THE UNIVERSITY OF HULL

Fatty Acids and the Modulation of Glucose Metabolism

being a Thesis submitted for the Degree of Doctor of Medicine in the University of Hull

by

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Dedication

To

My wife Helen, my daughter Yasmeen and son, Hani

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List of abbreviations:

8-iso-PGF2α: 8-iso-prostaglandin F2α

A1C: haemoglobin A1C

AACE: American Association of Clinical Endocrinologists

ACCORD: Action to Control Cardiovascular Risk in Diabetes

ADP: adenine diphosphate

AGE: advanced glycaemic products

AHA: American Heart Association

ALA: alpha linolenic acid

ANOVA: analysis of variance

Apo C 3: apolipoprotein C3

ARA: arachidonic acid

ATP: Adult Treatment Panel

AUC: area under the curve

BG: blood glucose

BHT: butylated hydroxytoluene

BMI: Body Mass Index

CaHPO4: calcium hydrogen phosphate

CCl4: carbon tetrahydrochlorate

CETP: cholesteryl ester transfer protein

CGMS: continuous glucose monitoring system

-CH-: methylene group

CHD: coronary heart disease

CI: confidence interval

CIGMA: continuous infusion of glucose with model assessment

CoA: coenzyme A

CONGA: continuous overlapping net glycaemic action

COPD: chronic obstructive pulmonary disease

CV: coefficient of variation

CVD: cardiovascular disease

DAG: diacylglycerol

DCCT: Diabetes Control and Complications Trial

DG: diglyceride

DGAT: diacylglycerol acyl transferase

DGLA: dihomo gamma linolenic acid

DHA: docosahexaenoic acid

DPA: docosapentaenoic acid

ED: effective dose

EDIC: Diabetes Interventions and Complications

EE: ethyl ester

EGIR: European Group for the study of Insulin Resistance

EIA: enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

EPA: eicosapentaenoic acid

ETG: enzymatically synthesised triglycerides

FADH: flavin adenine dinucleotide

FAME: fatty acid methyl esters

FPG/FPG: fasting plasma glucose

FSIVGT: Frequently Sampled Intravenous Glucose Tolerance Test

G6P: glucose 6 phosphate

GLA: gamma linolenic acid

GLC: Gas Chromatography

GLUT: Glucose Transporter

HbA1c: Haemoglobin A1c

HDL: High Density Lipoprotein

HDL-C: High Density Lipoprotein cholesterol

HNE: 4-hydroxyalkenals

HOMA: Homeostatic Method of Assessment

HOMA-IR: Homeostatic Method of Assessment of insulin resistance index

HPLC: high performance liquid chromatography

HSP: hexosamine pathway

HZ: hazard ratio

I: insulin

ICU: Intensive Care Unit

IDF: International Diabetes Federation

IDGU: insulin dependent glucose utilization

IFG: impaired fasting glycaemia

IGT: impaired glucose tolerance

INF: glucose infusion rate

IR: insulin resistance

IRS: insulin receptor substrates

ISI: insulin sensitivity index

JNK: c-Jun N Terminal Kinase

$KHCO_3$: Potassium Bicarbonate

LA: linoleic acid

LC: Liquid Chromatography

LCPUFA: long chain polyunsaturated fatty acid

LDL: Low Density Lipoprotein

M: mean

M: Peripheral glucose utilization (during glycaemic clamps)

MAGE: mean amplitude of glycaemic excursions

MDA: malonaldehyde

MG: monoglyceride

MI: myocardial infarction

MODD: mean of daily differences

MR: magnetic resonance

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

MS: Mass Spectrometry

N-3 PUFA: omega 3 polyunsaturated fatty acids

NAD/NADH: nicotinamide adenine dinucleotide

NADP/NADPH: nicotinamide adenine dinucleotide phosphate

NAFL: non alcoholic fatty liver

NCEP ATP: National Cholesterol Education Program Adult Treatment Panel

NCI: negative ion chemical ionization

NEFA: nonesterified fatty acids

NF: Nuclear Factor

NHLBI: National Heart, Lung, and Blood Institute

NIDGU: non insulin dependent glucose utilization

NO: Nitric Oxide

NTG: natural triglyceride

OSM: oxidative stress markers

PAI-1: plasminogen activator inhibitor 1

PARP: poly adenine diphosphate ribose polymerase

PI3: Phosphoinositide 3 Kinase

PKC: Protein Kinase C

PPAR: peroxisome proliferator-activated receptor

PUFA: polyunsaturated fatty acids

Quicki: Quantitative Insulin Sensitivity Check Index

RAGE: receptors for advanced glycaemic endproducts

RCT: randomised controlled trial

RNA: ribonucleic acid

ROS: reactive oxygen species

RR: relative risk

RTG: reconstituted triglyceride

SC: space correction

SD: standard deviation

SEM: scanning electron microscopy

Si: glucose sensitivity

Sn: side chain of triglyceride

SREBP-1C: sterol regulatory element binding protein-1c

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TAG: triacylglycerol

TBA: thiobarbituric acid

TG: triglyceride

TGF β1: transforming growth factor β1

TLC: Thin Layer Chromatography

Tmax: time to maximum concentration

TNF: tumour necrosis factor

UDP: uridine diphosphate

UKPDS: United Kingdom Prospective Diabetes Study

VCAM-1: vascular cell adhesion molecule-1

VLDL: Very Low Density Lipoprotein

WHO: World Health Organisation

Acknowledgement

I have only been able to complete this project with the help of many individuals who deserve my gratitude. First and foremost, my profound gratitude has to be to my supervisor, Professor Stephen Atkin. His staunch support and tireless encouragement throughout this study were instrumental to its successful completion. He supported me in the planning, implementation and writing of each and every part of this project. His outlook on life in general and his vision and guidance during the work were pivotal to my success and to this achievement. I would also like to thank Professor Eric Kilpatrick, my co-supervisor, for the support in providing not only the material needed for my work, but also for his ingenious ideas and constructive feedback throughout my work. I am also grateful for the help and support of Dr Grahame Mackenzie and Alberto Diego Taboada from the Chemistry Department in Hull University for their time, dedication and effort in providing me with the spores used in this work.

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Publications

Two of the studies presented in this thesis have been published in peer-reviewed journals:

Wakil A, Mir M, Mellor D, Mellor S, Atkin SL Atkin: The bioavailability of eicosapentaenoic acid from reconstituted triglyceride fish oil is higher than that obtained from the triglyceride and monoglyceride forms, 2010Asia Pac J Clin Nutr.;19(4):499-505.

Wakil A, Mackenzie G, Diego-Taboada A, Bell JG, Atkin SL: Enhanced bioavailability of eicosapentaenoic acid from fish oil after encapsulation within plant spore exines as microcapsules, Lipids. 2010 Jul;45(7):645-9.

Abstract

Introduction:

Cardiovascular disease is the leading cause of death globally. This thesis has critically evaluated aspects related to two cardiovascular risk factors, namely hyperlipidaemia and hyperglycaemia, in healthy volunteers. Free fatty acids produced by visceral fat and transported to the liver in the portal circulation are thought to be one mechanism by which obesity contributes to increased insulin resistance and therefore reduced glucose disposal and a propensity to hyperglycaemia. The first study assessed the bioavailability of eicosapentaenoic acid, a cardio-protective fatty acid, in different forms of fish oil and then by incorporation with spores or exines. Potential use of the latter as a carrier of fatty acids to the portal circulation to increase insulin resistance and reduced glucose disposal was also studied. As short term plasma glucose fluctuations have been linked to an increased production of oxygen radicals, and thereby the microvascular complications of diabetes, a controlled assessment of this has been investigated in the final study.

Methods:

The first study examined in a double blind design the short term bioavailability, in the form of 24 hour area under the curve, of eicosapentaenoic acid obtained from 5 forms of fish oil. The second study was an open label study which tested the change in the bioavailability of eicosapentaenoic acid in the form of ethyl ester by their incorporation with spores or exines. The latter was used in the third open label study to test whether the exines are capable of delivering what is inside them directly to the portal circulation. This

could directly deliver oleic acid to the portal circulation thereby simulating the portal influx of fatty acid from the central adipose tissue.

In the final study, urinary isoprostane, a marker of oxidative stress, linked to the microvascular complications of diabetes was measured in three short term glycaemic states using the hyperglycaemic clamps in a cohort of healthy volunteers. This study aim was to establish whether glycaemic variability is associated with significant change in oxidative stress over and above that already present due to hyperglycaemia.

Results:

In the first study, a significant difference was found between the calculated median AUC of EPA from RTG (Median=31.3; 98.4% CI = 23.5-42.6) and that of the ETG (Median=10.7; 98.4% Confidence Interval (CI) = -8-29.5) and MG (Median=16.8; 98.4% CI = -2.8-30.4), z = -2.36 and -2.19, respectively, $p<0.05$. The mean area under the curve of eicosapentaenoic acid from ethyl ester incorporated to exines (M=19.7, SD=4.3) was significantly higher than that obtained from ethyl ester without exines (M=2, SD=1.4). The difference between means= 17.7, 95% CI=13.4-22.0, $p<0.01$; indicating enhanced bioavailability of eicosapentaenoic acid by the exines. In the third study, there was no significant change in the glucose disposal using the euglycaemic clamps after ingesting oleic acid with or without exines.

The fourth study also showed no significant change in urinary isoprostanes after 3 hours of glycaemic variability compared to patients clamped to have either consistent hyperglycaemia or euglycaemia.

Conclusion:

The reconstituted triglyceride form of eicosapentaenoic acid is better absorbed in the first 24 hours than the monoglyceride and enzymatically synthesised triglycerides. The standard therapeutic form of eicosapentaenoic acid is the ethyl ester and it can be enhanced by the incorporation into spores or exines. The exines-loaded oleic acid at a dose of 5 g was not associated with perceptible change in glucose disposal (and therefore presumably insulin resistance) as measured by the euglycaemic clamps. Finally, there was no significant change in urinary isoprostanes following short term glycaemic variability compared with hyperglycaemia or euglycaemia in healthy volunteers, suggesting short-term glycaemic variability may not be as potentially contributory to microvascular complications as has previously been suggested.

Format of the thesis

The first chapter is the “Introduction” which is a literature review setting the background for the studies done in this thesis. The second chapter describes the methods used in the studies. Chapters 3-6 describe the 4 studies in this thesis. Chapter 7 begins by summarising my conclusions and findings.

Aim of the work

1- To examine the short-term bioavailability of eicosapentaenoic acid from different types of fish oil oils and test whether the pollens (exines) are capable of improving the absorption.

2- To test the ability of exines to act as a carrier to oleic acid by indirectly testing the effect on the glucose disposal using the hyperinsulinaemic euglycaemic clamps in healthy volunteers.

3- To compare the effect of short term glycaemic fluctuations using hyperglycaemic clamps on the urinary isoprostane, an oxidative stress marker, in healthy volunteers.

Chapter 1: Introduction

1.1 Cardiovascular disease and the metabolic syndrome:

According to the World Health Organisation (WHO) report on mortality in 2004, cardiovascular diseases are the leading cause of mortality in the world accounting for almost 32% and 27% of death in women and men respectively [1]. It has been recognised for several decades now that patients with diabetes have a cluster of other disorders such as, obesity, hypertension and lipid disorders[2]. This had lead Stern and Haffner to raise the question of whether this clustering is a disorder which requires new clinical and public health control measures [3].

Historically, the constellation of hypertension, hyperglycaemia and gout was first described by Kylin while Vague suggested that the "android" obesity is associated with diabetes and cardiovascular diseases through common metabolic disorders[4, 5] . Now it is recognised that obesity and the metabolic syndrome are afflicting all societies and constitute a major public health challenge [6, 7].

1.1.1 Definitions:

In 1999 and after a report of the consultation group by Alberti *et al*, the World Health Organisation published its provisional definition of the Metabolic Syndrome, shown in Table 1[8, 9]. Although this definition emphasises the importance of predicting diabetes, it demanded tests beyond the routine clinical need to define the glucose status to diagnose the metabolic syndrome[10]. There are now various criteria for the diagnosis of the metabolic syndrome and they are summarised in Table 2 [11]. Despite the difference in the details of these criteria, they all encompass obesity, hypertension, glucose intolerance or diabetes and dyslipidaemia. In the WHO definition, glucose intolerance is the centre of the diagnosis but in those without impaired fasting glycaemia (IFG) or impaired glucose tolerance (IGT), a

euglycaemic hyperinsulinaemic clamp would be needed, a research tool which does not lend itself to routine clinical practice. In their executive summary, the 2001 National Cholesterol Education Program (Adult Treatment Panel [ATP] III) placed equal importance on glucose abnormalities and other features of the metabolic syndrome[12]. Their practical criteria made it easy for others, such as the American College of Endocrinologists to follow their model with minor alterations [13]. In 2005, the International Diabetes Federation adopted the ATPIII criteria but this time made obesity a central theme and a pre-requisite for the diagnosis[14]. The ATPIII criteria were adopted by the National Heart, Lung, and Blood Institute Scientific Statement (NHLBI) in 2005 with the only change being the lowering of the threshold for diagnosis of IFG to 5.6 mmol/L while including diabetes explicitly in the definition of hyperglycaemia criteria and including drug treatment for dyslipidaemia and antihypertensive medications for hypertension in the definition [10, 15]. The lowering of IFG threshold to 5.6 mmol/L was based on their Expert Committee analysis of the receiver operator characteristic curve of various baseline levels of fasting plasma glucose (FPG) ability to predict diabetes in four populations. They concluded that the mean FPG value closest to the ideal of 100% sensitivity and specificity over the glycaemic range of 4.5–7.0 mmol/L in the four populations was 5.6 mmol/L[15, 16]. Although different criteria emphasise different pathogenic cause for the metabolic syndrome, some studies suggest that central obesity is more important a predictor than insulin resistance for the development of the metabolic syndrome [17, 18].

Table 1: Criteria for the diagnosis of the Metabolic Syndrome.

Insulin resistance, identified by 1 of the following:

- Type 2 diabetes
- Impaired fasting glucose
- Impaired glucose tolerance
- or for those with normal fasting glucose levels (<110 mg/dL), glucose uptake below the lowest quartile for background population under investigation under hyperinsulinaemic, euglycaemic conditions

Plus any 2 of the following:

- Antihypertensive medication and/or high blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic)
- Plasma triglycerides ≥150 mg/dL (≥1.7 mmol/L)
- High Density Lipoprotein (HDL) cholesterol <35 mg/dL (<0.9 mmol/L) in men or <39 mg/dL (1.0 mmol/L) in women
- BMI >30 kg/m^2 and/or waist:hip ratio >0.9 in men, >0.85 in women
- Urinary albumin excretion rate ≥20 µg/min or albumin:creatinine ratio ≥30 mg/g

Table 2: The different criteria of metabolic syndrome diagnosis by different expert bodies[11].

Parameters	**NCEP ATPIII 2005**	**IDF 2005**	**EGIR 1999**	**WHO 1999**	**AACE 2003**
Required		Waist ≥94 cm (men) or ≥80 cm (women)*	Insulin resistance or fasting hyperinsulinemia in top 25 percent	Insulin resistance in top 25 percent‡; glucose ≥6.1 mmol/L (110 mg/dL); 2-hour glucose ≥7.8 mmol/L (140 mg/dL)	High risk of insulin resistance§ or BMI ≥25 kg/m^2 or waist ≥102 cm (men) or ≥88 cm (women)
Number of abnormalities	≥3 of	And ≥2 of	And ≥2 of	And ≥2 of	And ≥2 of
Glucose	≥5.6 mmol/L (100 mg/dL) or drug treatment for elevated blood glucose	≥5.6 mmol/L (100 mg/dL) or diagnosed diabetes	6.1-6.9 mmol/ (110-125 mg/dL)		≥6.1 mmol/L (110 mg/dL); ≥2-hour glucose 7.8 mmol/L (140 mg/dL)
HDL cholesterol	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women) or drug treatment for low HDL-C¶	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women) or drug treatment for low HDL-C	<1.0 mmol/L (40 mg/dL)	<0.9 mmol/L (35 mg/dL) (men); <1.0 mmol/L (40 mg/dL) (women)	<1.0 mmol/L (40 mg/dL) (men); <1.3 mmol/L (50 mg/dL) (women)
Triglycerides	≥1.7 mmol/L (150 mg/dL) or drug treatment for elevated triglycerides¶	≥1.7 mmol/L (150 mg/dL) or drug treatment for high triglycerides	or ≥2.0 mmol/L (180 mg/dL) or drug treatment for dyslipidemia	or ≥1.7 mmol/L (150 mg/dL)	≥1.7 mmol/L (150 mg/dL)
Obesity	Waist ≥102 cm (men) or ≥88 cm (women) #		Waist ≥94 cm (men) or ≥80 cm (women)	Waist/hip ratio >0.9 (men) or >0.85 (women) or BMI ≥30 kg/m^2	
Hypertension	≥130/85 mmHg or drug treatment for hypertension	≥130/85 mmHg or drug treatment for hypertension	≥140/90 mmHg or drug treatment for hypertension	≥140/90 mmHg	≥130/85 mmHg

*For South Asian and Chinese patients, waist ≥90 cm (men) or ≥80 cm (women); for Japanese patients, waist ≥90 cm (men) or ≥80 cm (women).
‡Insulin resistance measured using insulin clamp.

§High risk of being insulin resistant is indicated by the presence of at least one of the following: diagnosis of CVD, hypertension, polycystic ovary syndrome, non alcoholic fatty liver disease or acanthosis nigricans; family history of Type 2 diabetes, hypertension of CVD; history of gestational diabetes or glucose intolerance; nonwhite ethnicity; sedentary lifestyle; BMI 25 kb/m2 or waist circumference 94 cm for men and 80 cm for women; and age 40 years.
¶Treatment with one or more of fibrates or niacin.
#In Asian patients, waist ≥90 cm (men) or ≥80 cm (women).
*AACE: American Association of Clinical Endocrinologists; ATP: Adult Treatment Panel; BMI: Body mass index; CVD: cardiovascular disease; EGIR: Group for the Study of Insulin Resistance; HDL: High-density lipoprotein; HDL-C: HDL cholesterol; IDF: International Diabetes Federation; NCEP: National Cholesterol Education Program.

1.1.2 The risk of cardiovascular diseases (CVD) in the metabolic syndrome:

The metabolic syndrome confers five folds increase in the risk of developing type 2 diabetes[19]. A meta analysis has found that those with the metabolic syndrome had increased mortality from all causes (relative risk [RR] 1.35; 95% confidence interval [CI], 1.17-1.56) and cardiovascular disease (RR 1.74; 95% CI, 1.29-2.35) [20], while others have reached similar conclusions, making the metabolic syndrome an important risk factor in cardiovascular as well as all cause mortality[21]. The increased risk appears to be related to the risk factor clustering or insulin resistance and not simply obesity as shown by a study of the Framingham population that obesity with the metabolic syndrome increased the incidence of diabetes by 10 fold and CVD by 2 fold while on the other hand normal weight people with the metabolic syndrome had 4 fold increase risk of developing diabetes and 4 fold of developing CVD. [22]. Similarly, the highest prevalence of coronary heart disease was observed among those with both metabolic syndrome and diabetes while those with diabetes without the metabolic syndrome had similar prevalence of coronary heart disease to those who had neither diabetes nor the metabolic syndrome[23]. Nevertheless it is still not clear whether the metabolic syndrome adds further information to CVD since CVD risk associated

with the syndrome is no greater than the sum of the components of the metabolic syndrome and treatment of the syndrome is no different than the treatment for each of its components [24, 25]. For example the Framingham risk score was superior to the metabolic syndrome [26, 27] while the presence of previous CVD and low HDL were stronger prediction for CVD in older population[28]. From a therapeutic stance, there are no medications to treat all the defined abnormalities within the metabolic syndrome, perhaps reflecting the multi-factorial nature of its pathogenesis and until clinical trials are conducted demonstrating medications efficacy on all features of the metabolic syndrome, pharmacological treatment should be used within the current guidelines, i.e. treating individual component [25].

1.1.3 Central obesity is the key component in the metabolic syndrome:

It is estimated that overweight and obesity in the U.S has exceeded 60 and 30% respectively[29]. Obesity is defined as excess corporal adiposity[30]. Body weight/stature emerged as a practical way of measuring body fat in the clinical setting as simple weight measurement was erroneous indicator of total body fat and more difficult methods of measuring fat is not practical when assessing population fatness [31]. Overweight is defined as a body mass index (BMI) $\geq$25 kg/m^2 while obesity is a BMI $\geq$30 kg/m^2 [32]. Although BMI correlates well with body fat mass, it can over-estimate body fatness in athletes and under-estimate body fatness in Asians [33, 34]. Other less well known subtypes of obesity are identified such as the metabolically healthy but obese individual who have obesity-range BMI but none of the known metabolic derangement associated with obesity [35]. On the other hand the opposite subtype exists; the metabolically obese normal-weight individual with a normal BMI but obese-characteristics such as central obesity, low HDL, high triglycerides and elevated blood pressure [36]. Taking these facts into account, simple obesity per se can

not explain the metabolic abnormalities that are commonly associated with high fat mass and subsequent studies correlated visceral obesity with the metabolic derangements preceding the development of diabetes and CVD [30]. Visceral obesity which is also known as android type obesity, is defined as increase adipose tissue accumulation in the abdominal viscera[37]. In Caucasians a waist circumference of more than 102 cm in men and 88 cm in women is associated with higher odd ratios for the development of diabetes, hypertension dyslipidaemia and the metabolic syndrome [38]. It is well known now that central obesity is highly associated with coronary heart disease and cerebrovascular diseases and can better predict them than BMI [39, 40]. Central obesity is associated with the insulin resistance and Type 2 Diabetes Mellitus (T2DM)[41-44] and this could be partly due to hyperglycaemia, hyperinsulinaemia secondary to increased insulin secretion and to raised hepatic glucose production due to hepatic insulin resistance[44]. An explanation of why obesity causes insulin resistance is that visceral fat leads to excess non esterified fatty acid delivery to the liver via the portal vein causing hepatic insulin resistance[45]. The explanation of hypertension in obesity is not well understood but it is thought that insulin resistance causes over-activity of the central sympathetic and renin angiotensin systems which raises the blood pressure [46]. Finally, it is recognised that altered lipid transport is a strong component linking the CVD with central obesity thereby forming the deadly quartet, central adiposity, hyperinsulinaemia, dyslipidaemia and hypertension [47]. There is a correlation between high very low density lipoprotein (VLDL) [21] and a reduced insulin suppression of VLDL production by the liver [22] that correlates with the fatty liver (hepatic insulin resistance) leading to increased serum triglycerides[48, 49].

1.1.4 The specific dyslipidaemia of the metabolic syndrome:

Central adipose tissue has emerged in recent years as a powerhouse of metabolically active substances, termed adipokines. Adiponectin, resistin, leptin and TNF-alpha and others are released from adipocytes to regulate energy expenditure and utilization. In the metabolic syndrome, the adipose tissue seems to be oversaturated with fat and unable to hold more fat inside as well as releasing some of its fat contents to the portal circulation, all as a result of impaired insulin sensitivity which drive these processes normally. The result of that is a release of non-esterified fatty acids to the liver, thereby providing an ample source of VLDL which is rich in triglyceride. The lipid abnormalities in the metabolic syndrome are low HDL cholesterol and high triglycerides with a modestly raised Low Density Lipoprotein (LDL) cholesterol. The high triglyceride in VLDL will be exchanged for the cholesterol particles in HDL and LDL through the action of cholesteryl ester transfer protein (CETP) forming low cholesterol HDL and the highly atherogenic small LDL cholesterol particles [50-52]. One could entertain the question of why in the metabolic syndrome there is a high incidence of CVD despite a modest rise of LDL cholesterol. To answer this question, one needs to understand that first, the small LDL cholesterol particle is similar to the ordinary LDL cholesterol particle in that they both have one particle of apoprotein-B (which is the ligand needed for LDL cholesterol to be transported to tissues for formation of plaques[53]) and therefore a similar concentration of small LDL cholesterol in fact has more Apoprotein B than the same volume of a larger LDL cholesterol[54]. This is why apoprotein B measurement should provide more information than merely LDL cholesterol in assessing the risk of CVD in the metabolic syndrome [55]. Additionally, the dense LDL cholesterol has a lower affinity for LDL receptor in the liver thereby reducing its clearance[56]. Furthermore, the dense LDL cholesterol is more likely to penetrate the vascular membrane (LDL receptor-

independent binding sites), more adherent to the atheromatous plaques and more susceptible to oxidation [56-60]. Second, elevated LDL had a weaker association with coronary heart disease while low HDL had a stronger association with the incidence of coronary heart disease in the Framingham Study[61]. This is not only due to its ability to transport cholesterol from foam cells in plaques to the liver but also by prevention of oxidation of LDL through the paraoxonase enzymes and platelet-activating factor acetyl hydrolase found in its protein [62-64]. Reconstituted HDL cholesterol infusion has been also shown to reverse the endothelial dysfunction in hypercholesterolaemic men further adding to the evidence that HDL has multifaceted cardiovascular protective function [65].

1.1.5 The role of eicosapentaenoic acid and docosahexaenoic acid in cardiovascular diseases:

The beneficial effects of polyunsaturated fatty acids (PUFA) on CVD was noticed first by Dyerberg and Bang who found that Eskimos of Greenland have a lower CVD mortality than Western population despite eating the same amount of daily dietary fat [66, 67]. The fact that Eskimos diet contained a high portion of PUFA/total fat (0.84 as compared with 0.24 in Danes) was thought to be the main cause for different CVD mortality between the two populations[67]. Further prospective trials showed similar results [68, 69] but others did not [70, 71] and this discrepancy may be a result of different relative risk of coronary heart disease (CHD) in the populations studied [72]. Controlled trials that followed have shown a high PUFA diet is protective against CHD and mortality. For example, the GISSI-Prevenzione trial have concluded that supplementation with 1 g a day of PUFA within 3 months post myocardial infarction (MI) will result in 20% reduction in the risk of dying [73]. The fact that 2 years all-cause mortality in men after myocardial infarction was reduced by 29% with fish oil intake without a significant change in serum triglyceride is thought to be

due to the mechanism an anti-arrhythmic action of PUFA [74, 75]. There are two mechanisms suggested to this reduction in sudden death. First, PUFA has an anti-inflammatory effect on the atherosclerotic plaque which would stabilise the plaque (which readily incorporates the PUFA), [76]. Plaque stabilisation would prevent ischaemia-induced arrhythmias as the second mechanism to prevent sudden death from further ischaemia to the myocardium [77].

1.1.5.1 Sources of different polyunsaturated fatty acids:

N-3 PUFA are 18-22 carbon atom fatty acids containing 3-6 unsaturated double carbon bonds, the first of which is 3 carbon atoms distance from the terminal methyl group. In n-6 PUFA there are 2-5 double bonds situated 6 carbon atoms distance from the terminal methyl group, shown in Figure 1. In humans alpha linolenic acid (ALA) (18:3 n-3) is an essential fatty acid because it is not synthesized from saturated fatty acids, n_9 monounsaturated fatty acids, or n_6 polyunsaturated fatty acids[78]. Additionally, the conversion of ALA to eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6 n-3), are inefficient [79, 80]. The intake of essential n-3 PUFA has shifted from preventing deficiency of essential fatty acids to preventing disease risk especially CVD disease[78]. While 18:2(n-6) linoleic acid (LA) can be obtained from vegetables and meat, EPA and DHA are derived from marine algae consumed by fish and the latter represents the main source of n-3 PUFA in humans [81]. On the other hand, ALA is derived mainly from plant source e.g. flaxseed, linseed and soy oil [81].

