TRINITY COLLEGE DUBLIN

Postprandial Cardiometabolic risk in Autoimmune Thyroid Disease and Type 1 Diabetes Mellitus

By

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Declaration

This thesis is submitted by the undersigned to the University of Dublin, Trinity College for the Degree of Doctor in Medicine. The work herein contains no material that I have submitted for an award of any other degree or diploma in this or any other university, and to my best knowledge and belief, contains no material that has been previously published or written by another person except where due reference has been made in the text. The library of Trinity College Dublin has my permission to lend or copy this thesis upon request.

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Description of Thesis

This thesis has been prepared in a similar style to thesis by publication. Chapter 3 has already been published in a peer-reviewed international journal. Chapter 4 is currently undergoing the peer review process. For this reason, the thesis has been presented in this format. Although there is some overlap in the methodology of chapters 3 and 4; for completeness all methodology is included within each separate study.

Summary

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Atherosclerosis is implicated in the development of CVD. Postprandial lipaemia is independently predictive of atherogenesis and of future cardiovascular events. Postprandial studies can unmask lipoprotein abnormalities not present in the fasting state.

The overall CVD risk is increased in overt hypothyroidism (OH) and possibly in subclinical hypothyroidism (SCH). LDL-Cholesterol (LDL-C) is increased in OH but not SCH, which may partly explain the increased CVD risk in OH, but likely does not entirely explain it. It is not consistently clear what happens to triglyceride and HDL-Cholesterol (HDL-C) in OH and is even less clear in SCH.

Hence, our hypothesis for the Thyroid study:

- Postprandial dyslipidaemia is associated with lipoprotein derangements that may contribute to the increased cardiovascular risk observed in hypothyroidism in addition to that conferred by fasting LDL-C concentrations
- To examine fasting and postprandial HDL-C metabolism in both OH and SCH in an effort to elucidate the differences observed between OH and SCH.

In order to test these hypotheses, a clinical study was undertaken. The thyroid study was a cross-sectional study with 21 OH, 28 SCH and 44 controls. Plasma lipids with particular emphasis on intestinally derived lipoproteins (ApoB48), HDL cholesterol (HDL-C) and endothelial function, as assessed by flow-mediated dilatation (FMD) of the forearm were measured fasting and postprandially.

The original findings of these studies are that ApoB48 was increased postprandially in OH and to a lesser degree in SCH compared to a matched control group. FMD was decreased in OH only.

In addition, HDL-C was significantly lower postprandially in SCH only and CETP associated HDL-C was decreased in OH, which may account for the preserved HDL-C observed in this cohort.

In conclusion, postprandial lipoprotein and vascular abnormalities differ between OH and SCH. Although both are characterized by increased intestinally derived lipoprotein particles, HDL is reduced only in SCH. Maintained HDL in OH probably reflects reduced CETP activity, which was not observed in SCH. Postprandial endothelial

dysfunction is abnormal only in OH and this effect does not appear to reflect increased inflammation. These findings give additional insight into the pathophysiology of CVD associated with hypothyroidism.

The overall CVD risk is increased in type 1 diabetes mellitus (T1DM) and individuals with T1DM have accelerated atherosclerosis. Endothelial dysfunction precedes the development of atherosclerosis. Yet, in the presence of good glycaemic control, individuals with T1DM have a relatively preserved fasting and even favourable lipid profile. It is not known to what extent postprandial lipoprotein changes contribute to the increased CVD risk. Hence, our hypothesis for the T1DM study:

- Postprandial dyslipidaemia is associated with lipoprotein derangements and endothelial dysfunction that may contribute to the increased cardiovascular risk observed in T1DM
- Elucidate what glucometabolic and lipoprotein factors affect endothelial dysfunction in T1DM.

In order to test these hypotheses, a clinical study was undertaken. The T1DM study was a cross-sectional study with 20 T1DM and 24 controls. Additional controls subjects (n=98) were studied to further explore variables associated with endothelial function.

Plasma glucose and lipids with particular emphasis on intestinally derived lipoproteins (ApoB48) and endothelial function, as assessed by flow-mediated dilatation (FMD) of the forearm were measured fasting and postprandially.

The original findings of these studies are that ApoB48 was increased fasting and postprandially in T1DM only. Fasting FMD did not differ between groups but decreased significantly postprandially in T1DM subjects only. Analysis of the matched group (n=44) and the pooled data (n=142) revealed a positive correlation between peak glucose concentration and overall percentage FMD change and an inverse correlation between AUC HDL-C and percentage postprandial FMD change.

To conclude, postprandial lipoprotein and vascular abnormalities differ in T1DM compared to matched controls, resulting in endothelial dysfunction. These changes are possibly mediated through postprandial glucose excursions and maybe attenuated by HDL-C

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Study Contributors

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Anthropometry	√			
Blood pressure measurement	√			
Dietary assessment	√			
Meal administration	√			

Laboratory analysis				
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AMG, Anne McGowan; MW, Matt Widdowson; JG, James Gibney; AMNCH, Adelaide and Meath Hospital incorporating the National Children's' Hospital; hsCRP, high sensitivity c-reactive protein; Apo B, Apolipoprotein B; Apo B48, Apolipoprotein B48; FMD, Flow Mediated Dilatation; CIMT, carotid intimal media thickness; QUB, Queen's University Belfast

Presentations, Publications and Awards arising from this Thesis

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AMNCH	Adelaide and Meath Hospital incorporating the National Children's' Hospital
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
ApoB48	Apolipoprotein B48
AUC	Area under the curve
BMI	Body mass index
CETP	Cholesterylester transfer protein
CI	Confidence interval
CM	Centimeters
CRP	C-reactive protein
CV	Coefficient of variation
ECG	Electrocardiogram
FT4	Free Thyroxine
g	gram
HC	Hip circumference
HDL	High density lipoprotein

HMG co-A	3-hydroxy-3-methylglutaryl-coenzyme A
HOMA-IR	Homeostasis model assessment of insulin resistance
hsCRP	High sensitivity C-reactive protein
IR	Insulin resistance
JG	Dr James Gibney
Kg	Kilogram
Kj	Kilojoule
LPL	Lipoprotein Lipase
µmol/l	Micromoles per litre
mg/dl	Milligram per decilitre
mmHG	Millimetres of mercury
mmol/l	Millimoles per litre
mU/l	Milliunits per litre
nmol/l	Nanomoles per litre
NEFA	Non-esterified fatty acids
NS	Not significant
OH	Overt Hypothyroidism
SCH	Subclinical Hypothyroidism
SD	Standard deviation

SPSS	Statistics package for the social sciences
SREBP-2	Sterol regulatory element binding protein-2
TAG	Triacylglycerol
TC	Total cholesterol
TNFα	Tumour necrosis factor alpha
TPO	Thyroid Peroxidase Antibody
TSH	Thyroid stimulating hormone
VLDL	Very low density lipoprotein
WC	Waist circumference
WCC	White cell count
WHR	Waist:hip ratio
%BF	Percent body fat

1 Introduction Hypothyroidism

1.1 Definition of Overt and Subclinical Hypothyroidism

Gull first reported the clinical changes associated with atrophy of the thyroid gland in 1874 [1]. It was not for another four years that the term myxedema was coined by Ord, who described the characteristic thickening of subcutaneous tissue due to abnormal mucin deposits in the skin and distinctive facial features such as swollen lips and thickened nose [1]. The term myxedema is typically used to describe severe or complicated thyroid hormone deficiency. It is also used to describe the pathological changes associated with the accumulation of glycosaminoglycan in the subcutaneous and interstitial tissue [2]. Murray documented the first clinical response to patients with hypothyroidism treated with thyroid extract some 17 years later in 1891 [1]. Following the identification of Thyroxine in 1926 by Harrington, commercially available Thyroxine became available. However, it was many years later when synthetic Thyroxine became preferable to desiccated extract [1].

Since it's first description by Gull, hypothyroidism is characterised by the insufficient production of thyroid hormones. Primary hypothyroidism is caused by insufficient thyroid hormone production [3]. In addition, it can be categorised based on it's time of onset (congenital or acquired) and it's severity; overt or subclinical disease. Secondary hypothyroidism (central hypothyroidism) caused by insufficient stimulation of a normal thyroid gland due TSH deficiency from pituitary or hypothalamic disease.

Primary hypothyroidism is overtly present when TSH is elevated and free thyroid hormones (especially free T4 [FT4]) are low. Subclinical hypothyroidism (SCH) is defined when TSH is above the upper limit of the reference range with preservation of free thyroid hormones within their reference range [4]. In adult patients, overt hypothyroidism (OH) is defined as TSH >10mU/L with a low FT4 concentration. SCH is defined as TSH >10mU/L with a normal FT4 concentration.

1.2 Epidemiology of Hypothyroidism

Hypothyroidism can be endemic in iodine deficient areas but is also quite prevalent in iodine sufficient areas. The annual incidence of clinical hypothyroidism is 0.4% in women and 0.06% in men with a prevalence of 1.9% in women and 0.1% in men. There is an approximate 7-fold increase incidence in women occurring in the 4^{th} decade [5]. The frequency of chronic autoimmune thyroiditis or Hashimoto's thyroiditis increases with age and is more common in individuals with a history or family history of other endocrine and non-endocrine autoimmune conditions [6].

OH was found in 2% of the total 2779 individuals tested as part of the Whickham survey and was ten-fold more common in women than men [7]. Risk factors for the development of hypothyroidism include a TSH >2.5 iu/L and the presence of TPO antibodies. In the twenty-year follow up of the Whickham survey, 55%, 33% and 27% of women with elevated TSH and positive TPO antibodies, elevated TSH alone or positive TPO antibodies alone developed hypothyroidism respectively [7]. In iodine sufficient areas, the prevalence in SCH ranges from 3-

10% depending on the criterion used to diagnose subclinical disease [5, 7]. The prevalence increases with age to 5% to 20% in men and women older than 60 years of age [8, 9].

1.3 Causes of Hypothyroidism

Primary hypothyroidism can be broadly divided into two categories; loss of functioning thyroid tissue and defects in thyroid hormone biosynthesis or release (Table 1.1). Iodine deficiency is the commonest cause of hypothyroidism worldwide.

Chronic autoimmune thyroid disease (Hashimoto's thyroiditis) is the commonest cause of primary hypothyroidism in iodine replete areas. Hakaru Hashimoto, a Japanese surgeon working in Germany first described the replacement of normal thyroid tissue by lymphocytic and fibrous tissue, until insufficient thyroid tissue is present to maintain thyroid hormone production. Hashimoto's thyroiditis can coexist with other autoimmune conditions and is present in 10-15% of individuals with autoimmune polyglandular syndrome type 1 (APS 1). Autoimmune polyglandular syndrome type II, a term used interchangeably with Schmidt syndrome is characterised by the presence of autoimmune adrenal insufficiency, autoimmune thyroiditis and/or type 1 diabetes mellitus (T1DM). Thyroid autoantibodies (Anti TPO antibodies) are present in 30% of people with T1DM but only 2-5% are diagnosed with autoimmune thyroiditis. Definitive treatment of Graves' disease, solitary toxic nodule and multinodular goitre with radioiodine results in hypothyroidism in approximately 82%, 60% and 64% over a 20-year period respectively.

Other causes of loss of functioning thyroid tissue include thyroiditis, surgery, irradiation and infiltrative conditions. Congenital hypothyroidism resulting from defects in thyroid hormone synthesis or release.

Iodine excess in the setting of abnormal thyroid tissue may result in hypothyroidism due a failure of a normal physiological escape mechanism. Drugs such as Amiodarone, kelp and iodinated contrast media can inhibit iodine organification and subsequent thyroid hormone synthesis.

Table 1-1 Causes of Hypothyroidism

Loss of Functional thyroidal tissue	Chronic autoimmune thyroiditis Thyroiditis (silent and postpartum thyroiditis, cytokine-induced thyroiditis). Surgery and irradiation (131I or external irradiation) Infiltrative and infectious diseases Subacute thyroiditis
Functional defects in thyroid hormone biosynthesis and release	Congenital defects in thyroid hormone biosynthesis Iodine deficiency and iodine excess Drugs: Antithyroid agents, lithium, natural and synthetic goitrogenic chemicals, tyrosine kinase inhibitors

1.4 Pathology of Hypothyroidism

The pathological hallmark of hypothyroidism is accumulation of glycosaminoglycans in interstitial tissue resulting in the presence of mucinous non-pitting oedema or myxoedema. These deposits typically occur in the dermis but can occur in many tissues. Glycosaminoglycans, such as hyaluronic acid accumulation are due to the loss of thyroid hormone inhibitory effects on the synthesis of fibronectin and collagen by fibroblasts [10, 11]. The dermis becomes hyperkeratotic and oedematous and similarly affected skeletal muscles become swollen and oedematous. The same pathological changes that occur in skeletal muscle cells also occur within the myocardium causing interstitial oedema. In the absence of cardiac failure, central congestive fibrosis occurs in the liver [12]. Longstanding myxoedema results in the deposition of glycogen containing round bodies and deposition of mucinous material in the brain[13]. Untreated congenital hypothyroidism results in delayed myelination and neuronal hypoplasia. Macroscopic study of the kidney is normal in hypothyroidism. However, microscopic studies demonstrate glomerular and tubular basement membrane thickening [14]

1.5 Pathophysiology of Subclinical Hypothyroidism and Overt Hypothyroidism

The development of hypothyroidism is a graded phenomenon. Mild biochemical thyroid perturbations can be associated with little or no symptoms whereas significant biochemical abnormalities result in incipient myxedema coma.

The transition from euthyroid state to the hypothyroid state is first detected by a slight elevation in serum TSH concentration secondary to decreased T4 secretion from the thyroid.

This does not result in changes to serum T4 concentration. The maintenance of serum T4 within the reference range exists because the pituitary thyrotroph is acutely sensitive to even slight changes in serum T4 concentration. Serum T3 remains preserved even as the serum T4 declines further with a resultant rise in TSH. Subnormal T3 concentrations occur in the setting of very low T4 levels and markedly elevated TSH levels (Table 1.2). Additional laboratory findings that may be present and raise suspicion of underlying thyroid dysfunction including dyslipidaemia, hypertriglyceridaemia, hyperprolactinaemia hyperhomocysteinaemia, hyponatraemia, anaemia and elevated creatine phosphokinase.

Table 1-2 Grade of Hypothyroidism

	Serum TSH concentration	Serum FT4 concentration	Serum FT3 concentration
Subclinical Hypothyroidism	Elevated	Normal	Normal *
Mild Overt Hypothyroidism	Elevated	Decreased	Normal
Overt Hypothyroidism	Elevated	Decreased	Decreased

* Can be elevated in very early stages of subclinical hypothyroidism

1.6 Clinical Features of Hypothyroidism

Hypothyroidism is a condition for which there is a wide spectrum of clinical presentation, ranging from minimal symptoms to myxedema coma.

This is partly explained by the different TSH concentrations used as a cut-off in defining subclinical disease. In addition, factors such as dietary iodine intake, race, age and sex contribute to the heterogeneity observed.

1.6.1 Overt Hypothyroidism

OH is suspected in in the presence of a variety of symptoms including fatigue, dry skin, cold intolerance and constipation (Table 1.3). The sensitivity of individual symptoms are variable, ranging from 2.9% to 24.5% [8]. Clinical symptoms can be influenced by disease severity and duration coupled with the patient's age and concomitant disease [15]. The common symptoms of thyroid hormone deficiency include reduced physical ability and lethargy. A large prospective study found that patients with a history of thyroid disease but with normal TSH and FT4 concentrations experienced more fatigue than euthyroid individuals. The reason for this is not entirely clear but it is suggested that altered thyroid hormone secretion may contribute [16].

Weight gain is a commonly cited feature of hypothyroidism and is reported by up to two-thirds of patients. Typically the observed weight gain is attributable to fluid accumulation and not fat deposition.

