefore this is the first study to demonstrate severe platelet NO resistance in platelets in T2DM subjects.

Our study further reinforces the findings of increased oxidative stress and worsening of endothelial function in the post-prandial state in T2DM (83,195). Previous reports from other researchers have also indicated that increases in FFA may play a role in these phenomena as evidenced by intralipid infusions in healthy controls showing decrements in endothelial function (244). In vitro data has suggested that this may play a part in abnormal platelet responses also (245). New data from this study would however seem to indicate that platelet responsiveness to
NO donors does not change post-prandially in T2DM; however because responsiveness is already low in this cohort, it is possible that a small decrease of further impairment may have been missed. This finding could be specifically examined if higher doses of GTN/SNP were used so that both pre and post-prandial responses would be greater than 20%.

Nevertheless this raises the possibility that platelet NO resistance in T2DM subjects may be less sensitive to changes in oxidative stress than endothelial function. This would be consistent with the previous observations that in platelets from patients with stable angina, there are two mechanisms; increased superoxide anion production and soluble guanylate cyclase inactivation accounting for platelet NO resistance. Of these mechanisms Chirkov et al (242) was able to demonstrate only a decrease in soluble guanylate cyclase activity in T2DM.

Therefore measures to reduce vascular events in diabetics may be relatively insensitive to manipulation of oxidative stress (cf. Vitamin E arm of the HOPE study) (246), and should be directed at means of stabilizing soluble guanylate cyclase.

Weaknesses

There are weaknesses in the methodology of this section of my thesis. The use of one concentration of ADP although based on previous experimental data could potentially limit the NO donor/platelet responsiveness data. Full dose response curves may have illuminated the actual dose that a significant difference in NO donor resistance was demonstrated. Furthermore no PP platelet responses were performed in healthy controls which may have delineated the question of platelet responsiveness postprandially. Platelet aggregometry techniques do not necessarily correlate with other tests of platelet activation and may have serious limitations in extrapolating to in vivo measures. There is uncertainty whether the ex vivo conditions in the platelet aggregometer truly reflect in vivo aggregation.
Acknowledgements

I wish to thank Mr. Peter Gapper and Catherine Mumford for their technical support during this part of my thesis.
Figure 1. Fasting ADP induced platelet aggregation in T2DM and controls.

* $p<0.05$ vs normals

Figure 2. Fasting SNP and GTN responses in controls and T2DM subjects

$\dagger p<0.001$ vs Controls, * $p<0.002$ vs Controls,
Figure 3. Fasting markers of oxidative stress in controls and T2DM subjects.

* P<0.001 vs Controls

Figure 4. Fasting and postprandial SNP platelet responses in T2DM.

* p<0.001 vs Controls
Figure 5. Fasting and postprandial markers of oxidative stress

Figure 6. Fasting and post-prandial endothelial function (FMD) in T2DM subjects.
Table 1. Patients/normal subjects' characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=15)</th>
<th>T2DM subjects (i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.8 ± 12.2</td>
<td>53.5 ± 8.7*</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10M, 5F</td>
<td>8M, 4F</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.9 ± 1.5</td>
<td>6.2 ± 1.1</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.5 ± 0.6</td>
<td>1.9 ± 1</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.9 ± 0.3</td>
<td>13.2 ± 4.3*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 ± 1</td>
<td>8.2 ± 2.6*</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td>126 ± 6</td>
<td>128 ± 5</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>27 ± 4.2</td>
<td>32.8 ± 5 *</td>
</tr>
<tr>
<td>Oral medications</td>
<td>-</td>
<td>9 *</td>
</tr>
</tbody>
</table>

* p<0.05 vs normals

Table 2: Metabolic data

<table>
<thead>
<tr>
<th>T2DM</th>
<th>Fasting</th>
<th>4 hours PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>13.2 ± 0.1</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>6.3 ± 1.3</td>
<td>6.25 ± 1.4</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.9 ± 1.1</td>
<td>3 ± 0.8 *</td>
</tr>
<tr>
<td>Insulin (lu/l)</td>
<td>18.1 ± 2.1</td>
<td>39.3 ± 3.6 *</td>
</tr>
</tbody>
</table>

(* denotes P<0.05 vs fasted baseline).
Table 3. Correlation of fasting platelet NO responses to SNP and GTN with various parameters in the T2DM group.