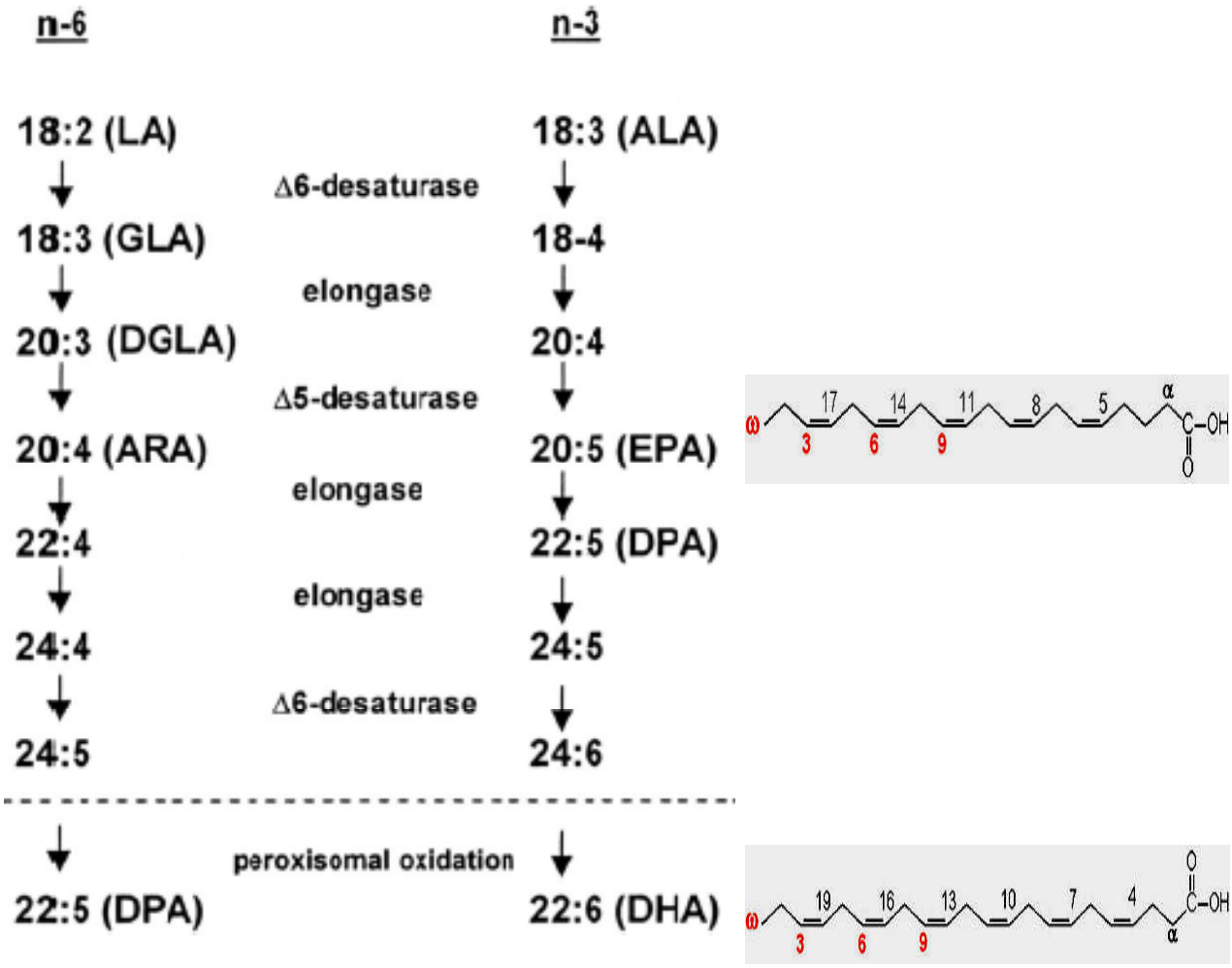


Figure 1: The enzymatic pathway for n-3 and n-6 PUFA. ALA, alpha linolenic acid; ARA, arachidonic acid; DGLA, dihomo gamma linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, _-linolenic acid; LA, linoleic acid. Adapted from L M Arterburn. [81]

1.1.5.2 Tissue distribution of n-3PUFA:

N-3 PUFA are present in the cell membranes in almost all tissues. Of all the n-3 PUFA, the DHA is the most abundant particularly in retinal and nervous tissue [81]. DHA is present 5-30 folds more than EPA in tissues but this ratio rises to several hundreds in the retina and nervous tissue [81]. On the other hand, in the adipose tissue, the principal n-3 PUFA is ALA

accounting for 1% only of all fatty acids in the triglycerides of adipose tissue while EPA and DHA are only present in small quantities suggesting that they are not stored in abundance in the human body[82]. N-3PUFA are incorporated into phospholipids and sphingolipids in plasma membranes and the higher the number of unsaturated bonds the more flexible is the plasma membrane [83] and therefore the highly unsaturated fatty acids can alter membrane physical properties such as fluidity and deformability which in turn contributes to their ability to play key roles in cellular functions [84, 85].

1.1.5.3 Effects of n-3 PUFA:

N-3 PUFA serves multiple functions in addition to their role in signal transduction through cell membranes. They act as a substrate for the cyclooxygenase and lipoxygenase enzymes producing mediators of anti-inflammatory substances such as resolvin and protectin[86]. Additionally they act as ligands for nuclear receptors such as the peroxisome proliferator-activated receptor (PPAR) gamma and alpha thereby influencing gene transcription [87, 88]. They were also found to have a beneficial effect on platelets aggregation in response to platelet activating factors which could be one of their mechanisms of preventing CVD events [89] .

Perhaps the most well understood and clinically applicable function is their effect on lipid metabolism. A supplementation with 4 g of n-3 PUFA a day can lead to the reduction of triglyceride levels by 25-30% with a concomitant increase in LDL of 5-10% and Both DHA and EPA supplementation for 7 weeks at high doses result in lowering triglyceride by 26 and 21% respectively[90, 91]. N-3 PUFA reduces triglyceride mitochondrial and peroxisomal oxidation in the liver and muscles through their action on PPARα and this can indirectly influence glucose uptake by these organs, i.e. insulin sensitivity [92, 93]. They also inhibit the

translocation of sterol regulatory element-binding protein 1 from the endoplasmic reticulum to the nucleus thereby interfering with their action on sterol regulatory element responsible for gene transcription of enzymes used in fatty acids synthesis [94]. Finally, EPA but not DHA is also responsible for decreasing triglyceride secretion and synthesis by the liver through reducing diacylglycerol acyl transferase activity and stimulating fatty acid oxidation [95].

1.1.5.4 Absorption of fish oil and methods employed to improve it:

After consumption of fat, physiological events lasting up to 24 hours will take place[96]. In humans, hydrolysis of fat begins mainly in the stomach as lingual lipase is not a major source of fat hydrolysis in humans [97] [98]. Very short-chain (C < 8 or 10) fatty acids are absorbed directly from the stomach into the venous circulation in rats while C14- to C18-chain-length fatty are passed into the intestinal lumen for further processing by pancreatic lipase, colipase with the help of bile to create the digestive micelles [99]. The majority of the n-3 fatty acids are absorbed in the small intestine[100]. Lingual lipase and pancreatic lipase liberate fatty acids from the 1 and 3 positions of natural triglycerides while gastric lipase tends to preferentially cleaves triglyceride in the side chain 3 (sn-3), leaving the 2-monoglyceride intact [101]. It should be noted that a variable amount of long-chain free fatty acids can be absorbed by the portal circulation and the amount absorbed by this route may vary with types and other nutrients being digested at the same time [97, 102] [103]. Monoglycerides, are absorbed by the enterocytes of the intestinal wall by passive diffusion[103]. In the enterocyte, the lipolysis products are re-esterified before incorporating into chylomicrons[104]. N-3 PUFA from fish especially DHA is located on side chain 2 (sn-2) of the triglyceride while re-esterified fish oil supplements could contain fatty acids on side chain 1 and 3 thereby

influencing their absorption as the pancreatic lipase action may be hindered by the presence of a double bond close to the site of its action. i.e. n-3 PUFA in sn-1 or 3 [105].

Since the main beneficial fatty acids in fish oil were EPA and DHA and they are exclusive to fish oil, which can be unpalatable to some, it was not surprising to see the food industry developing supplements of EPA and DHA. Currently, nutritional recommendations for risk prevention in the UK advises that at least two portions of fish is consumed every week, which is rich in omega-3 PUFAs, or take >200 mg/ day of omega-3 PUFAS although this is rarely done[106] in practice and therefore making supplementation a suitable alternative[107]. Ethyl ester forms of EPA are more stable than free fatty acids which can potentially release toxic methanol upon methyl hydrolysis and PUFA esters were the main studied form of administered PUFA[108]. Although in vitro studies showed ethyl ester absorption to be slow, it tends to be a complete process by pancreatic lipase in animals[109]. In fact, up to 6 grams a day of ethyl ester have been completely hydrolysed by pancreatic lipase [109-111]. Recently the main emphasis has been on emulsification as a way to improve the absorption of ethyl ester forms of EPA and DHA. Recent studies have been done to show that pre-emulsification is effective [112]in improving absorption of fish oil and that it was better than encapsulation as well as better tolerated[112, 113]. In the past, comparisons in animals and humans have been made between the different chemical structures of EPA and DHA; diacylglycerol, triglycerol and ethyl ester and other forms [108, 114]. Some showed poor absorption of ethyl esters of n-3 fatty acids in the small intestine as compared to free fatty acids in humans [108]. On the other hand, others showed acceptable absorption result from ethyl ester especially when the latter is taken with a high fatty meal[115]. One study on animals showed diglyceride (DG) and monoglyceride (MG) n-3 fatty acids to be better absorbed than triglyceride (TG) and ethyl ester (EE) [114]. New food technologies exist nowadays and fish

oil supplements could vary in the side chain position of EPA and DHA which could affect their absorption and in turn result in variable plasma EPA/DHA levels after intake. There is a paucity of trials directly comparing the absorption of purified EPA/ DHA existing as ethyl ester, triglyceride (natural and chemically modified to incorporate sn-2 only), diacylglycerol or even free fatty acids.

1.1.5.5 Use of fish oil in cardiovascular disease:

As mentioned earlier, randomised clinical trials have convincingly shown fish oil to be effective as secondary prevention measure following myocardial infarction[73]. The recommendation from the American Heart Association (AHA) states that patients with a history of coronary artery disease should take 1 g/day (EPA) plus docosahexaenoic acid (DHA) but those with documented hypertriglyceridaemia should increase their daily intake to 2-4 grams/day[116]. the National Institute of Clinical Excellence recommends using fish supplement, Maxepa or as a second option Omacor (provided that the first is clinically equivalent to the second) if dietary sources is not effective in lowering triglyceride[117].Omacor is a prescription fish oil preparation. Omacor is composed of approximately 90% omega-3 fatty acids (465 mg EPA, 375 mg DHA, and 60 mg other omega-3 fatty acid esters), for a total of 900 mg of omega-3 fatty acids per 1-g capsule [118]. In a double blind RCT, Omacor was shown to reduce triglyceride from baseline by 27% while in another large 2x2 multifactorial trial there was a reduction of triglyceride by 19% with 10% increase in LDL and both studies showed no positive effect on re-stenosis after angioplasty [119, 120]. The positive effect on mortality post MI has been attributed to anti-arrhythmic properties of n3-PUFA, see discussion before. However, there are other minor benefits in relation to cardiovascular health for n-3 PUFA. They were shown to cause a small

dose dependent reduction in blood pressure although the higher doses needed to achieve meaningful clinically important reduction makes this intervention questionable[121]. Similarly, their positive effect on platelet aggregation is not clinically significant at doses below 3 g/day [89, 122]. The anti-atherogenic properties of fish oil are due to their negative competitive effect on the cyclooxygenase pathway; inhibiting the production of thromboxane, prostacyclines and leukotriens leaving the biologically inactive prostaglandins derived from EPA pathway intact[122]. Studies also showed that EPA and DHA favourably alter vascular reactivity as measured by flow mediated dilatation, possibly due to incorporation into the endothelial membranes altering their fluidity and possibly nitric oxide release [123].

1.2 Insulin resistance (IR):

1.2.1: Background:

The term insulin sensitivity first appeared in relation to diabetes in 1939 although the concept of syndrome X which then evolved to become the insulin resistance was introduced in 1988 at the Banting medal award[124-126]. Although insulin has multiple metabolic actions, the one that is most understood is its action on glucose metabolism. The broad and accepted definition of insulin resistance is a diminished sensitivity of end organs to the action of insulin. In terms of glucose metabolism, this implies that for a given concentration of insulin, there is reduced biological activity or glucose sensitivity (Si). Kahn has proposed two mechanisms for insulin resistance; the first is reduced insulin responsiveness and the second is reduced insulin sensitivity[127]. Insulin responsiveness is the Vmax (maximum reaction can be achieved) while insulin sensitivity is the Effective Dose $(ED)_{50}$ i.e. the concentration of insulin that is needed to achieve half Vmax. Kahn argued that the lower Vmax is due to

post receptor defect and the lower sensitivity is due to defect in insulin interaction with its receptor.

Regardless of where the defect is, to be able to define insulin resistance one needs to quantify it i.e. determine the glucose disposal to tissue as a result of insulin action.

1.2.2 Overview of insulin action and molecular pathogenesis of IR:

Insulin is a peptide hormone secreted by the β islet cells of Pancreas. Its metabolic action is mediated by the insulin receptor, a cell surface glycoprotein receptor of 2 α and 2 β chains with the alpha exclusively on the cell surface and the beta traversing the cell membrane, thereby anchoring the alpha chains[128]. Upon attachment to the alpha chains insulin causes a conformational change which will bring them together to allow the ATP to phosphorylate specific tyrosine residues on the β chain[129]. This initiates a cascade of autophosphorylation of other tyrosines (tyrosine kinase activity) on the receptor as well as other protein substrates in the cell. One of the most important of these intracellular protein substrates for the tyrosine kinase activity of the insulin receptor are Insulin Receptor Substrates (IRS) of which many forms have been identified[130, 131]. Mouse studies have shown the IRS 1 and IRS 2 to play the main role in glucose homeostasis in skeletal muscles, adipose tissue and pancreatic β cells [132, 133]. The IRS molecule has a central domain with multiple potential tyrosine residues which undergo multiple phosphorylation reactions catalysed by the IR tyrosine kinase[130]. Upon phosphorylation, this central domain can bind to several adaptor proteins (such as the regulatory subunits of phosphatidylinositol kinases, PI3 Kinase) and enzymes (such as kinases and phosphatises), thereby apposing different proteins to effect the glucose metabolism[134]. The activated PI3 kinase will phosphorylate the inositol ring at the 3’ position of phosphatidylinositol in the adjacent cell membranes generating

Phosphatidylinositol (2,3,4)phosphate 3 (PIP3) which acts as a second messenger binding to proteins and activating them for downstream regulation of glucose metabolism among other metabolic effects[134]. One of these proteins is the important kinase; phosphatidylinositide 3 dependent kinase which in turn activates the Akt/PKB pathway responsible for many of the insulin metabolic action including translocation of glucose transporter (GLUT4) into the cell membrane[135].

This divergent and expanding chain of reactions initiated by insulin can be interrupted at any stage and the result would be insulin resistance. Hyperinsulinaemia can be due to downregulation of the number of insulin receptors thereby reducing insulin sensitivity. For example, obesity causes high level of plasma differentiation factor 1 which interferes with the B subunit phosphorylation[136]. Other mechanisms such as serine – threonine phosphorylation and protein tyrosine phosphatases can interfere with the binding of insulin receptors and IRS molecules, thereby causing insulin resistance[134]. Serine – threonine phosphorylation is a major mechanism of inhibition of insulin signalling by interfering with the activity of tyrosine kinase of IR and the binding of IRS molecules. There is evidence that obesity, cellular stress, high fat diet as well as pro-inflammatory cytokines are associated with serine – threonine phosphorylation processes, albeit through different pathways such as the JNK (c - Jun N - terminal kinases) and PKC (Protein Kinase C) pathways [137-142]. Although insulin has multiple actions, it is customary to refer to insulin resistance as the resistance to the specific action on glucose utilisation by the whole body (adipose tissue, liver cells and muscles).

1.2.3. Methods of measuring insulin resistance:

1.2.3.1 Background:

Measuring insulin sensitivity has always been viewed as a research tool due to the variations and inaccuracies of the simple methods and the complexity of the more accurate methods to quantify it. Broadly speaking there are two types of methods to assess insulin resistance; indirect and direct methods[143]. The latter involves insulin administration (e.g. insulin clamp) while the former does not require that (e.g. Homeostatic Method of Assessment of insulin resistance index- HOMA-IR). Perhaps the simplest method is fasting plasma insulin measurement which reflects the level of biological activity of insulin. i.e. insulin sensitivity since a low activity is reflected by a parallel increase in insulin secretion by the β cells of pancreas[144]. Arguably, this is not true when diabetes ensue where β cell function usually declines. Another inherent problem with this simple method is the pulsatile nature of insulin secretion and inaccuracy of immunoradioassay which cross reacts with other molecules secreted in insulin resistance such as pro-insulin[145, 146]. To correct for the change in glucose level as a result of varying degree of insulin resistance both glucose and insulin measurement in the fasting level were employed to calculate insulin resistance[147].

1.2.3.2 HOMA

A computer-solved model has been used to predict the homeostatic concentrations which arise from varying degrees β cell deficiency and insulin resistance, using fasting, steady state, insulin and plasma glucose concentration. This method is termed Homeostasis Model Assessment or HOMA. Comparisons of fasting values with a computer generated model predictions of homeostatic concentrations reflecting various degrees of insulin resistance were used to quantify the insulin resistance index or HOMA IR. In the original report,

HOMA correlated well with the hyperinsulinaemic euglycaemic clamp (Rs = 0.88) and with the fasting insulin concentration (Rs = 0.81). It also correlated with the hyperglycaemic clamp in measuring β cell dysfunction (Rs = 0.69). However, it had a wide precision in the original report with a coefficient of variation (CV) of 31% and 32% for insulin resistance and β cell function[147]. Although later, using modern insulin assays, the Coefficient of Variation (CV) found to be between 7.8% and 11.7% [148, 149].

The original equation for the HOMA-IR is HOMAIR= (FI×FG)/22.5, where FI= fasting insulin and FG= fasting plasma glucose. The HOMA-β index is the model assessment for β Cell function; HOMA-B index = (20 × FI)/(FG − 3.5), where FI is fasting insulin and FG is fasting plasma glucose[150]. The HOMA IR cut off was found to be different in different populations; it was found to be 3.8 in a Spanish population while the normal range was found to be lying between 1.21-1.45 in the original HOMA IR paper [147, 151]. Again the fasting insulin is a determinant of the HOMA IR and therefore the same argument against the validity of insulin level also applies here[152]. This together with the fact that it does not correlate accurately with the insulin resistance in hyperglycaemic individuals measured by the clamp method restricts its usefulness to normoglycaemic individuals [152-154]. Modifying the original formula was also attempted to achieve a better correlation with hyperinsulinaemic euglycaemic clamps [149]. HOMA2 is a non-linear HOMA taking into account the following[155]:

1- Variations in hepatic and peripheral glucose resistance[156]
2- Accurate calculations of insulin secretion when plasma glucose is above 10 mmol/L[157]
3- Proinsulin level thereby allowing the use of either total or specific insulin levels[155].
4- Renal glucose losses which allows for hyperglycaemia[155].

1.2.3.3 Quantitative Insulin Sensitivity Check Index (QUICKI)

Another mathematical method is the quantitative insulin sensitivity check index (Quicki), which uses the following formula to obtain information on insulin sensitivity: (QUICKI = 1/[log(I(0)) + log(G(0)) where I and G are fasting insulin and glucose levels respectively [158]. Quicki has correlated strongly with the clamp method [158]. The Quicki equation uses the logarithms and reciprocals of the same parameters used in the HOMA (insulin and glucose) equation which makes it more accurate over wider sensitivity range[159] although a study has shown that the variability of HOMA, measured by coefficient of variation, is not improved by the changes to the equation made in the Quicki equation[160]. Other methods using similar information are the glucose insulin ratio which was found to be a good population screening method for insulin resistance in polycystic ovary syndrome [161, 162].

1.2.3.4. Methods involving glucose administration

Continuous infusion of glucose with model assessment (CIGMA) is another method whereby a steady state post-prandial insulin and glucose concentrations in the last 15 minutes of 1 hour glucose infusion is assessed against a healthy population computer model to quantify insulin resistance and β cell function[163]. This method was found to be highly correlated with the euglycaemic hyperinsulinaemic clamps (Rs=0.89) when assessing insulin resistance although variability of 21% was observed[163]. The advantages of CIGMA over HOMA is that it deals with higher levels of insulin thereby avoiding any poor precision of insulin assays while the higher insulin concentration steady state glucose uptake is a better reflection of peripheral insulin sensitivity[152]. Other methods used to assess insulin resistance are indices applied to

the oral glucose tolerance test such as the Matsuda index among many others [164] while direct methods such as insulin tolerance test and measurement of glucose disappearance was found to be closely correlated with the clamp method[165].

1.2.3.5 The glycaemic clamps:

The gold standard method to assess insulin sensitivity is considered to be the hyperinsulinaemic euglycaemic clamp described by Defronzo first in 1979[166]. Here, an insulin infusion at a rate of 40 mU/m2 is used to inhibit hepatic glucose output contribution to any glucose homeostasis for 2 hours. To prevent hypoglycaemia a concurrent 20% dextrose infused to correct any deviation from the glucose level aim set at the beginning of the clamp. Plasma glucose will need to be measured every 5 minutes from a retrograde cannula in a warmed hand to arterialise the vein and obtain accurate plasma glucose prior to tissue metabolism. The correction to the dextrose infusion is made either using a formula provided by Defronzo in his original paper or using the researcher's experience to manually adjust the rate of dextrose infusion to keep the plasma glucose close to the target[167]. In a steady state glucose equilibrium, the rate of glucose being infused will equal the rate of its metabolism by the peripheral tissue (M). M is usually calculated for a 20 minutes period and corrected for any minor difference of the plasma glucose at the beginning and the end of that period. Total M is the mean of the five consecutive Ms between 20 and 120 minutes of a two hour euglycaemic clamp. In lean normal people, M is between 4.7-8.7 mg. Kg -1 .min -1 [152, 153, 168]. Other calculations to correct for insulin level during the clamp can be made such as the M/I index, which is total M divided by the average insulin concentration and multiplying the ratio by 100[166]. The euglycaemic clamp has been criticised for the supraphysiologic level of insulin level it employs and the fact that it does not measure the

insulin-independent disposal which can be high in patients with insulin resistance[152]. The expense and the requirement of needing a highly trained researcher limit this method to research purposes only. However, it has a CV of less than 0.10[169]. The use of radiolabelled glucose allows the quantification of both hepatic glucose output and whole body glucose utilization which enables the researcher to measure hepatic insulin resistance[170]. Invasive methods to obtain blood samples from the hepatic artery will enable the measurement of splanchnic glucose uptake [171].

The hyperglycaemic clamp is another method used to quantify both insulin resistance and β cell function. In this method, only 20% dextrose is infused intravenously to obtain a square wave of hyperglycaemia to a desired level. Plasma glucose is measured every 5 minutes and the result of the test is used to alter the dextrose infusion. The glucose that needs to be infused at steady state (usually in the last hour) hyperglycaemia is equal to the glucose metabolised (M) and if divided by the average plasma insulin the insulin sensitivity index (ISI) is obtained[172] . Plasma insulin is measured every 2 minutes for the first 10 minutes and every 10 minutes thereafter to determine early and late phase insulin secretion by β cells in response to hyperglycaemia [166, 173]. The hyperglycaemic clamp has also been used to estimate the non insulin dependent glucose utilization (NIDGU) in tissues when insulinopaenia was induced by exogenous somatostatin simulating hyperglycaemia effect on inducing NIDGU [173, 174].

1.2.3.6 Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGT) and Minimal Model

This is a dynamic method to quantify insulin resistance first described by Bergman in 1979[175]. It involves the rapid infusion of 50% dextrose (0.3 g/kg body wt) over 2 minutes

with labour intensive repeated blood sampling for glucose and insulin over 3 hours period. Blood samples are taken for plasma glucose and insulin measurements at _10, _1, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, and 180 min. The data is then input into a computer program, MINMOD[176], to calculate insulin sensitivity (Si) based on two equations and four possible models parameters assuming a single glucose compartment and a remote insulin compartment. The Si is defined as fractional glucose disappearance divided by the insulin concentration and expressed in min^{-1} / Microunit per mL. Studies showed Si to be varied from as high as 7.6 in young Whites to 2.3 obese non-diabetic subjects [177]. In addition to measuring the insulin dependent glucose utilization (IDGU; Si), this method can be used to calculate glucose effectiveness (i.e. NIDGU; Sg) by measuring glucose disposition in the first 20 minutes where the change in plasma glucose is independent of plasma insulin. The addition of tolbutamide or insulin [178, 179] is now employed since the original study used in dogs that had a slower pancreatic response to hyperglycaemia allowing a clear demarcation between IDGU and NIDGU. The use of insulin and tolbutamide when plasma glucose is low during the test made it possible to use this method in people with insulin resistance or type two diabetes with B-cell dysfunction where Si is small or even negative while it also improved their correlation coefficient when compared to the euglycaemic clamps [179-181]. Additionally, the FSIVGT also allows the assessment of β cell function [182, 183]. The correlation of this method with the clamp method is strong in non diabetic; r = 0.84[184] and 0.89 [181]. In subjects with impaired glucose tolerance and diabetes this correlation was weak (0.48 and 0.41, respectively) [185]

Although it is considered easier than the more labour intensive clamps method, it still requires frequent blood sampling for 3 hours which is also labour intensive. This however did not interfere with its use in a large trial to correlate insulin sensitivity (n>1600) with intima media

thickness measure of atherosclerosis[186]. The CV of this method is approximately between 16.9% for Si[187]. Attempts to reduce the number of blood sampling have resulted in higher CV compared to the original extended version[188]. A major criticism to this method is the oversimplification of the glucose compartment and the multiple assumptions by the procedure which all could lead to systematic error especially in insulin resistant subjects rendering the clamp method superior to the FSIVGT[180]. For example assuming a single compartment resulted in persistent under estimation of Si in those with insulin resistance and its improvement by assuming the two compartment model [189-191].