A decrease in tissue specific calorigenesis results in cold intolerance. The skin is dry, thick and feels cold, resulting from cutaneous vasoconstriction. Subcutaneous tissue can be thick

and pitting oedema of the lower limbs is not uncommon. Delayed wound healing has been described in myxoedema.

Along with skin changes, hair texture can be altered resulting in dry and brittle hair. Coupled with this, hair growth is decreased and readily falls out. Loss of eyebrow hair is described and classically begins at the lateral border. However, loss of eyebrow hair is not uncommon in elderly euthyroid women. Thickening of the nails also occur [17]. Two factors result in changes to speech quality and rarely articulation including macroglossia and thickening of the lips.

Various gastrointestinal symptoms result from slowing of the bowel in one form or another. Disordered oesophageal motility can result in heartburn. Nausea, altered appetite, or vomiting may be a result of delayed gastric emptying [18]. Bacterial overgrowth is more prevalent in patients with overt hypothyroidism compared to controls (54% compared to 5% respectively), which can cause abdominal bloating and flatulence. Symptoms improve with decontamination of bacterial overgrowth [19]. Prolonged intestinal transit time and reduced peristalsis can cause constipation that can result in ileus. Intestinal absorption is also slowed.
The presence of circulating gastric parietal cell antibodies and coincident pernicious anaemia is present in 25% and 14% of individuals with Hashimoto's thyroiditis [20].

Menstrual irregularity is reported in premenopausal women with hypothyroidism. Compared to controls, 23% of women experience irregular menses compared to 8% of controls [21]. Menorrhagia is the commonest menstrual irregularity reported.

Table 1-3 Symptoms and Signs of Hypothyroidism

	Symptoms	Signs
Skin	Dry skin Hair loss Brittle nails	Puffy facies Non-pitting oedema of hands, face, and ankles Peri-orbital swelling Yellowish skin Coarse hair
Neurological	Weakness Muscle cramps Joint pain Headache Paraesthesia Deafness Vertigo or tinnitus Sleep apnoea	Delayed relaxation of deep tendon reflexes Carpal tunnel syndrome Cerebellar ataxia
Cognitive Function	Reduced attention span Memory deficits Calculation difficulties	Somnolence Slow speech
Psychiatric	Depression	
Respiratory	Hoarse voice	Pleural effusion
Musculoskeletal	Myalgia Muscle weakness Stiffness	Joint effusions Carpal tunnel syndrome Delayed linear bone growth in children
Bone		Reduced bone turnover
Cardiovascular	Decreased exercise tolerance Angina	Bradycardia Diastolic Hypertension Cardiomegaly Pericardial effusion Low-voltage ECG Peripheral (non) pitting oedema
Gastrointestinal	Anorexia Nausea Constipation	Macroglossia Prolonged intestinal transit time Delayed intestinal absorption Gallbladder hypotonia
Reproductive	Reduced fertility Menstrual irregularities	

1.6.2 Subclinical Hypothyroidism

Many of the symptoms and signs associated with OH are not present in SCH. The diagnosis of hypothyroidism has changed over the last two to three decades and given the frequency of thyroid function testing; subclinical disease is increasingly detected prior to the development of symptoms. Older clinical scores validated for the use in detecting OH and SCH do not always correlate with the biochemistry but a clinical score developed by Zulewski et al. correlates with serum FT4 and TSH concentration in SCH [22]. It is more difficult to identify symptoms and signs of SCH in the elderly and clinical signs are poor predictors of SCH in the elderly [23].

Two studies have examined the presence of symptoms in patients with SCH. Amongst 40 patients with SCH, the most common hypothyroid symptom was fatigue (83%) and weight gain (80%) and 50% had elevated anxiety scores [24]. The presence of hypothyroid symptoms was higher in SCH patients compared to euthyroid-matched controls [25]. Controversy exists regarding the increased presence of depression in patients with SCH. An interview survey of 825 individuals with a mean age of 74 years did not self-report increased rates of depression or symptoms in those with SCH compared to people with a normal TSH concentration. A study in healthy females reported a higher lifetime frequency of depression in those with SCH compared to controls. Some of these studies may be confounded by the fact that individuals with poorer quality of life are more likely to seek medical attention and undergo routine thyroid screening. Larger population studies have demonstrated no reduction in quality of life in those with SCH.

1.7 Cardiovascular Disease and Hypothyroidism

1.7.1 Cardiac Function and Hypothyroidism

The link between thyroid disease and the heart was first described in 1825. Caleb Parry noted that a number of women presenting with heart failure and possibly the first description of atrial fibrillation had enlarged thyroid glands. A number of years later, Robert Graves' described 3 cases of women presenting with palpitations, goitre and exophthalmos. The association between hypothyroidism and the heart was not reported until 1918. Hermann Zondek described "Das Myxodemherz", noting the reversibility of some of his patient's heart failure with thyroid hormone replacement and an increase in pulse rate. A number of years later, George Fahr observed "dilatation of the heart, slow indolent heart action, a normal blood pressure, loss of electrocardiographic complexes and reversibility of the abnormalities following thyroid administration" in 17 cases of myxoedema.

Adequate thyroid hormone concentrations are necessary to maintain normal cardiovascular function. T3 exerts inotropic effects on myocardial contractility and vascular function. Hypothyroidism is associated with a decreased pulse rate that occurs more or less in tandem with reduced metabolic rate. In addition, stroke volume is also reduced resulting in decreased cardiac output. Hypothyroidism is associated with left ventricular diastolic dysfunction at rest and with systolic and diastolic dysfunction on exertion, which can result in decreased exercise capacity. Hypothyroidism in animal models leads to maladaptive changes in cardiac myocytes and ultimately the development of heart failure [26]. Heart failure secondary to hypothyroidism is uncommon in individuals without underlying ischaemic heart disease and typically occurs in

individuals who are T4 deplete for a prolonged period. Hypothyroid individuals who were thyroid hormone deplete had reduced myocardial oxidative metabolism as measured by positron emission tomography (PET), reduced cardiac contractility and increased peripheral vascular resistance compared to when they were hormone replete [27]. The increased systemic vascular resistance results in diastolic hypertension, that typically improves following treatment with Thyroxine therapy [28]. It is suggested that reduced T4 to T3 conversion resulting in low serum T3 concentration, so called low T3 syndrome, which is a predictor of mortality in patients with severe heart disease [29, 30].

Hypothyroidism is associated with effusions in many tissues including the pericardium, pleura and peritoneum. When present in the pericardium, it is associated with cardiomegaly however; it is rarely associated with tamponade. A physiological study carried out in 12 hypothyroid individuals in 1958 reported slightly elevated mean pressures in the right atria and pulmonary arteries and end diastolic right ventricular pressures suggested underlying pericardial effusions rather than underlying myocardial disease [31]. ECG findings typical but not pathognomonic of hypothyroidism include bradycardia, low voltage complexes, increased P-R interval, widening of QRS complexes, flattening or inversion of T waves; so called “u” waves. ECG changes associated with hypothyroidism resolve on treating hypothyroidism.

Individuals with hypothyroidism are at increased risk of cardiovascular disease, possibly secondary to increased risk of coronary artery disease and heart failure [32]. This is also the case in SCH, as reported more recently [33]. Similar to OH, impaired left ventricular diastolic function is observed in SCH, characterised by impaired ventricular filling and delayed

myocardial relaxation[34]. Diastolic dysfunction is reversible with Thyroxine replacement [35]. It is not clear if left ventricular systolic dysfunction occurs at rest in SCH but there is emerging evidence using newer imaging techniques that it is impaired. The balance of evidence suggests that systolic function is also reversible with Thyroxine treatment, although one study reported improved systolic function compared to baseline but not compared to controls [36].

1.7.2 Hypothyroidism and Coronary Artery Disease

Hypothyroidism is implicated in the development of coronary artery disease and this occurs in two ways. Firstly, hypothyroidism is associated with the development of dyslipidaemia and hypertension both of which are involved in the pathogenesis of atherosclerosis. Secondly, hypothyroidism has a negative inotropic and chronotropic effect on the heart. This results in reduced myocardial oxygen demand and recovery, which can exacerbate underlying coronary ischemia. However, despite the increased prevalence of cardiovascular risk factors in the hypothyroid population, there is a relatively low incidence of angina pectoris and myocardial infarction. This is partly explained by overall reduced metabolic demands placed on various organs including the heart in the setting of hypothyroidism.

1.7.3 Hypothyroidism and Dyslipidaemia and Atherosclerosis

Dyslipidaemia and more specifically postprandial lipaemia are implicated in the development of atherosclerosis. Overt and to a lesser extent subclinical hypothyroidism is associated with elevated concentrations of LDL-C, ApoB and possibly triglyceride

concentrations. HDL-C metabolism is less clear but in OH is typically normal or increased, resulting in an unfavourable LDL: HDL-C ratio. Dyslipidaemia is present in 90% of cases of OH but reversal occurs on treating with Thyroxine therapy, unless dyslipidaemia is present outwith of thyroid dysfunction. Total and LDL-C concentrations are increased by 30% in individuals with OH.

The mechanism for elevated LDL-C in OH is partly meditated by decreased LDL receptor (LDLR) activity, resulting from reduced sterol regulatory element binding protein-2 (SREBP-2), which is mediated by triiodothyronine [37]. The decrease in LDL-C receptors leads to reduced clearance of LDL-C from the serum. Increased cholesterol absorption occurs in OH via the action on Nieman-Pick-C1-like 1 protein (NPC1P) in the gut. Although, hepatic cholesterol synthesis is reduced in OH, the overall net effect is increased LDL-C in OH (Figure 1). Thyroid hormone increases lipoprotein lipase (LPL) activity and it is decreased in OH, resulting in elevated triglyceride concentrations. Cholesterol ester transfer protein (CETP) concentrations are decreased in OH, giving rise to altered HDL-C concentrations.

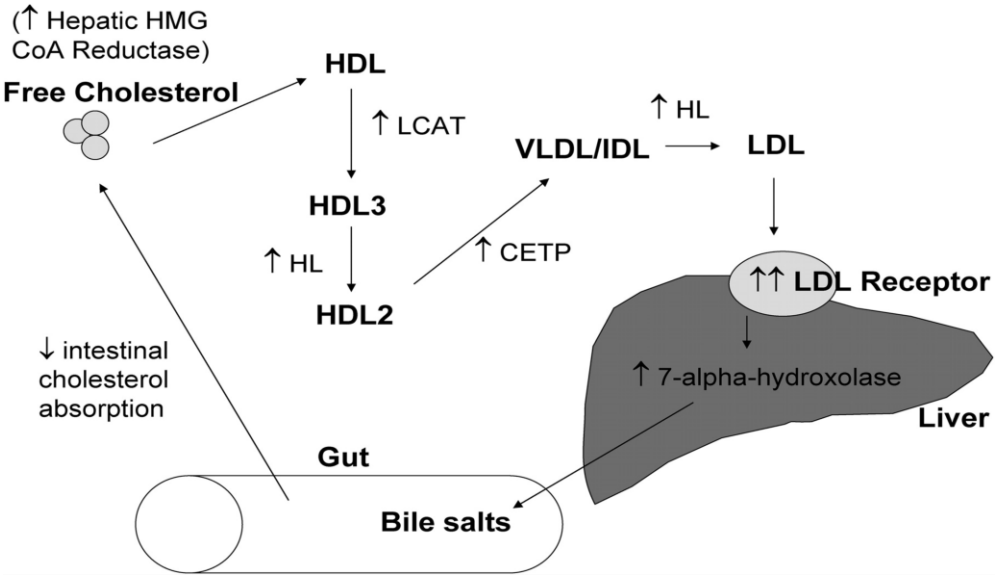

Figure 1-1 Lipid Metabolism
Adapted from Pearce EN, JCEM 97(2):326-333

Lipid abnormalities in SCH are less well defined. Cross sectional studies report variably increased total and LDL-C but HDL-C results are inconsistent, either normal or slightly reduced. Chylomicrons remnants and VLDL remnants are triglyceride rich lipoproteins, typically present in the postprandial state and are implicated in the development of atherogenesis. Chylomicrons remnants are increased in OH, changes that are reversible with Thyroxine therapy. Altered lipid concentrations are one cardiovascular risk factor associated with both OH and SCH. The combination of diastolic hypertension, increased arterial stiffness and elevated CRP also play a role in the development of atherosclerosis. Increased homocysteine concentrations are observed in OH only and not in SCH. Hypothyroidism accelerate atheromatous changes in animal studies and human post-mortem studies show severe atherosclerosis in patients with hypothyroidism.

Surrogate markers of atherosclerosis, such as carotid intima media thickness (CIMT) are increased in both overt and subclinical disease [38, 39]. Endothelial dysfunction is reported in OH and to a lesser extent in SCH [40]

2 Introduction TYPE 1 DIABETES MELLITUS

2.1 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide [41]. Atherosclerosis is implicated in the development of CVD. Risk factors associated with CVD are also associated with the development of atherosclerosis; including dyslipidaemia, smoking, hypertension and diabetes. Dyslipidaemia, in particular elevated levels of low-density lipoprotein (LDL) cholesterol are widely thought to play a key role in the atherosclerotic pathway[42]. There is increasing evidence that postprandial lipaemia is independently predictive of atherogenesis and of future cardiovascular events. Postprandial studies can unmask lipoprotein abnormalities not present in the fasting state. The overall CVD risk is increased in type 1 diabetes (T1DM).

2.2 Definition of Diabetes

Diabetes mellitus is characterised by increased blood glucose concentrations resulting in a chronic systemic condition. The meaning of the word Diabetes originates from the Greek word "*diabainein*", meaning siphon or pass through referring to the large volume of urine observed in untreated cases. In 1675, an English Physician, Thomas Willis added the word mellitus, derived from the Latin "*me*l" or honey referring to the sweet smell from the urine. Diabetes results from decreased insulin production from pancreatic β-cells or decreased effect of insulin on target tissues or a combination of both. Although it primarily affects carbohydrate metabolism, diabetes also disturbs protein and lipid metabolism. Diabetes is defined as a fasting glucose ≥7.0mmol/l

or 2-hour plasma glucose of ≥11.1mmol/l following an oral glucose tolerance test (OGTT) or HbA_{1C} ≥6.5% (≥48mmol/mol) or random plasma glucose of ≥11.1mmol/l in a patient with classic symptoms of hyperglycaemia [43].

2.3 Classification of Diabetes

In order to treat the pathogenesis of the hyperglycaemia and treat accordingly, diabetes is broadly classified based on its pathogenesis. T1DM results from cellular mediated autoimmune destruction of the insulin secreting β-cells of the islets of Langerhans in the pancreas, leading to insulin deficiency. The rate of β-cell decline is variable; rapid in some cases (mainly infants and children) and can be slower in others, resulting in preservation of some β-cell function over many years. It accounts for 5-10% of all cases of diabetes. One or more of the autoantibodies involved are present in 85-90% of cases. T1DM is also associated with HLA, with linkage to DQA and DQB genes. Patients with T1DM are prone to developing other autoimmune conditions. Latent Autoimmune Diabetes of the Adult (LADA) occurs in older adults and is characterised by autoimmune destruction of the β-cells. Unlike T1DM, patients with LADA do not always require insulin at presentation but approximately 80% of patients will require insulin a number of years following diagnosis.

Type 2 diabetes mellitus (T2DM) is characterised by increased insulin resistance, primarily in adipose tissue, liver and skeletal muscle and/or relative lack of insulin secretion from β-cells. It accounts for 90-95% of all cases of diabetes.

Maturity Onset Diabetes of young (MODY) is characterised by monogenic defects in β-cell function, typically due to impaired insulin secretion with minimal defects in insulin action. Abnormalities on six different genetic loci have been identified to date and inheritance is in an autosomal dominant fashion.

