

Title:

**Glycaemic Influences on the ATP-Binding
Cassette Transporter A1 (ABCA1).**

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Abstract

Increased glucose levels are associated with increased risk of vascular disease. The risk is elevated 2-4 fold in type 2 diabetes and there is a positive relationship between glucose (or HbA1c) levels and vascular disease in the general population. We tested the hypothesis that there is a glycaemia-mediated impairment of reverse cholesterol transport (RCT) by studying an early key step, notably the expression and activity of the ATP binding cassette transporter-A1 (ABCA1). This protein exports cellular cholesterol to apolipoprotein A1 (ApoA-I), thereby forming nascent HDL. In this thesis, it is shown that ABCA1 gene expression in leukocytes in men is reduced in Type 2 diabetes and correlates negatively with circulating HbA1c levels and fasting glucose. This confirms our earlier findings in healthy men. An independent relationship between glycaemia and leukocyte ABCG1 gene expression was not seen. ABCA1 protein concentrations in blood leukocytes were reduced in patients with diabetes. ApoA-I-mediated cholesterol efflux in cultured fibroblasts taken from subjects was studied as a measure of ABCA1 function. This negatively associated with fasting glucose at the time of sampling as well as adiposity. Cellular cholesterol removal was reduced in subjects with diabetes compared with controls. There was a positive relationship between both ABCA1 function and leukocyte protein concentration with circulating HDL cholesterol. These relationships were independent of expression of liver X receptor- α (LXR α) and peroxisome proliferator-activated receptor- γ (PPAR γ). These data imply a persistent glycaemia-mediated suppression of an early step in RCT which may contribute to the excess vascular disease observed in such patients. It further proposes impaired cholesterol efflux may contribute to the low

circulating HDL cholesterol which is commonly seen in patients with impaired glucose regulation.

Declaration of Contributors

Liberation of fibroblasts from biopsy material was performed by Dr Celine Pourreyron and Ms Rosalind Hannen (Centre for Cutaneous Research, Barts and the London, Queen Mary's School of Medicine and Dentistry).

Assistance in leukocyte pellet extraction from whole blood and measurement of plasma non esterified fatty acid (NEFA) concentration was provided by Dr Darrel Pavitt.

Assistance in RNA extraction was given by departmental students, Ms Kroopa Joshi and Ms Haji Dhamba (Imperial College London). Development of a cholesterol efflux assay using human cell lines was assisted by Julia Feldmeyer (M.Sc student, Department of Physiology, Technical University, Munich) and Kroopa Joshi (B.Sc. student, Imperial College London).

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Carotid Ultrasound images and measurements were taken in collaboration with senior radiographer, Surinder Dhanjil (Irvine Vascular Unit, St Mary's Hospital).

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ABBREVIATIONS

ACAT	acyl-coenzyme A:cholesterol acyltransferase
ABC	Adenosine triphosphate binding cassette transporter
ATP	Adenosine triphosphate
ABCA1	ATP Binding Cassette Transporter A1
ABCG1	ATP Binding Cassette Transporter G1
AMP	adenosine monophosphate
AGE	Advanced glycated endproducts
AV	AnnexinV ^{FITC}
Apo	Apolipoprotein
BSA	Bovine serum albumin
ChEBP	Carbohydrate responsive element binding protein
CVD	Cardiovascular disease
CIMT	Carotid Intima-media thickness
CETP	Cholesterol ester transfer protein
CV	Coefficient of variance
cDNA	Complementary DNA
CHD	Coronary heart disease
CRP	C reactive protein
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Phenol-red-free Dulbecco's modified Eagle medium
ER	Endoplasmic reticulum
EL	Endothelial lipase
ELISA	enzyme linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FPG	Fasting plasma glucose
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOI	Gene of interest
GTPase	Guanosine triphosphatase
HDL-C	High density lipoprotein cholesterol