1.2.4. Insulin resistance and fatty acids:

In 1963, Randle described the glucose fatty acid cycle. It hypothesizes that a primitive hormonal-independent mechanism exists and that it maintains glucose control in animals that feed intermittently[192]. He also hypothesised that this reciprocal competitive interaction between lipid (Fatty acids and ketones) and carbohydrates (glucose) exists at the tissue phase. More specifically, the availability of glucose as a substrate for energy shuts down the use of fatty acids as an energy source and promotes the uptake of fatty acids for re-esterification into triacylglycerol. On the other hand, the abundance of fatty acids at the tissue level will switch off glucose metabolism as a source of energy. In reaching their conclusion, he followed two lines of observations; first that fatty acid production does change in different tissues in experimental and disease conditions and second that fatty acids are able to induce changes in glucose metabolism similar to what happens in diabetes and other conditions. Randle *et al* went further to suggest that the modifying action of insulin on glucose metabolism is altered by the inhibitory effect of fatty acids at the muscle and adipose tissue. The excess nonesterified fatty acids (NEFA) acts via malonyl coenzyme A (malonyl-CoA)-mediated

inhibition of carnitine palmitoyltransferase 1, thereby promoting the diversion of hepatic NEFA from adipose tissue away from oxidation towards re-esterification[193]. Randle in his observation asserted that concentration of glucose 6 phosphate (G6P) increases as a result of reduced utilization of glucose by the glycolytic pathway when NEFA is available as a substrate. This is made possible by inhibition of pyruvate dehydrogenase by Acetyl CoA and Nicotinamide adenine dinucleotide (NADH) and inhibition of phosphofructokinase by citrate. The accumulation of G6P will enhance glycogen synthesis in muscles in those with diabetes. However, measuring glycogen and G6P in muscles is difficult in vivo and in vitro measurement of glycogen and G6P is affected by in vitro hypoxia[194]. Using magnetic resonance spectroscopy made it easier to measure these metabolites in vivo[195]. In contrast to Randle hypothesis and using Magnetic Resonance Spectroscopy (MRS), G6P and glycogen were found to be low in those with diabetes and lean relatives of T2DM with insulin resistance [195-197]. Infusion of lipids with heparin to stimulate lipoprotein lipase in healthy volunteers was found to reduce G6P and caused insulin resistance as measured by the hyperinsulinaemic euglycaemic clamps[198]. Thus, in healthy individuals, high fatty acids caused insulin resistance and was associated with a defect in either glucose transport or phosphorylation activity, not an impairment in glycolysis[194]. Subsequent studies linked the high fatty acids effect on insulin sensitivity to the reduction of insulin-mediated glucose uptake by adipose tissue and muscles possibly as a result of reduced insulin signalling post IR phosphorylation [199-201]. One mechanism for this inactivation of insulin signalling is through the activation by diacylglycerol (DAG) of serine threonine phosphorylation by the novel and conventional PKC[201]. The diacylglycerol and not triglyceride is likely to be responsible for the insulin resistance as both animal and human studies seem to indicate. Intramyocellular diacylglycerol can be depleted by increased energy expenditure[202]. Also

transgenic mice that lack leptin but have high adiponectin seem to have normal insulin sensitivity despite being heavy as a result of triacylglycerol accumulation giving further evidence that DAG and not TG is the cause of insulin insensitivity[203]. Another mechanism for metabolising DAG is by the diacylglycerol kinases to phosphatidic acid, a membrane lipid, or by Diacylglycerol acyl transferase (DGAT) 1 which makes triacylglycerol[194]. Overexpression of the latter is similar to endurance exercise athletes who have high intramuscular Triacylglycerol (TAG) with high insulin sensitivity[204]. Although the DAG theory clearly explains the presence of low insulin sensitivity in obesity, it fails to explain why lean relatives of T2DM have similar insulin resistance. This could be due to low lipid oxidation due to mitochondrial depletion[205]. Similarly, age related reduction in mitochondrial density was found in elderly using MRS studies which are not confined to muscles but also to neuronal cells, indicating the possibility that it could be a widespread age-associated phenomenon, thereby playing a role in β cell dysfunction [206-208].

1.2.5 The mechanism of hepatic insulin resistance:

It is well known that non alcoholic fatty steatohepatitis is an insulin resistance-associated condition[209]. Peterson *et al*, found 60% reduction in glycogen storage after a carbohydrate rich meal in people with insulin resistance and this paralleled a 60% increase in postprandial triglyceridaemia and a 20% reduction in HDL when compared to controls matched for BMI, %fat and abdominal obesity blood pressure and activity[210]. They hypothesized that insulin resistance in muscle causes less glucose uptake and glycogen synthesis in muscles and that carbohydrate will be diverted to the liver where they will undergo lipogenesis[205]. It became evident from others that intrahepatic and intramuscular adiposity and not visceral adiposity is associated with insulin resistance[211, 212] .One mechanism for the increased hepatic

triglyceride is a polymorphic apolipoprotein C3 (apo C3) which lead to a 30% increase in apo C3 and consequent increase in hepatic clearance of triglyceride from chylomicron postprandially[210]. One mechanism of how hepatic steatosis is linked to the development of insulin resistance is the novel PKC$^{\varepsilon}$ which interferes with the insulin receptor kinase activity[210]. Using molecules that can block PKC$^{\varepsilon}$ has been found to improve insulin sensitivity in rats who were fed high fat diet and had excess hepatic triacylglyceride and diacylglycerol[213]. The presence of obesity, visceral and subcutaneous, and steatohepatitis in patients with the insulin resistance makes it difficult to discern the effect of steatohepatitis in isolation on insulin resistance[194]. However, lipodystrophy offers a solution to this problem as subjects with lipodystrophy has hypoleptinaemia and associated hyperphagia which results in postprandial hypertriglyceridaemia and ectopic fat deposition in muscles and liver [194]. Those subjects were found to have insulin resistance which is attenuated by leptin which also caused a reduction in the intrahepatic and intramuscular triglyceride content that paralleled the improved hepatic and muscular insulin responsiveness[214].

1.2.6 Fish oil and insulin resistance:

Long chain polyunsaturated fatty acids (LCPUFAs), as represented by arachidonic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are essential fatty acids that can only be obtained from diet. In contrast with the protein content of the cellular membrane which is largely determined by genetic factor, the fatty acid composition is modulated by both genetic factors as well as dietary factors and physical activity [215, 216]. Since LCPUFAs are readily incorporated into the cell membrane it is therefore not unreasonable to think that they play an important role in modulating insulin sensitivity. By their incorporation into the cellular membrane they can change not only the fluidity of the cell membrane but also the

different membrane signals including insulin receptors and oxidative capacity [217-219]. Furthermore, both n-6 and n-3 fatty acids have been found to suppress hepatic lipogenesis, hepatic triglycerides output while on the other hand enhance ketogenesis, and fatty acid oxidation in the liver and the skeletal muscle[220]. Insulin sensitivity may improve as a result of the effects of fatty acid intake on membrane fluidity, although the improvement in glucose uptake after membrane enrichment with LCPUFA is apparently related to an increase in the number of glucose transporters type 1 and 4 (GLUT1 and GLUT4) in the plasma membrane [220, 221]. Further interventional studies in overweight and obese subjects without diabetes but with the metabolic syndrome phenotype have shown that fish oil supplementation is associated with improvement in insulin resistance [222, 223]. However, studies on n-6 PUFA in humans have failed to show any effect on insulin resistance[224]. Therefore it seems that n-3 PUFA (e.g. EPA) and not n-6PUFA has a positive effect on insulin sensitivity and action. There is now interest in the effect of Omega 3 fatty acids on insulin resistance and in particular hepatic insulin resistance[225]. Studies on the use of PUFA in animals who were fed a high fat diet showed that hepatic insulin resistance is ameliorated by PUFA by the effect on PPARα and adiponectin induced PPARγ effect thereby reducing the diacylglycerol detrimental effect on insulin sensitivity [194, 226, 227]. Another mechanism for the plausible beneficial effect of the PUFA is their negative modulation of the sterol regulatory element binding protein-1c (SREBP-1c) which is a transcription factor required for the activation of de novo lipidogenesis as described earlier[228]. Human studies showed that using PUFA in subjects with non alcoholic fatty liver (NAFL) is associated with improvement in their liver enzymes, serum triglycerides and liver ultrasound echogenicity [229-231]. Therefore, there is strong evidence to suggest beneficial effect of n-3 PUFA on insulin action in healthy subjects

or those with NAFL. The same however does not apply to those with type 2 diabetes[232, 233].

1.2.7 Fish oil and the incidence of diabetes:

In the 1970s epidemiological observations by Bang and Dyerberg concluded that the lower death rate due coronary heart disease in Greenland Eskimos when compared to Danish population correlated with lipoproteins and cholesterol levels in Greenland Eskimos and their higher intake of Omega-3 fatty acids [66, 234].

It is well documented in epidemiological studies that fish intake is associated with lower incidence of diabetes with lower prevalence being attributed mainly to diets rich in n-3 LCPUFA [235, 236]. Both the incidence and prevalence of T2DM was found to be lower in the Icelandic population than their Western European counterparts [237]. There was also an inverse relationship between PUFA intake and hyperinsulinaemia in the Zutphen (town in Eastern Netherland) elderly study, an epidemiological longitudinal study [238]. Another observation in a cohort of Icelandic men was the inverse relation between T2DM and heart disease with n-3 PUFA and n-3PUFA/n6- PUFA ratio in their milk. Icelandic milk has a higher content of n-3PUFA mainly due to fish meal feeding of the cows in Iceland. Therefore it is estimated that 500 ml of Icelandic milk provides the same amount of EPA found in 100 gram of sea trout[239].

1.2.8 Fish oil effect on glucose control in diabetes

In contrast to the beneficial effect of PUFA on insulin resistance in healthy subjects, PUFA does not seem to reverse or ameliorate insulin resistance in subjects with diabetes [240, 241]. Additionally, there is ongoing debate on the ingestion of fish oil and n-3 fatty acid and metabolic control in patients with diabetes. Studies on fish oil supplementation in subjects

with Type 1 diabetes showed no effect on glucose metabolism and one study showed a reduction in the transcapillary albumin escape rate [242-244]. In a randomized trial of 1 year duration, eighty nine patients with T2DM were given either EPA plus DHA 2.6 g/day for the first 2 months and then 1.7 g/day for the next 4 months or olive oil as a control[245]. This is followed by a non blind continuation of intervention for 12 months. In this study there was no significant difference observed between the two groups in fasting glucose and insulin levels. Further supplementation with fish oil (1.7 g/day) to all patients for 6 months produced no deterioration of glucose control. Although there was a deterioration in the metabolic control of some subjects this may have been part of the natural history of diabetes rather than n-3 fatty acids[245]. Although it is far from clear, it is plausible to infer that a reduced insulin secretion without change in insulin sensitivity means that insulin effectiveness (sensitivity) is enhanced by n-3 fatty acids ingestion.

On the other hand, some studies on subjects with type 2 diabetes showed deterioration in the glucose control, although it has been noted that these studies had either few participants or the dose of fish oil given was high (> 3grams) [220, 246]. Another methodological error which may have contributed to these findings is the different total energy intake in the fish oil and control arms[247]. For example a study on 59 obese subjects with T2DM showed an increase of fasting glucose from baseline (1.4+/- 0.29 mmol/L) after 4 grams of EPA supplementation for 6 weeks when compared with a diet of olive oil. In that study glycated haemoglobin did not increase, although 6 weeks is arguably too short period to detect a change in glycated haemoglobin[246]. Glauber found the meal-stimulated insulin secretion is reduced while fasting plasma glucose increased after supplementation with n-3 fatty acids[248]. In another study using either 4 grams or 7.5 grams of fish oil for a month in subjects with T2DM significantly increased the fasting glucose and glycated haemoglobin by 20 and 12%

respectively in the high dose of fish oil only[249]. However, despite the change in fasting blood glucose, insulin sensitivity did not change and in some studies the metabolic clearance rate of glucose was increased [247, 250, 251]. More recently Mostad *et al* assessed the metabolic effect of high dose (8.5 grams) of fish oil with a high DHA to EPA (contrary to many available fish oil formulations) in patients with type 2 diabetes without hypertriglyceridaemia. They reported 1 mmol/L increase in home glucose monitoring and a reduced glucose uptake by the euglycaemic clamps. There was a paradoxical increase in free fatty acid and fat oxidation which the authors attributed to the resistance to insulin action in adipose tissues that might have been induced by the high fish oil intake [252]. Nevertheless, the authors of that study concurred that a lower dose than that may have no deleterious effect on insulin action.

There is no conclusive explanation as to why in some studies glucose homeostasis deteriorated after n-3 fatty acid supplementation. Some postulated mechanisms are an increase in hepatic glucose output due to increased gluconeogenesis or change in the insulin or glucagon secretion rate as well as a change in the sensitivity of the liver to the actions of these hormones[248, 253, 254]. On the other hand the beneficial effect of fish oil on insulin action is possibly mediated by skeletal muscles insulin mediated glucose uptake[232].

1.3 Exines

1.3.1 Exines morphology and structure:

Spores are the reproductive structures of plants[255]. They generally consists of one or two cells (sporoplasm) containing various fats, many vitamins and some proteins and carbohydrates. They are usually between 1 – 250µm and are protected by a remarkably

complex and robust double-layered wall. The inner layer of this wall, intine, is acetolysis susceptible and mainly composed of the polysaccharides cellulose. The outer layer is acetolysis resistant , also called exine, consists largely of biopolymer belonging to the sporopollenin family[256]. Sporopollenins chemical structures have not been characterised completely due to their unusual chemical stability and inertness[257]. They are very resistant to chemical, physical and biological degradation, making sporopollenin, extremely resistant natural organic material [257]. The exines have pores which play an important role in dehydration, dispersal and rehydration by determining the amount of water loss during these processes[258].

In this review the spores mentioned are those derived from algae, fern, mosses and fungi (cryptogams) as opposed to spores from seeded plants (spermatophyte) and bacterial endospores[255].

Spores from Lycopodium species (club moss), so called because of their resemblance to wolf's foot, have been used in pyrotechnic displays due to their even suspension, artificial lightening[259] and as a dusting agent[260].

The wall of the spores have a multi-layered structure as seen on Figure 2 and 3, although some layers may be absent in some species[261].

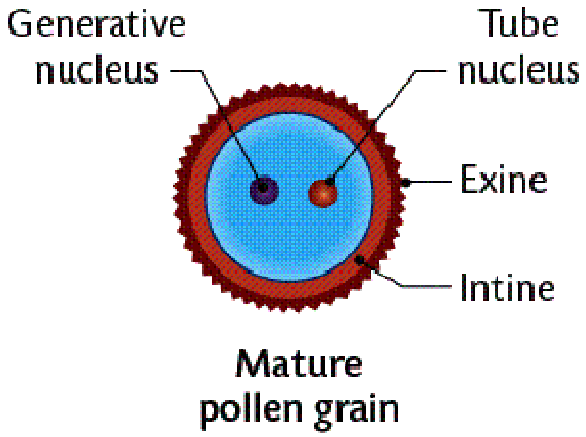


Figure 2: A diagram showing a mature pollen grain with its different layers.[262]

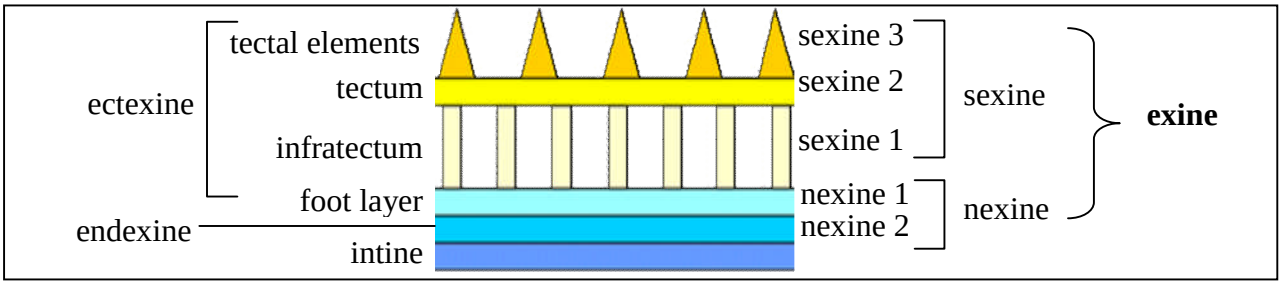


Figure 3: A cross section of the spore wall showing the different layers, taken from Punt *et al*.[263]

The *intine* is the innermost layer of the spore wall, also called the *endospore*. The *Exine* is the outer layer of the pollen grain wall.[263, 264] The exine can be divided according to morphology into the sculptured and non sculptured parts, *sexine* and *nexine*, respectively. While on the basis of their staining, development stages and texture they can be subdivided into an outer *ectexine*, and an inner *endexine*; the *ectexine* stains positively with basic fuchsine in optical microscopy and has higher electron density in conventionally prepared

Transmission Electron Microscopy sections. The ectexine is the highly resistant s*poropollenin* part of exine, whereas endexine is a thin heterogeneous lamellated layer [261]. Therefore, Sporopollenins is "the resistant non-soluble material left after acetolysis"[265]. This chemical inertness has been used to isolate the exines since acetolysis result in stripping off the core and leaving the skeleton (the exines) of the spores. However, it is beyond this work to discuss the different chemical methods for exines isolation.

1.3.2 Characteristics of exines:

Exines are monodispersable and have a very uniform species-specific size. [255, 266] They are largely insoluble in most of the common solvents[267]. Some of the biological properties of the resulting particles are dependent on their size. Therefore co-workers at Sporomex Ltd. are currently developing studies on several species, so as to cover a broader size range which can influence the choice of their use[268]. Species under investigation by Sporomex in Hull University are summarised in Table 3.

Table 3: Species under investigation by Sporomex Ltd. and their spore/pollen size[268].

Species	**Vernacular name**	**Spore/pollen size (µm)**
Lycopodium spec.	club moss	40
Lycopodium clavatum L.	club moss	25
Cannabis sativa L.	hemp	25
Lolium perenne L.	ryegrass	20
Ambrosia trifida L.	giant ragweed	15
Aspergillus niger Tiegh.	n/a	4

A testimony of the exines exceptional resistance to different chemical, and physical destructive forces is their ability to survive intact in ancient sedimentary rocks, dating back from more than 500 million years[266, 269]

Although the exines are resistant to strong acids (e.g. phosphoric acid), alkalis (e.g. concentrated potassium hydroxides) and organic solvents (e.g. methanol)[270-274], they can be degraded by oxidisers such as nitric acid, ozone and nitrobenzene[275]. However, it is also crucial for the germination to occur that sporopollenin is biologically degradable. As an example, a number of bacteria are capable of degrading sporopollenin under certain PH conditions[276-278], while the intine can be degraded by a collection of enzymes such as acid phosphatase, ribonuclease, esterase and amylase[276].

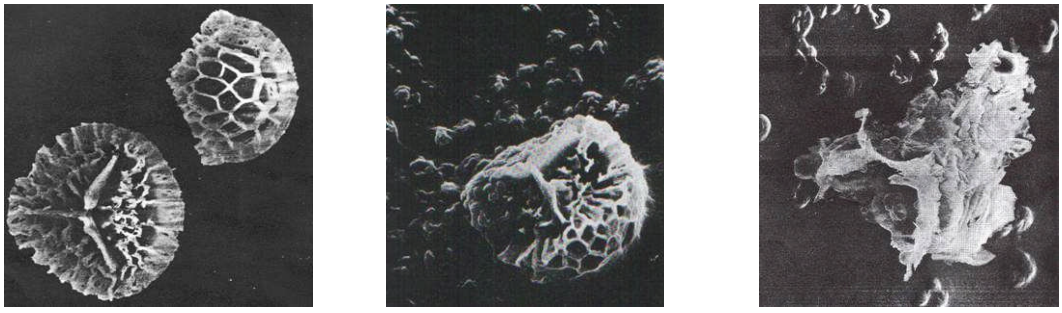
1.3.3 Uses of Spores as a carrier:

Spores and pollen grains can be found in many food sources such as mushrooms or honey. Some even sell bee-collected pollen as an energizer (e.g. CC Pollen®, USA) and others propose *Chlorella vulgaris* as a nutraceutical[279]. Pollen is being actively marketed for alleviating certain health afflictions and as a beneficial dietary supplement. Pollen tablets are also used as “natural food” and it has not reported that they are harmful when ingested.

Maack has patented sporopollenin use with an emulsifier and hydrophilic solvent to be used for cosmetic use, pharmaceutical purposes, as a chelating agent and as a food supplement[280].

In general, chemicals can cross the gastrointestinal tract barrier by two main uptake pathways: transcellular (mucosal lining cells) and paracellular (passing between cells) transports. It is the paracellular method that, although less active, may allow macromolecules and even particles such as spores to enter the blood stream[281-285]. Volkheimer *et al.*[284, 286-291] termed this mechanism *"persorption"*. Persorption was later restricted to the paracellular transport of micrometric solid colloids. Jorde and Lenskin however found that spores do undergo persorption[292, 293]

Once inside the circulation, direct enzymatic catabolism occur in the blood [294]as detailed below. Hence, spores and pollen grains can be eliminated by human body, and their exine completely removed[268]. Their degradation in blood was studied by Jorde and Linskens,[292] whose SEM pictures are displayed in Figure 4.



before oral ingestion 30 min after ingestion 60 min after ingestion

Figure 4:Degradation of sporopollenin in the blood stream – SEM.[268, 292]

As a consequence of its non-toxicity, uniformity from any one species and hollow microparticulate form as described above, sporopollenin can be envisaged for cosmetic, food or pharmaceutical applications, as a non-toxic microcapsule. The applications envisaged

could target body oils or ω-3 oils protection, and oral delivery of specialised food or drugs, for example.

1.3.4 Possible use of sporopollenin as a contrast agent:

Although sporopollenin is extremely robust, enzymes present in human plasma are capable of partially digesting the capsule and releasing the contents[295, 296]. More importantly, studies on exines have shown the encapsulated agent can be dosed and transported intact into the blood[294, 297]. This was the basis to the experimentation with loaded exines in contrast agents delivery. Lorche *et al* from University of Hull demonstrated that the sporopollenin could be loaded with the contrast agent and detected using microimaging[296]. They monitored the release of contrast agent release from the spores *in vitro* using human plasma. They collected human plasma for a period of 30 minutes following loaded exines intake and they found that some of the sporopollenin particles are partially digested, thereby offering a route to release of the spore contents. Most importantly, however, they were able to record higher magnetic resonance (MR) intensity of contrast mixed with plasma when compared to a control solution *in vitro*. The group now intends to study the exines loaded Magnetic resonance imaging (MRI) contrast *in vivo*.

1.3.5 Possible use of sporopollenin for targeted delivery of active compounds:

It was recognised that sporopollenin can be used to develop novel drug and nutraceutical delivery systems[298]. Mackenzie *et al* developed a range of applications of sporopollenin derived from *Lycopodium clavatum* based on filling of the exines with fat (Omega 3 fatty acids), proteins, vitamins and enzymes[298, 299]. Although a large proportion of oils can be encapsulated, in some cases of low solubility compounds, lower loadings resulted. The method of loading sporopollenin involves physical adsorption of the loading material on the sporopollenin surface and/or penetration of the active component into its interior by diffusion

through the pores of the outer membrane, up to 40 nm in diameter [299-302]. Paunov *et al*[299] have studied methods of loading sporopollenin with inorganic or organic nanoparticles synthesised *in situ*. The principle behind this method is to use the sporopollenin particle as a host where a chemical reaction used to generate a large amount of a product of low solubility inside the shell. When that reaction produces a nanoparticle larger than the size of the exine, then it will be trapped inside the shell. They described loading the exines with a magnetite nanoparticles (Fe_3O_4) and an active substance such as a drug, vaccine etc which can then be directed by an external magnetic field to deliver the active substance to a location of interest[299]. Another example of this method is the loading of *Lycopodium clavatum* with calcium hydrogen phosphate ($CaHPO_4$) which can then be used in fortified food to improve bioavailability of calcium and other minerals. The final example they provided in their paper was the loading of exines with nanoparticles involving a reaction of cationic and anionic dyes. They envisaged that some ionic charged drugs can be precipitated with a hydrophobic counter-ion inside the sporopollenin capsules forming hydrophobic nanocrystals. These have low solubility and dissolve slowly from the capsules to the continuous phase thus supporting a constant concentration of the active component such as a drugs or a vaccine[299].

1.3.6 Possible use of sporopollenin to mask the taste of unpleasant flavours:

The exines have also been used to mask the unpalatable taste of Cod Liver Oil in a 1:1 weight ratio. In a double blind trial, respondents were unable to identify the fish oil among sun flower and water loaded exines. When the ratio of fish oil to exines was increased (2:1 or 4:1), the respondents were able to identify the fish oil readily. It was also noted that at this high weight of exines (1:1), they continued to retain the free flowing powder consistency which is an important property if they are to be mixed with other components. This simple

procedure is not costly and can be used at industrial quantities to improve the taste of fish oil[303].

1.4 Oxidative stress markers

1.4.1 Free radicals

Oxygen is essential for life but has toxic effects[304]. A radical is defined as a molecule with highly reactive unpaired electron in an outer orbital which can initiate chain reactions by removal of another electron from another molecule to complete its own orbital[305]. Therefore free radicals are molecules or molecular fragments with unpaired electrons [304, 306]. The chemical structure of oxygen favours its reduction by the addition of one electron at a time thereby leading to the formation of oxygen radicals, the first of which is the superoxide anion($O_{2}^{\cdot -}$) [305]. Adding a second electron to the latter will form the peroxide ion (O_2^{-2}) which will immediately protonate to the hydrogen peroxide (H_2O_2). The latter can undergo homolytic fission at the O-O bond thereby forming two hydroxyl radicals $^{\cdot}OH$, the most active and unstable oxygen radical[304]. Additionally $^{\cdot}OH$ can be formed when either iron (through the Fenton reaction) or Copper salts are close to H_2O_2[304, 307]. The hydroxyl radical can react with whatever is nearby (sugars, amino acids, phospholipids, DNA bases and organic acids) to produce secondary radicals of variable reactivity by three ways; abstracting hydrogen atom, addition or transferring an electron[304]. The sources of free radicals are many but the most prominent one is the mitochondrion where electron transport chain and oxidative phosphorylation occur with small amount of electron leakage forming the superoxide. Others are the peroxisomes, Cytochrome P450 system and also the phagocytic cells during respiratory burst while killing invading bacteria[305]. Other radicals are sulphur-

centred $RS^{\cdot}$, carbon-centred ($CCl_3^{\cdot}$) and nitric oxide ($NO^{\cdot}$)[308]. Reactive oxygen species (ROS) include the superoxide radical anion $O_{2-}^{\cdot}$, hydrogen peroxide H_2O_2 , the hydroxyl radical $^{\cdot}OH$, lipid peroxides, the peroxyl radicals $RO_2^{\cdot}$ the alkoxy radicals $RO^{\cdot}$, the radicals of nitric oxide $^{\cdot}NO$ and nitrogen dioxide $^{\cdot}NO_2$, ozone O_3 and possibly singlet oxygen, either in its low-energy form $^1\Delta O_2$ or in its high-energy form $^1\sum O_2$[309].

1.4.2 Lipid Peroxidation

Fatty acids are present in the plasma and mitochondrial membranes. The process of lipid peroxidation therefore could have a major role in the pathogenesis of disease processes such as atherosclerosis[310].

When two radicals meet, they combine their unpaired electrons and form a covalent bond. However most of the biological substances are non-radicals and therefore upon meeting with the reactive radical, that non-radical substance becomes a radical. This can then change another non-radical to a radical and the process will propagate to become a chain reaction; a feature of a reaction between radical and non-radical[308]. Lipid peroxidation is a process which can be initiated by abstracting a hydrogen atom in the methylene group (-CH-) by a free radical of sufficient reactivity, usually $^{\cdot}OH$. In polyunsaturated fatty acids, the methylene bond next to the double bond (allylic hydrogen bond) is weak and susceptible for abstraction of hydrogen atom giving rise to a carbon radical as the hydrogen atom leaves an unpaired electron behind. The carbon radical will then undergo stabilization by molecular rearrangement to form conjugated dienes which further react with O_2^- to form hydroxyperoxyl ($C\text{-}O\text{-}O^{\cdot}$).

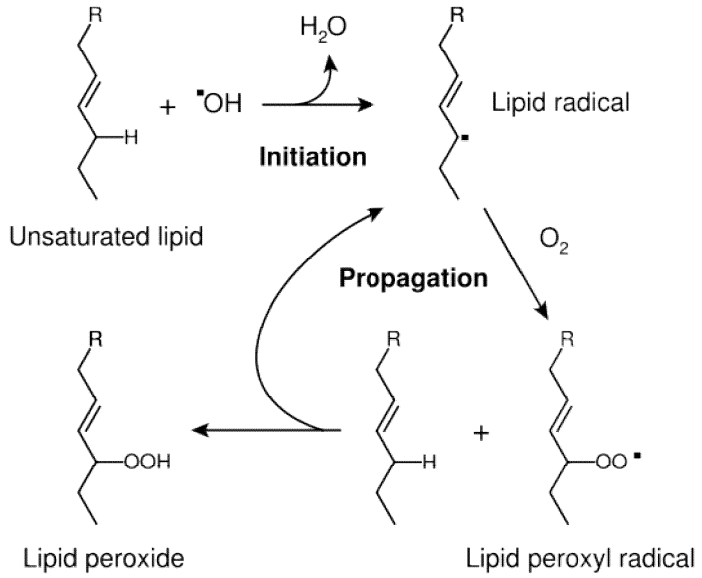


Figure 5: The propagation of lipid peroxidation.[311].

The Peroxyl group, in the presence of metals such as iron or copper, can abstract another hydrogen from an adjacent fatty acid molecule forming a hydroperoxide and carbon radical thereby initiating a chain reaction as seen in Figure 5 [308]. This self propagating reaction is only terminated when a free radical scavenger like vitamin A react with the peroxyl radical[308].