A heterogeneous group of genetic conditions characterised by disorders of insulin action result from insulin receptor mutations that can present in child or adulthood and are associated with insulin resistance. The presentation of hyperglycaemia associated with insulin resistance occurring in pregnancy is referred to as gestational diabetes and carries an increased risk of developing T2DM. Finally, the causes of secondary forms of diabetes are varied from medication induced to pancreatic insufficiency.

2.4 Pathogenesis of Type 1 Diabetes Mellitus

T1DM result from T-cell mediated autoimmune destruction of the insulin producing β-cells in the islets of Langerhans and is characterised histologically by insulitis. Inflammation of the islets in T1DM is characterised by a pancreatic infiltrate composed of T-lymphocytes, B-lymphocytes and macrophages and diminished insulin-producing β-cells. Autoimmunity in T1DM is usually identified by the presence of autoantibodies directed against β-cell autoantigens. Amongst the most common autoantibodies are autoantibodies to islet cells, glutamic acid decarboxylase (GADAs), insulin (IAAs), Zinc transporter 8A molecule (ZnT8As) and against insulinoma associated autoantigen 2 (IA2As). These autoantibodies are present at presentation in 70-80% of T1DM cases at presentation and can be present in the preceding years

prior to the onset of the disease. Conversely, only 20-25% of children that are autoantibody positive will go on to develop the condition [44]. The overall risk of developing T1DM is not accounted for by autoantibodies alone.

Additional insights can be gained from familial and aggregation studies. Among individuals with first-degree relatives with T1DM, the risk of developing T1DM was 1 in 20 compared to 1 in 300 in the background population [45]. The concordance amongst monozygotic twins is 30-50% and amongst dizygotic twins is 6-10%. Despite the apparent genetic association, approximately 85% of individuals with T1DM have no family history of the condition [46]. No single gene is in itself sufficient to predict the development of T1DM. Most of the 40 identified susceptible loci are thought to involve immune responses. Human Leukocyte Antigens (HLA) complex provides the approximately 60% of the overall genetic susceptibility. HLA-DR3 and DR4 class II antigens are present in 30% of patients with T1DM and in Caucasians; the DR3/DR4 phenotype confers the highest risk of T1DM. The proportion of newly identified cases with T1DM presenting with HLA genotypes is decreasing and other factors including the environment are thought to have increased [47]. So although it occurs in genetically susceptible individuals, the triggers for the autoimmune process are not clear but environmental triggers appear to play a role.

There is no direct evidence that infections play a role in the development of T1DM but there are reports of a relationship between B cell autoimmunity and enteroviral infections [48]. Increased anti-enteroviral antibodies and immunohistochemistry detection of enteroviruses have been observed in pancreatic tissue of individuals with T1DM but whether this is causal or not is

a matter of debate. Dietary factors, specifically low levels of breast-feeding and early introduction of cow's milk have been linked to the development of T1DM. Additional larger studies are needed to examine the relationship between exposure to gluten and certain cereals and the development of T1DM, as the evidence is weak at present [49]. The highest incidence of T1DM is in Northern Europe, which has led some investigators to wonder if low vitamin D concentrations are associated with the development of T1DM. In support of this, more cases are diagnosed in the Autumn and Winter [49]. The incidence of T1DM is increased amongst children who move from a region of low to high incidence of T1DM. It is clear environmental factors play a role in the pathogenesis of T1DM, although additional collaborative research is needed to elucidate this further as much of the work to date are focused in small studies.

2.5 Epidemiology of Type 1 Diabetes Mellitus

Both males and females are affected equally in childhood but men are more commonly affected in early adulthood. The highest incidence is in Northern Europe, specifically Finland, which has an incidence of 64 per 100000 <15 years of age in contrast to Venezuela with only 0.1 per 100000 per year. T1DM is increasing worldwide yet the exact mechanism for this remains unclear. Finland observed a non-linear increase in children developing T1DM and rates doubled from 1980 to 2005 but appear to have plateaued [50]. It is not clear why this increase is occurring. According to the "Spring Harvest Theory", the increased incidence observed in children may accompany decreased incidence in older adults and this appeared to be the case in Sweden and Belgium but not Finland.

2.6 Cardiovascular Risk in Type 1 Diabetes Mellitus

T1DM is associated with an increase in all cause mortality, more marked in women than men, compared to the general population. Cardiovascular disease is still the leading cause of mortality amongst patients with T1DM [51-53]. It was largely felt that this was due to prolonged duration of glycaemic exposure. The 17-year follow-up of the diabetes control and complications trial/epidemiology of diabetes interventions and complications trial revealed a 58% risk reduction in non-fatal myocardial infarction, stroke and cardiovascular disease related death in those originally assigned to intensive glycaemic control [54]. Thus, good glycaemic control is important in the prevention of cardiovascular disease. Hypertension and renal disease are important contributing factors to the progression to cardiovascular disease in people with T1DM. In the FinnDiane cohort, the presence of microalbuminuria and macroalbuminuria conferred a 3-fold and 9-fold excess mortality compared to the background population. Similar results were observed in the Pittsburgh EDC cohort, followed for 20 years [55] .

It is unclear why women with T1DM have a particularly high relative risk of cardiovascular disease compared to men with T1DM and to the population at large, despite improved practices in care over the last number of years. Altered body fat distribution and elevated blood pressure in women with T1DM may convey a more insulin resistant type picture in this group contributing to the increased relative risk. An elevated CRP in women with T1DM compared to men may reflect insulin resistance at some level. Coronary artery calcification is increased in women with T1DM compared to men [56]. Nearly a quarter of asymptomatic patients with T1DM between 35-60 years of age have evidence of ischemia on either exercise

electrocardiography or dynamic perfusion scintigraphy[57] . Coronary artery disease in T1DM is diffuse and distal stenosis is more common in major vessels, precluding bypass grafting at times.

2.7 Dyslipidaemia in Type 1 Diabetes Mellitus

In the absence of concomitant renal disease, patients with T1DM a favourable fasting lasting lipid profile compared to the general population and have a normal or high HDL-C and normal or low triglyceride concentration. Low-normal triglyceride and LDL-C concentration is likely due to diminished very low-density lipoprotein (VLDL) as a consequence of subcutaneous insulin treatment. In addition, peripheral hyperinsulinaemia increases LPL activity, lowering triglyceride concentrations further [58]. Intraperitoneal insulin administration via an implantable insulin pump mimics physiological insulin replacement, preventing peripheral hyperinsulinaemia and hepatic hypoinsulinaemia that occurs with subcutaneous insulin. Studies examining lipid alterations following administration of insulin intraperitoneally have been inconclusive [59]. Although the lipid profile maybe favourable in T1DM, lipoprotein abnormalities present in T1DM have been shown to be a better predictor of mortality than conventional lipid measurements in T1DM [60].

2.8 Atherosclerosis and Type 1 Diabetes Mellitus

Atherosclerosis occurs earlier and is accelerated in T1DM. Surrogate markers of atherosclerosis such as carotid intima media thickness (CIMT) are increased in T1DM even in teenagers with poor glycaemic control [61].

Postprandial lipoprotein abnormalities are independently predictive of atherosclerosis. Postprandially, the majority of the circulating triglycerides exist in 2 forms; ApoB48 rich chylomicrons and the apolipoprotein-B100 (ApoB100) rich VLDL particles that are synthesized in the liver. Abnormalities in chylomicron synthesis and clearance are thought to play a role in the development of atherosclerosis. *In vitro* work has demonstrated that chylomicron remnants may have an immunomodulatory role and be involved in inflammation and oxidation, seen in the pathogenesis of atherosclerosis. Chylomicron particles have been found in the atherosclerotic plaque and apoB48 receptors found on the surface of the macrophage. A delayed chylomicron clearance response is seen in patients with coronary heart disease and in patients with both T1DM and T2DM [62, 63]. Irrespective of a conventional lipid profile, patients with known coronary artery disease were found to have a higher concentration of chylomicron remnants in their plasma than patients with normal coronary arteries, which may partly explain why they are felt to be atherogenic.

2.9 Endothelial Function and Type 1 Diabetes Mellitus

The endothelium is a monolayer of cells covering the vascular lumen, acting as a barrier between the vasculature and underlying tissue. Historically, it was thought the endothelium was

relatively inert. It is now recognised that the endothelium is metabolically active and is responsible for the maintenance of vascular tone [64].

When the endothelium is exposed to stress including hyperglycaemia, a negative intracellular cascade occurs resulting in endothelial dysfunction. Nitric oxide (NO) is a key mediator in the maintenance of vascular tone and integrity.

Hyperglycaemia results in a decrease in NO availability in a number of ways. Firstly, high intracellular glucose alters the intracellular redox state leading to Protein Kinase C activation and a subsequent decrease in cellular NADPH pool. NADPH is necessary for the production in NO. Hyperglycaemia induces excess of electrons that leak from the oxidative chain and are captured by oxygen generating superoxide excess. Excess superoxide production uncouples eNO synthase (eNOS) resulting in decreased NO concentration. Superoxide excess also results in up regulation of proinflammatory cytokines including ICAM and VCAM via NFKB signalling. It also results in up regulation of Thromboxane A2 and ultimately the coagulation cascade. Chronic hyperglycaemia leads to non-enzymatic glycation of proteins and macromolecules. The final common pathway is that foam cell derived inflammatory cytokines maintain vascular inflammation as well as proliferation of smooth muscle cells accelerating the atherosclerotic process [65].

Endothelial dysfunction precedes development of atherosclerosis in diabetic and nondiabetic subjects, possibly mediated through inflammation. Postprandial glucometabolic changes impair endothelial function in normal and T2DM subjects. It is not clear from the literature whether endothelial dysfunction in T1DM is due to the diabetic milieu or whether it is

a marker of vascular damage. The balance of evidence suggests that T1DM predisposes to the occurrence of endothelial dysfunction but is not necessarily causative and is likely that other factors are involved [66, 67]

2.10 Hypotheses and aims of this thesis

The overall CVD risk is increased in overt hypothyroidism (OH) and possibly in subclinical hypothyroidism (SCH). LDL-Cholesterol (LDL-C) is increased in OH but not SCH, which may partly explain the increased CVD risk in OH, but likely does not explain it entirely. It is not consistently clear what happens to triglyceride and HDL-Cholesterol (HDL-C) in OH and is even less clear in SCH.

Hence, our hypothesis for the Thyroid study:

- Postprandial dyslipidaemia is associated with lipoprotein derangements that may contribute to the increased cardiovascular risk observed in hypothyroidism in addition to that conferred by fasting LDL-C concentrations
- To examine fasting and postprandial HDL-C metabolism in both OH and SCH in an effort to elucidate the differences observed between OH and SCH.

The overall CVD risk is increased in type 1 diabetes mellitus (T1DM) and individuals with T1DM have accelerated atherosclerosis. Endothelial dysfunction precedes the development of atherosclerosis. Yet, in the presence of good glycaemic control, individuals with T1DM have a

relatively preserved fasting and even favourable lipid profile. It is not known to what extent postprandial lipoprotein changes contribute to the increased CVD risk.

Hence, our hypothesis for the T1DM study:

- Postprandial dyslipidaemia is associated with lipoprotein derangements that may contribute to the increased cardiovascular risk observed in T1DM and endothelial dysfunction
- Elucidate what glucometabolic and lipoprotein factors affect endothelial dysfunction in T1DM.

3 HYPOTHYROIDISM AND DYSLIPIDAEMIA

Postprandial studies uncover differing effects on HDL particles of overt and subclinical hypothyroidism

3.1 Introduction

Elevated levels of total cholesterol, LDL cholesterol (LDL-C), Apolipoprotein B (Apo B) and possibly triglycerides are associated with overt hypothyroidism (OH) and to a lesser extent with subclinical hypothyroidism (SCH) [68]. Although hepatic cholesterol synthesis is reduced in OH, the overall effect is increased LDL-C [69]. This is partly mediated by decreased LDL receptor (LDLR) activity resulting from reduced sterol regulatory element binding protein-2 (SREBP-2), which is mediated by triiodothyronine [37]. Studies in hypophysectomised rats have demonstrated markedly increased intestinal cholesterol absorption, which is normalized following replacement with thyroid hormone but not with cortisol or growth hormone [70]. However, it is likely that other mechanisms are involved in the dyslipidaemia of hypothyroidism since in LDLR knockout mice thyroid hormone reduces LDL-C at least as markedly as in wild-type mice. In the same studies, thyroid hormone was also demonstrated to reduce circulating Apo B48, a specific marker of intestinally-derived lipoproteins, and hepatic production of Apo B while increasing triglyceride production [71]. Further actions of thyroid hormone that potentially influence LDL-C and triglyceride metabolism include increased hepatic expression of

hydroxymethylgluaryl coenzyme A (increased cholesterol synthesis) and increased lipoprotein lipase (hydrolysis of triglyceride-enriched lipoproteins).

The effects of hypothyroidism on HDL-C are less clear. Levels have been reported to be normal or slightly increased in OH [72], and normal or reduced in SCH [73-75]. Consistent with the latter, studies in euthyroid subjects have demonstrated associations between greater thyroid hormone levels and greater HDL-C concentrations. Mechanisms through which thyroid hormone could influence HDL-C include increased cholesterol ester transport protein (CETP) (transferring cholesterol from HDL-C to LDL and VLDL particles) [76], increased hepatic lipase (influencing HDL subfractions) and increased cholesterol efflux from macrophages to HDL via the ABCA1 transporter [77].

For a number of reasons, studies in the postprandial phase enhance our understanding of these processes and their clinical importance. Firstly, postprandial lipoprotein abnormalities are considered to be independently predictive of atherogenesis, in the case of triglycerides more markedly than fasting levels [78, 79]. Secondly, meal ingestion can act as a challenge to accentuate abnormalities that are difficult to detect under fasting conditions. To date very few postprandial studies have been carried out in OH or SCH. One study using the retinyl palmitate technique demonstrated an increase in intestinally derived lipoprotein particles following L-thyroxine replacement in OH [80]. Another demonstrated a greater frequency of postprandial hypertriglyceridaemia levels in OH with also a possible effect in SCH [81]. We considered that in view of the prevalence of SCH (4-10% of the adult population) [15] and the observations that cardiovascular risk is increased in these patients, studies of postprandial lipoprotein metabolism

is SCH were justified. Furthermore, we considered that studying triglyceride and HDL metabolism under postprandial conditions would provide further insights into how lipoprotein physiology is altered in hypothyroidism.

We carried out detailed postprandial studies in subjects with OH and SCH and matched controls. Measurement of ApoB48 was used to determine the proportion of intestinally derived lipoprotein particles in circulation. HDL-C was sub fractionated into HDL_2 and HDL_3 and their isolates were assessed for a variety of lipid and protein components including CETP. Endothelial dysfunction was determined using flow-mediated dilatation of the brachial artery [82, 83].

3.2 Methods

3.2.1 Experimental subjects

Ninety-three subjects were recruited. Subjects with OH (n=21) and SCH (n=28) were recruited from General Practitioner referrals to the Endocrine laboratory or Endocrinology outpatients. Control subjects (n=44) were recruited by local advertisement. OH was defined as thyroid stimulating hormone (TSH) concentration ≥10 mU/L and free thyroxine (FT_4) concentration ≤11 pmol/L, and SCH as TSH concentration ≥4 and ≤10 mU/L and normal circulating FT_4 concentration.

Exclusion criteria included a prior history of ischemic heart disease, stroke, diabetes, head injury, epilepsy, psychiatric illness, significant visual impairment or pregnancy. Subjects were also excluded if they had a body mass index <18 or >35 kg/m^2 or taking medication that was likely to influence the results. All participants gave written informed consent before

participating in the study, which was approved by the Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland).

3.2.2 Study design

This was a cross-sectional study. All subjects attended the Diabetes Day Centre in the Adelaide and Meath Hospital, Tallaght, Dublin after a 12-hour fast and having avoided excessive exercise and alcohol for the previous 24 hours. A mixed meal consisting of three slices of toasted white bread, 3 x 7 g packs of standard butter, a standard commercially available blueberry muffin, and a 330 mL carton of flavoured milk was consumed within 15 minutes. Nutritional content of this meal was 940 kcal consisting of 27 g of protein, 140 g of carbohydrate (40 g of sugar and 36 g of fat).