<table>
<thead>
<tr>
<th></th>
<th>SNP Correlation Coefficient</th>
<th>SNP Significance P value</th>
<th>NTG Correlation Coefficient</th>
<th>NTG Significance P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.465</td>
<td>0.128</td>
<td>0.189</td>
<td>0.557</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>-0.315</td>
<td>0.319</td>
<td>0.079</td>
<td>0.807</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>-0.404</td>
<td>0.193</td>
<td>-0.273</td>
<td>0.390</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>-0.438</td>
<td>0.154</td>
<td>-0.263</td>
<td>0.409</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>-0.441</td>
<td>0.152</td>
<td>-0.028</td>
<td>0.931</td>
</tr>
<tr>
<td>Insulin (IU/l)</td>
<td>-0.391</td>
<td>0.209</td>
<td>-0.417</td>
<td>0.177</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>-0.307</td>
<td>0.332</td>
<td>-0.474</td>
<td>0.119</td>
</tr>
<tr>
<td>EID (%)</td>
<td>-0.363</td>
<td>0.247</td>
<td>-0.005</td>
<td>0.988</td>
</tr>
<tr>
<td>EPR (Units)</td>
<td>-0.543</td>
<td>0.068</td>
<td>-0.515</td>
<td>0.087</td>
</tr>
<tr>
<td>TBARS (μmol/l)</td>
<td>-0.179</td>
<td>0.579</td>
<td>-0.294</td>
<td>0.354</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>0.331</td>
<td>0.289</td>
<td>0.398</td>
<td>0.212</td>
</tr>
</tbody>
</table>
Conclusions

In summary, postprandial lipaemia in healthy individuals is associated with endothelial dysfunction and increased oxidative stress. The studies have shown that the degree of oxidative stress correlates with TG enriched VLDL but not with the magnitude of post-prandial hypertriglyceridaemia per se. HDL-C levels appear to be inversely correlated to the increase in oxidative stress.

In diabetic subjects, a fatty meal results in greater PPL than healthy controls and there is fasting endothelial dysfunction consistent with previous data. PPL induces a greater degree of oxidative stress in association with endothelial dysfunction. In diabetics, TG-VLDL and also TG-LDL are correlated with the degree of PP deterioration in endothelial dysfunction; like healthy individuals, TG-VLDL is correlated with the PP rise in oxidative stress.

It is unclear whether the source of this increased oxidative stress, is via dietary ingestion of lipid peroxides or through induction of an oxidative process in the vasculature causing endothelial dysfunction that impairs vascular dilatation with production of peroxidised and oxidised lipoproteins. Changes in lipid radicals in plasma and in lipoproteins in response to a fatty meal in diabetics were delineated by using the technique of electron paramagnetic resonance spectroscopy in plasma and the lipoprotein subfractions.

In the post-prandial state, increases in lipid derived free radicals were observed in both the chylomicron/VLDL and HDL/LDL fractions, which generally correlated with markers of secondary lipid peroxidation in each subject. These increases were not limited to the chylomicron/VLDL fractions, and so it is unlikely that the PP increases in lipid peroxidation simply resulted from the incorporation of dietary hydroperoxides into these particles, followed by peroxidation induced by these species. Rather, it would indicate that there is a generalised increase in oxidative stress possibly at an endothelial level; although it may be due to circulating
monocytes and neutrophils within the bloodstream in the post-prandial state, resulting in initiation of lipid peroxidation within all classes of lipoproteins.

Attenuating the magnitude and duration of exposure of the endothelium to atherogenic lipoproteins may be of benefit post prandially, with reduced TG rich VLDL particles and increased HDL-C levels being of particular importance. The studies also demonstrate that ciprofibrate attenuated PPL, decreased TG enrichment of lipoproteins and in particular reduced TG rich VLDL and LDL. This led to a significant improvement in fasting and PP endothelial function, and also attenuated PP oxidative stress. Although ciprofibrate improved endothelial function both in the fasting state and post-prandially, it did not however blunt the decrement in endothelial function post-prandially, suggesting other mechanisms to be involved.

Other investigators have shown that endothelial function after a fatty meal in healthy individuals has normalised by 6 hours. In the diabetic subjects in our study, there is prolonged endothelial dysfunction up to 8 hours after fatty meal ingestion. The antioxidant vitamin C improves endothelial function at all time points but attenuates PP induced oxidative stress at 4 hours only. Although vitamin C therapy improves baseline and PP endothelial function, there still remained some decrement in function. This decrement in PP endothelial function despite vitamin C therapy indicates that lipoprotein interactions with the endothelium may continue to blunt some endothelial function. This suggests that both processes acting together are likely to play a role in PP endothelial dysfunction and that strategies to alter both lipid metabolism and oxidative stress need to be employed with fatty meal ingestion to completely abolish endothelial dysfunction PP in T2DM.

The use of chronic insulin therapy in T2DM results in reduced oxidative stress and improved fasting and PP vascular reactivity, with augmented effects on endothelial function and oxidative stress produced by additional vitamin C. The underlying mechanisms may include increased HDL-C concentrations; modified TG metabolism with attenuated PPL and reduced
levels of TGRL with supplemental effects of vitamin C on antioxidant defences. The effects of insulin appear to be independent of changes in glycaemic control.