HL	Hepatic lipase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Inter-cellular adhesion molecule
IGH	Impaired glucose homeostasis
IMT	Intima-media thickness
KO	Knock out
LpA	lipoprotein A
LCAT	Lecithin-cholesterol acyl transferase
LSC	Liquid scintillation counting
LXR α	Liver X Receptor alpha
LDL-C	Low density lipoprotein cholesterol
MM	Master Mix
mRNA	Messenger RNA
MS	Metabolic syndrome
MI	Myocardial infarction
NO	Nitric oxide
NF-kB	Nuclear transcription factor kappa-B
OGTT	Oral glucose tolerance test
PPAR α	Peroxisome proliferator activated receptor alpha
PPAR γ	Peroxisome proliferator activated receptor gamma
PBS	Phosphate buffered saline
PL	Phospholipid
PS	Phosphatidylserine
PLTP	Phospholipid transfer protein
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
PEST	Proline-Glutamate Serine-Threonine
RXR	Retinoid-X receptor
RCT	Reverse cholesterol transport
RT	Reverse transcription
SAA	Serum amyloid A
SRB1	Scavenger receptor B1
SREBP	Sterol regulatory element binding protein

TD	Tangier disease
TRIS	tris(hydroxymethyl)aminomethane
DM	Type 2 diabetes mellitus
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
WHAM	Wisconsin hypoalpha mutant

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1.0 INTRODUCTION

1.1 The association of circulating glucose levels and cardiovascular disease

Cardiovascular disease (CVD) remains the main cause for premature death in the western world. The alarming rise in the incidence of obesity worldwide is expected to increase the burden of diabetes, which currently accounts for 6% of total global mortality. 50% of these deaths are attributed to CVD (1).

Type 2 Diabetes Mellitus (DM) which is characterised by insulin resistance and defective insulin signalling is a known risk factor for developing CVD (2). It is estimated the risk of coronary heart disease (CHD) is increased two to four fold with established DM. A meta-analysis of multiple studies demonstrated patients with DM had a 3.4 fold higher risk of fatal CHD compared to those without diabetes (3). A controversial study by Haffner et al., reported the incidence of CHD events in men with DM was similar to that in non-diabetic men who had already suffered a myocardial infarction (4). This proposed a concept of DM being considered as a coronary risk equivalent. This concept has not been replicated by later studies and others have implicated that the degree of risk from DM is dependent on age (5;6). However, a more recent large population based study supported CHD risk equivalence in treated diabetic patients over the age of 30 years (7). These reports provide evidence for the need for aggressive primary prevention strategies to prevent CHD in people with DM.

Excess CVD risk has also been attributed to lesser degrees of blood glucose elevation. A prospective European study demonstrated excess cardiovascular mortality in people with elevated glycated haemoglobin (HbA1c) concentration below the threshold of DM (8). These results suggest circulating blood glucose levels, similar to blood pressure and cholesterol, increase CVD risk with a

continuous relationship. Observations using global data, estimates that 21% of all coronary deaths and 13% of fatal stroke may be attributable to high blood glucose concentrations (9). A large meta-analysis of prospective studies confirmed a two-fold excess risk of cardiovascular disease in patients with diabetes mellitus and a modest association in people with impaired fasting glycaemia (10).

The timing of hyperglycaemia in relation to food intake has also been investigated. HbA1c represents both fasting and post-prandial glucose, may not distinguish between these glucose measurements. Post-prandial hyperglycaemia has been implicated to be a stronger risk factor in the development of CHD (11;12). Impaired glucose tolerance (IGT), represents a pre-diabetic condition with fasting glucose concentration of $<7\text{mmol/l}$ and elevated glucose response during an oral glucose tolerance test (OGTT) of 7.8-11.0 mmol/l.

Studies have illustrated the association of post load glucose and CVD in both type 2 diabetic and non-diabetic populations (13-15). The DECODE study showed no increase in CVD based on fasting glucose measurements comparing diabetic to non-diabetic glucose levels. However, there was a significantly greater risk when post-OGTT glucose levels were used and this appeared to be an independent predictor of CVD mortality (16). Moreover, specific treatment of post meal hyperglycaemia with an α -glucosidase inhibitor has been shown to reduce cardiovascular events (17).

1.11 The effect of glucose lowering treatments on cardiovascular outcomes.

In general, glucose lowering treatments have shown contrasting effects on CVD outcome measures. This may reflect the type of agents used and the severity of disease. The UK Prospective Diabetes Study (UKPDS) demonstrated that patients taking an insulin sensitising therapy, adenosine monophosphate (AMP) kinase activator-Metformin had a 39% lower risk of developing cardiovascular disease and a reduction in all-cause mortality (18). This potential protective effect has been confirmed in a subgroup, in a 10 year follow-up study (19). However, this beneficial effect has not been consistently observed, when metformin was supplemented in patients established on insulin secretagogue therapy.