Fasting blood samples were taken for analysis of plasma lipids including Apo B48 and HDL subfractions and flow-mediated dilatation of the fore-arm was measured. Postprandial samples were taken at time 15, 30, 60, 90, 120, 240, 360 and 480 minutes following the mixed meal. Flow mediated dilatation was measured in the postprandial setting at T240 minutes. All subjects were included in the final analysis resulting in 49 cases and 44 controls, matched for age, sex, BMI, waist hip ratio.

3.2.3 Measurement of body composition

All subjects underwent estimation of body composition using auxiological methods and bioimpedance analysis. Height (measured with a Harpenden stadiometer) and weight were

measured in a hospital gown. Waist (WC) and hip (HC) circumferences were measured with a non-distensible flexible tape measure at the waist and hip. A Health and Lifestyle questionnaire was completed by each participant, which included details on smoking history and alcohol consumption, which could potentially influence markers of inflammation. Percentage body fat was estimated using a body fat analyser (TBF-300; Tanita Corp., Arlington Heights, IL). Percentage fat was calculated using the manufacturer's programmed equations. The same observer made all measurements on all study days.

3.2.4 Laboratory methods

Total cholesterol (TC), TG and HDL-C were measured by an enzymatic colorimetric method (Cobas Roche diagnostics Ltd., UK) on the Roche P Module (Roche, Stockholm, Sweden). Glucose was measured by an enzymatic method on the Roche P Module. Insulin was measured by electrochemiluminesence immunoassay (Cobas Roche diagnostics Ltd) on the Roche E Module (Roche, Stockholm, Sweden). LDL-C was calculated using the Friedwald equation.

3.2.5 Measurement of Hormones

TSH and FT_4 were measured by standard chemiluminesence immunoassays (Cobas Roche diagnostics Ltd) on the Immulite 2500 analyser (Siemens). In our laboratory, the normal hormone reference range is 0.4-4.0 mU/L and 9-22 pmol/L respectively.

3.2.6 Measurement of markers of inflammation and cytokines

Total white cell count and differential were measured by laser flow cytometry. High sensitivity C reactive protein (hsCRP) was measured by an enzyme linked immunosorbent assay (ELISA) using a commercially available kit according to the manufacturer's instructions (BioCheck Inc., USA).

3.2.7 Measurement of Lipids

3.2.6.1 Measurement of Apo B48

ApoB48 was measured by ELISA using a commercially available kit (Gentaur, Belgium).

3.2.6.2 Isolation and sub fractionation of HDL_2 and HDL_3 from serum

HDL_2 and HDL_3 were isolated from serum by rapid ultracentrifugation according to a method previously established [84]. This method is a 3-step, 6-hour long procedure; crude (c)HDL was isolated by a 2-hour rapid sedimentation method. This cHDL was then sub fractionated into HDL_2 and HDL_3, by two, 2-hour sequential rapid flotation ultracentrifugation procedures.

3.2.6.3 Apolipoprotein A1 (ApoA1) Determination

ApoA1 concentration in the HDL subfractions was determined using single radial immunodiffusion (SRID) (19).

3.2.6.4 SAA concentration

SAA in serum and associated with HDL_2 and HDL_3, was analysed by an ELISA assay (Invitrogen^{TM} UK, KHA0011), as per the manufacturer's instructions. Analysis was performed using a Grifols TRITURUS system (Italy).

3.2.6.5 CETP and LCAT activity

The activities of CETP and LCAT were measured in serum and in HDL_2 and HDL_3 using commercially available fluorometric assays, as per manufacturer's instructions (CETP catalogue no. RB-CETP; LCAT catalogue no. RB-LCAT; Roar Biomedical, NY, US).

3.2.6.6 Total Protein Determination

Protein concentration within HDL_2 and HDL_3 was determined by a spectrophotometric assay [84]. Total protein concentration was utilised to standardise SAA, CETP and LCAT within HDL_2 and HDL_3.

3.2.8 Flow Mediated Dilatation

Flow-mediated dilatation (FMD) of the brachial artery was completed at two time-points during the study; fasting and at 240 minutes postprandially. FMD was carried out on the subjects' right arm, with measurements of the brachial artery diameter and flow parameters taken at rest, and at 30, 60, 90 and 120 seconds after the performance of a hyperaemic manoeuvre. A sphygmomanometer cuff was inflated to at least 20 mmHg greater than systolic pressure on the

upper arm for 5 minutes before release, with images taken at the time points mentioned above. Calculation of percentage change of the arterial diameter was calculated based on resting diameter compared to the maximal increase seen over these time-points.

3.2.9 Statistical analysis

Statistical analysis was performed using SPSS for windows Version 18.0 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD). Skewed variables were logarithmically transformed to normalise data prior to analysis. Absolute p values are displayed to allow the reader to interpret results of borderline significance. ANOVA was the primary statistical tool used. Mixed between-within subject ANOVAs were used to compare measurements across different time points between the 3 groups. Where significant main effects were observed ($p<0.05$), subsequent one-way ANOVAs compared the dependent variable (measurement) between the groups. Where significant between-group differences were observed, post-hoc analyses were performed to explore the differences further. A Bonferroni adjustment was applied to these comparisons to maintain a reasonable α level across all tests. The relationship between TSH and FT4 concentrations and all dependent variables were evaluated using Pearson's correlation coefficient

3.3 Results

3.3.1 Baseline Characteristics

OH, SCH and control subjects were closely matched for age, sex, BMI, WHR and percent body fat (Table 3.1). There were no significant between-group differences in HOMA-IR, Hb_{A1c}, insulin AUC or glucose AUC (Table 3.2).

3.3.2 LDL-C, HDL and Triglycerides

There was no significant effect of group or time for LDL-C or triglyceride concentration (Table 3.2). There was a significant effect of time for HDL concentration, all groups showing a significant decrease postprandially ($p<0.001$) (Figure 3.1). There was also a significant between-group effect for HDL concentration ($p <0.01$). Post-hoc analysis revealed significant differences between SCH subjects and controls for both peak ($p <0.05$) and AUC HDL ($p=0.001$) concentrations. Differences between SCH and OH approached significance ($p=0.07$) in post hoc analysis.

Table 3-1 Baseline demographic and anthropometric measurements in control, SCH and OH subjects.

	Control (n=44)	SCH (n=28)	OH (n=21)	p value
Age (years)	47.3 ± 9.7	47.3 ± 8.6	43.8 ± 11.6	NS
Sex (M/F)	16/28	10/18	9/12	NS
Height (m)	1.68 ± 0.09	1.68 ± 0.08	1.70 ± 0.12	NS
Weight (kg)	79.9 ± 14.4	80.1 ± 12.1	77.8 ± 16.3	NS
BMI (kg/m^2)	28.1 ± 4.32	28.4 ± 3.96	26.4 ± 4.17	NS
WHR	0.93 ± 0.12	0.93 ± 0.06	0.92 ± 0.10	NS
Body Fat (%)	34.5 ± 8.8	34.2 ± 8.5	32.4 ± 8.2	NS

Data expressed as mean ± S.D. p value derived from mixed between-within subject ANOVAs. NS= non-significant. WHR=Waist Hip Ratio.

Table 3-2 Biochemical characteristics in control, SCH and OH subjects.

	Control (n=44)	SCH (n=28)	OH (n=21)	p value
Fasting Glucose (mmol/L)	5.07 ± 0.68	5.16 ±0.42	4.76 ± 0.41	NS
Fasting Insulin (μIU/L)	9.19 ± 6.74	10.00 ± 7.71	7.15 ± 3.71	NS
AUC Glucose (mmol/L)	86.07 ± 9.95	90.43 ± 12.18	82.34 ± 16.09	NS
AUC Insulin (μIU/L)	715.54± 460.07	822.17 ± 798.79	692.65 ± 671	NS
HOMA IR	2.03 ± 1.56	2.23 ± 1.86	1.44 ± 0.89	NS
Fasting Triglyceride (mmol/L)	1.48 ± 1.08	1.50 ± 0.76	1.25 ± 0.57	NS
AUC Triglyceride (mmol/L)	15.36 ± 8.38	15.64 ± 7.90	13.85 ± 6.90	NS
Fasting LDL-C (mmol/L)	3.06 ± 0.82	2.96 ± 0.90	3.36 ± 1.22	NS
AUC LDL-C (mmol/L)	21.32 ± 7.96	18.73 ± 6.16	22.74 ± 8.68	NS
TSH (mU/L)	1.9	5.5	53.7	0.000
FT_4 (pmol/L)	15.72 ± 2.17	14.8 ± 2.19	8.77 ± 3.06	0.000

Data expressed as mean ± S.D or median for skewed data. p value derived from mixed between-within subject ANOVAs. NS= non-significant: WHR=Waist Hip Ratio

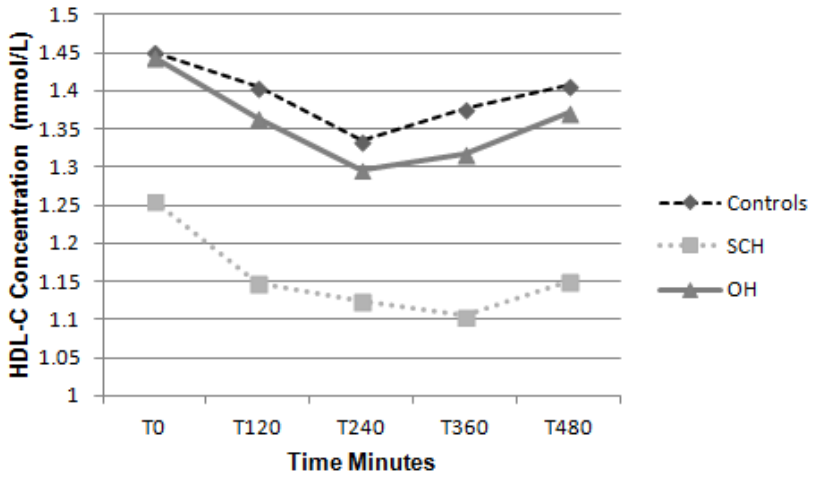

Figure 3-1 Mean HDL-C concentration fasting and T120, T240, T360 and T480 postprandially in Control (n=44), SCH (n=28) and OH (n=21) subjects. Post-hoc analysis revealed significant differences between SCH and control subjects for both peak ($p < 0.05$) and AUC HDL-C ($p=0.001$) concentrations

3.3.3 Apo B48 Concentration

There was a significant effect of time for Apo B48 concentration, all groups showing a significant increase postprandially ($p<0.001$) (Figure 3.2). There was also a significant between-group effect for Apo B48 concentration ($p<0.001$). Post-hoc analysis revealed significant differences between OH subjects and controls for fasting ($p<0.005$), peak ($p=0.001$) and AUC Apo B48 ($p<0.001$) concentrations. Post-hoc analyses also demonstrated a significant difference between SCH subjects and controls for peak ($p<0.05$) Apo B48 concentration.

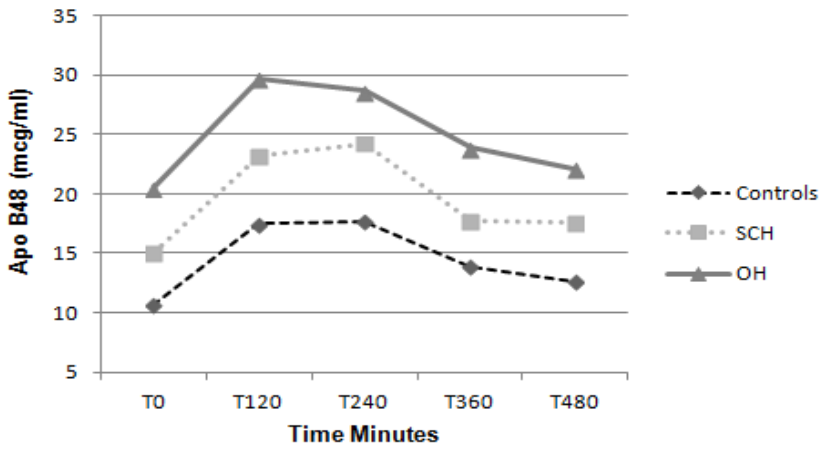

Figure 3-2 Mean ApoB48 concentrations fasting and T120, T240, T360 and T480 postprandially in control, OH and SCH subjects. Post-hoc analysis revealed significant differences between OH and control subjects for fasting ($p<0.005$), peak ($p=0.001$) and AUC Apo B48 ($p<0.001$) concentrations. Post-hoc analysis also demonstrated a significant difference between SCH and control subjects for peak ($p<0.05$) Apo B48 concentration.

3.3.4 HDL_2 analyses

3.3.4.1 HDL_2 total protein Concentration

There was no significant effect of time for HDL_2 total protein concentration, but it did approach significance ($p=0.07$). Although there was no significant between-group effect, AUC HDL_2 total protein concentration approached significance ($p=0.056$) between groups.

Markers of HDL_2 Function

There was no significant effect of time for HDL_2 associated CETP activity. There was a significant between-group effect for HDL_2 associated CETP activity ($p<0.0001$). Post-hoc analysis revealed significant differences between OH and controls for fasting ($p<0.01$), peak ($p<0.005$) and AUC HDL_2 associated CETP activity ($p<0.005$) (results not shown). There was a significant effect of time for CETP/ApoA1 per HDL_2 concentration ($p<0.01$) but no significant

between group effect. There were no significant differences in LCAT activity (results not shown).

3.3.5 HDL3 analyses

3.3.5.1 HDL_3 total protein Concentration

There was a significant effect of time for HDL_3 total protein concentration ($p<0.05$), both OH and SCH groups demonstrating a significant increase postprandially (Figure 3.3). There was also a significant between-group effect for HDL_3 total protein concentration ($p<0.05$). Post-hoc analysis revealed significantly lower concentrations between SCH subjects and controls for fasting HDL_3 total protein concentration ($p<0.005$) and differences that approached significance for peak ($p=0.052$) and AUC HDL_3 total protein ($p=0.06$) concentration.

3.3.6 Markers of HDL3 Function

There was a significant effect of time for HDL_3 associated CETP activity, all groups showing an overall significant increase postprandially ($p<0.001$) (Figure 3.4). There was also a significant between-group effect for HDL_3 associated CETP activity ($p<0.05$). Post-hoc analyses revealed significant differences between OH subjects and controls for fasting ($p<0.05$), peak ($p<0.05$) and AUC HDL_3 associated CETP activity ($p<0.001$). There was a significant effect of time for CETP/ApoA1 per HDL_3 concentration ($p<0.01$) but no significant between group effect (results not shown). There were no significant differences in LCAT (results not shown).

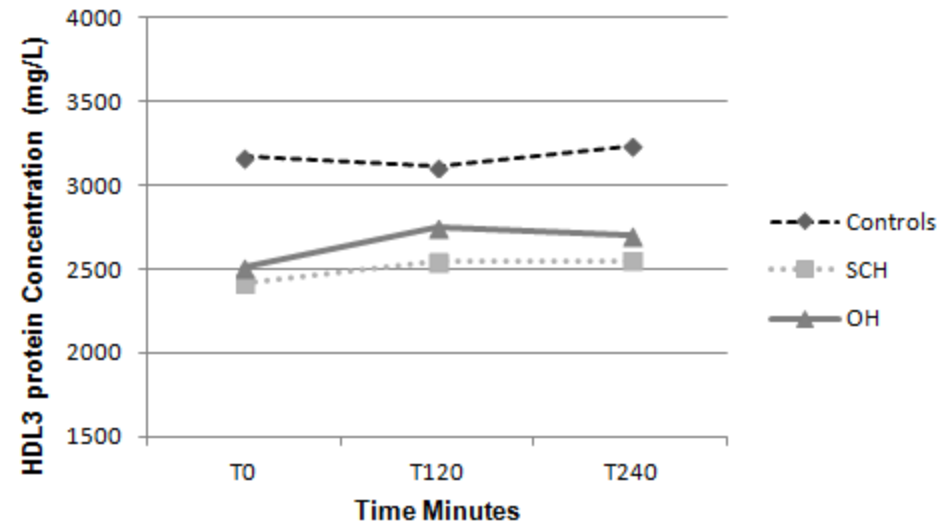

Figure 3-3 Mean HDL-$_3$ total protein concentrations fasting and T120, T240, T360 and T480 postprandially in Controls, OH and SCH subjects. Post-hoc analysis revealed significant differences between SCH and control subjects for fasting HDL$_3$ total protein concentration ($p<0.005$) and differences that approached significance for peak ($p=0.052$) and AUC HDL$_3$ total protein ($p=0.06$) concentrations.