Insulin is responsible for other lipid changes that could enhance endothelial function, in particular reduced FFA levels. This may prove beneficial since FFA's are considered to be directly toxic to the endothelium and potentiate the effects of TG rich VLDL. These effects may be particularly relevant during PPL, which is associated with fluxes of increased FFA. TG depletion of VLDL following insulin correlated with improvements in endothelial function. Thus by reducing levels of TG rich VLDL, improved endothelial function following insulin may be partly due to attenuation of the atherogenic effects of these particles.

It appears that LPL may hold a key role in controlling lipoprotein/FFA interactions at the endothelial interface. PP endothelial dysfunction and arterial injury may result from a direct effect of circulating lipoprotein lipolysis causing elevated circulating FFA levels released at the endothelial cell surface. This PP endothelial dysfunction may be overcome by using different fat products. Fish oils and α-linolenic acid (ω-3) based meals, did not cause significant PP endothelial dysfunction and resulted in a lesser absolute rise in plasma TG and also did not result in PP endothelial dysfunction. Therefore the source of the fat ingested (and its susceptibility to oxidation) that is incorporated within the lipoproteins metabolised seems to be of importance.

In the platelet section of this thesis, marked abnormalities of platelet function in T2DM were demonstrated. There was both increased platelet aggregation to ADP and decreased platelet NO responsiveness in T2DM. The magnitude of this impaired NO responsiveness was even greater than that previously observed in subjects with angina pectoris. This study also indicates that platelet NO responsiveness is associated with increased oxidative stress but unlike the observations concerning endothelial function in this and previous studies, does not appear critically sensitive to incremental oxidative stress.
Discussion

The modern lifestyle with a tendency for high fat diets combined with a reduction in physical activity can be detrimental to health. There is also a trend towards increasing urbanisation which is directly associated with an increase in dietary fat. These trends highlight the importance that alimentary or PPL may have in daily life. Indeed given the duration that the PP phase lasts for 6 hours and up to 8hrs in T2DM, repeated meals high in saturated fat mean that the vast majority of a 24 hour period could be ‘post prandial’. A period during which, the endothelium receives a complex combination of insults.

Several potentially atherogenic changes occur in healthy individuals after a fatty meal. Endothelial function is transiently disturbed, probably via increased oxidative stress which attenuates bioavailable NO resulting in pro-vasoconstrictor forces. This phenomenon can be blunted by manipulation of NO activity by antioxidants, ACE inhibitors and folate supplements. In diabetics this phase is exaggerated and prolonged where even in the fasting state there are lipoprotein changes that are similar to the ‘postprandial phase’ in healthy individuals. Whether the endothelial dysfunction seen is due to generated oxidative stress or by locally released free fatty acid toxicity at an endothelial cell level is uncertain.

The influence on the fatty meal in disease states, particularly coronary artery disease, and T2DM can potentially be quite profound. There is emerging evidence that a fatty meal can influence myocardial perfusion as shown by PET imaging (247), it may even induce changes in coronary flow reserve (coronary microcirculation) in healthy individuals (248). PPL in patients with CAD can worsen endothelial function and like T2DM can be blunted by the addition of vitamin C (249). However there is some rather limited evidence that ACE inhibitors and fibrates may not blunt the PP deterioration in endothelial function in CAD patients unlike that in healthy controls and diabetics (250). Further studies are needed to clarify these changes more clearly in coronary artery disease.
Acutely, a number of physiological processes can lead to plaque rupture and progression to coronary artery occlusion. Vascular endothelial cells influence platelet function and thrombosis as well as smooth muscle tone, processes that may all be disturbed during PPL. PPL is associated with transient activation of factors VII and XII (85). Activated factor VII is a potent procoagulant, initiating the thrombotic response to atheromatous plaque rupture after its formation by complexing with exposed tissue factor to activate the coagulation cascade. This finding perhaps supports the positive association between activated factor VII and the risk of fatal CAD (86). Increased PP factor VII activation may thus raise the likelihood that plaque rupture leads to occlusive coronary thrombosis.

In adults taking a diet rich in long-chain saturated fatty acids (87), activated factor VII increases after a meal containing more than 90g fat (88). Factor VII activation correlates quantitatively with the degree of PP triglyceridaemia and seems to be related to FFA production during lipolysis of TG-rich lipoproteins by LPL on the endothelial cell surface (89). It appears that large PP TG-rich lipoproteins are required in vivo (90) and that long chain saturated fatty acids provide the surface that activates the coagulation system at the endothelial bound LPL/lipoprotein interface (91). The PP rise in activated factor VII can be attenuated however. There is limited evidence that acutely, a meal containing n-3 polyunsaturated fatty acids does not increase activated factor VII (92) or induce endothelial dysfunction (81), suggesting that the two outcomes may be linked.