A recent class of treatments-thiazolidinedione may also influence the development of CVD. A trial using Pioglitazone, a Peroxisome proliferator-activated receptor- α (PPAR α) and Peroxisome proliferator-activated receptor- γ (PPAR γ) agonist, demonstrated a decrease in secondary cardiovascular endpoints in over two thousand patients (20). In contrast, a meta-analysis of Rosiglitazone, predominantly a PPAR γ agonist, suggests this agent may be associated with adverse cardiovascular outcomes (21). This remains controversial and mechanisms underlying this observation as well as the magnitude of effect are currently unknown.

Recent trials investigating the effects of intensive glucose lowering in patients with a 10 year history of DM show a possible deleterious effect on the incidence

of CVD (22). Autoimmune insulin deficiency (Type 1 diabetes) is also associated with an increased risk of CHD, but long term intensive insulin therapy has been associated with a 42% reduction in cardiovascular events (23).

Several common mechanisms have been proposed for the detrimental effect of hyperglycaemia, including free radical damage and protein glycosylation (see section 1.3) although definitive mechanisms that lead to atherosclerosis are yet to be clarified.

1.2 Diabetes and surrogate markers of cardiovascular disease

Carotid Intima-media thickness

Surrogate markers have been used as non-invasive tools to assess atherosclerotic burden as well as to predict clinical cardiovascular events. The most validated technique is the measurement of arterial wall intima-media thickness (IMT) using high resolution B-mode ultrasound. This was originally used to assess atherosclerotic changes in the aorta (24). Large prospective studies have confirmed Carotid IMT (CIMT) to correlate with conventional cardiovascular risk factors; blood pressure, smoking status and lipid profile and predict future coronary events (25;26). It should be noted that while atherosclerosis predominantly affects the intima, ultrasound imaging cannot discriminate between the intima and media of the vessel wall (27). The Rotterdam study in over seven thousand patients estimated a 1.43 fold relative risk for future myocardial infarction (MI) with each standard deviation increase (0.16mm) in CIMT (26). Absolute IMT has also been demonstrated to predict

coronary events. Hodis et al., similarly showed each 0.13mm increment in IMT was associated with a relative risk of 1.4 (28).

The positive association between CIMT and glycaemia is well established. The Insulin Resistance Atherosclerosis Study demonstrated diabetes is associated with increased carotid artery thickness (29). Newly diagnosed patients with DM appear to have increased CIMT (30). Tropeano and colleagues have shown glucose to be a major independent determinant of CIMT both in patients with impaired fasting glucose and DM, confirming a continuous relationship between glucose and atherosclerosis (31). A current study in normal women further suggest a relationship with postprandial glucose levels (32).

We, and others, have shown CIMT is increased in young patients with type 1 diabetes compared to non-diabetic subjects and there is evidence to show similar association with glycaemia as that seen with DM (33-35). CIMT predicts CHD in asymptomatic DM patients over a 5 year period. Using regression analysis, CIMT improved prediction of events compared to traditional Framingham risk scoring (36). This suggests CIMT may have improved predictive value beyond conventionally measured cardiovascular risk factors.

Several glucose lowering agents including thiazolidinediones, metformin, acarbose & some insulin secretagogues have been shown to slow progression of CIMT (37-40). This is suggestive of anti-atherogenic potential, although the mechanisms by which this is achieved have not been characterised. In one study, using Pioglitazone, increases in high density lipoprotein cholesterol (HDL-C) predicted the improvement in CIMT (41). Moreover, in a randomised trial, Pioglitazone was demonstrated to slow the rate of atherosclerosis

progression compared to glimepiride, using both CIMT and direct endoscopic ultrasound of coronary vasculature (39;42). This provides evidence of potential anti-atherosclerotic activity in this class of glucose lowering drugs.