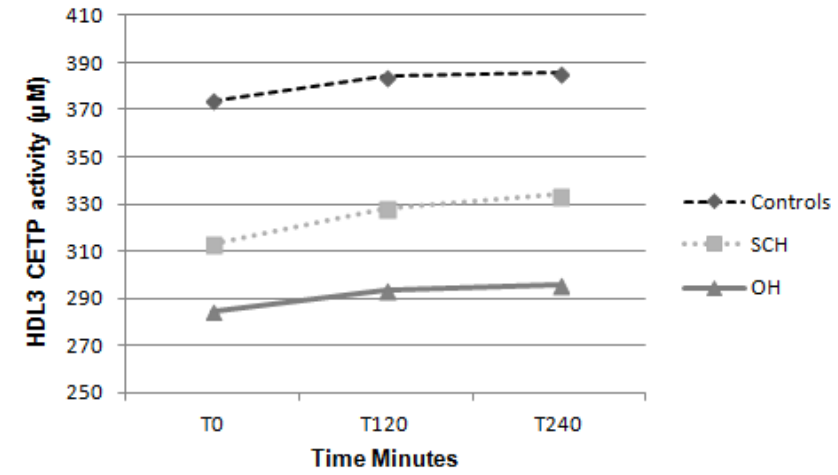

Figure 3-4: Mean HDL3 associated CETP concentrations fasting and T120, T240, T360 and T480 postprandially in control, OH and SCH subjects. Post-hoc analysis revealed significant differences between OH and control subjects for fasting ($p<0.05$), peak ($p<0.05$) and AUC HDL3 associated CETP activity ($p<0.001$).

3.3.7 Inflammation and endothelial function

There was no significant time- or group effects for WCC, neutrophil count, hsCRP, SAA, systolic or diastolic BP (results not shown).

There was no significant effect of time or between-group effects for fasting brachial artery diameter or %FMD. The %FMD change is an indication of endothelial function. There was a significant effect of time for postprandial %FMD change ($p<0.05$). There was a significant between-group effect for postprandial %FMD change ($p<0.05$). Post-hoc analysis revealed significant changes between OH subjects compared to controls ($p<0.05$) (Table 3).

Table 3-3 Brachial artery measurements and percent flow-mediated dilatation in control, SCH and OH subjects.

	Control (n=44)	SCH (n=28)	OH (n=21)	p value
Fasting Brachial Artery Diameter	3.85 ± 1.83	3.26 ± 0.65	3.05 ± 0.54	NS
Postprandial Brachial Artery Diameter	3.65 ± 0.65	3.32 ± 0.61	3.14 ± 0.57	NS
Fasting %FMD	5.79 ± 3.90	5.92 ± 3.30	4.46 ± 1.68	NS
Postprandial %FMD	5.88 ± 3.93	5.52 ± 2.28	3.65 ± 1.93	<0.05*

Data expressed as mean ± S.D. p value derived from mixed between-within subject ANOVAs, where significant main effects were observed ($p<0.05$). *Significant between OH vs. control subjects.

NS= non-significant: FMD=Flow mediated dilatation of the brachial forearm

3.3.8 Correlations

A positive correlation was observed between TSH and fasting and peak ApoB48 ($p<0.005$ and $p<0.05$ respectively) and AUC Apo B48 concentration (Table 4). A negative correlation existed between TSH and AUC CETP HDL_2 and HDL_3 and the %FMD change postprandially (Table 4). A negative correlation was observed between FT4 and AUC Apo B48 concentration and a positive one between it and AUC CETP HDL_2 and HDL_3 (Table 4). A number of additional correlations existed including AUC Apo B48 negatively correlating with AUC CETP HDL_3 and %FMD change postprandially (Table 4) and also a positive correlation between with AUC CETP HDL_2 and %FMD change postprandially (Table 4)

Table 3-4 Correlations between TSH, FT4, Apo B48, CETP associated HDL_2 and $HDL_3$and FMD

	TSH	FT4	AUC Apo B48	AUC CETP HDL_2	AUC CETP HDL_3	% FMD Change postprandially
TSH			0.337**	-0.483*	-0.278*	-0.215*
FT4	-	-	-0.251**	0.390*	0.297*	NS
AUC Apo B48	0.337**	0.251**	-	NS	0.422**	-0.241*
AUC CETP HDL_2	-0.483*	0.390*	NS	-	-	0.301**
AUC CETP HDL_3	-0.278*	0.297*	-0.422**	-	-	NS
% FMD Change postprandially	-0.215*	NS	-0.241*	0.301**	NS	-

R values for Pearson's product moment correlation coefficient $p<0.05$* or $p<0.005$** for all correlations

3.4 Discussion

This is the most detailed study yet reported of the postprandial phase in OH and SCH, and the first to include postprandial effects on Apo B48, functional aspects of HDL and endothelial dysfunction. The results demonstrate postprandial abnormalities in both OH and SCH that were not evident under fasting conditions, and suggest that the effects of SCH are not simply similar but less severe effects of OH.

Both OH and SCH were characterized by increased intestinally derived lipoprotein particles, demonstrated by increased levels of ApoB48, in the postprandial phase. Postprandially, the majority of circulating triglycerides exist in 2 forms; ApoB48 rich chylomicrons and apolipoprotein-B100 (ApoB100) rich VLDL particles that are synthesized in the liver. Abnormalities in chylomicron synthesis and clearance are thought to play a role in the development of atherosclerosis [85]. Chylomicron particles have been found in the atherosclerotic plaque and Apo B48 receptors found on the surface of the macrophage [86]. A delayed chylomicron clearance response is seen in patients with coronary heart disease [87]. The mechanism through which Apo B48 levels are increased in OH could be due in part to their increased secretion or decreased clearance and/or degradation. Animal studies have shown decreased clearance of chylomicron rich lipoproteins in hypothyroid rats [88, 89]. In LDLR knockout mice, both Apo B100 and Apo B48 levels were reduced by administration of T3[71]. A decrease in intestinally derived lipoprotein particles following L-Thyroxine therapy in OH subjects was observed using retinyl palmitate as a means of extracting the chylomicron rich lipoproteins [80]. However, retinyl palmitate is not as accurate a measurement of postprandial

intestinal lipoproteins properties as ApoB48 measurement by ELISA [90]. Hepatic lipase facilitates the uptake and degradation of chylomicron remnants and a number of studies have found that hepatic lipase is more responsive to changes in FT4 levels than lipoprotein lipase [91-93]. Total Apolipoprotein B (Apo B) kinetics were studied in detail in 6 individuals with OH and the authors observed an increase in hepatic lipase activity on replacement of thyroid hormone [94]. Gut transit time increased in subjects with hypothyroidism when replaced with thyroid hormone [95]. This may in part explain in this study the failure of Apo B48 levels to fully return to fasting levels at time 8 hours. Fasting Apo B48 levels have correlated positively to TSH levels in two previous studies [81, 96]. Supporting these studies, both fasting and postprandial Apo B48 levels positively correlated with TSH concentration and negatively with FT4 concentration in our study. Peak Apo B48 was significantly increased in SCH compared to controls and both fasting, peak and AUC Apo B48 was significantly increased in OH compared to controls in this study.

Plasma HDL levels in OH did not differ from control subjects either fasting or postprandially, However, although fasting HDL levels did not differ in SCH from control subjects, a highly significant difference emerged postprandially. Fasting HDL has been reported to be increased [97, 98], similar [99] or decreased [100] in OH. A number of studies have reported a decrease in fasting HDL concentration in treated OH subjects, including a decrease in HDL by 20% in OH treated subjects [94, 97, 98, 101]. Conversely, fasting HDL has been reported to be similar [102] or decreased [97] in hyperthyroidism. An inverse relationship exists between plasma HDL concentration and coronary artery disease [103]. Observational data suggests that the incidence of coronary artery disease increased by 2-3% for every 1% decrease in HDL [104]. However, the

concept that increased HDL translates into cardiovascular disease reduction has been challenged by recent genetic studies, suggesting that mutations causing an increase in HDL do not confer a reduction in myocardial events [105].

When we examined the subfractions of HDL, we found that HDL_2 and HDL_3 associated total protein differed subtly between groups. HDL_3 associated protein was significantly lower in the fasting SCH group compared to controls only. In contrast, there was no significant difference in HDL_2 associated protein between groups. The difference between HDL_3 and HDL_2 associated proteins are difficult to explain, given the lack of significant change in ApoA1 concentration. The smaller HDL_3 particle incorporates ApoA1, while the larger HDL_2 particle incorporates ApoA1 and ApoA2. A previous study documented increased fasting HDL_2 associated protein and ApoA1 levels in OH compared to euthyroid controls, which reversed on thyroid hormone replacement [106] and although in this study the concentration of ApoA1 was increased in OH compared to SCH and control subjects, both fasting and postprandially was not significant. This differing total protein concentration in HDL_3 between the SCH and control subjects may be related to changes in other HDL associated proteins that were not measured in the current study, especially paraoxonase-1 (PON-1). This enzyme, mainly associated with HDL_3, is responsible for the major antioxidant properties of HDL, thus a decrease in its concentration would reduce the antioxidant capacity of this subfraction.

In addition, this difference may possibly be due to the decreased CETP activity observed fasting and postprandially in the OH group. Decreased CETP activity has previously been reported in fasting subjects with OH [76, 107]. Further insights can be gained by considering

HDL metabolism and its role in reverse cholesterol transport. Due to the action of $ABCA_1$, the nascent HDL molecule removes cholesterol esters from foamy macrophages and matures to HDL_3 due to the addition of ApoA1. LCAT facilitates further maturation to the HDL_3 particle, to the larger HDL_2 subfraction, conferring more protective properties. CETP facilitates the mass gradient transfer of cholesterol in exchange for triglycerides from HDL_2 to the Apo B-containing lipoproteins, LDL and VLDL [108]. The observed decreased CETP activity was more marked in HDL_2, which may reflect decreased CETP activity in HDL_2, as this is where it exerts most of its action. Additionally, this latter result also suggests that the mass of CETP was not different between the groups (due to a lack of change in total protein) but that its activity function was influenced by the presence of OH.

The relationship between HDL, CETP and atherogenesis are complex. CETP activity has been described as both pro and anti-atherogenic [109]. Animal studies have reported a reduction in atherosclerosis by inhibiting CETP activity [110] and CETP gene transfer induced atherosclerosis in mice [111]. Genetic CETP deficiency results in large HDL particles that are cholesterol saturated and thus poor cholesterol acceptors [112] and is not associated with significantly decreased rates of cardiovascular disease [113]. Data from the Honolulu Heart study suggests that CETP deficiency associated with HDL concentrations between 1.06-1.55 mmol/L was associated with risks of coronary artery disease similar to that in non-CETP deficient subjects and HDL levels of >1.6 mmol/L appear to confer anti-atherogenic effects [114]. Further insight can be gained from the CETP inhibitors. The first phase 3 study (ILLUMINATE; NCT00134264) of a CETP inhibitor, Torcetrapib commenced in 2004 and

terminated in 2007 due to increased incidence of the primary cardiovascular disease endpoints in the treated group. A less potent CETP inhibitor, Dalcetrapib (Dal-OUTCOMES; NCT00658515) entered phase 3 trials in 2008 and was discontinued in 2012 following interim analysis due to futility. A number of prospective observational cohort studies have demonstrated an inverse relationship between the incidence of cardiovascular disease and plasma CETP concentration [115-120]. Reduced CETP concentrations, as observed in the OH group in this study are associated with larger HDL particles and concentration, but this does not reflect the functionality of HDL in reverse cholesterol transport.

There was no difference in the HDL-associated inflammatory marker SAA, either in serum or associated with HDL_2 and HDL_3 between groups, perhaps indicating that although HDL is preserved in OH, it is not conferring a reduction in systemic inflammation. There was no significant time- or group effects for WCC, neutrophil count, hsCRP or systolic or diastolic BP. However, a correlation was observed between TSH and the percentage change in FMD postprandially and the absolute postprandial change decreased significantly in the OH group compared to both controls and SCH. FMD is a validated tool used to measure endothelial function and is independent predictor of future cardiac events [121]. In one previous study, following an oral fat load, FMD was reduced in both controls and SCH and OH subjects and remained decreased in OH only at eight hours [40]. Unlike two other studies that used fasting measurements, we did not show a significant change in FMD in the SCH group [122, 123]. Postprandial FMD inter-correlated with fasting, peak and AUC Apo B48 in this study and although other studies have demonstrated a reduction in FMD in OH, no study has measured

Apo B48 in tandem with FMD, either fasting or postprandially and this information gives additional insight into the postprandial phase in SCH and OH subjects. There was a strong inter-correlation between CETP associated HDL_2 and HDL_3 and the absolute and percentage postprandial change in FMD and although HDL is preserved in OH, reduced FMD indicates likely postprandial inflammation occurring, perhaps mediated in some way by reduced CETP activity.

4 TYPE 1 Diabetes Mellitus and Endothelial Dysfunction

Postprandial studies unmask endothelial dysfunction in subjects with type 1 diabetes

4.1 Introduction

A reduction in mortality due to cardiovascular disease (CVD) and coronary heart disease (CHD) has been observed in people with and without diabetes over the last 40 years [124]. Mortality has decreased in people with T1DM over the last two decades but despite this, the overall risk of CVD is increased two to threefold in men and three to five fold in women for people with T1DM compared to people without diabetes [125]. At the age of twenty, men and women with T1DM can expect to live 13 and 11 years less than their peers without diabetes respectively. The majority of the estimated loss in life expectancy is related to ischaemic heart disease [126].

Prolonged exposure to hyperglycaemia contributes to the increased risk observed in T1DM. The follow-up of the Diabetes Control and Complications Trial demonstrated a 58% risk reduction in non-fatal myocardial infarction, stroke and cardiovascular disease related death in individuals with T1DM originally assigned to intensive glycaemic control [54].

However, glycaemic control alone does not fully account for the increased incidence of CVD and hypertension, renal disease and dyslipidaemia are also implicated [127]. In the absence of renal disease, subjects with T1DM have a relatively preserved lipid profile in the fasting state and have been reported to have a normal or high HDL concentration and normal or low triglyceride concentration [128]

Lipoprotein abnormalities are also present in T1DM and have been shown to be a better predictor of mortality than more conventional lipid measurements in T1DM [60].

Atherosclerosis, which is associated with CVD and predicts future cardiovascular events, is accelerated in T1DM [129, 130]

Endothelial dysfunction precedes the development of atherosclerosis and various factors associated with endothelial dysfunction including hyperglycaemia, fasting LDL cholesterol (LDL-C), smoking [130-132], postprandial hypertriglyceridaemia [133], triglyceride rich lipoproteins [134, 135] and hypertension [136] are also associated with CVD.

Postprandial lipoprotein abnormalities are independently predictive of atherosclerosis and in the case of triglycerides more markedly than fasting levels [78, 79]. Abnormalities in either synthesis or clearance of Apo B48 rich chylomicrons, which are derived from postprandial triglycerides are associated with in the development of atherosclerosis [85]. A delayed chylomicron clearance rate was observed in individuals with T1DM but this study used extracted radiolabelled cholesterol to isolate chylomicron remnants [63].