Not only does PPL induce a prothrombotic tendency but a fatty meal can also decrease the fibrinolytic capacity in the blood. Tissue-type plasminogen activator (t-PA) is the principal fibrinolytic mechanism that operates in the coronary vascular bed (93). Therefore any increase in the natural inhibitor of this plasminogen activator inhibitor-1 (PAI-1), will intimately influence coronary thrombosis in vivo. The importance of the fibrinolytic system being highlighted recently with PAI-1 and tPA levels correlating with risk of reinfarction in both men and women (251).
Increased plasma TG levels chronically are associated with increased PAI-1 activity due to activation of the promotor gene by VLDL (94). This undermines the principal fibrinolytic protection in the coronary vasculature in chronic conditions eg T2DM. There is however evidence that PAI-1 activity and PAI-1 antigen is increased acutely after a fat load by 76 and 64% respectively. This infers relative fibrinolytic resistance in the aftermath of a fatty meal (95). Two and four hours after a fatty meal in CAD patients there is also a significant rise in plasma fibrinogen again reinforcing the procoagulant milieu (96).

There is evidence of altered platelet function after fatty meal ingestion. The membrane expression of platelet P-selectin is enhanced PP (252). This suggests that platelets are sensitised to thrombin and collagen after a meal (253), a finding of potential importance to thrombus formation after plaque rupture. Platelet P-selectin actually increases platelet/leucocyte adhesion. Indeed in acute coronary syndromes, increased P-selectin actually facilitates microembolization distally in the coronary artery, as this process is thrombin and collagen dependent (254). It also stabilizes glycoprotein IIb/IIIa-fibrinogen interactions allowing the formation of large stable platelet aggregates (255). It may even predict unsuccessful thrombolysis in patients with acute myocardial infarction (256). Increased platelet sensitivity is detectable three hours after a fatty acid meal and follows a similar time course to endothelial dysfunction.

If a fatty meal in itself does not trigger, through its myriad changes in the vasculature, acute coronary syndromes then it certainly may facilitate its continuation if it occurs in the PP phase. If plaque rupture occurs in the context of a PP phase there may be expected to be a milieu of endothelial dysfunction, vasospasm with resulting low flow, low fibrinolytic activity, a procoagulant state, high levels of fibrinogen and reactive platelets. These processes clearly can promote thrombus formation, and its subsequent stabilisation and occlusion of the coronary arteries.
In 1989 in an article published in Circulation Muller et al. (257) examined the still unknown triggers for acute coronary syndromes/MI they felt that there is a;

"Need to re-examine the possibility that the onset of MI and sudden cardiac death is frequently triggered by daily activities’’

‘Increases in coagulability or vasoconstriction triggered by daily activities, contribute to complete occlusion of the coronary artery lumen’

This year, discussing plaque rupture and acute coronary syndromes in Circulation Schoenhagen et al (258) felt that;

‘Presumably patients with ACS have an underlying (temporary?) biochemical milieu predisposing them to the development of widespread plaque degradation and/or accelerated subsequent thrombus formation’’

My hypothesis proposes that fatty meal ingestion can be one of these temporary (?) triggers of daily activity.

I propose that as a result of ingesting fatty meals as a part of everyday living, there occurs a constellation of changes in the vasculature that result in both a hypercoagulable and a pro-vasoconstrictor state. These acute changes in endothelial function, prothrombosis, and platelet activation can potentially trigger, facilitate and propagate the forces that drive acute coronary syndromes. In T2DM, adverse PP phenomena are exaggerated and prolonged and may therefore be expected to contribute significantly to the excess risk of acute coronary syndromes and atherosclerotic development. Further evaluation of these issues merits investigation, as simple dietary means may effect profound benefits.
Future Directions

Attempting to delineate the role of oxidative stress or locally released FFA’s causing endothelial dysfunction is difficult to achieve. However heparin (which releases LPL from its endothelium bound anchor) may help. By releasing LPL and increasing transient TG metabolic capacity in the context of a meal and simultaneously dissociating the atherogenic molecules from the endothelial cell surface, this may help in our mechanistic understanding of PPL induced endothelial dysfunction. Future studies in CAD patients with particular reference to platelet and coagulation disturbances postprandially may also further our understanding.

Furthermore prospective dietary questionnaires to all infarct and ACS patients of what they ate in the 12 hours prior to the index event may shed light on potential dietary triggers. Clearly much work needs to be done to clarify the mechanisms of postprandial disturbance in endothelial dysfunction and ways to ameliorate this, particularly in disease states. The next phase of research in this area may most profitably be spent looking at its role in the genesis of disease states.
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168


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