Newer imaging techniques including carotid magnetic resonance and coronary calcification scoring have been more recently evaluated as surrogate markers of CVD. Coronary artery calcium (CAC) signifies the presence of atherosclerosis and correlates with total plaque burden. However, the pathogenesis of vascular calcification is not fully understood and not all atherosclerotic plaques contain calcium. The clinical determinants of CAC progression are also unclear (43). There is limited evidence that treatments significantly slow or reverse progression of CAC (44). However, CAC progression has been shown to correlate with CIMT measurements in middle aged men after controlling for standard risk factors (43) In a cohort with DM, high CAC score predicted first CVD endpoints better than conventional risk scoring. CAC scores in this study only related to baseline age, waist-hip ratio and duration of diabetes (45) Another study in patients with DM confirmed CAC measurement was superior to traditional risk factors in identifying patients with subclinical myocardial ischaemia (46) There is emerging evidence, similar to that established with CIMT, that significant CAC may be present in people with pre-diabetes (47)

1.3 Mechanisms of cardiovascular disease in hyperglycaemia.

Accelerated atherosclerosis in DM may have multiple possible underlying mechanisms. Excess cholesterol deposition is recognised to cause atherosclerosis. Approximately two-thirds of cholesterol in man is carried by the class of lipoprotein, named Low density lipoprotein cholesterol (LDL-C). Excess

LDL cholesterol is usually cleared from the plasma by interaction with a cell surface LDL-receptor which internalises and routes cholesterol to the endoplasmic reticulum (ER) for storage or recycling (endosomes). The hepatic LDL-receptor is predominantly accountable for clearing excess LDL-C from the plasma (48) Excess circulating LDL-C is prone to undergo modification, making it more likely to be taken up by scavenger receptors in macrophages. Cholesterol-loaded macrophage foam cells represent the hallmark of an atherosclerotic plaque.

At the blood vessel wall, activation of nuclear transcription factor kappa-B (NF- κ B) by hyperglycaemia leads to altered gene expression of several inflammatory genes. This process is shown to promote monocyte adhesion to endothelial cells in early lesion development (49) In murine models, there has been co-localisation of regions prone to develop atherosclerosis and increased components of NF- κ B (50) At present it is not clear whether NF- κ B has modulatory effects on macrophage cholesterol influx or removal.

Chronic hyperglycaemia leads to the formation of non-enzymatic glycated proteins and lipids. These advanced glycated end-products (AGE) can generate reactive oxygen species as well as bind cell surface receptors to modulate gene expression. This process is thought to lead to oxidative stress and tissue damage. Endothelial nitric oxide (NO) production has been shown to be inhibited by hyperglycaemia and AGE resulting in reduced endothelium relaxation (51) This process is considered to represent an early defect leading to vascular injury. AGE-modified albumin has been shown to reduce selective cholesterol removal from HDL-C interfering with reverse cholesterol transport (RCT), the process by which cellular cholesterol is removed by the body (52)

Alteration in vascular proteoglycan architecture has been reported in subjects with diabetic atherosclerotic lesions which may lead to susceptibility for lipoprotein retention (53) Specifically, glycation of collagen increases its ability to bind LDL-C (54) Glycation of LDL particles is associated with decreased clearance by the LDL receptor, resulting in prolonged transit (55-57) and increased likelihood to be taken up by macrophage scavenger receptors (57)

There are significant changes in plasma lipoproteins due to DM, leading to diabetic dyslipidaemia. This will be explored in more detail in section 1.6.

The hallmark of DM, insulin resistance, may directly contribute to atherogenesis. Vascular insulin resistance has been shown to be associated with impaired NO-mediated arterial dilatation (58) At a cellular level, increased macrophage ER stress and consequent apoptosis and increased cell-surface scavenger receptor expression are additional mechanisms that may contribute to advanced lesion development in insulin resistant states (59)

The metabolic syndrome (MS) is a clinical constellation of factors associated with insulin resistance; these include hypertension, central obesity, fasting hyperglycaemia and dyslipidaemia (60) A recent European prospective study showed this disorder predicted CVD events regardless of the presence of impaired glucose homeostasis (IGH) or DM (61) As individual components of MS have been independently associated with CVD, it is difficult to ascertain the specific contribution of insulin resistance per se.

1.4 Cholesterol Transport

Cholesterol cannot be converted directly into an energy source. The primary role of cholesterol in mammals is that of a membrane constituent and a

precursor for steroid hormone synthesis. The main sources are dietary cholesterol and hepatic *de novo* synthesis. Dietary cholesterol is transported to the liver and secreted with triglycerides as very low density lipoproteins (VLDL) which contains an apo-lipoprotein B (ApoB) particle. Peripheral metabolism of triglyceride content and cellular uptake of cholesterol esters via the LDL receptor largely enables the transfer of this potential energy source to tissues. With the exception of the liver and steroidogenic tissues, cellular cholesterol cannot be metabolised. Cellular cholesterol removal is based on the formation of HDL-C and export occurs via the plasma membrane by either diffusion or active transport mechanisms.