Studies in the postprandial phase enhance our understanding of these processes including their clinical significance. Meal ingestion acts as a challenge to accentuate abnormalities that may not be present under fasting conditions. To date, very few postprandial lipoprotein studies have been carried out in T1DM. One study examining the effect of consecutive meals on postprandial endotoxaemia and lipoproteins used the Augmentation Index as a measure of arterial stiffness [137]. We considered that detailed postprandial studies were warranted specifically examining factors affecting postprandial endothelial function.

Measurement of Apo B48 was used to measure the proportion of intestinally derived lipoprotein particles in circulation. Endothelial dysfunction was determined using flow-mediated dilatation of the brachial artery fasting and postprandially. In order to strengthen the study, we chose to analyse variables independently associated with endothelial dysfunction and so included a larger cohort of individuals who were studied fasting and postprandially.

4.2 Methods:

4.2.1 Experimental Subjects and Study Design for Case Control Study

Forty-four subjects were recruited. Subjects with T1DM (n=20) were recruited from the Diabetes outpatients. T1DM was diagnosed based on the National Diabetes Data Group criteria [138]. Control subjects (n=24) were recruited by local advertisement.

Subjects were excluded if they had a body mass index <18 or >35 kg/m^2 or taking any medication that was likely to interfere with the results. Additional exclusion criteria included a prior history of stroke, ischaemic heart disease, head injury, epilepsy, psychiatric illness or pregnancy. Women were excluded if they were taking the oral contraceptive pill. The study approved by the Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland) and all participants gave written informed consent.

This was a cross-sectional study. All subjects attended the Diabetes Day Centre following a 12-hour fast, having avoided alcohol and excessive exercise in the preceding 24 hours. Subjects with T1DM were advised to administer their usual basal insulin prior to and during the study day.

In the fasting state, blood samples were taken for analysis of plasma glucose; lipids including Apo B48 and FMD of the forearm were measured. A breakfast mixed-meal with a nutritional content of 900 kcal consisting of 18g of protein, 78g of carbohydrate and 46 g of fat was consumed within fifteen minutes.

Subjects with T1DM were made aware of the meal content in advance and calculated their bolus insulin requirements based on their carbohydrate ratio, which they administered prior to receiving the meal.

In the postprandial state, samples were taken at time (T) 15, 30, 60, 90, 120 and 240 minutes following the breakfast mixed meal. FMD of the forearm was measured in the postprandial setting at T240 minutes.

Following these measurements, a lunch mixed-meal with a nutritional content of 940kcal consisting of 17g of protein, 100g of carbohydrates and 53g of fat was consumed within fifteen minutes. Additional samples were taken following the lunchtime mixed meal at T360 and T480 minutes from baseline. All subjects were included in the final analysis resulting in 20 cases and 24 controls; matched for age, sex, BMI and WHR.

4.2.2 Experimental Subjects and Study Design for Pooled Study

In addition to the case control study, additional subjects were recruited (n=98) to assess independent variables associated with endothelial function. These subjects were recruited as part of a study characterizing the association of early markers of vascular disease and intestinal gene expression. They were randomly recruited from the routine hospital upper gastrointestinal

endoscopy waiting list, with enrolment occurring by telephone on the day preceding the procedure and were consented for the collection of two additional duodenal biopsies (D2) at the time of their routine upper GI endoscopy. In addition, subjects consented to attend a study day within one month of the endoscopic procedure. Similar exclusion criteria to the case control study applied to this study.

Subjects attended the Diabetes Day Centre following a 12-hour fast, having avoided alcohol and excessive exercise in the preceding 24 hours.

In the fasting state, blood samples were taken for analysis of plasma glucose; lipids including Apo B48 and FMD of the forearm were measured. A breakfast mixed meal with a nutritional content of 940kcal consisting of 27g of protein, 140g of carbohydrate and 36g of fat was consumed within 15 minutes.

In the postprandial state, samples were taken at time (T) 15, 30, 60, 90, 120 and 240 minutes following the breakfast mixed meal. FMD of the forearm was measured in the postprandial setting at T240 minutes. Additional blood samples were taken at 360 and 480 minutes.

4.2.3 Measurement of body composition

Body composition was measured in all subjects using auxiological methods and by bio impedance analysis. Height (measured with a Harpenden stadiometer) and weight were measured by the same observer. Waist (WC) and hip (HC) circumferences were measured with a non-distensible flexible tape measure at the waist and hip. Percentage body fat was estimated using a body fat analyser (TBF-300; Tanita Corp., Arlington Heights, IL) and was calculated using the

manufacturers programmed equations. The same observer made all measurements on all study days. A Health and Lifestyle questionnaire was completed by each participant, which included details on smoking history and alcohol consumption, which could potentially influence the results.

4.2.4 Laboratory methods

Serum glucose was measured by an enzymatic colorimetric method (Cobas Roche diagnostics Ltd., UK) on the Roche P Module (Roche, Stockholm, Sweden). In addition; total cholesterol (TC), TG and HDL were measured on the Roche P Module. LDL-C was calculated using the Friedwald equation. Insulin was measured by electrochemiluminesence immunoassay (Cobas Roche diagnostics Ltd) on the Roche E Module (Roche, Stockholm, Sweden).

4.2.4.1 Measurement of markers of inflammation

Total white cell count and differential were measured by laser flow cytometry. High sensitivity C reactive protein (hsCRP) was measured by ELISA using a commercially available kit according to the manufacturer's instructions (BioCheck Inc., USA).

4.2.4.2.Measurement of ApoB48

ApoB48 was measured by ELISA using a commercially available kit (Gentaur, Belgium).

4.2.4.3 Flow Mediated Dilatation

FMD of the brachial artery was completed at two time-points during the study; fasting and at 240 minutes postprandially. A sphygmomanometer cuff was inflated on the subjects' right arm to at least 20 mmHg greater than systolic pressure on the upper arm for 5 minutes before release. Measurements of brachial artery diameter and flow parameters were taken at baseline and 30, 60, 90 and 120 seconds following release of the sphygmomanometer. Calculation of percentage change of the arterial diameter was calculated based on resting diameter compared to the maximal increase seen over these time-points.

4.2.5 Statistical Analysis

Statistical analysis was performed using SPSS for windows Version 18.0 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD). Skewed variables were logarithmically transformed to normalise data prior to analysis. Absolute p values are displayed to allow the reader to interpret results of borderline significance. Analysis of variance (ANOVA) was the primary statistical tool used. Mixed between-within subject ANOVA were used to compare measurements across different time points between the two groups. Where significant main effects (measurements) were observed ($p<0.05$), subsequent one-way ANOVA comparing the dependent variable between the groups was carried out. Where significant between-group differences were observed, post-hoc analysis were performed, exploring the differences in more detail using a Bonferroni adjustment in order to maintain a reasonable α level across all tests. The relationship between endothelial function and dependent variables including glucose,

insulin, lipids, and Apo B48 concentrations were evaluated using Pearson's correlation coefficient. Where significant correlations were observed, regression analysis was performed in an attempt to establish factors contributing to those variables.

When data was pooled, one way between group ANOVA was carried out and where significant differences were observed, additional post hoc analyses was performed to explore the between group differences.

4.3 Results:

4.3.1 Baseline Characteristics

Subjects with T1DM and control subjects were closely matched for age, sex, BMI, WHR and percent body fat (Table 4.1). There were significant group differences in glucose AUC between the two groups (Figure 4.1 and Table 4.1).

Table 4-1 Baseline demographic and anthropometric measurements in subjects with T1DM and controls

	T1DM (n=20)	Controls (n=24)	p value
Age (years)	39.7 ± 10.8	43.1 ± 10.8	NS
Sex (M/F)	15/5 ± 0.44	15/9 ± 0.49	NS
Height (m)	176.5 ± 8.36	175.5 ± 9.26	NS
Weight (kg)	81.9 ± 12.8	86.1 ± 16.4	NS
BMI (kg/m^2)	26.16 ± 3.27	28.0 1± 5.37	NS
WHR	0.92 ± 0.06	0.93 ± 0.06	NS
Body Fat (%)	27.1 ± 6.4	30.2 ± 8.9	NS

Data expressed as mean ± S.D

p value derived from mixed between-within subject ANOVAs.

NS= non-significant. WHR=Waist Hip Ratio.

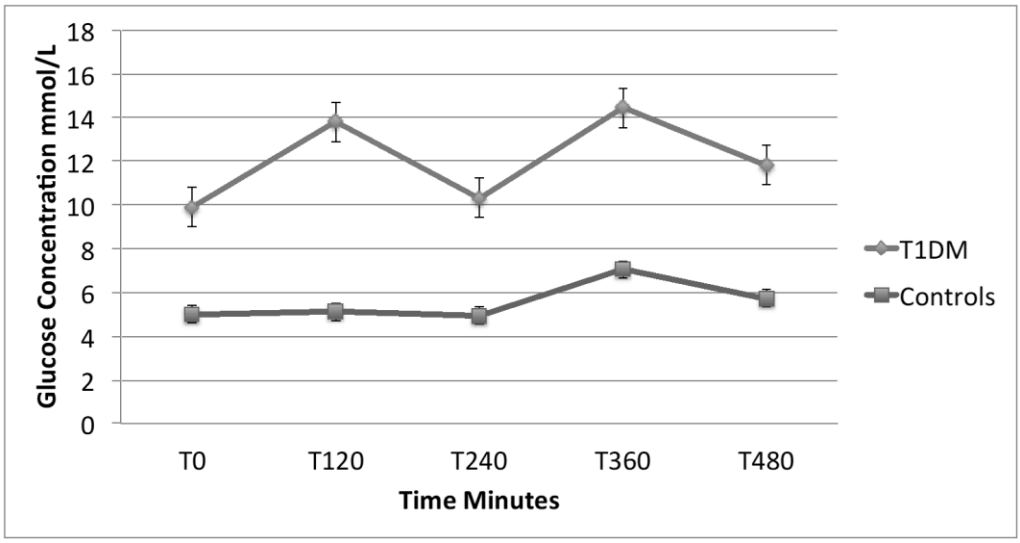

Figure 4-1 Mean glucose concentration fasting and T120, T240, T360 and T480 postprandially in Control (n=20), T1DM (n=24) subjects. Post-hoc analysis revealed significant differences between T1DM and control subjects for both peak ($p < 0.05$) and AUC glucose ($p = 0.001$) concentrations

4.3.2 LDL-C, Triglyceride and HDL Concentration

There was a significant difference in fasting LDL-C between groups but this was not demonstrated when assessed using the mixed between within ANOVA (Table 4.2). There was no significant difference in triglyceride concentration between groups (Table 4.2). There was change in HDL concentration across different time points but no significant between group effect was demonstrated (Figure 4.2).

Table 4-2 Biochemical characteristics in subjects with T1DM and controls

	T1DM (n=20)	Controls (n=24)	p value
Fasting Glucose (mmol/L)	9.8 ± 4.22	5.0 ± 0.44	<0.0001
AUC Glucose (mmol/L)	216.1 ± 5.4	80.6 ± 25.9	<0.0001
HbA1C (mmol/mol)	8.17 ± 0.95	5.29 ± 0.25	<0.0001
Fasting LDL-C (mmol/L)	2.36 ± 0.47	2.82 ± 0.83	0.04
AUC LDL-C (mmol/L)	16.81 ± 3.50	20.66 ± 8.54	NS
Fasting TG (mmol/L)	1.02 ± 0.47	1.25 ± 0.83	NS
AUC TG (mmol/L)	12.4 ± 6.4	16.5 ± 13.6	NS

Data expressed as mean ± S.D or median for skewed data.

p value derived from mixed between-within subject ANOVAs.

NS= non significant

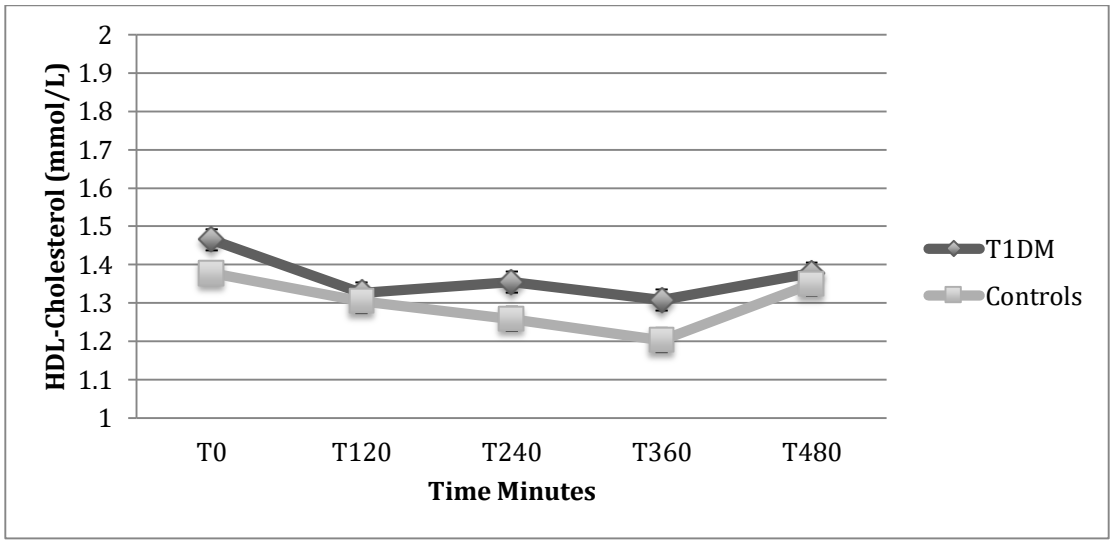

Figure 4-2 Mean HDL-C concentration fasting and T120, T240, T360 and T480 postprandially in Control (n=20), T1DM (n=24) subjects. Post-hoc analysis revealed no significant differences between T1DM and control subjects

4.3.3 Apo B48 Concentration

There was a significant effect of time for Apo B48 concentration, both groups showing a significant increase postprandially ($p < 0.005$) (Figure 4.3). There was also a significant change in Apo B48 concentration between groups ($p < 0.05$). Post-hoc analysis revealed significant differences between T1DM subjects and controls for fasting ($p < 0.005$), T120 ($p < 0.05$), T240 ($p < 0.05$) and T360 ($p < 0.01$) Apo B48 concentrations but not AUC Apo B48 concentration.

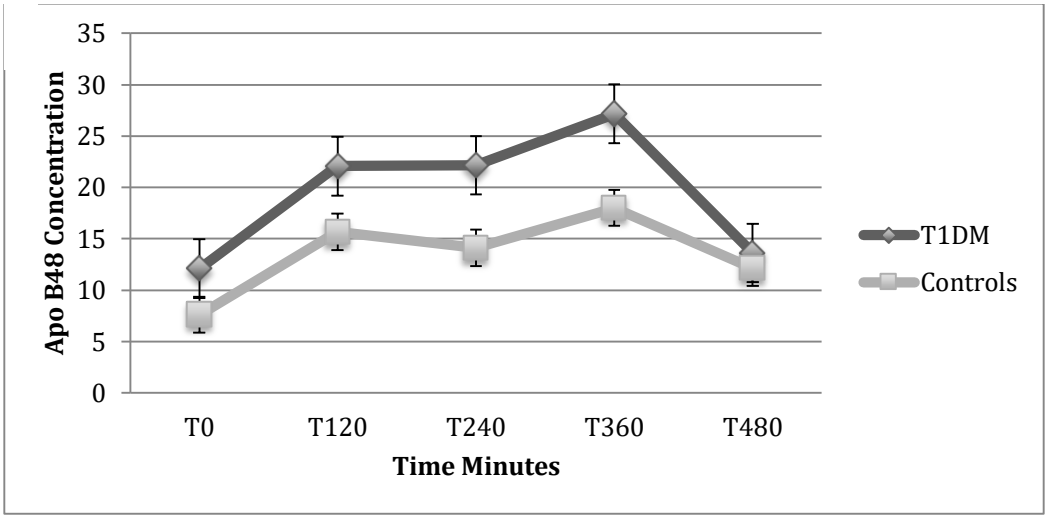

Figure 4-3 Mean ApoB48 concentration fasting and T120, T240, T360 and T480 postprandially in Control (n=20), T1DM (n=24) subjects. Post-hoc analysis revealed significant differences between T1DM and control subjects for fasting ($p < 0.005$) and T120, T240, T360 ApoB

4.3.4 Inflammation and Endothelial Function

There was no significant difference between fasting and postprandial brachial artery diameter between groups. There was no significant change in fasting FMD in the T1DM or in the control group. There was a significant decrease in the postprandial FMD in subjects with T1DM only ($p < 0.005$) (Figure 4.4).