It is now known that hydroperoxide is the primary peroxidation product while secondary peroxidation products exist from the cleavage of hydroperoxides containing fatty acids which are thought to act on distant foci[312]. These are mostly alkanes and alkenes free radicals. Lipid peroxidation is thought to be the basis of many diseases pathophysiological pathways. An example is the observation that metabolites of the toxic substance carbon tetrahydrochlorate (CCl_4), haloalkanes free radicals, is linked to the peroxidation in liver microsomal lipids [306, 313].

The chain reaction can be terminated in the presence of a free radical scavenger (chain terminator) such as alpha-tocopherols which is capable of transferring an hydrogen atom to lipid peroxyl group[314]. It is the balance between the free radicals scavengers (e.g. vitamin E, C and D) and the rate of free radical production that will determine if the former will be overwhelmed while the free radical initiating cell injury.

1.4.3. Measuring free radicals effect; oxidative stress:

Oxidative stress can be defined as the imbalance between the production and disposal of reactive oxygen species[315]. When this occurs, free radicals can initiate damage to many molecules; lipids, DNA, proteins and these peroxidation products can be measured in the biological system. In a multilaboratory study to assess different peroxidation (lipid, DNA and proteins) products in rats after CCl_4 exposure to establish specificity, only lipid peroxidation end products measured in plasma and urine (malondialdehyde and F_2-isoprostanes, 8-iso-Prostaglandin $F_2\alpha$ i.e. 8-iso-$PGF_2\alpha$) were found to be significantly correlated with the oxidative damage[316].

1.4.3.1 MDA:

Previous methods used to measure the product of lipid peroxidation such as detection of conjugated dienes, lipoperoxides, and aldehydes in plasma are poorly reproducible due to the reactivity and instability of the species[317]. The most studied aldehydes are malonaldehyde (MDA) and 4-hydroxyalkenals (HNE). There are various methods to measure free and/or bound form MDA in human plasma, blood or serum. They may be either reactions leading to coloured or fluorescent products or chromatography such as Thin Layer Chromatography (TLC) or high performance liquid chromatography (HPLC) or GC (Gas Chromatography) with or without derivatization [318-322]. Colorimetric or fluorimetric determination of MDA

or MDA-like materials by the thiobarbituric acid (TBA) assay whereby the addition of the latter result in a pink-coloured products which absorb in 500-550 nm range[321]. Despite this method's simplicity, it lacks specificity as MDA-like substances can form during the addition of TBA to precursors of lipid peroxidation. Additionally substances like sugars, biliverdin and pharmaceuticals can form complexes with TBA which absorb in 530-535 nm range [323, 324]. Even the use of more accurate and expensive methods such as gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) is not going to solve the problem as MDA is not a specific product of lipid peroxidation and it represents less than 1% of lipid peroxides[325, 326] [327].

1.4.3.2 Isoprostanes:

The F_2-Isoprostanes (F2-IsoPs), isoprostanes containing an F type prostane ring, are a family of 64 prostaglandin F_2-like compounds generated in vivo by nonenzymatic free-radical–mediated peroxidation of arachidonic acid and then cleaved and released into the circulation by phospholipase(s) before excretion in the urine as free isoprostanes[328]. There are many types of isoprostanes but the ones that received attention is the F_2-isoprostanes. Morrow and Roberts showed that compounds similar to the prostaglandin F2 (F2-isoprostanes, F2-IsoPs) form in vivo and in vitro by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid, independently of the cyclooxygenase[329, 330]. They are relatively stable and are excreted in urine making their measurement in plasma and urine the gold standard method for quantifying oxidative stress [312, 316, 331]. Using GC–MS (Gas Chromatography-Mass Spectrometry) to measure plasma F2-isoprostanes is a highly specific and sensitive method to quantify lipid peroxidation [332, 333]. However, measurement requires a relatively large volume of plasma (0.5–1.0 mL) and is a multi-step complex

process with the potential for errors at many points[332]. Radioimmunoassay methods to measure F2-isoprostanes do exist but they lack sensitivity but the relatively low cost have expanded research in the field of measuring oxidative stress [332, 334]. Studies have shown conflicting results on the correlation between Immunoassay and MS methods to measure F2-isoprostanes[335-337]. There is on the other hand some evidence that they lack accuracy. Their inhibition by cyclooxygenase inhibitors and the fact that platelets and monocytes can synthesise isoprostanes points to an enzymes-mediated production[338-340]. However, these amounts appear to be trivial and therefore do not detract from the isoprostanes accuracy in assessing lipid peroxidation by ROS[325, 341]. All tissues have isoprostanes in them and they are also found in measurable quantities in most of the biological fluids analysed, including plasma, urine, synovial fluid, bronchoalveolar fluid, bile, lymph etc[328]. Plasma and urine are the sample types that are commonly analysed, being the most convenient to obtain and the least invasive[328]. F2-IsoPs exist in plasma in 2 forms: the more abundant esterified to lipids and the free acid form while only the stable hydrolysed form appears in urine; making urinary test more stable method[328]. Furthermore the abundance of isoprostanes in body fluids and tissues means that urinary isoprostanes can give an authentic picture about total body oxidative stress[334].

1.4.3.3 The role of isoprostanes in diseases:

The evidence is accumulating that isoprostanes are not just a mere biomarker of oxidative stress, but also have an active biological role in diseases. For example 15-F2t-IsoPs (isomer of the F2-IsoPs) was found to induce vasoconstriction in renal glomerular arterioles through a prostanoid receptor also activated by thromboxane A2[342, 343]. This was then seen in vitro in the aorta, pulmonary, coronary as well as the cerebral vessels[344-348]. Isoprostanes were also found to increase retinal vessels endothelin 1 and thromboxane production in the retina

which could contribute to retinopathy of prematurity and may be relevant to the revascularization of retinopathy in diabetes[312, 349-351]. They have also been implicated in mesangial cells proliferation in diabetic rats through the production of transforming growth factor-β1 (TGF-β1)[352]. Additionally, they are thought to have a role in atherosclerosis through platelet activation and it is plausible that 8-iso-PGF2α can induce aggregation of platelets in response to subthreshold concentrations of platelet agonists, for example in diabetic subjects [350, 353, 354].

Recently the isoprostanes have been also implicated in pulmonary pathology. Isoprostanes especially those of E_2 conformation (not the one that is usually measured) were found to cause airway hyper-responsiveness possibly through specific Prostaglandin E_2 receptors[355, 356]. The E ring were also found to cause vasoconstriction in pulmonary and bronchial vasculature probably through the Prostaglandin E_3 receptor and that may account for their involvement in pulmonary hypertension and acute lung injury [357-359]. The role of E_2 ring form in Asthma is supported by the findings of cholinergic stimuli augmentation and increasing Cl^- conductance accounting for mucus hypersecretion in asthma and COPD [360, 361].

Therefore it seems that different isoprostanes can have effects on different cells and hence are potentially have an active role in the pathogenesis of diseases and not just serving as a marker of the primary insult i.e. oxidative stress.

1.5 Glycaemic variability:

Diabetes Mellitus refers to a group of common metabolic disorders that share the phenotype of hyperglycaemia, with hyperglycaemia being a cardinal feature for all the metabolic disturbances and complications related to diabetes [362]. Furthermore, the degree of

glycaemia is closely related to the known complications of diabetes[363]. Glycated haemoglobin is a retrospective indicator of the average glucose concentration over the previous 6 – 8 weeks. Approximately 50% of the variance in Haemoglobin A1c (HbA1c) is determined by the average blood glucose concentration over the previous month, 25% by the concentration over 30 – 60 days and the remaining 25% by the concentration from 60 to 120 days [134, 364]. However, clinical experience suggests that in practice a fall from an elevated HbA1c can be demonstrated within a few days of a change in therapy and certainly within 2 weeks[365] or 4–8 weeks [366, 367].

Recently the mechanism through which glucose can induce vascular damage has been linked to a unifying theory; the overproduction of reactive oxygen species by the mitochondria caused by hyperglycaemia[368, 369]. Although all cells are exposed to hyperglycaemia in diabetes, only certain cells are affected. This is due to the difference in intracellular glucose concentration in different cells partly due to different glucose transporter proteins (GLUT) on their surfaces. Therefore cells like the vascular smooth cells where intracellular glucose transport does not change when there is extracellular hyperglycaemia are not damaged by hyperglycaemia when compared to the vascular endothelial cells which fail to downregulate their intracellular glucose content[134].

1.5.1.1 Mechanisms of cellular damage caused by hyperglycaemia:

There are five proposed pathways to explain how glucose could potentially cause cellular damage; the polyol pathway, Advanced Glycation Endproducts (AGE), increased expression of receptors for the AGEs pathway, activation of Protein Kinase C (PKC) isoforms and overactive hexosamine pathway[134, 370]. However, one upstream mechanism has been hypothesized to link all of the above and that is reactive oxygen species overproduction by the mitochondria as a result of intracellular hyperglycaemia.

1.5.1.2 The polyol Pathway:

Aldose reductase is a cytosolic enzyme that catalyses the reduction of variety of glucose using Nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor to sorbitol which is changed to $NADP^+$. The latter is then oxidized to fructose by the enzyme sorbitol dehydrogenase which uses NAD^+ and reduces it to NADH. It is important to note that the affinity of aldose reductase to the glucose is low and therefore it will only be influential when intracellular hyperglycaemia occur[370].

It has been suggested that the depletion of NADPH which is needed for the production of reduced glutathione (an important ROS scavenger) is behind the damaging effect of the polyol pathway [371]. However sorbinil (aldose reductase inhibitor) was not found to be of benefit in diabetic retinopathy[372] while another study showed positive effect of the potent aldose reductase inhibitor, zenarestat, on neuropathy; thereby confounding the picture[373]

1.5.1.3 AGEs:

Intracellular hyperglycaemia initiates AGEs. AGEs are products of interaction between a very reactive dicarbonyls and of intracellular and extracellular proteins. The reactive dicarbonyls are formed from auto-oxidation of glucose to glyoxal, decomposition of the Amadori product to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal[370].

These products can then be involved with a variety of intracellular and extracellular pathways to induce tissue damage. As an example, AGEs have been linked to retinopathy and nephropathy in diabetes[134, 374] through their influence on angiogenesis[375].

In general there are three mechanisms whereby intracellular AGEs can cause cellular damage: modification of function of intracellular proteins, modification of extracellular matrix which in turn reacts with other cellular matrix proteins and finally by combining with plasma proteins and binding to AGE receptors. The latter is present in macrophages and can induce production of reactive oxygen species and can also changes gene expression[134].

1.5.1.4 Activation of Receptors of AGEs (RAGE)

Proteins modified by AGEs can act through specific receptors for AGEs; such as those of monocytes, macrophages and glomerular mesangial cells. AGE protein binding to these receptor stimulates macrophages to produce cytokines, such as interleukin-1, Tumour Necrosis Factor alpha (TNF α) among many others [376, 377]. AGE receptors have also been identified on glomerular mesangial cells inducing changes likely to be responsible for nephropathy[378]. Receptors for AGEs were also identified in the vascular endothelial cells [379] . RAGE is then thought to mediate a variety of pathways such as reactive oxygen species production[380] which can in turn mediates other deleterious effects[380]. Furthermore the activation of RAGE can alter the expression of genes responsible for procoagulation factors such as the vascular cell adhesion molecule-1 (VCAM-1) , thereby causing adhesion of inflammatory cells to the endothelial vasculature[381]. Finally the activation of RAGE can promote endothelial permeability possibly through endothelial growth factor [382].

1.5.1.5 Increased protein kinase C (PKC) activation

The group of PKCs consist of many isoforms and the activity of the classic isoforms requires calcium ions and phosphatidylserine and is enhanced by the presence of Diacylglycerol

(DAG)[134]. Persistent activation of PKC is thought to be caused by increased intracellular levels of DAG. The latter is caused by raised intracellular glucose with consequent increased synthesis of DAG via dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, of the glycolytic pathway[383].

Activation of PKC-B isoforms has been shown to mediate a decrease in retinal and renal blood flow [384] perhaps by depressing the production of the vasodilator NO [385]while inhibitors of PKC-B were found to improve vascular function[385-387]. The increased in endothelial permeability has also been linked to PKC alpha [388] through vascular endothelial growth factor[388].

1.5.1.6 Increased hexosamine pathway (HSP) flux:

There is evidence that intracellular hyperglycaemia result in some of the glucose being used in the hexosamine pathway [389, 390]. This is a minor pathway which results in the fructose 6 phosphate being diverted to form Uridine diphosphate **(**UDP)-*N*-acetylglucosamine (UDP-GlcNAc) which is one of the building blocks of the glycosyl side chains of glycoproteins, glycolipids, proteoglycans and gangliosides[391] . Although the exact mechanism of HSP induced vascular complications is not completely understood, there is evidence that it its mediated through tissue growth factor (TGF) β1[392, 393] and plasminogen activator inhibitor 1 (PAI-1) [394]. The primary mechanism hypothesized is the role of acetylgalactosamine (GlcNAc) which acts as a substrate for *O*-GlcNAc transferase that is responsible for the reversible posttranslational protein modification. In this reaction GlcNAc is transferred in *O*-linkage to serine/threonine residues of proteins. These proteins could be transcription proteins for PAI-1 or other protein [395-397]. Among the protein that could be afflicted by this O- GlcNACation is the insulin receptor substrate, GLUT4 or the

phosphatidylinositol signalling thereby also causing insulin resistance[398-401]. The fact that the Ribonucleic acid (RNA) polymerase II can undergo GlcNACation at specific serine/threonine sites means that many of the gene expression processes will be glucose determined through this pathway upon intracellular hyperglycaemia[402, 403]. Additionally nuclear and cytoplasmic proteins can be modified by *O* - GlcNACation thereby interfering with their normal function. An example being, phosphorylated endothelial nitric oxide which can be inactivated by O- O-GlcNAc in erectile dysfunction caused by diabetes [404].

1.5.2 Unifying Theory:

Brownlee in 2001attributed the damage caused by the 5 pathways mentioned above to one single process; the overproduction of ROS by the mitochondria caused by intracellular hyperglycaemia[370]. In 2000, two important discoveries showed that superoxide radicals by mitochondria is involved in the HSP flux and that normalizing this can block three other hyperglycaemia-induced pathways[395, 405]. Although it was previously shown that hyperglycaemia can induce ROS formation[406], the exact mechanism was not very well understood. The Unifying theory by Brownlee proposed a threshold above which further influx of NADH, $FADH_2$ (caused by glucose entry to glycolytic and tricarboxylic pathways) to the electron transport chain of the mitochondria will result in the generation of superoxide[407]. This is due to blocking complex III in the electron transport chain [395, 407, 408]. Overproduction of superoxide will overcome mitochondria ability to convert superoxide to hydrogen peroxide and water by superoxide dismutase and catalase[409-411]. The excess superoxide will diffuse to the nucleus and will cause DNA damage which will activate the Poly ADP Ribose Polymerase (PARP) enzyme [412, 413]. The latter is responsible for polymerizing the key enzyme in the glycolytic pathway, glyceraldehydes 3 Phophate

dehydrogenase which will interfere with its function [413]. When this occurs, all the upstream reactions in the glycolytic pathway will more or less seize[134]. This means the glucose 6 phosphate will be diverted to fructose 6 phosphate and the hexosamine pathway while intracellular glucose will be non-enzymatically changed to sorbitol through the polyol pathway[134]. The conversion of glyceraldehydes 3 phophate into methylglyoxal (AGE) nonenzymatically will also take place. This AGE will activate the RAGE while DAG (a product of glyceraldehydes 3 phosphate) will activate the PKC pathway[134].

1.5.3 Haemoglobin A1c (HbA1c):

HbA1c is a stable product of the nonenzymatic addition of glucose at the N-terminal of Valine amino group of the β chain of haemoglobin Ao (N-[1-deoxyfructosyl] haemoglobin) which irreversibly rearrange into a stable ketoamine [414-416]. There are two main methods of assaying glycated haemoglobin; assaying the charge difference between different haemoglobins such as cation-exchange chromatography or those which utilise the structure of glycogroup on haemoglobin such as affinity chromatography and immunoassay[417, 418]. As Glucose is free to enter erythrocyte, the glycated haemoglobin level will be influenced by the ambient glucose level in the previous 120 days (the lifespan of erythrocyte).

A direct relationship exists between HbA1c and mean glucose; in the Diabetes Control and Complications Trial, an HbA1c of 6% corresponded to a mean plasma glucose level of 7.5 mmol/L, and every 1% increase in HbA1c resulted from an increase in the mean of 2 mmol/L[419]. However, as mentioned earlier, the glycation of haemoglobin is not uniform over the life span of erythrocytes and recent glycation tend to be overrepresented in HbA1c result[414].

Confounders to the result of HbA1C include: Haemoglobin variants, uraemia, short erythrocyte life span or processes that lengthens life span of erythrocytes [414, 420-423]. The

HbA1c has been used in landmark prospective trials as a measure of good glycaemic control and its correlation with clinical outcomes is well established. As an example in the Diabetes Control and Complications Trial (DCCT), a reduced mean A1c by 1.8% in the intensively treated group resulted in a 76% decrease in the development of new retinopathy and a 39% reduction in microalbuminuria[363].

Subsequent follow-up of the DCCT cohort in the Epidemiology of Diabetes Interventions and Complications (EDIC) has however shown a very intriguing observation. The intensively treated group maintained lower level of microvascular complications many years after the initial period of intensive therapy. This occurred despite a worsening of diabetes control after the initial intensive therapy period and also the mixing of the two groups after the initial period. Despite the merging of the two groups, the positive differences in microvascular complications between the initial intensive and standard therapy groups were sustained for many years[424]. In fact this difference was even more pronounced in later years. This was subsequently termed Hyperglycaemia Memory. Similarly subsequent follow-up of United Kingdom Prospective Diabetes Study (UKPDS) cohort with T2DM showed similar “legacy” effect [425, 426].

How is it then possible to explain these findings? It has been hypothesised that short term intracellular hyperglycaemia causes increased ROS which can have a long lasting gene modifying effect (epigenetic effects) through increase in the activity of P65, a nuclear transcription factor and a member of the Nuclear Factor (NF) κB - subunit[134, 427, 428]. These effects have been seen in lymphocytes of patients with T1DM and were also linked to pro-inflammatory processes in vascular smooth muscle cells [429-431]. The effect of transient hyperglycaemia on gene expression and the potential effect on microvascular

complications have been viewed by Brownlee and others as an evidence of HbA1c - independent risk factor for diabetic complications [134, 368].

1.5.4 What is glucose variability:

According to Monnier *et al*, dysglycaemia of diabetes have two components; chronic hyperglycaemia and the acute fluctuation of blood glucose (glycaemic variability)[432]. However defining glycaemic variability is a complex issue since there are many sources for glucose fluctuation including variability in the type of treatment, the metabolic rate, lifestyle, compliance, meal and the method used in assessing variability[433]. Glycaemic fluctuation is assumed to have two components; the intraday glycaemia represented on the vertical axis and the inter-day fluctuation over time represented on the horizontal axis[432]. Another unknown issue is the lack of knowledge on the level of fluctuations needed to cause diabetic complications (microvascular or macrovascular)[432]. Therefore, glycaemic instability is difficult to define as fluctuation of glucose could occur over any period of time; minutes, hours, days or even weeks and it is far from clear which of these have more impact on the development of diabetes complications [434].

1.5.5 Methods of measuring glycaemic variability:

Currently there is no agreement on the mathematical method suited best to assess glycaemic variability. Researchers have used different variability measurement to account for glycaemic variability; standard deviation, coefficient of variation and descriptive statistics such as the median and interquartile range [435-437]. Some also used the daily delta BG (calculated as the mean of daily [maximum- minimum] BG), hyperglycaemic and hypoglycaemic index (area under the curve for a defined hypo and hyperglycaemic values)[438]. However two

important concepts were brought about by Service *et al* and these are the mean amplitude of glycaemic excursions (MAGE) and mean of daily differences (MODD)[439, 440]. MAGE is the average of differences of values above and below a normal range of readings in 24 hours and it refers to intraday variability. MODD on the other hand refers to the mean of absolute differences in values taken at the same time in two successive days[439, 440]. Recently, McDonnell *et al* introduced another concept and that is the $CONGA_n$ (continuous overlapping net glycaemic action) where n= hour (1-4 hours). Here the signed differences of values separated by n hours is averaged to obtain another index of intraday variability[441]. New methods for the assessment of glucose variability from the Continuous Glucose Monitoring System (CGMS) have been proposed by Rodbard *et al*. Here they use a modified ANOVA (Analysis of Variance) to estimate total variability (SD_T), within day variability (SD_w), between time points variability for the glucose profile averaged over days ($SD_{hh:mm}$), within series variability ($SD_{ws\ h}$), between daily means variability (SD_{dm}), between days–within time points variability ($SD_{b\ hh:mm}$), and between day–within time points variability after correction or adjustment for changes in daily mean glucose ($SD_{b\ hh:mm\ //\ dm}$)[442]. These are easily calculated from an Excel spreadsheet supplied as an appendix with the paper[443]. They found the SD_w and $SD_{b\ hh:mm}$ to be very close to SD_T while the SD_{dm} was smaller than the SD_T. They also suggested that in situations where SD_w and SD_{dm} are the predominant sources of variability, then plotting them against each other in a graph where SD_w is the horizontal axis and the SD_{dm} is the vertical access can give a better picture on the patient's glycaemic variability[442]. The reason why SD has been chosen by this group as a method to assess variability is the fact that clinicians are well versed in the concept of SD. On the other hand, ANOVA and other measures of glycaemic variability (MAGE and CONGA) may not be known to all clinicians to apply into their practice.

By analysing the 5-minutes interval blood glucose result obtained from 85 subjects over a period of 8 days[444-446], Rodbard *et al* concluded that there are essentially two measures of glycaemic variability; the intraday and interday variability[442].The former represented by MAGE, SD_w, $SD_{hh:mm}$, $SD_{ws\ h}$ for small values of h (e.g., =1–6), and CONGA*n* for small values of n (e.g., n=1–3); all are strongly correlated with each other. The interday variability is represented by MODD, MODDd, SD_{dm}, $SD_{b\ hh:mm}$, $SD_{b\ hh:mm\ //\ dm}$, and $CONGA_{24}$ with strong correlation among each other. However, there is a weak correlation between the interday and intraday variability measurements methods[442].

1.5.6 Can glycaemic variability add to the risk of diabetic complications?

In reviewing the DCCT results, Brownlee and Hirsch saw the higher incidence of microvascular complications in a subgroup of the conventional arm with similar A1c to the intensive group as an evidence of factors other than A1c in causing these outcomes[368]. However, the DCCT, in a subsequent more accurate analysis using more accurate statistical model, found that A1c accounts for 96.2% of retinopathy and even higher for other outcomes. The DCCT group defended that DCCT analysis original paper did not directly assess whether subjects at the same A1c level have a higher risk of retinopathy progression with conventional versus intensive therapy[447]. Furthermore, they argued that the original analysis was only for the effect of time on outcomes and it did not take into account the A1c effect. Therefore the new direct analysis of the DCCT using accurate statistical model showed no significant differences in the incidence of retinopathy between any of the conventional and intensive groups who had the same glycaemic range (6.5-7.49, 7.5-8.49 and 8.5-9.49)[447]. Additionally, after adjustment for the updated mean A1c for each subject as a time-dependent covariate, they concluded that only 6.6% of the retinopathy was explained by treatment group

effect and that 96.2% was as a result of the difference in A1c between the two groups[447]. The other piece of evidence that is widely viewed as a testimony to the effect of glucose variability on retinopathy is that from Mohsin *et al*. They found that adolescents who were treated intensively with insulin had little change in HbA1c while retinopathy incidence was halved between 1990 and 2002, a time when insulin therapy changed from conventional to intensive after the publication of the DCCT [448].

1.5.7 Evidence for the role of glycaemic variability in diabetic complications:

In the last few years, there has been a debate among researchers on the subject of glycaemic variability and its contribution to the development of diabetic complications over and beyond the concept of hyperglycaemia. Since postprandial hyperglycaemia in subjects with impaired glucose tolerance seems to be associated with a risk of cardiovascular events even before frank hyperglycaemia of diabetes, this was taken as evidence to support the theory that variable glucose is a risk for macrovascular complications.

Epidemiological studies have shown that 2 hours postprandial blood glucose after a glucose challenge is a powerful predictor of cardiovascular disease[449-451]. In a population based prospective study, the diabetes intervention study, participants were followed for 11 year to identify future risks of myocardial infarction as well as all cause mortality. In multivariate analysis age, blood pressure and smoking were independent risk factors for myocardial infarction and male sex, age, blood pressure, triglycerides, postprandial blood glucose and smoking for death[452]. Postprandial blood glucose was found to be a predictor of early cardiovascular disease (intima-media thickness) in another study by Hanefeld *et al*[453] .

Apart from the evidence sited above, Monnier and Ceriello groups have published extensively on the matter *in vivo* and *in vitro*[454, 455]. For example, they found that glycaemic fluctuation causes endothelial cells apoptosis via PKC in umbilical veins[455] supporting the findings from another group that glycaemic variability has a harmful effect on renal mesangial cells[456]. In a euinsulinaemic hyperglycaemic clamp study on healthy volunteers and subjects with T2DM, Ceriello *et al* oscillated blood glucose between euglycaemia (4-6 mmol/L) and hyperglycaemia (15 mmol/L) 6 hourly for 24 hours. Their outcomes were endothelial function (flow mediated dilatation) and oxidative stress markers (plasma 3-nitrotyrosine and 24-h urinary excretion rates of free 8-iso PGF2α)[457]. They also did constant hyperglycaemic clamps for 24 hours at two levels (10 and 15 mmol/L). They found:

1- Increase in oxidative stress markers in a concentration-dependent, fasting glucose-independent manner in the normal and diabetic subjects.
2- Oscillating glucose between 5 and 15 mmol/L for 6 hours over 24 hour period resulted in further significant increases in endothelial dysfunction and oxidative stress compared with either continuous hyperglycaemia at 10 or 15 mmol/L.

It is worth mentioning that 6 hours of hyperglycaemia and euglycaemia do not resemble real-life glycaemic fluctuations in patients with diabetes where BG could change from minute to minute and hour to hour as seen in the CGMS of many studies.

Monnier *et al* studied the variability in subjects with T2DM and matched control using CGMS, measuring the MAGE and AUC pp (area under the Curve postprandial glucose). They found that urinary Free 8-iso PGF2α (using enzyme immunoassay)are not only elevated in subjects with T2DM but that it is highly correlated in multiple linear regression analysis to both the MAGE and AUC pp[458].

A recent study on normal subjects and subjects with diabetes and the metabolic syndrome showed that glycaemic variability (using CGMS and measuring CV) is an independent risk factor for poor endothelial function and carotid intima-media thickness in all groups. However, this was not the case when the MAGE was used as a measurement of glycaemic variability[459]. Interestingly, the HbA1c as well as the mean blood glucose was significantly different between the groups. Both the two stratified groups with high mean blood glucose with low CV and the low mean blood glucose with high CV had similar endothelial function as well as carotid intima-media thickness[459].

Further evidence listed by the proponents of the relation between glycaemic variability and macrovascular complications comes from Intensive Care Units (ICU) studies [460-462]. In these studies glycaemic variability was found to be higher in non-survivors in Intensive Care Unit (ICU) compared to those who survived. In an analysis of the two critical illness intensive insulin treatment by Van de Berghe, Meyfroidt *et al* found a significant increase in blood glucose SD among non survivors when compared to survivors (42 vs. 33 mg/dl; 2.3 vs. 1.8 mmol/L) which were close to that found by another study[438, 460]. Furthermore, there was a stepwise increase in mortality with higher SD in the medical ICU patients; 22.9%, 44.8%, and 54.5% for those with SD <32, 32 to 47, and >47 mg/dL, respectively with no correlation between SD and severity of illness [438, 463]. However, the mortality benefit of intensive insulin arm seen in the Leuven interventional trials have been attributed to decrease in glycaemic levels rather than to a decrease in glycaemic variability[463]. In fact the mean daily delta blood glucose (BG) increased by intensive insulin therapy from 59 to 72 mg/dL (3.2 to3.9 mmol/L) in the surgical patients and there was insignificant increase in glycaemic variability in the medical patients [463, 464]. Finally, severe hypoglycaemia (BG < 2.2 mmol/L) occurred among 4% of the survivors and among 15% of the nonsurvivors,

conferring an independent risk of mortality, with an odds ratio of 3.22 (95% confidence interval, 2.25– 4.64; p < 0 .0001)[464]. These data confirm the findings from two recent, large, retrospective studies on hypoglycaemia and the risk of mortality in ICU patients [463, 465-467]. There are also speculations that in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial hypoglycaemia may be implicated in causing increased mortality in intensively treated patients with diabetes [468].

As to the microvascular complications and the association with glycaemic variability, there is not a lot of evidence besides the often sited interpretation by Brownlee and Hirsch of the DCCT result as well as the findings of Mohsin *et al* (mentioned earlier)[368, 448]. Therefore it is plausible to conclude from the above evidence, that glucose variability with hypoglycaemia can be a risk in certain clinical settings giving rise to harm and mortality.[469]

1.5.8 Evidence against the role of glycaemic variability in diabetic complications:

Kilpatrick and DeVries groups have been ardently defending this stance in the last few years. Kilpatrick *et al* have analysed the publicly available DCCT data in recent years to provide evidence to back their position that glycaemic variability does not add to the risk of microvascular complications beyond the mean glucose in T1DM[470-475]. In a Multivariate Cox regression the intra-day and inter-day variability in BG did not have influence on the development or progression of retinopathy[470]. Their results supported two other published work on the DCCT data. The first was a similar analysis of mean blood glucose, biological variability of A1c as well as the SD of BG and their relative impact on the A1c variability. They found the first two play a major role in determining the A1c variability while the SDBG had little influence[476]. The other piece of evidence is the one emerged from the DCCT and

EDIC statisticians who re-analysed the data of the original study. They concluded that contrary to Brownlee and Hirsch assertion, the difference in retinopathy in those with similar HbA1c in the two groups have been largely due to A1c and not the groups assignment[447]. Interestingly, however, the concept of A1c variability was raised by Kilpatrick *et al* who found it to be strongly correlated with the risk of development and progression of retinopathy and nephropathy in a multivariate Cox regression. For every 1% increase in A1c SD, there was an increase in the hazard ratio (HR) for retinopathy of 2.26 [95% CI 1.63-3.14], $P < 0.0001$) and nephropathy (1.80 [1.37-2.42], $P < 0.0001$)[472]. Monnier *et al* have criticised the methodology adopted [470] indicating that the SD around the mean is unlikely to yield major fluctuations in blood glucose and that only the gold standard "MAGE", which requires CGMS, could be relied on to measure accurately major fluctuations in blood glucose[477]. However, a further analysis by Kilpatrick *et al* of the 7 point quarterly blood glucose measurements in the DCCT confirmed that only the mean blood glucose and A1c were predictors of retinopathy and nephropathy at year 4 follow-up of the EDIC while the MAGE and SD were not[471].

Further evidence contradicting Monnier and Ceriello's findings was the failure to establish a link between glycaemic variability in T1DM and urinary oxidative stress markers by DeVries *et al*. Wentholt *et al* could not confirm a relationship between glycaemic variability (MODD, MAGE and $CONGA_{-1}$) and urinary 8-iso-PGF2α (measured by HPLC TMS)in 25 patients with T1DM[478]. The same group have repeated the study by Monnier *et al* in T2DM but they were unable to reproduce a relationship between glucose variability and oxidative stress ($r = 0.12$; $P = 0.53$)[474, 479]. More recently, Monnier *et al* observed that urinary 8-iso-PGF2α in patients with diabetes on insulin (both type 1 and 2) was significantly lower than those with T2DM on OHA, concluding that insulin has an inhibitory effect on the generation

of ROS[480]. It could be debated that their conclusion is flawed, since being on insulin meant high variability and lower oxidative stress markers (OSM) secretion, which points to factors other than glycaemic variability impacting the generation of ROS. Also in this study plasma insulin was not measured to objectively define that correlation and inhibitory effect of insulin. Finally, one of the criticisms to this group methods is the analysis of 8-iso-PGF2α using radioimmunoassay which can detect unrelated cross reacting molecules[474]. A more recent cross-over interventional trial comparing inhaled meal time insulin vs. basal insulin glargine showed significant improvement in the urinary 15(S)-8-iso-PGF2α in both groups although there was no improvement in glycaemic variability as measured by the MAGE and MODD. Again, there did not seem to be a correlation between the 15(S)-8-iso-PGF2α and glycaemic variability[481].

Kilpatrick *et al* cite in their debate evidence from other studies which fail to show a special value of postprandial hyperglycaemia beyond fasting hyperglycaemia in predicting cardiovascular mortality[482, 483]. In a 5.2 year follow-up of subjects with IFG, IGT and T2DM, although both IFG and IGT were associated with higher HR for mortality, only IFG was an independent predictor of cardiovascular death[484]. In another widely criticised interventional trial for the lack of power, two different treatment strategies with basal insulin or prandial insulin have failed to show a significant difference in the rate of cardiovascular mortality [483, 485, 486]. A visual evaluation of the 7 point glucose profile collected during the study showed a difference in glycaemic variability in favour of the prandial insulin group; questioning whether glycaemic variability is a contributor to cardiovascular death in this negative study[474].

With regards to the glycaemic variability in critically ill patients (adults and paediatrics), it has been shown that ICU patients without diabetes with glycaemic variability have increased mortality[460, 487]. However glycaemic instability (SD) bear no relation to survival in a subset of patients with diabetes, probably indicating adaptation of diabetic subjects to glycaemic variability [460, 474]. Large studies on non diabetic subjects in ICU have shown contradictory findings of intensive insulin therapy in ICU patients and mortality with one showing improved mortality (Van den Berghe *et al*) and the other showing excess mortality (NICE-SUGAR)[461, 467]. Two factors were implicated in these differential findings; the improved glucose variability in the intensive group in the first study (SD of early morning glucose) and the high incidence of hypoglycaemia in the second[474]. However, as mentioned earlier, the improved survival in the intensively treated ICU patients from the two trials by van den Burgh were attributed to improved mean blood glucose and not improved glycaemic variability[438, 463].

It is therefore not unreasonable to conclude that there is evidence of glucose variability being a predictor of cardiovascular mortality in non-diabetic critically ill patients although other factors such as hypoglycaemia as a result of intensive glycaemic control could offset this theoretical benefit. On the other hand, there is no convincing evidence that glycaemic variability predicts or causes macrovascular outcomes in patients with diabetes[474].

1.6 Hypotheses examined by the thesis:

In the subsequent chapters, the thesis will examine in four clinical studies four hypotheses:

1- Is the bioavailability of EPA from natural fish oil triglyceride significantly different from that of reconstituted triglyceride, enzymatically synthesised triglyceride, diglyceride or monoglyceride forms obtained after chemical modification of natural fish oil?
2- Whether the bioavailability of the EPA from ethylester fish oil can be improved when it is encapsulated by exines?
3- Whether there is a significant difference between peripheral glucose disposal two hours after a single dose non-encapsulated and exine-encapsulated oleic acid in a four hour hyperinsulinaemic euglycaemic clamp experiment?
4- Is the 24 hours urinary excretion of 8-Isoprostane F2α significantly different between the three glycaemic states; euglycaemia, hyperglycaemia and variable glycaemia in healthy volunteers?

Chapter 2: Methods

2.1 Laboratory methods and reagents

2.1.1 Bioavailability studies:

2.1.1.1 Fish oil supplements bioavailability

All baseline serum samples were taken after overnight fast. Serum was then taken at 2, 4, 6, 8 and 24 hours following the fish oil intake. Samples were separated by centrifugation at 2000 g for 15 min at 4 ℃, and the aliquots stored at –80 °C within 1 h of collection, before shipping for batch analysis for fatty acids in total serum lipids. Serum was withdrawn into a 13 x 100 mm open top test tube. Water was added to make up a volume of 200 ul and 25-50 ug of 17:0 PC was added as internal standard. Then 2ml of chloroform/methanol and vortex was added for 15 seconds [488, 489] and 200 ul water and slightly vortex sample was added for approximately 3 seconds. The sample was then centrifuged for 5 minutes at 3000 rpm. Most of the lower Chloroform phase was removed with a pipette into a 4ml glass vial before drying down using nitrogen. Fatty Acid methyl esters were prepared as described before[490] and before being analysed by Varian Model 3400 using a 60m X 0.32mm ID X 0.15 micron film thickness DB-23 column.[491]

2.1.1.2 Bioavailability of ethyl ester fish oil with exines:

All baseline serum samples were taken after overnight fast. Serum was then taken at 2, 4, 6, 8 and 24 hours following the fish oil intake. Samples were separated by centrifugation at 2000 g for 15 min at 4 ℃, and the aliquots stored at –80 °C within 1 h of collection, before shipping for batch analysis for fatty acids in total serum lipids by the Nutrition Group, Institute of Aquaculture, University of Stirling, Stirling UK, as described before[492]. 0.5 ml serum was extracted by the Folch *et al*. method [488], using chloroform/methanol (C/M; 2:1

v/v). The extracted lipid was dissolved in 0.8 ml of C/M, 2:1 v/v and dried under nitrogen in a pre-weighed glass vial, was and desiccated for 16h. Final lipid extracts were re-suspended in C/M (2:1 v/v) + 0.01% (w/v) butylated hydroxytoluene (BHT), at a concentration of 10 mg/ml and stored at -70 ºC.

Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of 0.5 mg of total lipid and 50 µg of 17:0 internal standard in 2 ml of 1% (v/v) H_2SO_4 in methanol at 50 ºC overnight[493]. Samples were neutralised with 2% Potassium Bicarbonate ($KHCO_3$) and extracted twice with 5 ml isohexane/diethyl ether (1:1 v/v) + BHT and finally dissolved in 0.3 ml of isohexane prior to FAME analysis.

Measurement of serum fatty acids

FAME were separated and quantified by glc (Fisons 8160, Carlo Erba, Milan, Italy) using a 60 m × 0.32 mm × 0.25 µm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, England). Hydrogen was used as carrier gas (flow rate of 4.0 ml/min) and the temperature programme was from 50 to 150 ºC at 40 ºC/min then to 195 ºC at 2 ºC/min and finally to 215 ºC at 0.5 ºC/min. FAME were identified using well characterised in house standards and commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England). Blood was withdrawn after 30 minutes and examined under a confocal microscope to investigate for the presence of exines, which are naturally fluorescing

2.1.2 Glycaemic variability and oxidative stress markers:

8-iso-PGF2α was measured in a 24 hour urine collection post-clamp and expressed in ng/24h. To allow comparison of 8-iso-PGF2α levels between the pre-clamp random urine sample and post-clamp 24h urine collection, 8-iso-PGF2α concentration was expressed as a ratio to

creatinine (pg/mmol creatinine). 8-iso-PGF2α was measured by a urinary isoprostane Enzyme immunoassay (EIA) kit supplied by Oxford Biomedical Research (Oxford, US). This assay is a competitive enzyme-linked immunoassay based on competition between 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$-horseradish peroxidase for a polyclonal goat anti-8-iso-$PGF_{2\alpha}$ antibody. Samples were analysed without purification according to the manufacturer's instructions.

2.2 Statistical Analysis:

2.2.1 Fish oil Supplements Bioavailability:

Area under the curve ($AUC_{0\text{-}24h}$) was used to determine the bioavailability of eicosapentaenoic acid (EPA) from the different fish oil supplements. The mean AUC for EPA for each fish oil supplement was calculated using the linear trapezoid method, baseline levels were normalised to 0. Since the subjects number was small (n=7), potentially jeopardising the strong assumption of normality required for a parametric test, a non-parametric statistical test, the Wilcoxon signed rank test, was used to compare the AUCs of reconstituted triglycerides and natural triglyceride versus other fish oil supplements using SPSS software version 15.

2.2.2 Bioavailability of EPA ethyl ester with and without exines

The AUC for the EPA was measured as described above. The maximum concentration (T_{max}) was calculated visually. Both the AUC and T_{max} for the ethyl ester with and without the exines were compared using paired sample t-test.

2.2.3 Oleic acid effect on glucose disposal with and without exines

The glucose metabolised (M) reflecting total insulin resistance during different time intervals of the euglycaemic hyperinsulinaemic clamps before and after each intervention (oleic acid

alone and oleic acid with exines) were measured as described by Defronzo *et al[166]*. M= INF-SC where INF is the glucose infusion rate and SC is the space correction (difference between plasma glucose at the start and end of the M period) and all are expressed in mg/ (kg. min)[166]. A comparison of the change in M after oleic acid or exine-loaded oleic acid was made using paired t-test.

2.2.4 Glycaemic variability and its effect on urinary isoprostanes:

The results of 24 hours urinary isoprostanes lacked normality and therefore a non-parametric test, Kruskal-Wallis H, was selected to statistically analyse the data using SPSS 16.

2.2.5: Sample size calculation:

2.2.5.1 Sample size calculation for oleic acid exine encapsulation study:

This study is a pilot study as there are no data available from a similar study to do the power analysis. However, based on the study by Boden et al [494] the following analysis was performed using N-Query software. Powered specifically for the change in hepatic glucose output the minimum difference worth detecting observed difference was 0.80 mg. Kg^{-1}. Min^{-1}, estimated within a group SD was 0.23; therefore, for 80% power and a significance level of 5%, a sample size of 6 was calculated. At the conclusion of the study the data will allow a proper power analysis to be determined in the future.

2.2.5.2: Sample size calculation for the glycaemic variability study:

A power calculation of sample size using the findings of McGwan et al[495] was made using N-Query software. Powered specifically for the change in isoprostanes F2α the minimum difference worth detecting/observed difference in the hyperglycaemic group from that study was 8.89 ng/mg, estimated within a group SD was 0.71; Therefore, for 90 % power and a significance level of 5%, a total sample size of 4 was calculated. If a high (~50%) drop out rate is to be adjusted for then a total of 6 patients would need to be recruited. To increase the power to detect difference between the variable group and each of the other two groups (euglycaemia and hyperglycaemia), which was not performed before, a target number of 10-12 subjects was aimed for.

2.3 Ethics

All subjects signed a written consent prior to enrolling in the studies which were approved by the Hull and East Riding Local Research Ethics Committee and South Humber Local Research Ethics Committee.

2.4 Regulatory approval

Since the fish oil was considered a food supplements and not a medicinal product, there was no need to seek approval from the Medicines and Health products Regulatory Agency of the United Kingdom.

2.5 Sponsorship:

Sponsorship for all studies was granted by Hull and East Yorkshire Hospitals NHS Trust and permission to conduct the trials was sought from the Research and Development Department.

Chapter 3: The bioavailability of eicosapentaenoic acid from reconstituted triglyceride fish oil is higher than that obtained from the triglyceride and monoglyceride forms.

3.1 Introduction

Omega-3 long chain polyunsaturated fatty acids (LCPUFAs) have been increasingly recognised to modify inflammatory and autoimmune diseases like atherosclerosis, rheumatoid arthritis, asthma, Alzheimer's disease among other conditions,[496-498] although Cardiovascular disease modifying effects are the most well known. [499-501] Recently, the nutritional requirements for n-3 fatty acids has shifted to their adequate intake in order to reduce disease risk rather than to correct or prevent nutritional deficiency.[78] This was illustrated by the JELIS study, in which eicosapentaenoic acid (EPA) added to statins for 4.6 years in hypercholesterolaemic Japanese resulted in 19% relative risk reduction in the rate of major cardiovascular events.[502] Currently, there is an omega-3 prescription medicinal product in the market (i.e. Omacor®/Lovaza®) that is licensed as adjuvant treatment in secondary prevention after myocardial infarction and to reduce serum triglycerides in subjects with hypertriglyceridaemia. EPA and docosahexaenoic acid (DHA) are the most important LCPUFA and can only be obtained from marine rich diet. Their precursor, alpha linolenic Acid (ALA), on the other hand is widely available in plant oil,[503] but the conversion of ALA to DHA and EPA is very inefficient.[80] Therefore, the consumption of fish is recommended to achieve adequate intake of EPA and DHA, but the amount of fish required to achieve the therapeutic benefits derived from EPA and DHA may be offset by the poor palatability. In the UK it is recommended to eat two to three portions of fish a week and each portion is thought to provide between 200 and 500 mg of EPA and DHA, respectively. Supplementation with fish oil containing high purity concentration of DHA and EPA with low levels of contaminants, like dioxins or methylmercury, which might be found in oily fish [504] is one method used to raise plasma levels of EPA and DHA. An alternative strategy is to optimise the absorption and bioavailability of EPA and DHA to attain a higher plasma

concentration of EPA and DHA.

DHA and EPA supplements can be given as free fatty acids, natural and reconstituted triglycerides and ethyl esters. The natural fish oil triglycerides (NTG) correspond to 100 % triglycerides whereas chemically reconstituted triglycerides (RTG), as defined in the European Pharmacopeia[505] are a mixture of monoglyceride (MG), diglyceride (DG) and triglycerides with triglyceride being the main component (> 60 %). Also in NTG, DHA is mainly located in the position sn-2 while EPA is in position sn-1and sn-3 which may have influenced their bioavailability.[506] In the case of RTG, EPA and DHA are randomly distributed in the three positions of the glycerol backbone.

The bioavailability of RTG of LCPUFA is not well studied and their absorption may well be altered by their incorporation into different forms of fat molecules. Studies on the different components of RTG in rats showed contradictory results. While one study showed n-3 DHA fatty acid from (DG) and (MG) to be better absorbed than triglyceride and ethyl ester [114], another one showed no substantial differences in DHA bioavailability between DHA-DG (31.8 %1,2-DG, 67.8 % 1,3-DG), DHA triglyceride (94 %) and a mixture of DHA-DG and triglyceride (69 % triglyceride, 8.2 % 1,2-DG, 22.6 % 1,3-DG).[507] However, there is a lack of evidence in man.

Aim of the Study:

Therefore, in order to study the effect of changing location of omega-3 fatty acids in the glycerol back bone on the bioavailability of EPA and DHA, this study was undertaken to compare the short term bioavailability in healthy humans of different fish oils given as NTG,

RTG and the individual components of RTG, i.e. MG, DG and enzymatically synthesised triglycerides (ETG). ETG is produced enzymatically to yield high triglyceride content (Figure 6).

3.2 Materials and Methods

Subjects and methods

This was a double blind cross over trial. Exclusion criteria were chronic or recent illnesses, regular concomitant or over the counter medications, allergy to fish, and pregnancy. Ethical approval for the study was obtained from The Hull & East Riding and South Humber Local Research Ethics Committees, UK. Twelve healthy volunteers were recruited from an advert in the local paper. All subjects signed a consent form prior to taking part. They had a dietary counselling by a dietician to avoid fish or omega 3 fatty acid containing diet one week before and during the course of the trial. Coffee, flax seed and alcohol were avoided a day prior, during and a day after each visit. A 7 day run in period was followed by 5 visits to take the fish oil supplements. There was at least 7 days wash-out period between each fish oil supplement. Subjects were asked to fast for 10 hours prior to each visit. At the start of each visit a polyethylene catheter was inserted and baseline blood samples taken for fatty acids analysis. This was followed by the intake of a random fish oil supplement and repeated blood sampling at times 2,4,6,8 and 24 hours. A fish-free, low fat lunch was provided after time 4 hours blood sampling. Subjects were free to eat another meal after the 8 hour blood sample provided they adhered to the above instructions. Subjects fasted for 10 hours prior to the final 24 hour blood sample. Anthropometric measures, blood pressure and pulse rate were taken at the first visit. Samples were separated by centrifugation at 2000 g for 15 min at 4 ℃, and the aliquots stored at –80 °C within 1 h of collection, before shipping for batch analysis for fatty acids in total serum lipids.

Fish oil

The fish oil supplements were provided by Croda Europe Ltd, Goole, UK, shipped in dark containers in amber vials frozen at 0-4 ºC and ready to be thawed before consumption. Each vial had 4.5 grams of fish oil. Randomisation of the fish oils supplements was done by Croda Europe and each supplement had a code (A-E) and given in a randomly to participants. The investigators, participants and analysing lab were blinded to the type of fish oil supplements throughout the trial. Blinding of fish oil supplements was provided by Croda and the decoding was done before data analysis. It is worth emphasizing that the chemically RTG are a mixture of mono-, di- and triglycerides where the triglyceride is the main component, whereas the ETG are triglycerides with almost no MG or DG (Table 5). The distribution of the molecular species across the range of supplements was established by gel permeation chromatography. The supplements involved in the trial consisted of NTG – sardine and anchovy oil and the four other molecular species obtained by modification of this batch of oil. The fatty acid composition of the five species is therefore similar across the product range under test and is detailed in Table 4. RTG were obtained by glycerolysis and also served as the feed to obtain MG and DG by thin film distillation under high vacuum. Finally, ETGs were obtained by enzymatic glycerolysis.

EPA analysis

EPA analysis was performed by Nutrasource Diagnostics Inc, University of Guelph Research Park, Ontario, Canada. Serum was withdrawn into a 13 x 100 mm open top test tube. Water was added to make up a volume of 200 ul and 25-50 ug of 17:0 PC was added as internal standard. Then 2ml of chloroform/methanol and vortex was added for 15 seconds [488, 489]

and 200 ul water and slightly vortex sample was added for approximately 3 seconds. The sample was then centrifuged for 5 minutes at 3000 rpm. Most of the lower Chloroform phase was removed with a pipette into a 4ml glass vial before drying down using nitrogen. Fatty Acid methyl esters were prepared as described before[490] and before being analysed by Varian Model 3400 using a 60m X 0.32mm ID X 0.15 micron film thickness DB-23 column.[491]

Statistical analysis

Area under the curve ($AUC_{0\text{-}24h}$) was used to determine the bioavailability of EPA from the different fish oil supplements. The AUC for EPA for each fish oil supplement was calculated using the linear trapezoid method, baseline levels were normalised to 0. Since the subjects number was small (n=7), potentially jeopardising the strong assumption of normality required for a parametric test, a non-parametric statistical test, the Wilcoxon signed rank test, was used to compare the AUCs of RTG and NTG versus other fish oil supplements using SPSS software version 15. The median, 98.4% confidence interval (CI) and quartile range were calculated using Microsoft Excel® and analyse-it® softwares.

3.3 Results

The clinical characteristics of the seven healthy volunteers are summarised in Table 6. The mean age, blood pressure, BMI and waist circumference were 33 years, 123/76 mm Hg, 23 and 76 cm, respectively. Two subjects withdrew due to nausea and intolerance of the first fish oil supplement while 3 others did not finish the trial citing other commitments as a cause for withdrawals, see Figure 7. Figure 8 shows the median concentration of EPA in mg/100 ml of serum plotted against the 24 hour time for the 5 different fish oil supplements. A significant difference was found between the calculated median AUC of EPA from RTG (Median=31.3;

98.4% CI= 23.5-42.6) and that of the ETG (Median=10.7; 98.4% CI= -8-29.5) and MG (Median=16.8; 98.4% CI = -2.8-30.4), z = -2.36 and -2.19, respectively, p<0.05. There was no significant difference in the median AUC of RTG when compared with those obtained from DG and NTG. The median AUC of EPA taken from NTG (Median=26.3, 98.4% CI= 8.4-40.7) was significantly higher than that obtained from ETG, z = -2.19, p<0.05, Figure 9.

3.4 Discussion

These data show that the highest median area under the concentration curve for EPA was from RTG. Furthermore, when this was compared to the other median AUCs we found it to be significantly higher than MG and ETG. There was no significant difference in the median AUC of RTG when compared with those obtained from DG and NTG. To the best of our knowledge this is the first report in humans on the short-term bioavailability of the different components, i.e. MG, DG and ETG, of reconstituted triglycerides.

The data on the position of the LCPUFA (including EPA) in the glyceride molecule in the different supplements was not available though the process of manufacture was likely to place them randomly. The higher bioavailability of RTG compared with ETG may be due to the RTG components (DG and MG) facilitating the intestinal phase digestion and acting emulsifying agents in the stomach [508] and thereby increasing the absorption and bioavailability from RTG.[107] On the other hand, in the case of NTG versus ETG, since both forms are almost 100% glycerides and the only difference is that EPA is mainly located in positions sn-1and 3 in NTG whereas it is randomly distributed in all three glycerol positions in the ETG, this implies that short-term bioavailability of EPA in the positions sn-1 and 3 of the glycerol backbone is higher than in sn-2. This is possibly related to the accessibility of sn-1 and 3 fatty acids to pancreatic lipase compared to the inaccessibility of sn-2 fatty acids although others have shown that after a quick absorption of LCPUFA in the

sn-2 position of TG in the first few hours, the overall 24 hour lymphatic appearance in the rats was not affected by the intramolecular position of the LCPUFA[509-511]. It remains unclear why the RTG absorption was preferentially better than the MG but not the DG, since both MG and DG should have more or less the same random distribution of LCPUFA in the triglyceride molecule as they were subjected to a similar conversion process (Figure 6). This study also confirms that serum EPA concentration supplemented as triglyceride reaches a peak a few hours after supplementation[107, 512] and concurs with other reports showing that EPA from reconstituted triglycerides are not less absorbed than those taken from natural fish oil.[513]

It is well known that prolonged intervention with fish oil (EPA and DHA) lowers postprandial triglyceride levels, while an acute intake of EPA with a mixed fat meal is not associated with a change in postprandial triglyceridaemia. This study was designed to compare the bioavailability of EPA from different fish oils formulas prepared and given as a single test dose in the fasting state and is not comparable.[514] Furthermore, a previous study reported the percentage of EPA fatty acid in chylomicrons after 6 hours of fish oil ingestion to be comparable to the EPA in total lipids taken after 24 hours of the same EPA rich test meal; 19. 7 vs. 22.6.[515] Therefore we used the EPA in serum lipids instead of EPA of chylomicrons TG, the latter being more precise in the first few hours when a fat rich meal is given, which was applicable in the study here.