There were no significant time or group effects for white cell count (WCC) or high sensitivity CRP (HsCRP), systolic or diastolic blood pressure (results not shown). Postprandial neutrophil count was increased in T1DM subjects compared to controls and approached significance ($p = 0.052$).

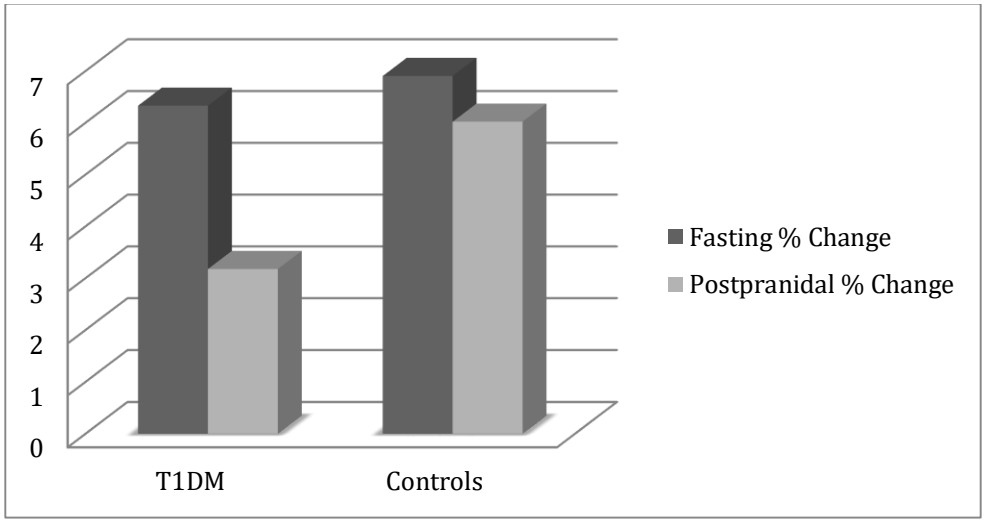

Figure 4-4 Fasting and postprandial percentage change in Control (n=20) and T1DM (n=24) subjects. Post hoc analysis revealed significant difference T1DM and Controls ($p<0.001$ between T1DM and Controls postprandial change) and $p<0.01$ between fasting and postprandial in T1DM

4.3.5 Correlations

Fasting and peak glucose concentration correlated positively with fasting, postprandial and peak Apo B48 concentration ($p<0.05$). Similarly, AUC glucose concentration correlated positively with fasting ($p<0.01$) and AUC Apo B48 ($p<0.05$) concentration. There was no significant correlation observed between fasting or postprandial Apo B48 concentration and WCC, neutrophil or HsCRP levels. In addition, a positive correlation existed between fasting and AUC TG and postprandial and AUC Apo B48 concentrations ($p<0.05$ for all correlations). A positive correlation was observed between fasting, peak and AUC glucose concentration and the change in FMD postprandially ($p<0.05$) and overall postprandial change; between fasting and postprandial ($p<0.001$) (Table 4.3). There was a positive correlation between the overall

postprandial percentage FMD change and postprandial ($p<0.001$), peak ($p<0.01$) and AUC ($p<0.05$) neutrophil count (Table 4.3). A negative correlation existed between AUC HDL-C and the percentage postprandial FMD change ($p<0.001$) only.

4.3.6 Additional Control Subject Characteristics and Analysis

Additional controls subjects (n=98) were studied to explore what independent variables were associated with endothelial function. The average age was 50.3 years (SD: 7.96), average BMI 29.5 kg/m2 (SD: 5.9), and HbA1C of 5.6% (DCCT) (SD: 0.7). Males constituted 57% of subjects.

A positive correlation was observed between fasting and peak glucose concentration ($p<0.05$) and postprandial FMD change (not overall change and not AUC). There was no significant correlation between postprandial FMD change or overall FMD change and AUC glucose, TG or Apo B48 concentrations.

Table 4-3 Correlations between fasting, peak AUC glucose, peak Neutrophil, Apo B48 concentration and overall percentage FMD change

	Fasting Glucose	Peak Glucose	AUC Glucose	Peak Neutrophil	Peak Apo B48	Overall % change in FMD
Fasting Glucose	-	0.77 **	0.78 **	0.31 *	0.35 *	0.42 **
Peak Glucose	0.77 **	-	0.97 **	0.32 *	0.32 *	0.43 **
AUC Glucose	0.78 **	0.97 **	-	NS	NS	0.47 ◆
Peak Neutrophil	0.31 *	0.32 *	NS	-	NS	.39 *
Peak Apo B48	0.35 *	0.32 *	NS	NS	-	NS
AUC HDL-C					NS	NS
Overall % change in FMD	0.42 **	0.43 **	0.47 ◆	0.39 *	NS	-

R values for Pearson's product moment correlation coefficient $p<0.05$*, $p<0.005$◆ or $p<0.001$** for all correlations

4.3.7 Pooled Data Analysis

All data was combined (n=142) and FMD analyzed at all time-points. One way between group ANOVA was carried out. Significant group differences were noted with respect to postprandial FMD change ($p<0.05$) and the overall change in FMD from fasting to postprandial

state ($p<0.001$). Post hoc analysis revealed significant differences between subjects with T1DM and the overall group of control subjects (Figure 4). Between group difference were observed with respect to fasting, peak and AUC glucose concentrations ($p<0.0001$) and post hoc analysis noted these changes were between subjects with T1DM and all control subjects. Positive correlations were observed between fasting, peak and AUC glucose and the percentage postprandial FMD change and the overall change from fasting to postprandial state (Table 4.4). A negative correlation existed between AUC HDL-C and the percentage postprandial FMD change ($p<0.05$) only.

Table 4-4 Pooled data correlations between fasting, peak AUC glucose, AUC HDL and overall percentage FMD change

	Fasting Glucose	Peak Glucose	AUC Glucose	AUC HDL	% FMD Change postprandially
Fasting Glucose	-	0.77**	0.78**	0.35*	0.42**
Peak Glucose	0.77 **	-	0.97**	0.32*	0.43**
AUC Glucose	0.78**	0.97**	-	NS	0.47◆
Peak Neutrophil	0.31*	0.32*	NS	NS	.39*
Peak Apo B48	0.35*	0.32*	NS	-	NS
Overall % change in FMD	0.42**	0.43**	0.47◆	NS	-

R values for Pearson's product moment correlation coefficient $p<0.05$*, $p<0.005$◆ or $p<0.001$** for all correlations

4.4 Discussion

These are the most detailed postprandial studies to date in T1DM reporting the postprandial effects on endothelial function in a real-life setting. T1DM was characterized by abnormalities in endothelial function in the postprandial state that were not present in the fasting state. The results suggest postprandial hyperglycaemia plays a significant role in this process

and analysis of subjects from a much larger heterogeneous cohort demonstrate an association between hyperglycaemia and changes in endothelial function postprandially, effects that may be attenuated by postprandial HDL-C concentration.

T1DM was characterized by similar fasting and postprandial triglyceride concentration but increased intestinally derived lipoprotein particles, both in the fasting and postprandial state. Postprandially, the majority of the circulating triglycerides exist in 2 forms, Apo B48 rich chylomicrons and the Apolipoprotein-B100 (Apo B100) rich VLDL particles that are synthesized in the liver. Consonant with this, we reported an association between postprandial TG concentrations and postprandial Apo B48 concentrations.

We also demonstrated an association between fasting and peak glucose concentrations and fasting and peak Apo B48 concentrations. In conjunction with this, we demonstrated a significant association between AUC glucose and AUC Apo B48 concentration. Elevated Apo B48 have been reported in individuals with type 2 diabetes mellitus (T2DM) and this study demonstrated reduced concentrations with improved metabolic control [139].

These particles are particularly atherogenic and a delayed chylomicron clearance has been reported in normolipidaemic patients with coronary artery disease [87].

Investigators have previously described elevated fasting and postprandial Apo B48 concentrations in a small number of T1DM subjects with confirmed coronary artery disease, who were under consideration for islet transplantation [140]. Similarly, another study demonstrated elevated fasting and postprandial Apo B48 concentrations in subjects with T1DM, not evident in control subjects [141]. Elevated Apo B48 may either be secondary to decrease clearance of these

remnant lipoproteins or due to increased production. Favouring the former, a reduction Apolipoprotein E (Apo E); the lipoprotein that is responsible for the catabolism of chylomicron-rich Apo B48 particles is decreased in T1DM [141]. It is not completely clear why subjects with T1DM have elevated Apo B48 concentrations in the fasting state. Insulin is involved in the clearance of chylomicrons by stimulating hydrolysis of chylomicron rich triglycerides and via hepatic uptake of chylomicrons by lipoprotein lipase [62]. It is conceivable that in a relatively insulin deficient state such as T1DM that this process is impaired resulting in delayed clearance of chylomicrons. Although this study showed evidence of lipoprotein abnormalities in individuals with T1DM, not present in controls; there was no clear association between postprandial lipoprotein changes and endothelial dysfunction.

In this study, there was no difference in endothelial function under fasting conditions between subjects with T1DM and controls but changes emerged in the T1DM under postprandial conditions. Notably, we did not find an association between endothelial dysfunction and fasting or postprandial lipids or lipoproteins. We did however show a significant clear association between glucose and endothelial dysfunction. From a practical perspective, the fasting and 4-hour pre-meal glucose concentration is similar but not reflective of the postprandial glucose excursion that occurred. In order to examine the effect on endothelial dysfunction in more detail, we analysed an additional 98 subjects to explore what variables are associated with endothelial function. In the pooled analysis, we reported that subjects with T1DM had a significantly lower FMD postprandially when compared to the unmatched individuals suggesting that this effect is quite pronounced given the much larger cohort of subjects included in this analysis. In

accordance with this, we found an association between fasting, peak and AUC glucose and postprandial endothelial dysfunction when all data was pooled (n=142). Hyperglycaemia has been shown to effect endothelial function in diabetic and non-diabetic subjects [142, 143]. Investigators reported that short-term poor glycaemic control was associated with reversible changes in endothelial function [144]. We have shown in this study, that changes in endothelial function occur following the challenge of a breakfast meal, replicating a real-life scenario. In a controlled setting, the reversal of endothelial dysfunction was achieved by the normalization of glucose and antioxidant therapy in smaller groups of individuals with T1DM depending on the duration of their disease [145]. Although we did not measure a marker of oxidative stress, we showed that HDL-C might play a role in attenuating endothelial dysfunction in the T1DM group. HDL-C is anti-atherogenic and removes cholesterol esters from foamy macrophages. A number of steps result in a net removal of cholesterol from the triglyceride rich lipoproteins. In this study, we have shown that HDL-C is inversely associated with postprandial endothelial dysfunction in the case control study and also confirmed these findings in the larger cohort of individuals. Although the absolute concentration of HDL-C was not significantly elevated in subjects with T1DM, it is possible that more subtle HDL-C lipoproteins are involved that were not measured as part of this study.

Furthermore, we have shown an association between postprandial endothelial dysfunction and postprandial neutrophil concentration. Postprandial neutrophilia has been reported previously in diabetic and non-diabetic subjects and perhaps the near significant elevated

concentrations observed here in T1DM may reflect the underlying exaggerated hyperglycaemia observed in this group.

4.5 Conclusion:

In conclusion, we have shown for the first time, the effects of multiple meals on lipid and lipoprotein metabolism and endothelial dysfunction in subjects with T1DM and demonstrated changes postprandially that were not present under fasting conditions. Additional examination of a much larger cohort of individuals demonstrated an association between hyperglycaemia and endothelial dysfunction. We also reported that this effect might be attenuated by HDL-C under postprandial conditions. These effects on endothelial dysfunction occurred following a standard meal, replicating a real-life day and studies are needed to evaluate the long-term effects of these findings in more detail.

5 Discussion

5.1 Thesis Overview

This thesis sought to explore the contribution of postprandial lipaemia specifically ApoB48 and HDL metabolism to cardiovascular risk in individuals with subclinical and overt hypothyroidism. It also sought to explore the contribution of postprandial lipaemia and inflammation to cardiovascular risk, specifically endothelial dysfunction in individuals with T1DM and explore variables associated with endothelial dysfunction in a larger cohort.

5.1.1 Postprandial lipaemia and cardiovascular risk in Hypothyroidism

Dyslipidaemia is an important risk factor in the pathogenesis of cardiovascular disease. There is increasing evidence that postprandial lipaemia is independently predictive of atherogenesis and of future cardiovascular events.

Overt and to a lesser extent subclinical hypothyroidism is associated with elevated fasting concentrations of LDL-C, Apo B and possibly triglyceride concentrations. HDL-C metabolism is less clear [72, 146]. Our study has clearly demonstrated altered fasting lipoprotein metabolism in hypothyroidism and also differing postprandial lipoprotein metabolism in hypothyroidism not evident under fasting conditions. We attempted to address the question of altered HDL-C in hypothyroidism and we clearly identified differences in OH compared to SCH.

To our knowledge, this is the most detailed postprandial study in individuals with overt and subclinical hypothyroidism. Specifically, the subjects with thyroid dysfunction were closely

matched to controls for BMI and waist-hip ratio in addition to the usual confounders of age and gender. Many studies have shown elevated LDL-C in OH and we found similar findings, although not significant between groups.

5.1.2 Apo B48 metabolism and Hypothyroidism

Given the association between elevated postprandial triglyceride rich lipoproteins and the pathogenesis of atherosclerosis, we chose to study Apo B48, which is specifically associated with chylomicrons. Abnormalities in chylomicron synthesis and clearance are implicated in atherogenesis. Only one study to date has attempted to address this by using a retinyl palmitate technique and did not include individuals with SCH [80]. Our findings that postprandial Apo B48 concentrations were increased to a greater degree in OH, compared to SCH and fasting are novel. The more pronounced differences observed in the hypothyroid group are interesting for a number of reasons. Firstly, were we to measure triglyceride concentrations fasting and postprandially alone, we would not have observed any differences between the three groups. This is important as postprandial hypertriglyceridaemia is implicated in the pathogenesis of atherosclerosis. Secondly, Apo B48 concentrations were greatest in the OH group, both fasting and postprandially. Although, Apo B48 concentrations failed to return to fasting levels in any group, this is likely to have the most deleterious effect in the OH group. Thirdly, it enhances our understanding of the pathophysiology of dyslipidaemia in hypothyroidism; possibly reflecting decreased intestinal clearance of lipoprotein particles. Interestingly, there was no difference in fasting or AUC glucose and insulin between groups and so the interplay between lipids and gut

transit is possibly more complex than a mechanical slowing of gut transit time. Finally, lipoprotein abnormalities are less well studied in SCH and our study highlights differences observed in lipoprotein metabolism between SCH and controls and also differences not previously observed between SCH and OH. In the case of Apo B48, it would appear that perhaps the differences in SCH are similar to those in OH but to a lesser degree.