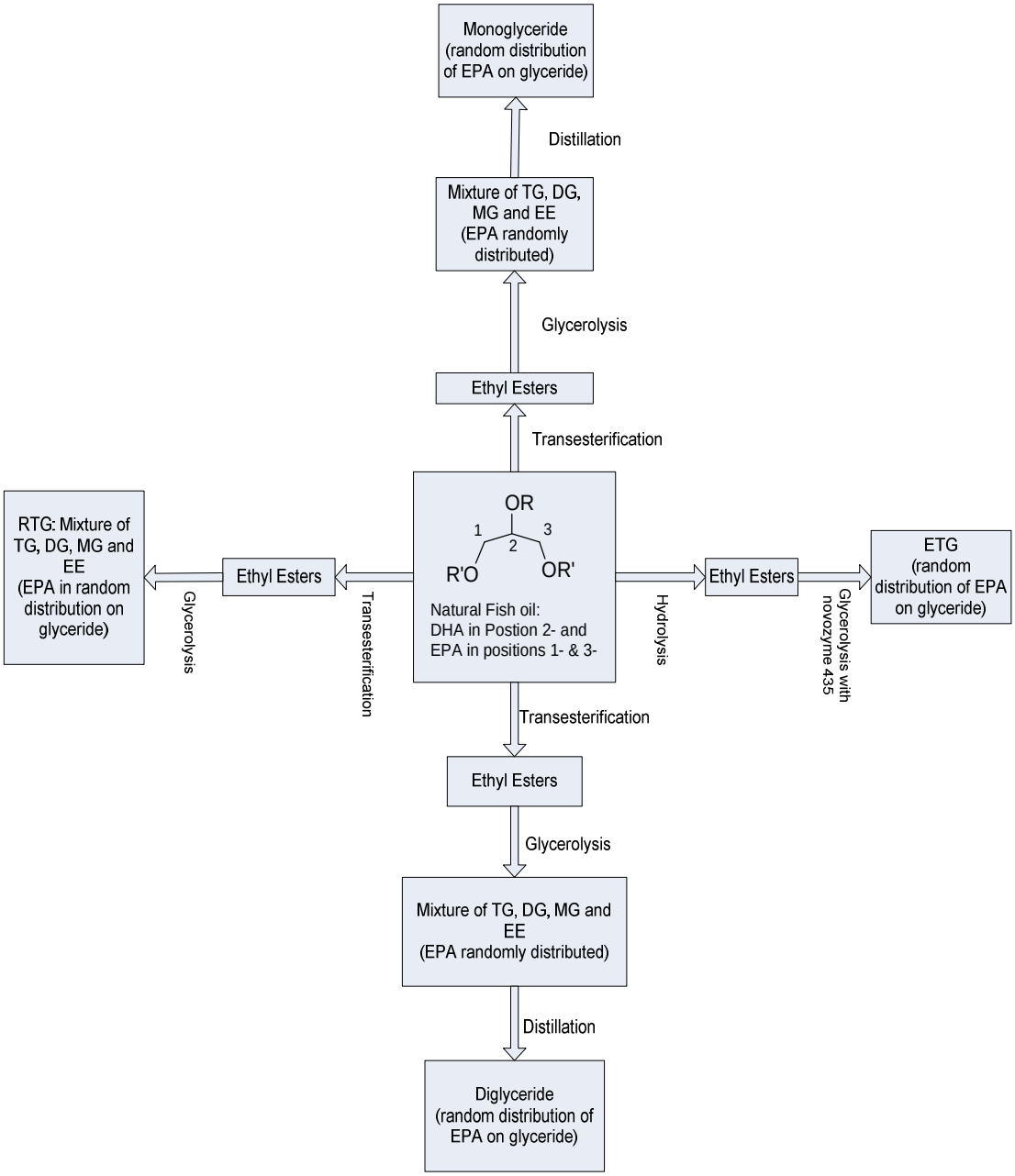
A limitation of our study was that the concentration of EPA and DHA used were lower than in other nutritional products, where higher concentrations of EPA/DHA are usually incorporated in the supplement, though the weight of the administered supplements (4.5 grams) meant that the total quantity was comparable. This concentration level was needed to have a direct comparison with the natural fish oil and thus establish the effect of the location

of the omega-3 fatty acids on the glycerol backbone on its bioavailability. Another limitation of this study was the lack of data on the position of the LCPUFA in the triglyceride in the different supplements, although this was likely to be random, this made interpretation of some of the data difficult.

This novel data is the first report in humans on the short-term bioavailability of the different components of MG, DG and ETG, of RTG. The results show that the EPA bioavailability over a 24 h period from chemically RTG i.e. the mixture of MG, DG and ETG where the triglyceride is the main component, was better than that obtained from ETG and MG alone, but was not significantly different from that of DG and NTG. These data may have important implications for the optimisation of LCPUFA absorption for their potential health benefits.

Source of funding: The University of Hull has received unrestricted grant from Croda Europe Ltd in support of conducting this research.

Figure 6: The process of conversion of natural fish oil to the 4 other fish oil supplements.



NTG: Natural Triglyceride, RTG: Reconstituted Triglyceride, DG: Diglyceride, ETG: Enzymatically synthesised Triglyceride and MG: Monoglyceride.

Figure 7: Flow chart of the progress through phases of double blind cross over (five scheduled visits, one week a part) of the EPA bioavailability from different fish oils.

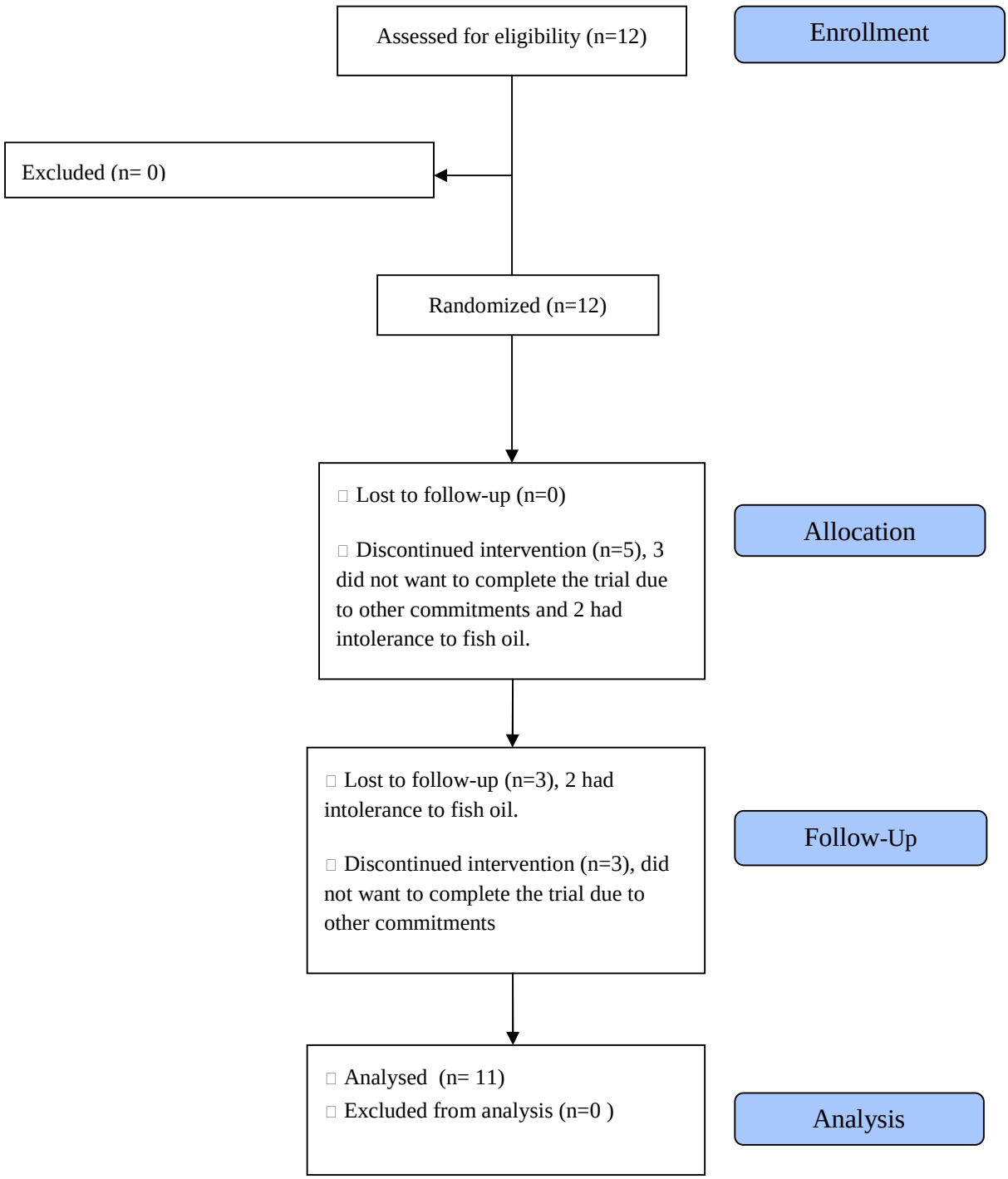
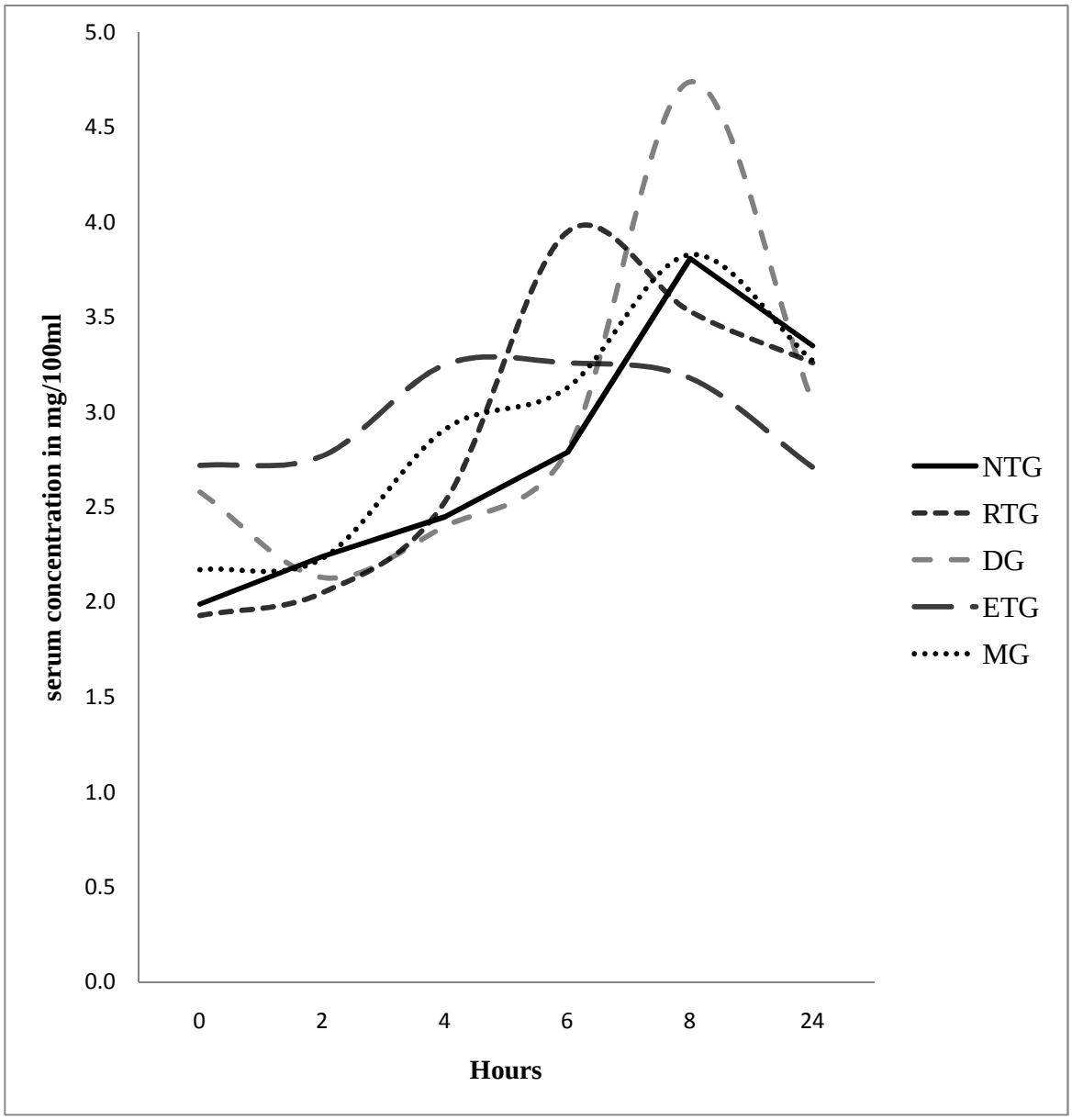
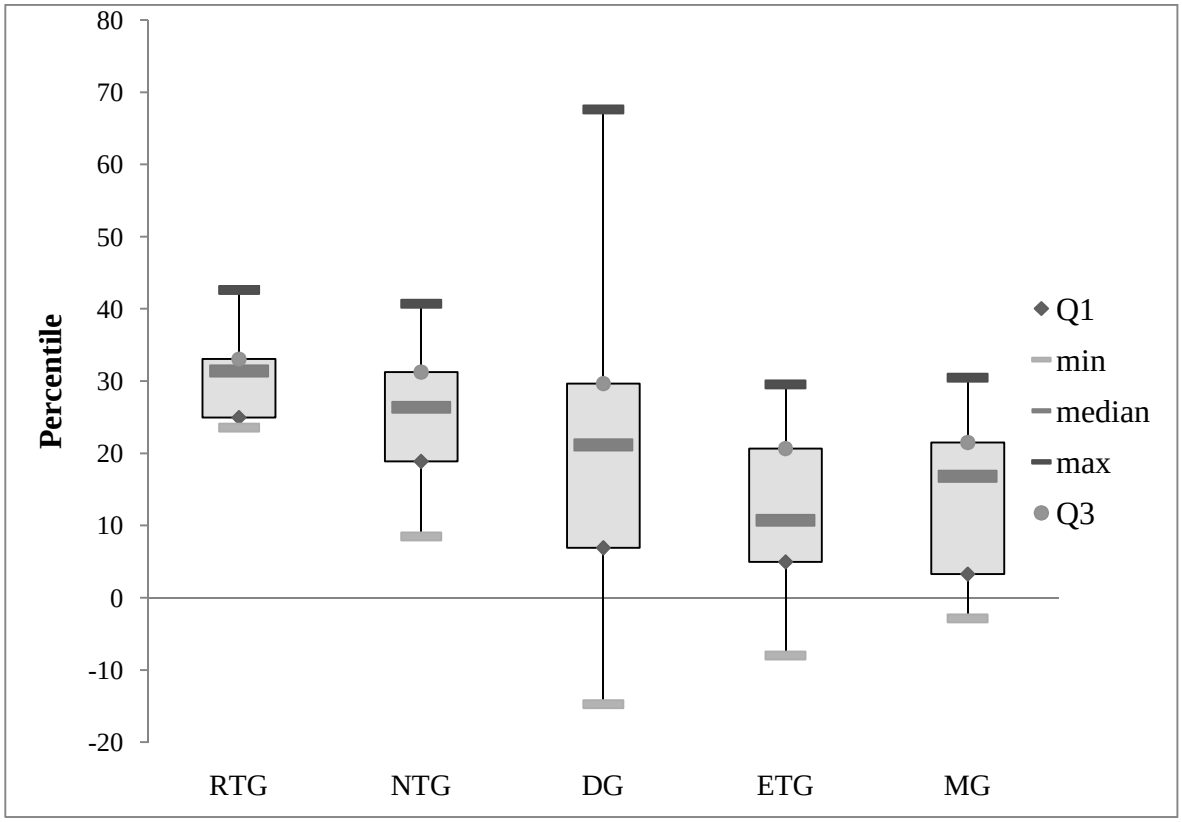


Figure 8: The median serum concentration in mg/100ml of different fish oils EPA plotted against time.



NTG: Natural Triglyceride, RTG: Reconstituted Triglyceride, DG: Diglyceride, ETG: Enzymatically synthesised Triglyceride and MG: Monoglyceride.

Figure 9: Box plot showing the median and interquartile ranges of $AUC_{0\text{-}24h}$ of the EPA from different fish oil supplements.



NTG: Natural Triglyceride, RTG: Reconstituted Triglyceride, DG: Diglyceride, ETG: Enzymatically synthesised Triglyceride and MG: Monoglyceride. Q1: 25th percentile, min: lower limit of 98.4% confidence interval of the median. Max: upper limit of the of the 98.4% confidence interval. Q3: 75th percentile.

Table 4: Typical fatty acid composition in fish oils provided by Croda Europe Ltd.

Fatty acid	Percentage
14:0	7.3
16:0	17.2
16:1(n-7)	8.6
16:2(n-4)	1.8
16:3(n-4)	2.3
16:4(n-1)	2.4
18:0	3.4
18:1(n-9)	9.6
18:1(n-7)	2.9
18:3(n-3)	0.8
18:4(n-3)	2.3
20:4(n-6)	1.1
20:4(n-3)	0.7
20:5(n-3)	**19.8**
22:1	1.6
22:5(n-3)	1.8
22:6(n-3)	**7.9**

Table 5: Molecular species in the different fish oils used in the bioavailability study of EPA from different fish oils.

Fish oil	TG (%)	DG(%)	MG(%)	FFA(%)	EE(%)
NTG	99.0	-	-	1.0	-
RTG	61.2	32.9	2.8	-	3.1
MG	-	5.7	94.3	-	-
DG	10.0	90.0	-	-	-
ETG	96.8	2.8	0.1	-	0.3

NTG: Natural Triglyceride, RTG: Reconstituted Triglyceride, DG: Diglyceride, ETG: Enzymatically synthesised Triglyceride and MG: Monoglyceride.

Table 6: Characteristics of healthy participants in the study of bioavailability of EPA from different fish oils supplements.

Total Number (n= 7)	
Female/male	4:3
Mean age in years (SD)	33 (8.4)
Mean systolic blood pressure in mm Hg (SD)	123 (7)
Mean diastolic blood pressure in mm Hg (SD)	76 (3)
Mean BMI in $kg/m^{2,}$ (SD)	23.2 (3)
Mean waist circumference in cm (SD)	78.6 (6)

mm Hg: millimetre of mercury, BMI: body mass index, Kg: Kilogram and m^2: square metre

Chapter 4: Enhanced bioavailability of eicosapentaenoic acid from fish oil after encapsulation within plant spore exines as microcapsules.

4.1 Introduction:

Eicosapentaenoic acid (EPA) and docosahexaenoic acid, the main long chain polyunsaturated fatty acids (LCPUFA), can only be obtained from a fish and shellfish rich diet. Recent trials have shown that EPA in the form of ethyl ester added to statins in hypercholesterolaemic Japanese resulted in 19% relative risk reduction in major cardiovascular events [502]. Instead of being taken to prevent nutritional deficiency they are now being taken to prevent diseases with an inflammatory pathology, including cardiovascular diseases[78]. For example, AMR101 is prescription grade, ultra-pure (>96%) ethyl ester of eicosapentaenoic acid (ethyl-EPA; an omega-3 fatty acid). AMR101 is being positioned as a best-in-class prescription medicine for treating patients with high or very high triglycerides[516]. One strategy to raise plasma concentration of LCPUFA is to optimise their absorption and bioavailability.

Microencapsulation has been used to mask unpleasant taste in food sciences as well as to protect against light and airborne oxidation [517, 518]. Pollen and plant spores, from mosses and ferns have an outer layer skeleton known as the exine that is composed of sporopollenin[519, 520]. Exine microencapsulation technology has been shown to provide excellent taste masking for fish oils[303], they have been investigated as a contrast agent[521], and attempts have been made to introduce them as a novel method of oral delivery of substances into the blood stream as opposed to the parenteral route[522].

Aim of the Study:

In this study we have investigated whether encapsulating the ethyl ester form of fish oil with exine microcapsules extracted from readily available and renewable *Lycopodium clavatum* spores, can enhance the bioavailability, measured by area under the curve, of EPA delivered as ethyl ester alone.

4.2 Experimental procedure:

This was an open labelled study. Six healthy volunteers without concomitant illnesses or medications were recruited from an advertisement for healthy volunteers in Hull University and Hull Royal Infirmary. The study protocol was approved by the Hull and East Riding Research Ethics Committee. All subjects received dietary counselling by an academic dietician to avoid fish or omega-3 fatty acid intake in their diet two weeks before and during the course of the trial. Coffee, flax seed and alcohol were avoided a day prior, during and a day after each visit. A run in period of 1 week was followed by 2 visits with 3 weeks between-visits wash-out period. Each subject ingested 4.6 grams of fish oil containing 20% of EPA in the form of the ethyl ester at each visit. In the first visit the fish oil was given in the form of a liquid immediately after defrosting. In the second visit the fish oil was encapsulated into exines and the subsequent powder was ingested. Blood samples were taken at baseline (prior to ingesting the fish oil preparations) for fatty acids and lipid analysis and again at 2, 4, 6, 8 and 24 hours from ingesting the fish oil for fatty acids analysis. Serum was instantly separated by centrifugation at 2000 g, and stored at –80 ºC before batch analysis of total serum fatty acid compositions by the Nutrition Group, Institute of Aquaculture, University of Stirling, Stirling UK, as described before[492]. 0.5 ml serum was extracted by the Folch *et al*. method [488], using chloroform/methanol (C/M; 2:1 v/v). The extracted lipid was dissolved in 0.8 ml of C/M, 2:1 v/v and dried under nitrogen in a pre-weighed glass vial, was and desiccated for 16h. Final lipid extracts were re-suspended in C/M (2:1 v/v) + 0.01% (w/v) butylated hydroxytoluene (BHT), at a concentration of 10 mg/ml and stored at -70 ºC.

Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of 0.5 mg of total lipid and 50 µg of 17:0 internal standard in 2 ml of 1% (v/v) H_2SO_4 in methanol at

50 ºC overnight[493]. Samples were neutralised with 2% $KHCO_3$ and extracted twice with 5 ml isohexane/diethyl ether (1:1 v/v) + BHT and finally dissolved in 0.3 ml of isohexane prior to FAME analysis.

Measurement of serum fatty acids

FAME were separated and quantified by glc (Fisons 8160, Carlo Erba, Milan, Italy) using a 60 m × 0.32 mm × 0.25 µm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, England). Hydrogen was used as carrier gas (flow rate of 4.0 ml/min) and the temperature programme was from 50 to 150 ºC at 40 ºC/min then to 195 ºC at 2 ºC/min and finally to 215 ºC at 0.5 ºC/min. FAME were identified using well characterised in house standards and commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England). Blood was withdrawn after 30 minutes and examined under a confocal microscope to investigate for the presence of exines, which are naturally fluorescing

Fish oil supplements were provided by Croda Europe, Goole, UK. Each vial had 4.6 grams of fish oil containing 20% EPA in the form of its ethyl ester. They were shipped in dark containers and kept in a –20 ºC freezer until ready for defrosting in visit 1. In visit 2, the defrosted oil was encapsulated with 4.6 grams of exines no more than 24 hours prior to ingestion and the dark container was filled with nitrogen to prevent oxidation. Exines extracted from *Lycopodium clavatum* spores were supplied by Sporomex Ltd, UK, and were prepared as detailed previously[303]. Microencapsulation was performed by mixing exines with oil (1:1 weight for weight) by gently stirring to form a homogeneous paste that was then subjected to a vacuum (ca. 10hPa) for 2h to facilitate passive loading of oil into the particles through the nano-porous sporopollenin walls.

Area under the curve ($AUC_{0\text{-}24h}$) was used to determine the bioavailability of EPA from the different supplements. The mean $AUC_{0\text{-}24h}$ for EPA was calculated using the linear trapezoid method and baseline levels were normalised to zero. We also observed visually the time of the maximum concentration (T_{max}). Comparisons of mean AUCs and T_{max} with and without exines were made using paired sample t-test *via* Microsoft Excel® and analyse-it® softwares.

4.3 Results:

All subjects completed the study without adverse effects, see Figure 10. The two male and four females' demographics are summarised in Table 7. The mean baseline of EPA percentage to total fatty acids in the six subjects was comparable to that reported in another study with healthy volunteers; M=0.69, SEM= 0.04% vs. M= 0.64, SEM=0.08% respectively[523]. There was no significant difference between the baseline concentration of EPA (mg/100ml) in the first visit (M=2.15, SD=0.6) and the second visit (M=2.0, SD=0.6, p=0.49). The mean AUC of EPA from ethyl ester with exine (M=19.7, 95% CI=12.7-24.3, SD=4.3) was significantly higher than that obtained from ethyl ester without exines (M=2, 95% CI=0.5-3.5, SD=1.4), difference between means= 17.7, 95% CI=13.4-22.0, $p<0.01$. When the mean concentration of EPA in serum over time was plotted, after subtracting the mean sera concentrations of the respective time from the mean baseline concentration, it was evident that microencapsulation in exines had significantly enhanced the EPA absorption as reflected by the serum concentration (Figure 11). The mean Time of maximum (T max) concentration for EPA when fish oil was encapsulated with exines (M=7.6 hours) was not different from the maximum concentration without exines (M=6.8, p=0.4), results not shown. Confocal microscopy (Bio-Rad Radiance 2100 laser scanning microscope equipped with Ar (488nm), Green HeNe (563nm) and Red diode (637nm) laser lines connected to a Nikon TE-2000E inverted microscope) showing an empty fluorescent exine before ingestion and an

apparently intact exine in blood plasma after ingestion (Figure 12). Micrographs of oil filled exines before ingestion and those recovered from blood, following ingestion, were also obtained using a Leica Cambridge Stereoscan 360 Scanning Electron Microscope (SEM) operated by Tony Sinclair, Institute of Chemistry for Industry, University of Hull, performed the SEM (Figure 13).

4.4 Discussion:

In this study, there was a significant rise in the bioavailability of EPA as measured by AUC_{0-24h} when the ethyl ester form of fish oil was encapsulated into the novel exine microcapsules, which has not been reported before. Previous studies have focused on the encapsulation of fish oil to preserve its qualities and prevent oxidation [518], rather than to enhance bioavailability. Although there are no previous studies on the effect of the bioavailability of EPA encapsulated into exines, encapsulation technology is commonly used in pharmaceutical preparations to improve bioavailability. For example, the use of a mixture of wax and fat has been used to achieve controlled drug release in the circulation[524] while the use of microspheres to produce mucoadhesive polymers can help maintaining intimate contact with the mucosa of the gastrointestinal tract thereby achieving improved bioavailability[525]. Exines have been used as a natural substance to mask-taste but this is the first pilot study to investigate its potential use to improve bioavailability of orally ingested fish oil in the ethyl ester form[303]. The mechanism by which exine microencapsulation can enhance oil absorption is unclear, but might be due to the protective structure of fish oil-enriched exines whereby the whole unit could travel unhindered through the mucosal lining without releasing its inner core until entering the circulation. This increase in bioavailability was independent of the T_{max} that is a measure of the time to achieve the maximum concentration, suggesting

that exines may enhance the absorption at the early stages and continue to do so throughout the 24 hour period, in contrast to a natural slower pace of absorption of EPA in the early period of supplementation.

Whilst it is difficult to cost this method, it is expected there would be no significant extra cost compared with other microencapsulation processes; however, no other technique has the advantage of anti-oxidant properties giving a long shelf-life, has been shown to taste mask and also to have a relatively high loading level. The preparation of the exines is simple and inexpensive with the total cost of the microencapsulation within the exines being less with readily available pollens such as that for rye or maize.

The major limitation to this pilot study is the small number of participants. However, as a proof of hypothesis, our results were highly significant and further *in vitro* and *in vivo* studies are warranted to explain this phenomenon.

In summary, this study showed that exines obtained from *Lycopodium clavatum* spores encapsulating fish oil in the ethyl ester are associated with an improvement in LCPUFA bioavailability as measured by the $AUC_{0\text{-}24h}$, that may be due to the oil being transported into the blood stream more efficiently by the intact exines, Another possibility is that exines improves the dispersion of oil in the duodenum and jejunum and this increases absorption of the oil.

Source of funding: The study was funded by the Diabetes Endowment Fund.

Figure 10: Flow chart of the progress through phases of open labelled sequential (two scheduled visits, two weeks a part) of the EPA bioavailability from encapsulated fish oil study.