5.1.3 HDL-C metabolism and Hypothyroidism

The relationship between HDL-C and hypothyroidism is less clear. Studies are conflicting in relation to abnormalities in fasting HDL-C metabolism in OH and in SCH with some studies reporting increased, no change or decreased concentrations. We found no difference between OH and controls with respect to HDL-C, HDL-2 and HDL-3 concentrations. Although we found no difference in fasting HDL-C, postprandial HDL-C was significantly reduced in SCH only compared to controls. On it's own, this result maybe initially surprising, especially in the context of preserved HDL-C in OH compared to controls. Some studies have demonstrated lower fasting HDL-C in SCH compared to OH [74, 75]. A Chinese study determining the relationship between serum TSH and the metabolic syndrome involving 1534 people reported elevated triglycerides and lower HDL-C in SCH compared to euthyroid individuals [147]. Other larger observational studies have shown no clear change in fasting HDL-C in SCH compared to controls. No alteration in serum HDL-C was observed following adjustment for age, sex and race among 8585 adults with subclinical hypothyroidism in the National Health and Nutrition Examination Survey III (NHANES) [148]. Similarly, data including 7000 individuals attending a

thyroid clinic found no difference in fasting HDL-C in a SCH group compared to euthyroid controls [149]. HDL-C concentrations were not altered in the Basel Thyroid study following 48 weeks administration of L-Thyroxine to individuals with SCH [73]. Our study attempted to address this discordance between studies and measured fasting and postprandial HDL-C and its various sub fractions in SCH, OH and euthyroid controls. HDL-C particles are categorised by size into the larger, more buoyant HDL_2 and the smaller, denser HDL_3. The larger HDL_2, likely in part reflecting increased ApoA1 confers more cardiovascular protection. We observed decreased HDL-C in SCH but also in HDL_3. In contrast there was no difference in HDL_2 associated protein between groups. When we looked in more detail, there was no difference between the ApoA1 concentrations between the subfractions. Previously it has been shown that ApoA1 and fasting HDL_2 associated protein concentrations are increased in OH and ApoA2 concentrations are unchanged [98]. It is suggested that the increase in fasting HDL_2 associated protein concentrations and ApoA1 are increased in OH in an effort to off set the increased LDL-C observed in overt disease. Interestingly, ApoA1 concentrations did not change among 26 individuals with SCH treated with Thyroxine. The same study demonstrated a reduction in ApoA1 among 13 individuals with OH treated with Thyroxine in tandem with a significant reduction in LDL-C [150].

5.1.4 HDL-C functionality and inflammation in Hypothyroidism

Increasingly, the focus on HDL-C leans less towards a cholesterol centric view towards alternative indices of HDL metabolism such as subclass distribution, particle size and measures

of HDL functionality. Our study attempted to address these indices in detail in both SCH and OH.

To our knowledge, this is the first study that has demonstrated significant HDL associated lipoprotein changes in the fasting and postprandial phase in both OH and SCH. In the cross-sectional analysis of individuals with SCH, a lower HDL-C was associated with preserved HDL_3 associated CETP activity. Although HDL associated CETP increased across all groups postprandially, it was significantly decreased in the OH group alone. We felt this may in part explain the preserved HDL-C concentrations observed in this group, both fasting and postprandially. Although CETP activity has been shown to be low in the fasting subjects with OH, this is the first study to demonstrate changes in the postprandial stage. CETP is involved in reverse cholesterol transport, facilitating the mass gradient transfer of cholesterol in exchange fro triglycerides from HDL_2 to LDL and VLDL. CETP activity was most markedly decreased in $HDL_{2,}$ which is where it exerts most of its effect. Notably, there is no consistent evidence that CETP exerts anti-atherogenic properties and it has been reported as both pro and anti-atherogenic. Reduced CETP activity does not necessarily confer reduced rates of cardiovascular disease [113]. CETP deficiency is associated with HDL molecules that are large and cholesterol laden and as a result do not accept cholesterol. Data from CETP inhibitor trials are disappointing and demonstrated no meaningful reduction in cardiovascular endpoints. Although reduced CETP concentrations were observed in the OH group, it does not necessarily reflect HDL functionality in reverse cholesterol transport. Paraoxonase-1 (PON-1) is a HDL associated esterase and exerting anti-oxidant and anti-atherosclerotic effects. PON-1 hydrolyses oxidised lipids in

lipoproteins and in atherosclerotic lesions. One of the hallmarks of atherosclerosis is macrophage foam cell formation. PON-1 contributes to a reduction in cholesterol within the macrophage by inhibiting cholesterol biosynthesis and also by inhibiting oxidised LDL via scavenger receptors [151]. It is conceivable that reduced PON-1 activity may result in reduced anti-oxidant properties of the associated HDL subfraction but was not measured in our study. However, other markers of HDL associated inflammation were not increased in serum or associated with HDL_2 or HDL_3 proteins. Specifically, serum amyloid A (SAA) was not different between groups. There was no difference in other measures of inflammation such as WCC or neutrophil concentration between groups, suggesting that although HDL-C was preserved in OH, it may not confer a reduction in systemic inflammation. The exact mechanism may be unclear but our study has shown significant differences in HDL-C, HDL associated proteins, CETP activity between subclinical and overt hypothyroidism and although HDL-C is preserved in OH, it may not confer anti-atherogenic properties given the deleterious changes in postprandial endothelial function we observed in this group.

5.1.5 Endothelial Dysfunction in Hypothyroidism

An alternative index of systemic inflammation is endothelial dysfunction and we used flow-mediated dilatation (FMD) of the brachial artery as a measure of endothelial function. FMD is a validated measure of endothelial function and endothelial dysfunction is predictive of future cardiovascular events [121]. Our original findings that following a mixed-meal, FMD decreased postprandially in subjects with OH only compared to a matched control group, indicating thyroid

dysfunction plays a role in the mediation of endothelial dysfunction. Congruent with this, a positive correlation existed between TSH and the overall and percentage postprandial FMD change. A previous study demonstrated reduced FMD in subjects with OH, eight hours following oral fat loading and found a positive correlation with serum triglycerides [40]. We found a positive correlation between postprandial FMD and fasting, peak and AUC Apo B48 concentrations. Postprandial lipaemia, specifically chylomicron remnants are associated with the development of atherosclerosis [152]. We used a novel method to measure ApoB48 and along with demonstrating increased fasting and postprandial concentrations in the OH group, postprandial values failed to return to fasting levels 8 hours postprandially. Amongst the gastrointestinal effects of hypothyroidism are prolonged intestinal transit time and delayed intestinal absorption. It is conceivable that a delay in ApoB48 clearance is contributing to the accelerated rates of atherosclerosis observed in this group, which ties in with our FMD findings. We wondered if the preservation of HDL-C was also contributing to the decreased FMD findings and found an association between CETP associated HDL_2 and HDL_3 and postprandial FMD change. This suggests that reduced CETP in OH may negate the protective properties of HDL-C, despite a total preserved concentration in this group and may be implicated in atherogenesis.

5.2 The discrepancy between cardiovascular risk and event rate in Hypothyroidism

The relationship between hypothyroidism and cardiovascular disease, specifically coronary artery disease manifests due to increased risk factors for atherogenesis and also resultant hemodynamic effects on the heart and coronary vasculature. Increased rates of dyslipidaemia;

specifically elevated LDL-C, hypertension, and homocysteine concentrations observed in hypothyroidism may account for the variably increased prevalence of atherogenesis observed in this group [153]. Hypothyroidism has a negative inotropic and chronotropic effect on the myocardium with resultant diminished myocardial oxygen demand and recovery can result in myocardial ischemia. Despite the occurrence of these risk factors, it contrasts with the relatively low incidence of angina pectoris and myocardial infarctions observed in the hypothyroid group as a whole. It is though the discrepancy results from decreased metabolic demand placed on the myocardium and for this reason is recommended that Thyroxine replacement be initiated in low dose in individuals with coronary artery disease. Among patients with hypothyroidism and coronary disease, replacement with T4 and/or T3 resulted in exacerbation of heart disease in only 16%, whereas the reminder were unchanged or improved [154]. A reduction in both preload (ventricular dimensions) and afterload (diastolic pressure) possibly counteract augmented myocardial oxygen demands induced by T4 therapy. Amongst hypothyroid patients undergoing coronary revascularisation, no increase in peri-operative death but higher rates of intraoperative hypotension and heart failure were observed [155].

The relationship between SCH and the development of coronary artery disease is less clear and data inconsistent. Although many of the same risk factors are present in SCH as in OH, interventional studies to date have concentrated on the effects of T4 therapy on surrogate markers of atherosclerosis or lipid reduction but have not examined the effect on cardiovascular events or mortality. Although randomised controlled trials (RCTs) are lacking, a meta-analysis a number of years ago demonstrated significantly increased risk of cardiovascular mortality and

morbidity observed in subjects with a TSH >10mU/L and potential effects with a TSH >7mU/L [32].

5.3 Recommendations for Screening and Therapy

Evaluation of thyroid dysfunction is recommended for all individuals with dyslipidaemia [156]. Thyroid function testing is recommended in individuals with symptoms and signs suggestive of hypothyroidism. Professional societies including the European Thyroid Association, American Thyroid Association, American Association of Clinical Endocrinologists and the Endocrine Society recommend L-Thyroxine monotherapy for individuals with OH to avoid progression of the disease and prevent adverse cardiovascular events. It is recommended that replacement therapy restore tissue and biochemical euthyroidism. Untreated overt hypothyroidism leads to increased risk of coronary heart disease, heart failure, pericardial and pleural effusions and the progression of atherosclerosis. In addition, the dyslipidaemia associated with OH is reversible on restoration of euthyroidism with L-Thyroxine therapy.

It is estimated that SCH is present in 1.4-11.2% of individuals with dyslipidaemia [157]. Given the prevalence of SCH among the general population, the American Thyroid Association recommend population screening in individuals >35 years of age but other professional bodies do not. Controversy remains whether or not to treat individuals with SCH but it is generally accepted that individuals with a TSH >10 mU/L be treated with L-Thyroxine. A number of studies have addressed the effect of L-Thyroxine replacement in SCH on serum lipid concentrations, with mixed results. A meta-analysis carried out a number of years ago found a

slight reduction in total plasma cholesterol (0.4mmol/L) in treated SCH patients [158]. Another more recent meta-analysis found smaller reductions in total cholesterol (<5%) in 6 of the 13 studies [159]. A number of studies have demonstrated improved diastolic function in patients with SCH treated with LT4 [35, 36]. Additionally, there is data to support improved LV function and exercise capacity with Thyroxine replacement in SCH. We found increased ApoB48 concentrations (Peak ApoB48) in the SCH group compared to controls even though the mean TSH concentration was 5.5mU/L in the group. Although, the magnitude is of overt lipid changes observed in many of the intervention studies is small, in many cases LDL-C or HDL-C only was measured and in the fasting state and is perhaps not fully representative of the underlying lipoprotein abnormalities present in SCH. Longitudinal data and intervention studies with Thyroxine examining the rates and risk of cardiovascular disease in SCH are warranted.

5.4 Limitations of Thyroid Study

A potential limitation of this study was the lack of intervention with Thyroxine therapy in the SCH and OH group. Although participants were matched for age, sex, BMI, WHR and % body fat, were we to demonstrate reversal of the lipoprotein abnormalities following treatment with Thyroxine, it would have added weight to the findings. We deemed inviting participants to attend another 8-9 hour study day would have been too time consuming.

It is also possible that altered fasting lipid and lipoprotein concentrations were not observed in the SCH group recruited had a relatively low mean TSH concentration, thus leading to a more restrictive SCH phenotype.

5.5 Postprandial lipaemia and cardiovascular risk in Type 1 Diabetes Mellitus

T1DM people with adequate glycaemic control have a relatively normal fasting conventional lipid profile. Yet, their burden of CVD risk is increased and is not fully explained by hyperglycaemia, hypertension and renal disease.

Our study has clearly demonstrated altered fasting lipoprotein metabolism in T1DM and also differing postprandial lipoprotein metabolism in T1DM not evident under fasting conditions. Specifically, ApoB48 was elevated fasting and postprandially in T1DM. These particles are particularly atherogenic and we showed an association between these atherogenic lipoproteins and AUC glucose concentration. This association may explain the underlying reason for these concomitant findings as insulin is involved in the clearance of chylomicrons by stimulating hydrolysis of chylomicron rich triglycerides and via hepatic uptake of chylomicrons by lipoprotein lipase and is involved in the clearance of hyperglycaemia. In an relatively insulin deficient state such as T1DM, this process is impaired resulting in delayed clearance of chylomicrons and hyperglycaemia.

5.6 Endothelial Dysfunction and Type 1 Diabetes Mellitus

Endothelial dysfunction predicts the development of CVD in non-diabetic and diabetic subjects. Hyperglycaemia can induce endothelial dysfunction in non-diabetic and diabetic patients [160]. Short-term hyperglycaemia induced endothelial dysfunction in individuals with T1DM. However, endothelial dysfunction is also present in the presence of normoglycaemia in T1DM. It has also been shown that oxidative stress plays a role in the development of endothelial dysfunction, which may be the common denominator in the various factors able to induce endothelial dysfunction. We found not difference in endothelial function under fasting conditions between subjects with T1DM and controls but changes emerged in the T1DM under postprandial conditions and we demonstrated a clear association between glucose concentrations and endothelial dysfunction in a real-life setting. As mentioned previously, despite near normalisation of plasma glucose concentrations between meals, this does not reflect glucose excursions. Analyses of the larger cohort demonstrated that subjects with T1DM had a significantly lower FMD postprandially when compared to the unmatched individuals suggesting that a pronounced effect given the much larger cohort of subjects included in this analysis. We found an association between fasting, peak and AUC glucose and postprandial endothelial dysfunction when all data was pooled. A small study showed that Vitamin C, an effective anti-oxidant improves endothelial dysfunction in T1DM but not in T2DM but a follow-up study demonstrated no significant effect on endothelial dysfunction by giving Vitamin C, only by rendering the patient euglycaemic with insulin [145]. We did not measure a marker of oxidative stress but demonstrated that HDL-C might play a role in attenuating endothelial dysfunction in

the T1DM group and in the larger cohort of patients. Although, there was no difference in HDL-C concentration between groups, this does not reflect the functionality of the HDL protein.

5.7 Limitations of Diabetes Study

A potential limitation of this study was the duration of diabetes amongst participants. The mean average time of onset of disease was 15 years. The length of diabetes may have an effect on the development of endothelial dysfunction. In order to confirm or refute our findings, rendering an individual euglycaemic using a clamp and performing the same study would determine the effect of postprandial glucose excursions on endothelial dysfunction.

5.8 Future Research

The identification of differing lipoprotein abnormalities, specifically HDL-C between OH and SCH is very interesting and require additional study. It may CETP related but additional parameters looking at HDL associated inflammation should be studied to see if the preserved HDL-C in OH confers anti-atherogenic properties. Our feeling is that this is not the case and HDL functionality is affected in OH despite a preserved concentration and is contributing to the increased CVD risk observed in this group. PON-1 is a marker of HDL related inflammation and is of potential interest in this group.

Similarly, additional studies in individuals with T1DM are required to ascertain if the endothelial dysfunction observed in this group is related to postprandial glucose excursions. By measuring oxidative stress in individuals with T1DM compared to a matched control group is a

consideration. What is clear is that postprandial studies provide additional information not evident under fasting conditions.

5.9 Conclusions

Individuals with hypothyroidism, irrespective of the grade have underlying lipoprotein and vascular abnormalities; changes that are not evident fasting and so perhaps a conventional lipid profile is misleading in determining these individuals cardiometabolic risk. This is a consideration when treating individuals with hypothyroidism especially those with subclinical disease.

Individuals with T1DM also have evidence of atherogenic lipoprotein particles, even in the fasting state. There is an association between ApoB48 and glucose concentrations. Similarly, vascular abnormalities in this group are related to hyperglycaemia. These findings provide additional evidence that controlling and maintaining euglycaemia in this group is paramount to prevent additional CVD risk.

6 References

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