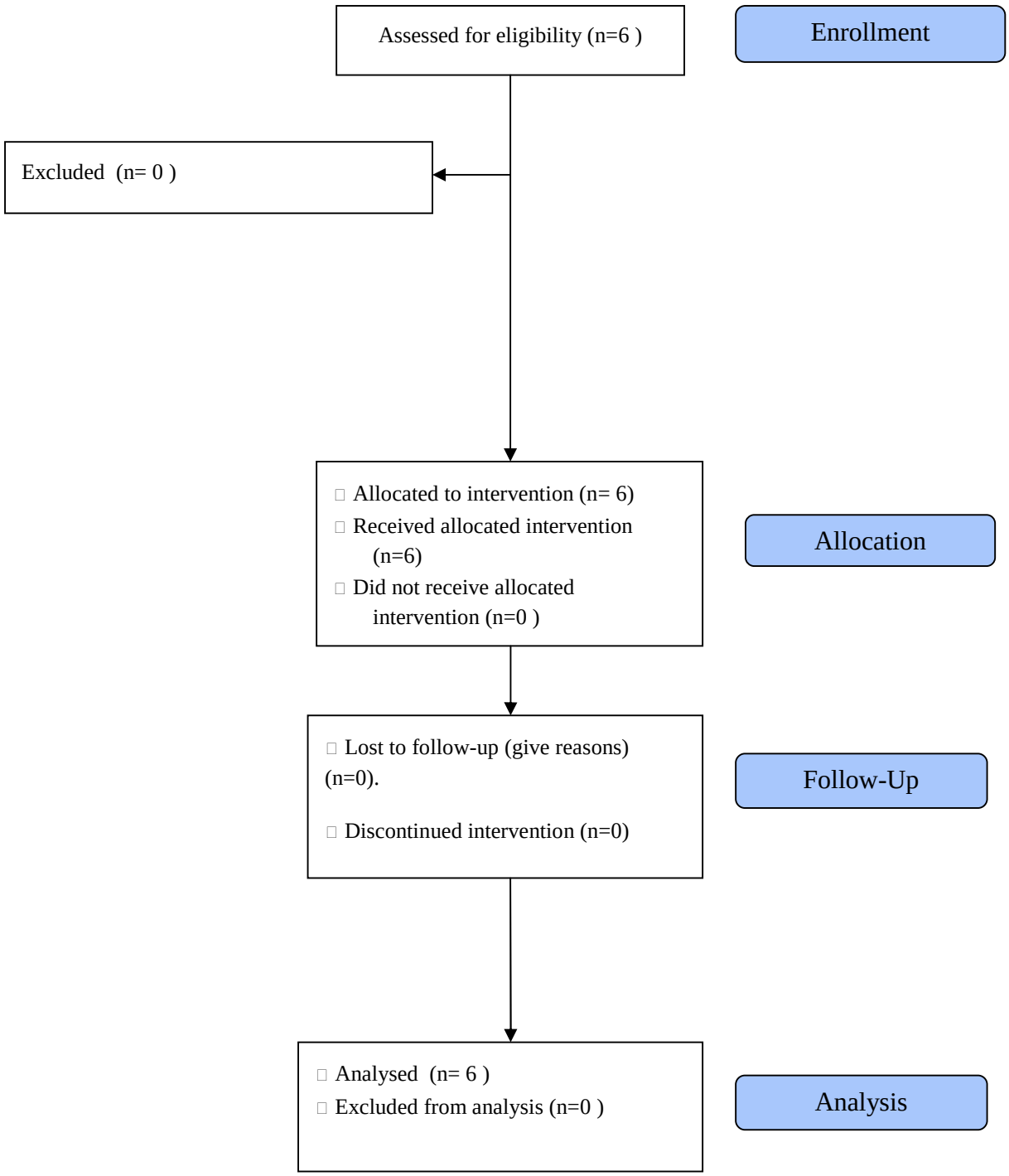


Table 7: Demographics of healthy participants in the study of EPA bioavailability from ethyl ester with and without exines.

Demographics	**Mean (SD)**
Systolic blood pressure (mm Hg)	131(7)
Diastolic blood pressure (mm Hg)	78 (4)
Total cholesterol (mmol/L)	4.7 (0.45)
Triglyceride (mmol/L)	1.06 (0.24)
High Density Lipoprotein (mmol/L)	1.25 (0.35)
Low Density Lipoprotein (mmol/L)	2.7 (0.84)
Total cholesterol/high density lipoprotein	4 (1.21)
Body Mass Index (Kg/m^2)	23.5 (2.2)

Figure 11: The change in mean EPA serum level over time obtained from the ethyl ester of EPA with and without exines.

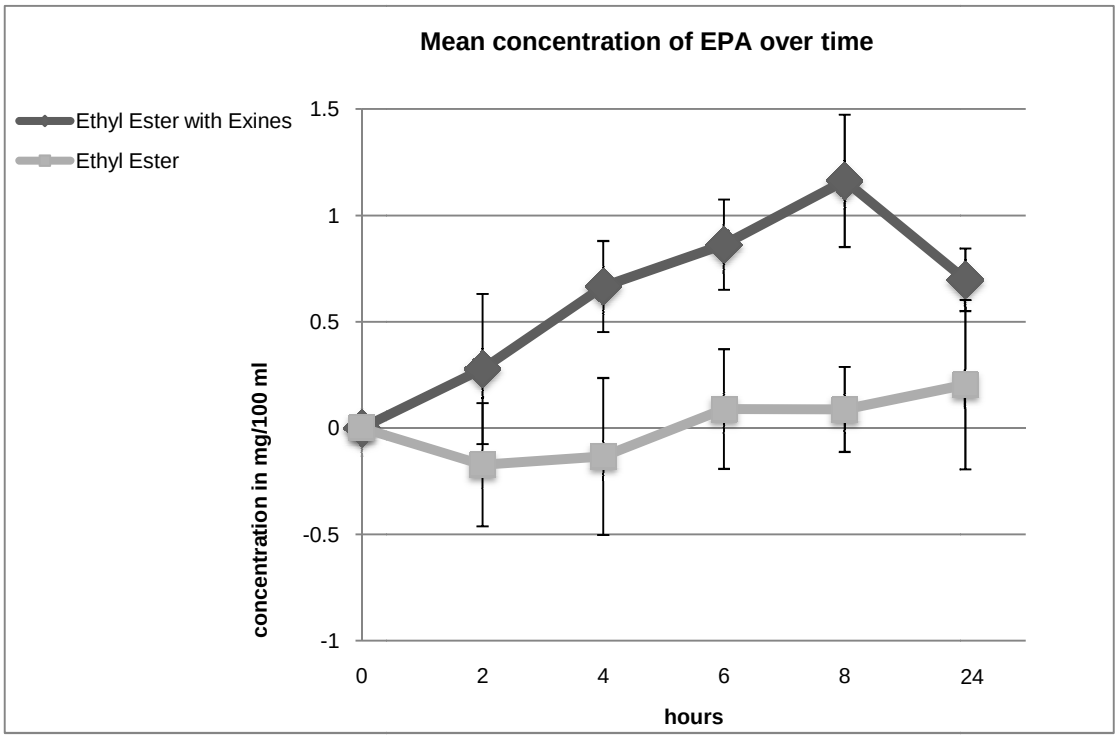
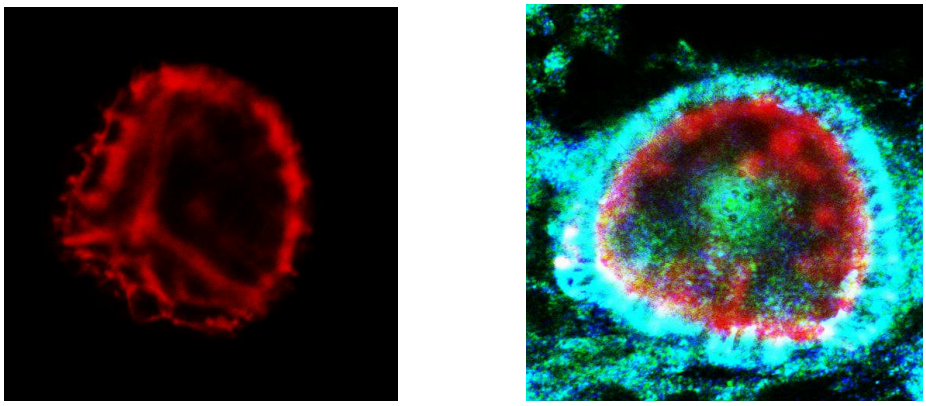


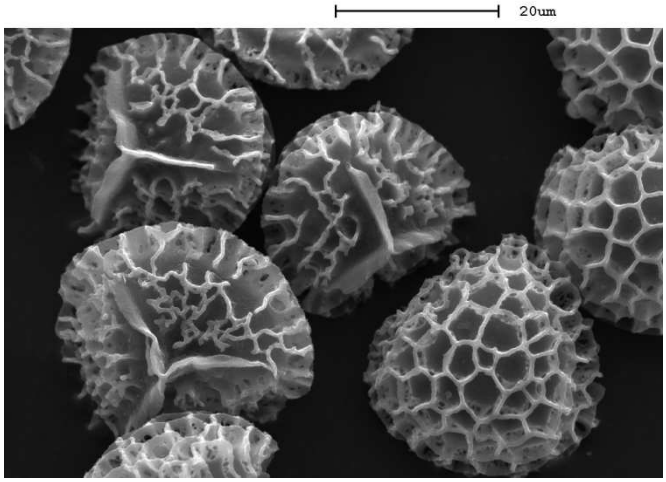
Figure 12: Confocal microscopy of sporopollenin prefilled and postfilling after 30 minutes of ingestion.

A. Confocal microscopy (magnification x60) of an empty sporopollenin exine (red) showing its architecture and fluorescence. B. An exine (red) previously filled with fish oil, found in the blood, 30 minutes after ingestion showing accumulation of material on the outside (blue).



A **B**

Figure 13: SEM images of exines (25µm) from L. clavatum spores prior to ingestion, filled with oil (A) and those recovered in vivo 30 minutes after ingestion (B and (C).



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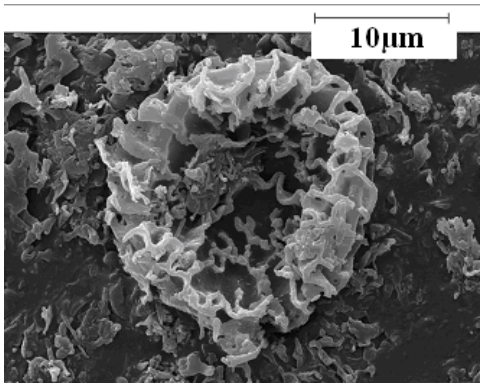
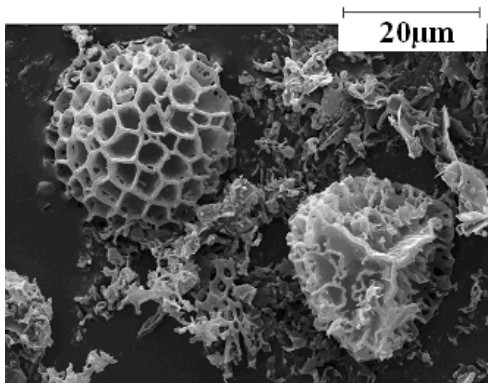




B C

Chapter 5: A Pilot study on the effect of oleic acid and oleic acid-rich exines on insulin resistance using hyperinsulinaemic euglycaemic clamp.

5.1 Introduction:

Since the publication of Randle's work on the glucose fatty acid cycle and their contribution to the metabolic disorder and the development of diabetes, researchers were able to demonstrate via multiple indirect methods that fatty acids delivery to the portal circulation is associated with suppression of insulin-mediated glucose suppression [526, 527]. Studies have utilised the infusion of fatty acids to the general circulation but not to the portal circulation in their quest to raise plasma free fatty acids and study the effect of it on insulin resistance[528]. They were able thus far to show that raised plasma free fatty acids can cause endogenous glucose production mainly through gluconeogenesis rather than decreased glucose utilisation [526, 529]. The demonstration of this glucose insensitivity to insulin directly using fatty acids delivered to the portal circulation was not attempted in humans due to ethical as well technical considerations[530].

Exines are the outer shell of pollens and they are very resistant to environmental insults. We have shown in a previous study that they enhance the bioavailability of eicosapentaenoic acid in fish oil supplements[531]. Although the mechanism is not clear, one suggested way is that exines act as a carrier to the substance encapsulated inside them and in doing so they directly enter the portal circulation, avoiding the lacteals and the lymphatic system. Based on this theory, the use of exines as a carrier of a long chain saturated fatty acid such as the oleic acid, the main fatty acid mobilised from visceral fat[532], could potentially create a novel method whereby the fatty acid could be directly delivered to the portal circulation for the purpose of studying their effects on insulin resistance in humans.

Aim of the Study:

Therefore this proof of concept study was designed to see if the intake of oleic acid-enriched exines results in a change of insulin sensitivity using the hyperinsulinaemic euglycaemic clamp.

5.2 Methods

This is an open labelled sequential study. Six healthy subjects without personal or family history of diabetes were recruited through a local advert in the University of Hull and Hull Royal Infirmary. Exclusion criteria were impaired fasting glycaemia, impaired glucose tolerance, previous gastric or intestinal illnesses or surgeries, presence of concomitant diseases as well as intolerance to dairy products. Subjects who fulfilled the criteria in the screening visit were invited to attend the Research Centre at Michael White Diabetes Centre at Hull Royal Infirmary on two occasions. Each visit was done in the post-absorptive (10 hours fasting) state. After taking anthropometric measures, a blood sample was taken at baseline and a two hours euglycaemic hyperinsulinaemic state was commenced as described before[533]. In brief, two intravenous cannulae were inserted, one in antecubital vein and the other in a retrograde direction in the back of the other hand which was kept in a 60 °C heat box to arterialise the vein. The latter was used for blood sampling every 5 minutes to determine the plasma glucose that was fedback into an Excel sheet which was (based on equation described first by Defronzo) to guide the dextrose infusion for the next 5 minutes[166]. The antecubital cannula was used for administration of Humilin S® at a rate of 40 mU/m^2 body weight and 10% dextrose at a rate determined by the plasma glucose results. After two hours of steady-state euglycaemic clamping, 5g of plain oleic acid was given to the subject mixed with 120 ml of yoghurt drink while the clamp resumed for another two hours to assess the change in insulin sensitivity after the intake of oleic acid. After two weeks, subjects

attended for their second visit and the same procedure described above was followed but this time the 5g oleic acid was encapsulated into the inner core of exines before mixing them with 80 ml of plain yoghurt. After each clamp subjects were given lunch before leaving the department. The protocol was approved by the Hull and East Riding Local Ethics committee. The highly purified (99%) oleic acid was provided by Croda®, Goole, UK. We used the HemoCue® glucose 201^{+} with plasma glucose conversion (Angelhom, Sweden) to measure plasma glucose at regular intervals [534, 535].

We used the total glucose metabolised (M) as described by Defronzo to calculate glucose utilization 1 hour before and two hours after oleic acid intake. We also computed M at 20 minutes intervals during these three hours to detect any potential change in M at any given time following oleic acid ingestion. A comparison of the change in M after oleic acid or exine-loaded oleic was made using paired sample t-test *via* Microsoft Excel® and analyse-it® softwares.

5.3 Results:

All recruited subjects finished the study without adverse events, see Figure 14. Subjects' characteristics are presented in Table 8. The change in M before each intervention is depicted in Figure 15. The mean and SD of glucose metabolised (M) at baseline were not significantly different in the two visits, being 5.59 +/-1.2 and 5.73 +/- 1.4 $mg.kg^{-1}.min^{-1}$, before oleic acid and oleic acid loaded exines respectively. The difference between mean M at baseline was -0.13; 95% CI= -1.4-1.1; p= 0.8. The mean and SD of M changed from the above baseline values to 5.07 +/-1.6 and 5.2 +/- 1 $mg.kg^{-1}.min^{-1}$ after taking the oleic acid and oleic acid loaded exines, a respective change of 0.52 and 0.53 $mg.kg^{-1}.min^{-1}$ which was statistically not significant (difference between means=0.006, 95% CI= -2-2.1, p = 0.9). There was also no significant change in M before and after taking either the oleic acid or the oleic acid loaded

exines, neither was there a significant difference between the calculated M values in the two hours post oleic acid or oleic acid loaded exines intake. Additionally, the change in M was not significantly different between oleic acid and oleic acid loaded exines in the following periods: 0-20, 20-40, 60-80, 80-100 and 100-120 minutes post intervention and the 20 or 60 minutes M prior to that intervention (data not shown). Furthermore, on analysing the dextrose infusion rates over the duration of the clamps, there was no difference between the two interventions and there was no difference in infusion rates before and after giving oleic acid and oleic acid loaded exines, data not shown.

5.4 Discussion:

In this study, the metabolised glucose (M) did not significantly change from baseline after taking either 5g of plain oleic acid or oleic acid encapsulated into exines. Furthermore, the change in M between the two interventions was not significant. This was also reflected by the lack of change in the rate of dextrose infusion before and after each intervention. Numerous studies undertaken in the past have been able to show that the infusion of free fatty acids influences various aspects of glucose metabolism [494, 528, 536, 537] . On the other hand, indirect evidence exists that visceral fat and abdominal subcutaneous fat are more deleterious than other subcutaneous fat sites in the development of insulin resistance and type 2 diabetes [527, 538, 539]. There is evidence that exines from *Lycopodium clavatum* (Club moss) could act as a carrier of what is encapsulated within their inner cores and while they are extremely resistant, plasma, but not serum, was found to be capable of lysing the exines in vivo and in vitro [296, 299, 531, 540]. It then became very intriguing to investigate whether the exines can transport what is encapsulated (even long chain fatty acids) within them to the portal circulation directly. If proven, this could be a novel way of delivering fatty acids to the portal circulation directly and study their effects on insulin sensitivity using the hyperinsulinaemic

euglycaemic clamp method. Potentially, the positive outcome of this experiment could serve two purposes; first it would have supported the previous evidence that exines are capable of carrying substances inside them directly to the blood stream and that even substances which are normally destined to be absorbed through the lacteals to the peripheral circulation like the 18 carbon mono unsaturated oleic acid, could reach the portal circulation aided by the exines. The second is to establish the effect of oleic acid delivery to the portal circulation on insulin sensitivity.

The negative outcome of this study, however, could be due to a combination of reasons; first the possibility that the exine types used did not carry the oleic acid to the portal circulation or did not carry the oleic acid to a sufficient concentration to demonstrate a change in insulin sensitivity. Second, the insulin sensitivity was measured peripherally and we did not determine hepatic insulin sensitivity, a more sophisticated method which was not planned within our protocol, after the delivery of oleic acid aided by the exines. Third, the amount of oleic acid used was not high enough to cause the change in insulin sensitivity. As an example, Intralipid® was infused in a dose of 1.85 $ml.Kg^{-1}.hour^{-1}$ to obtain metabolic effect[541]. If this is computed for two hours Intralipid® infusion, it will equate to 9-15 grams of oleic acid given during that period which is 2-3 times the weight given in our trial. Furthermore the actual amount of fatty acid in the hypothesized infusion above would have been 51 g, 44-62% of which is the unsaturated omega 6 linoleic acid [542, 543]. Arguably we could have used larger weight of oleic acid but this would have resulted in subject retention issues due to the poor tolerance of high volumes of purified oleic acid and technical difficulty in loading this high amount of oleic acid.

In summary, we did not find a significant change in insulin sensitivity as a result of ingesting 5 g of oleic acid whether in the plain or exine-encapsulated forms. Further confirmatory

studies into the ability of exines to carry substances within them to the portal circulation or inventing novel carriers to do so will be needed before an experiment similar to ours could answer the question of whether fat delivered to the portal circulation is associated with change in insulin sensitivity above and beyond fat delivered to the peripheral circulation.

Source of funding: The study was funded by the Diabetes Endowment Fund.

Figure 14: Flow chart of the progress through phases of sequential open labelled (two scheduled visits, two weeks a part) of the encapsulated oleic acid and its effect on insulin sensitivity.

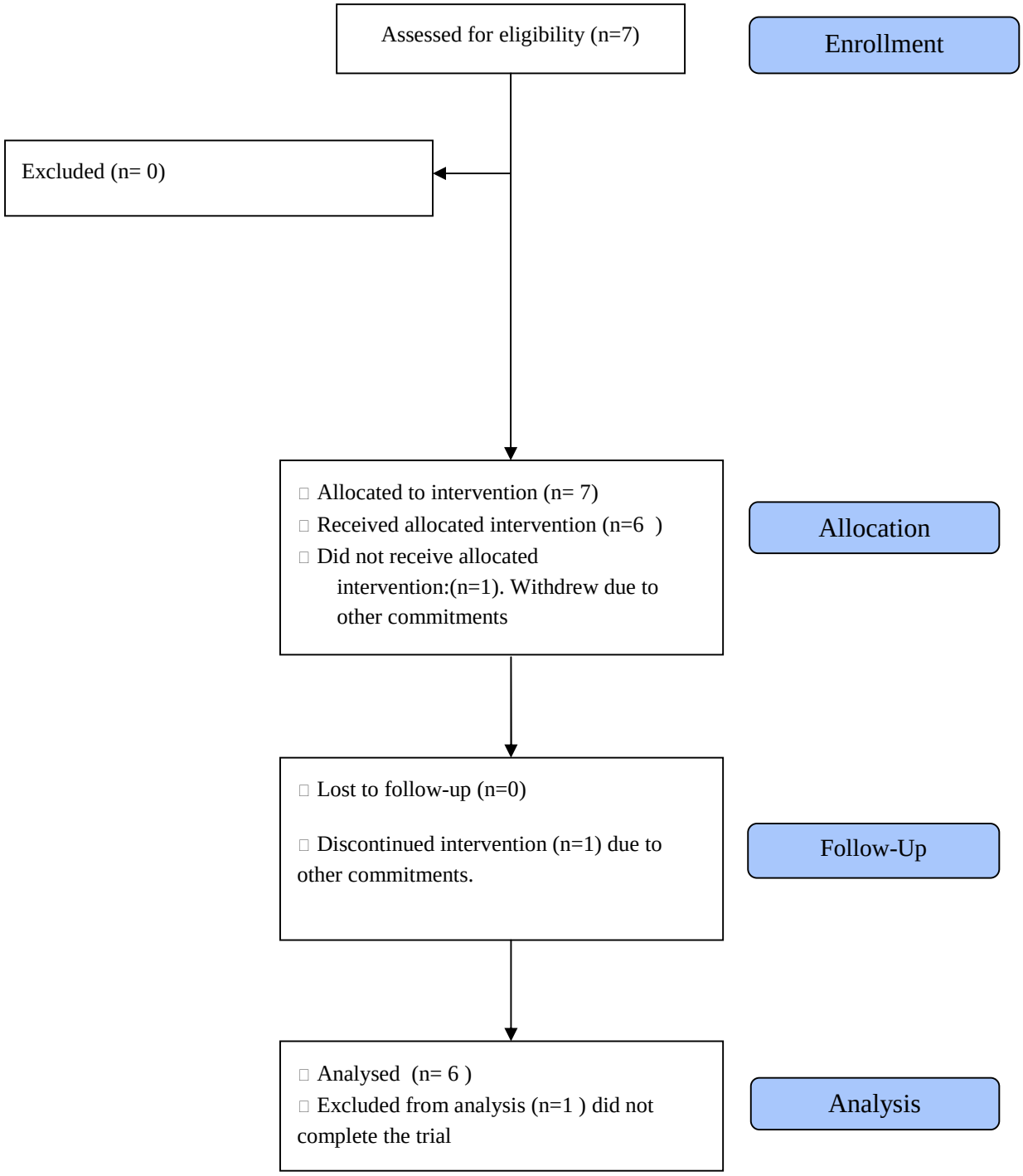
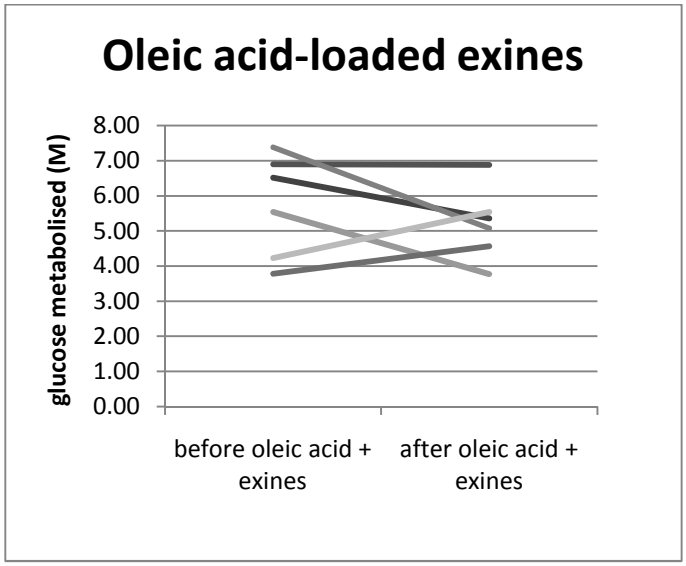
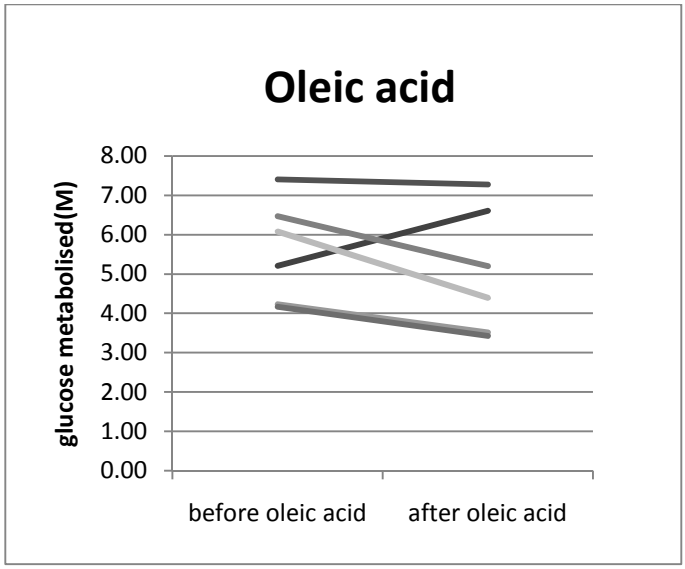


Table 8: Characteristics of healthy participants in the study of insulin resistance modification by oleic acid mixed with exines.

	Mean, (SD)
Weight in Kg	81.3 (13)
BMI in Kg/m^2	24.8 (3)
Fasting plasma glucose in mmol/L	4.3 (0.5)
Age in years	28.5 (6)
Systolic Blood Pressure in mm Hg	130.8 (7)
Diastolic Blood Pressure in mm Hg	74.5 (9)

Figure 15: The change in glucose metabolised after oleic acid and oleic acid-rich exine intake.





Chapter 6: Short term glucose variability in healthy volunteers is not associated with raised oxidative stress markers.

6.1 Introduction:

Oxidative stress describes the cellular damage caused by excess reactive oxygen species (ROS) which are not adequately inactivated by antioxidant systems. There are many markers of oxidative stress and these are often lipid peroxidation end products such as 8-iso-PGF2α. This marker is a member of the isoprostane family which are metabolites of arachidonic acid peroxidation, of which 8-iso PGF2α is the most widely used marker of oxidative stress. The measurement of urinary 8-iso PGF2α over a 24 hour period is preferred since it reflects an integrated assessment of oxidative stress over the 24 hours period [316].

It has been shown that constant hyperglycaemia leads to an excess production of markers of free radical damage [544]. In turn, a unifying hypothesis for the development of microvascular complications has been proposed based on the increased formation of reactive oxygen species (ROS) [545]. It is unknown whether fluctuations in blood glucose add to the risk of developing microvascular complications of diabetes over and above purely the mean glucose value for a patient. Few human studies have focussed on the effect of glycaemic variability on oxidative stress in free living individuals but not in a laboratory adjustment of glycaemia.

Using continuous glucose monitoring, variability seemed to be strongly associated with the excess production of urinary 8-iso-PGF2α, a marker of oxidative stress, in patients with type 2 diabetes [458, 546]. However, this has not been found to be the case in a similar study performed in patients with type 1 diabetes[478]. Also, retrospective analyses of the DCCT dataset have found no evidence to suggest that glucose variability was predictive of microvascular complications in their type 1 patients[547].More recently, Ceriello *et al* reported worse endothelial function and higher oxidative stress markers when the glucose was

varied in comparison to a constantly elevated glucose[548]. The differences between these studies are compounded by the fact that they employ differing methods for measurement of 8-iso-PGF2α. It has been previously shown that these methodologies do not produce comparable results [549].

Aim of the Study:

The aim of this study is to compare oxidative stress by measurement of 24 hour urinary 8-iso-PGF2α in healthy subjects in three laboratory-like glycaemic states (hyperglycaemia, euglycaemia and variable glucose) controlled by glucose infusion.

6.2 Study Design and Methods:

Study design and patients:

This was an open labelled sequential study. The study protocol was approved by the South Humberside local ethics committee. Twenty healthy volunteers answered an advert placed in Hull Royal Infirmary and Hull University. Eleven men satisfied the inclusion criteria of being healthy with no chronic illness or recent illness within the last three months. Exclusion criteria were the use of any medication, a first degree family history of diabetes as well as estimated Glomerular Filtration Rate below 60 ml/min/1.73m^2. All consenting subjects attended in the post-absorptive state after 12 hour fasting. Each subject was provided with a dietary diary to avoid high anti oxidant containing diet 72 hours before and on the day of each experiment. They were also provided with standard meals to consume on the day of each experiment to avoid variability among the three experiments. The standard meals provided a daily allowance of 2527 Kcal providing 392, 1352 and 783 grams in proteins, carbohydrates and fat respectively.

Anthropometric measurements were collected prior to each glycaemic state. Each subject collected urine overnight and for 24 hours post-glycaemic state to determine the urinary $PGF_{2\alpha}$ before and after each glycaemic state. Additionally six subjects were asked to collect urine at timed intervals (3, 9 ,15, and 24 hours) from commencing the clamp to determine if there is any significant variability in the rate of excretion of $PGF_{2\alpha}$ in different periods after the three glycaemic state. Three subjects had continuous glucose monitor system (Glucoday S, Menarini Diagnostics, Firenze, Italy) to measure interstitial glucose from the start of the clamp and for 24 hours afterwards to objectively detect significant glucose variability following the glycaemic states [550, 551].

Glycaemic states:

Each subject underwent three glycaemic states for three hours with a washout period of 2 weeks between each state. In the hyperglycaemic state, the plasma glucose was kept between 12-13 mmol/L for three hours using a hyperglycaemic glucose clamp while in the euglycaemic state the plasma glucose was kept between 4 and 6 mmol/L for three hours by modifying the hyperglycaemic clamp procedure i.e. infusing dextrose only at a slow rate to maintain euglycaemia. In the glucose variability state, the plasma glucose was oscillated between normoglycaemia (4-6 mmol/L) and hyperglycaemia (12-13 mmol/L) twice over the 3 hour period using the hyperglycaemic clamp. The hyperglycaemic clamp was based on the method described by Defronzo[166]. In brief, two venous accesses were obtained, one in each arm. The retrograde cannula was used to sample arterialised blood (by warming the hand in a heat box at 60 degree Celsius), the result of which was fedback to Defronzo equation to alter the rate of 20% glucose infused in the other venous access. A HemoCue® glucose 201^{+} with plasma glucose conversion, (Angelhom, Sweden), was used to obtain instantaneous accurate

plasma glucose result every 5 minutes. In the normoglycaemic state, the Defronzo equation was used to alter the 20% glucose infusion to achieve a plasma glucose level between 4 and 6 mmol/L according to 5 minute-interval plasma glucose measurements. The glucose variability state was achieved through the use of hyperglycaemic clamp to elevate plasma glucose for one hour followed by stopping the dextrose infusion for 30 minutes thereby allowing plasma glucose to drop between 4-6 mmol/L before restarting the hyperglycaemic clamp. The cycle was repeated to obtain two peaks and two troughs of glucose level in the three hours procedure.

Measurement of 8-isoPGF2α

8-iso-PGF2α was measured by a urinary isoprostane EIA kit supplied by Oxford Biomedical Research (Oxford, US). This assay is a competitive enzyme-linked immunoassay based on competition between 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$-horseradish peroxidase for a polyclonal goat anti-8-iso-$PGF_{2\alpha}$ antibody. Samples were analysed without purification according to the manufacturer's instructions.

8-iso-PGF2α was measured in a 24 hours urine collection post-clamp and expressed in ng/24h. To allow comparison of 8-iso-PGF2α levels between the pre-clamp random urine sample and post-clamp 24h urine collection, 8-iso-PGF2α concentration was expressed as a ratio to creatinine (pg/mmol creatinine). The collected data lacked normality and therefore a non-parametric test, Kruskal-Wallis H, was selected to statistically analyse the data using SPSS 16 and Microsoft Excel® with analyse-it® softwares.

6.3 Results

All recruited subjects completed the study protocols without adverse events, see Figure 16. The baseline characteristics of the volunteers are depicted in Table 9. The mean plasma glucose and standard deviation for each glycaemic state were 5.2 +/- 0.4, 11.9 +/- 0.7 and 10.5 +/- 2.5 mmol/L in the euglycaemic, hyperglycaemic and variable glucose states respectively. The 24 hours urinary 8-iso-PGF2α excretion results are depicted in Table 10. The median, 96.5% confidence interval (CI) and interquartile range of the 24 hour urinary 8-iso-PGF2α were (1373, 937-1598, 513), (996, 811-1172, 298) and (1227, 1059-1622, 472) for the euglycaemic, hyperglycaemic and variable states respectively. Statistical analysis, using Kruskal-Wallis test, showed that there was no significant difference in the urinary isoprostanes among the three different glycaemic states; (H (2)= 5.0, p= 0.08) with a mean rank of 19.5 for the euglycaemic state, 12.6 for the hyperglycaemic state and 18.8 for the glycaemic variability state.

Comparison of 8-iso-PGF2α concentration prior and after each glycaemic state is depicted in Figure 17. The median, 98.8% CI and interquartile range of the absolute difference of isoprostane concentration before and after each of the glycaemic states (9, -20 - 39, 36.5), (-9, -42-20, 39.5) and (5, -35-23, 35) for the euglycaemic, hyperglycaemic and variable states respectively. Using Kruskal-Wallis test, there was no significant difference in the absolute difference between the isoprostane concentration before and after each of the glycaemic states; (H (2) = 1.214, p= 0.545) with a mean rank of 18.8 for the euglycaemic state, 14.4 for the hyperglycaemic state and 17.7 for the glycaemic variability state. In those who collected urine at timely intervals for 24h post-clamp, there was no significant difference in 8-iso-PGF2α concentration in any of these timed collections compared to the pre-clamp urine sample. In the three subjects who had the CGMS, there was no significant variability in the

interstitial glucose among the three glycaemic states as measured by standard deviation, J-index[552], mean amplitude of glycaemic excursions (MAGE) [439] and continuous overlapping net glycaemic action (CONGA-2) [553].

6.4 Discussion:

There was no significant difference in 8-iso-$PGF_{2\alpha}$ in healthy subjects as measured in 24 hour urinary collection between the three glycaemic states; hyperglycaemia (12-13 mmol/L), euglycaemia (4-6 mmol/L) and two spikes and troughs of oscillating glucose level between hyperglycaemia and normoglycaemia, each over three hours period. There is ongoing debate on the impact of mean blood glucose and variable blood glucose on long term microvascular complications in patients with diabetes with researchers on both sides of the argument. Monnier *et al* has, in a case control study, reported a higher 24 hour urinary free $PGF_{2\alpha}$ in patients with type 2 diabetes compared to normal control with the high $PGF_{2\alpha}$ levels strongly linked in univariate analysis to the glycaemic swings as measured by the Mean Amplitude of Glycaemic Excursion (MAGE)[458]. Evidence against the role played by glucose variability in initiation of oxidative stress markers over and beyond chronic hyperglycaemia has been shown by the lack of correlation of glucose variability as measured by various methods and the increased oxidative stress markers seen in patients with type 1 diabetes as compared to healthy control[478]. More recently, Ceriello *et al* have tested the effects of hyperglycaemia and glucose variability on endothelial function and oxidative stress markers, two driving forces for the development of vascular diseases[548]. In this study, both healthy and type 2 diabetes patients were subjected to a glucose clamp for 24 hours at 10 mmol/L, 15 mmol/L and at 15 and 5 mmol/L alternately every 6 hours. They found increased endothelial

dysfunction and higher oxidative stress markers in the glucose variability group in comparison to the constant hyperglycaemia groups. Despite the contradictory results of these three studies, there was one unifying observation and that is elevation of blood glucose in patients with diabetes (type 1 or type 2) is associated with elevated oxidative stress markers when compared with healthy controls. However, this was not demonstrated in our results. There may be several explanations to why that is the case. First the subjects in our trial were healthy volunteers who may be intrinsically different from subjects with diabetes. There is evidence that subjects with diabetes could have defective antioxidant protective mechanism when compared to healthy people and therefore healthy subjects are less likely to exhibit raised 8-iso-$PGF_{2\alpha}$ after any type of short lived hyperglycaemia[554] although the findings of Ceriello *et al* would suggest differently[548]. The second explanation is the duration of each glycaemic state was shorter than that of Ceriello[548] and so this period, chosen to try and replicate physiological changes in glycaemia, may have been too short to impact on oxidative stress markers. In addition, there may be an association between oxidative stress and longer term variability; over a few hours (as seen in Ceriello's findings) to days or even weeks.. This is further supported by Kilpatrick *et al* who found HbA1c variability to be strongly correlated with the risk of development and progression of retinopathy in an analysis of the DCCT[472], while within-day glucose variability was not[547]. Finally, inhibition of endogenous insulin by somatostatin used[457] differs from the pathophysiological state of type 2 diabetes where the absolute insulin levels may, in fact, be high[555]. For these reasons we tried to oscillate the plasma glucose by glucose infusion alone and although the duration was short, we believe that bears more resemblance to hyperglycaemia in type 2 diabetes, where both insulin and glucose may be elevated. A recent cross-sectional study suggested that insulin could have anti-oxidative effect in patients with type 1 and type 2 diabetes when compared to those with

type 2 diabetes on oral hypoglycaemic agents[480]. In this study, there was no relation between the MAGE and 8-iso-$PGF_{2\alpha}$ in those who were on insulin while those who were on oral hypoglycaemic medications, association between glycaemic control (HbA1C) and glycaemic variability (MAGE) and 8-iso-$PGF_{2\alpha}$ was significant[480]. It is plausible therefore that in our healthy volunteers with normal glucose tolerance and insulin levels, that no change was seen in the $PGF_{2\alpha}$ as a result of the protective effect of insulin.

Finally, differences in methodology for measurement of 8-isoPGF2α between these studies may have an effect. 8-iso-PGF2α is an isobaric compound which can be measured by a number of methods including GC/MS, LC/MS/MS and Enzyme-linked immunosorbent assay (ELISA) [549], however cross reactivity and interference from related enzymatically and non-enzymatically formed prostaglandins can occur to varying levels between different methods. To circumvent this potential difference, urinary 8-isoPGF2α in 6 of the study subjects was also measured by an alternative ELISA and also an LC/MS/MS method. There was no significant difference between the different glycaemic states or between 8-iso-PGF2a concentration before and after the intervention.

Criticisms of this trial includes the small number of participants and potentially measuring the 24 hours 8-iso-$PGF_{2\alpha}$ when the period of intervention was only 3 hours could result in bias. To avoid this, we asked the participants to collect urine at specific times over the 24 hours including immediately after the clamps. This allowed us to analyse the 8-iso-$PGF_{2\alpha}$ in these timed collections which again did not significantly differ among the three glycaemic states (data not shown). By giving the participants standardised meals we attempted to omit the variability in glucose excursions post meals. We objectively measured in 3 subjects using continuous glucose monitor system (CGMS) interstitial fluid glucose during the three

different glycaemic states. When we compared the glucose variability in the post clamp period we did not find a significant difference between them (data not shown).

In conclusion, there was no difference in 24 hour urinary 8-iso-$PGF_{2\alpha}$ in healthy volunteers following three glycaemic states of euglycaemia, hyperglycaemia and variable glucose of 3 hour duration. Studies with longer period of glucose variability are needed to address the relative importance of mean plasma glucose and glucose variability on markers of long term diabetic complications.

Source of funding: The study was funded by the Diabetes Endowment Fund.

Figure 16: Flow chart of the progress through phases of sequential open labelled (three scheduled visits, two weeks a part) of the glycaemic variability and the urinary 8-iso-PGF2*α* study.

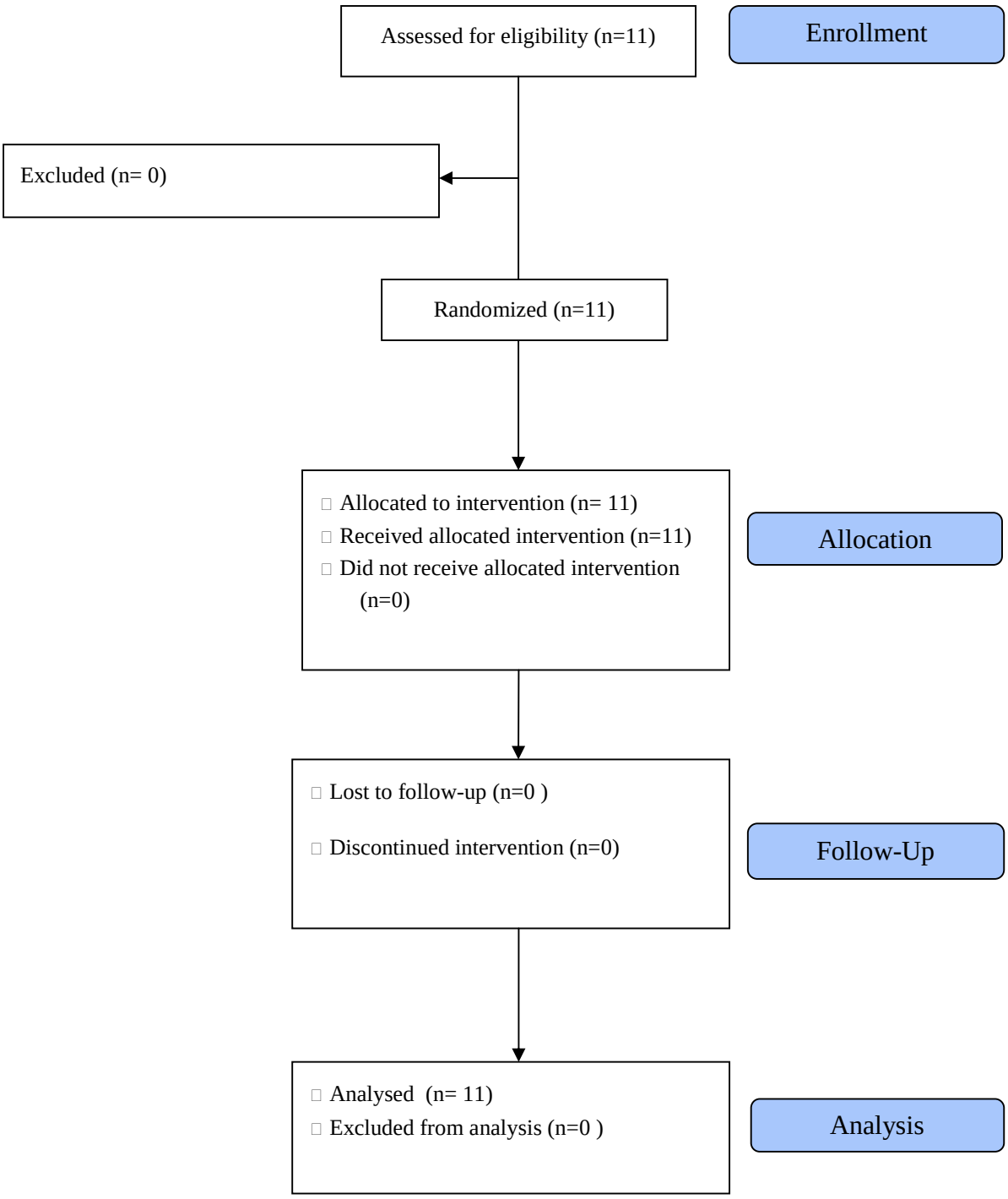


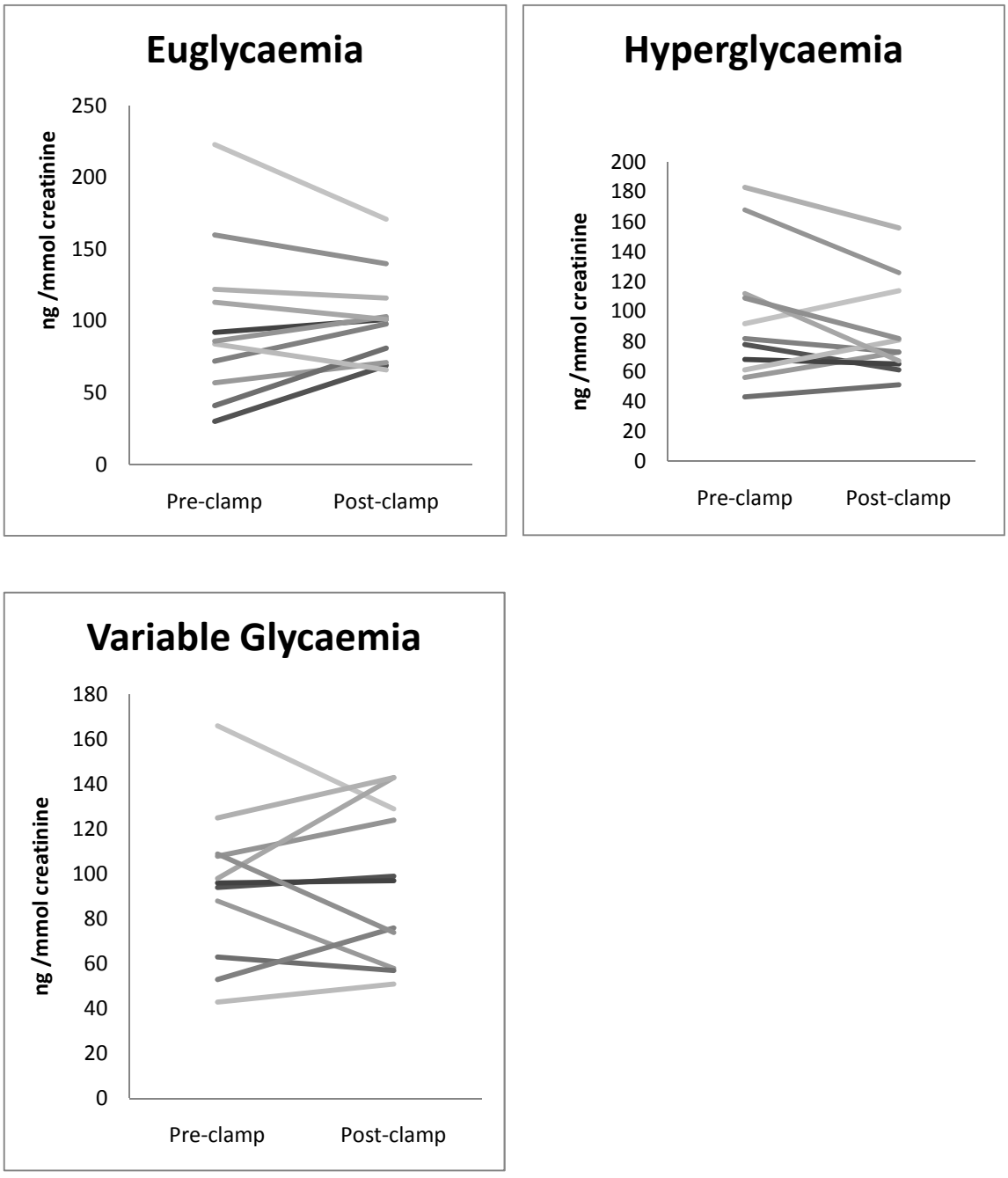
Table 9: Characteristics of healthy participants in the effect of glycaemic variability on oxidative stress markers.

	Mean, (SD)
Weight in Kg	80.3 (17)
BMI in Kg/m^2	24.2 (3)
total cholesterol in mmol/L	4.0 (0.5)
Fasting plasma glucose in mmol/L	5.0 (0.3)
Age in years	31.5 (7.2)
Systolic Blood Pressure in mm Hg	124.8 (8.1)
Diastolic Blood Pressure in mm Hg	71.1 (10)
Triglycerides in mmol/L	0.9 (0.28)
LDL in mmol/L	2.1(0.25)

Table 10: Results of 24h urinary output of 8-iso-PGF2α (ng/24h) following each clamp.

Subject	Euglycaemic	Hyperglycaemic	Variable
1	1271	958	1481
2	1367	1277	1151
3	1373	811	1059
4	896	863	1275
5	937	751	758
6	1175	1587	870
7	1598	1709	1735
8	1541	913	1371
9	1902	1172	4701
10	2443	996	1100
11	2000	1054	2343

Figure 17: Change in 8-iso-PGF2α prior and after each intervention expressed in ng/mmol creatinine.



Chapter 7: Summary Discussion

This thesis examines three questions related to the pathogenesis and modification of certain cardiovascular disease (CVD) risks in healthy volunteers. First; is the bioavailability of eicosapentaenoic acid (EPA) obtained from different fish oils and using sporopollenin to enhance it. Second; is the relation between the CVD risk marker, 8 iso-F2α, an oxidative stress marker, and glycaemic variability in healthy subjects. Third; is using sporopollenin to model long chain free fatty acid delivery to the portal circulation, a process implicated in the pathogenesis of conditions that are considered CVD risks such as the metabolic syndrome, insulin resistance and diabetes.

The metabolic syndrome is common and is associated with increased risk of cardiovascular diseases and Type 2 Diabetes Mellitus (T2DM). In epidemiological studies, the risk of having a stroke or coronary heart disease over 6.9 years of follow-up was increased by 3 folds when the metabolic syndrome was present at baseline[556]. On the other hand the presence of metabolic syndrome confers 6 folds increase in the development of T2DM[557]. There is strong evidence, from epidemiological and interventional trials, that n-3 fatty acids in fish oil have a beneficial effect on CVD [502, 558]. The underlying mechanisms thought to be responsible for this positive effect are their anti-arrhythmic, triglyceride lowering, blood pressure lowering as well as platelet anti-aggregation effects [559-561]. In recent years the use of n-3 fatty acids have changed from preventing dietary deficiency to their use in decreasing the risk of morbidity and mortality from long chain fatty acids-related chronic diseases such as cardiovascular diseases[562]. N-3 fatty acids, especially, EPA acid and docosahexaenoic acid (DHA) can only be obtained from marine diet. It is well known that daily consumption of fish oil is difficult due to compliance issues and therefore ways to overcome this problem include concentrating the n-3 fatty acids in fish oil supplements as well as increasing bioavailability of fish oils from the different supplements. There are many

factors influencing the bioavailability of n-3 fatty acids in fish oils. Among these are; the source of fish oil, the ratio of n-3 to n-6 fatty acid in the formulation, the chemical form (triglyceride *vs.* ethyl ester or free fatty acids), the position of n-3 fatty acid in relation to the side chain (sn) of triglyceride as well as food processing such as emulsification[563-567]. One method described recently is the emulsification of fish oil to achieve better digestion, bioavailability and palatability probably by enhancing lingual, and pancreatic lipase activity [107, 568]. In our first EPA bioavailability study, it was demonstrated that EPA in the form of reconstituted triglyceride (RTG) is better absorbed than enzymatically synthesised triglyceride and monoglyceride. This was probably related to the presence of the monoglyceride and diglyceride components in the RTG which were acting as emulsifying agents. In the nutraceutical industry, the fish oil is converted first to ethyl ester by micro distillation reaction to purify the product. However re-conversion to triglyceride is a costly process that increases the bulk price by 30-40%[565]. Omacor®, an ethyl ester of EPA and DHA, has been recommended by the National Institute of Clinical Excellence (NICE) as a cost effective measure for secondary prevention after myocardial infarction[117]. In our second bioavailability study, the relatively less expensive and widely available ethyl ester form of EPA was encapsulated using a novel method; sporopollenin of *Lycopodium clavatum*. The result was an enhanced bioavailability in the order of magnitude when compared to free EPA ethyl ester. Although the number of subjects (n=6) is too small to generalise the findings, the novelty of using this sustainable and green method to enhance the health benefit of n-3PUFA is encouraging and will need to be confirmed in a larger cohort[520]. Furthermore, the fact that sporopollenin can also mask the taste of fish oil is an added advantage of this novel method[520].

The interaction between the liver, skeletal muscles and adipose tissues, insulin sensitive organs, are behind the pathogenesis of insulin resistance and diabetes. The interaction between the liver and the central adipose tissue is a key component of the metabolic syndrome, insulin resistance and indeed diabetes, all of which are risks for CVD, but the exact mechanism is unknown[569]. Studies have investigated fatty acids effects on insulin resistance by infusing lipids peripherally but this usually elevates both the hepatic and intramyocellular fatty acid contents[570]. In order to investigate the effect of hepatic fatty acid accumulation alone, one needs to infuse them directly to the liver, an invasive method reserved for animal studies[571]. Preliminary studies in the University of Hull showed that sporopollenin can pass in an undetermined mechanism directly to the blood stream. This property coupled with their ability to carry substances, such as long chain fatty acids, was the basis for their use in a proof of concept study to model the non-invasive portal delivery of fatty acids and investigate the effect of this on insulin resistance. If successful, it would be a valuable method in investigating the effect of fatty acids on hepatic insulin resistance non-invasively. The negative results of this study could be due the small dose of oleic acid used or the size and types of exines used in our trial which could affect their absorption. Finally, measuring changes in total glucose disposal may not reflect changes in hepatic insulin resistance which is the intended goal. However, increasing oleic acid dose would have caused side effects, irritation to the pharynx and irritating cough, as well as increasing the volume of the mixture; both would undoubtedly have a negative effect on the retention of volunteers. Although measuring hepatic insulin resistance would have been a more accurate method in registering the effect of oleic acid loaded exines, it does require more sophisticated methods and using radiolabelled glucose which is difficult logistically[572].

The precise pathogenesis for the development of microvascular and macrovascular complications of diabetes is not well understood but randomised controlled trials have linked the degree of glycaemia, measured by haemoglobin A1c, with outcomes of log term diabetes complication[363, 573]. Reactive oxygen species (ROS) are hypothesised to be the underlying link between the different mechanisms by which glycaemia can initiate long term complications, mainly microvascular[370]. Studies in animals and in vivo have suggested that glycaemic variability is more detrimental than hyperglycaemia and this concept have been explored *in vivo* with mixed results [457, 458, 480, 481, 574, 575]. The best available method to assess ROS is the urinary or plasma 8 iso-F2α and studies showed conflicting outcomes on the relation of this marker with glycaemic variability and hyperglycaemia in subjects with diabetes [458, 575]. The main difficulty facing any study on glycaemic variability are our lack of knowledge on the best mathematical method to assess variability and the strength of this hypothetical method in predicting the adverse generation of ROS and diabetes complications. In our effort to reproduce short term laboratory glycaemic variability we used the hyperglycaemic clamp method to create three glycaemic states in 11 healthy volunteers. Our finding of negative association between the three glycaemic states and 8 iso-F2α was surprising but not an isolated observation by other groups[479]. In fact the positive studies in subjects with diabetes mellitus have been criticised for the lack of short term glycaemic variability as seen in diabetes mellitus or the measurement the measurement of 8 iso-F2α using radioimmunoassay, a less accurate method[474]. The negative finding of this study raises a few questions which need to be taken into consideration in futures studies on glycaemic variability. First, does short term variability, arguably mimicking that of DM, have a lesser role than longer term variability in generating ROS? Second, is it possible that ROS generated by short term glucose fluctuations or even hyperglycaemia are not capable of

overwhelming the ROS scavengers in healthy individuals? Third, can the presence of endogenous insulin, thought to have a protective property recently[480], in our subjects be a reason why we did not detect any change in 8 iso-F2α in our healthy cohort? To answer these questions, one can envisage a study design that compares short term *vs.* long term glycaemic variability in healthy individuals and in subjects with diabetes (type 1 and type 2).

Although the trials in this thesis had mixed positive and negative results, the questions and issues raised by these results give an indication to the potential use of sporopollenin in drug delivery while more work is needed to further elucidate the mechanism of their entry to the circulation. This may be crucial for any further investigations of their potential use in investigating portal delivery of long chain fatty acids. The issue of glycaemic variability and its relation to oxidative stress markers remain unanswered and future studies need to be designed with the aim of identifying the best mathematical method to measure glucose fluctuation as well as linking that to diabetes outcomes.

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