1.41 Reverse cholesterol transport

HDL-C can be formed by the removal of intracellular cholesterol and therefore serves as a crucial vehicle to return cholesterol to the liver for biliary excretion. This process is termed RCT (62-64). The ATP-Binding Cassette Transporter A-1 (ABCA1) is a member of the ATP-Binding Cassette (ABC) transporter family. Its principal function is the transfer of cellular unesterified (free) cholesterol and phospholipids across the plasma membrane to the surface of a lipid poor ApoA-I particle to form “nascent” or pre β -HDL (62;65;66). The step is considered rate limiting for HDL particle formation. Once ApoA-I is lipidated via the action of ABCA1, further cholesterol export is possible by other recently discovered transporters, ATP Binding Cassette Transporter G1 (ABCG1) and possibly Scavenger Receptor B1 (SR-B1) (67;68).

In addition to initiating cholesterol removal from arterial wall cells, ABCA1 is primarily responsible for native HDL formation. Animal studies suggest this

mainly occurs at the liver (70%) and intestine (30%) (69;70). In contrast, ABCG1 removes cholesterol from macrophages but is not thought to influence plasma HDL concentration (71;72).

1.42 The role of ABCA1, ABCG1 and SR-B1 membrane transporters

Transmembrane transporters, ABCA1 and ABCG1 act in concert in a unidirectional manner. ABCA1 lipidates lipid free or lipid poor ApoA-I to generate nascent or pre β -HDL. It only modestly stimulates cholesterol removal to small (HDL-3) molecules (73). Subsequent cholesterol export to these particles is facilitated by ABCG1 (68). These processes are thought to account for 60-70% of cholesterol removed by HDL or serum from cholesterol loaded macrophages (74). In contrast, Out R et al., report a 100% decrease in efflux without ABCA1 and ABCG1 (75). Any residual efflux to HDL may be due to passive diffusion or mediated by an as yet unknown transporter (76). *Yvan-Charvet* and colleagues suggest SR-B1 may not contribute significantly to net cholesterol efflux in macrophages (77).

In double *ABCA1* and *ABCG1* knockout (KO) mice models (A1-/G1-) there was dramatic evidence of increased macrophage cholesterol accumulation with foam cell formation despite low cholesterol levels using LDL KO mice (74;75). This provides good evidence that these transporters are vital in preventing early atherosclerotic plaque formation. However, when either *ABCA1* or *G1* are knocked down individually, there is a compensatory rise in expression of the complementary transporter, limiting atherosclerosis. This implies some redundancy in their function. The observed mechanisms leading to atherosclerosis in these models were decreased cholesterol efflux, decreased

apolipoprotein E (ApoE) secretion (a putative function of ABCA1), increased inflammatory gene expression and increased apoptosis (74).

ABCG1 is considered to have an additional, specific role of exporting 7-ketosterol and related oxysterols from macrophages. 7-ketocholesterol is the major toxic oxysterol found in oxidized-LDL and atherosclerotic plaques. As *ABCG1* is highly expressed in vascular endothelium, it has been implicated to protect against oxidized-LDL mediated endothelial dysfunction (78). Early SR-B1 knockout models in mice demonstrated increased atherosclerosis despite a doubling of HDL-C (79). Genetic over expression of SR-B1 resulted in greater removal of HDL associated cholesterol ester and increased HDL catabolism, resulting in lower HDL-C concentrations. Overexpression was associated with regression of atherosclerosis in LDL and ApoE KO mice. These experiments indicate discordance between HDL-C levels and atherosclerosis and indicate HDL-C flux may be a better marker of RCT than plasma HDL-C concentration. As SR-B1 functions as a bidirectional transporter it may not significantly contribute to net macrophage cholesterol efflux *in-vivo* (77;80). In mice, SR-B1 may function as a backup transporter to clear circulating remnant lipoproteins which may help to explain some of the conferred atheroprotection (81). Overall the contribution of SR-B1 to cholesterol efflux and atherosclerosis is currently uncertain. A summary of the steps in cellular cholesterol efflux by membrane transporters is shown in figure 1.1.

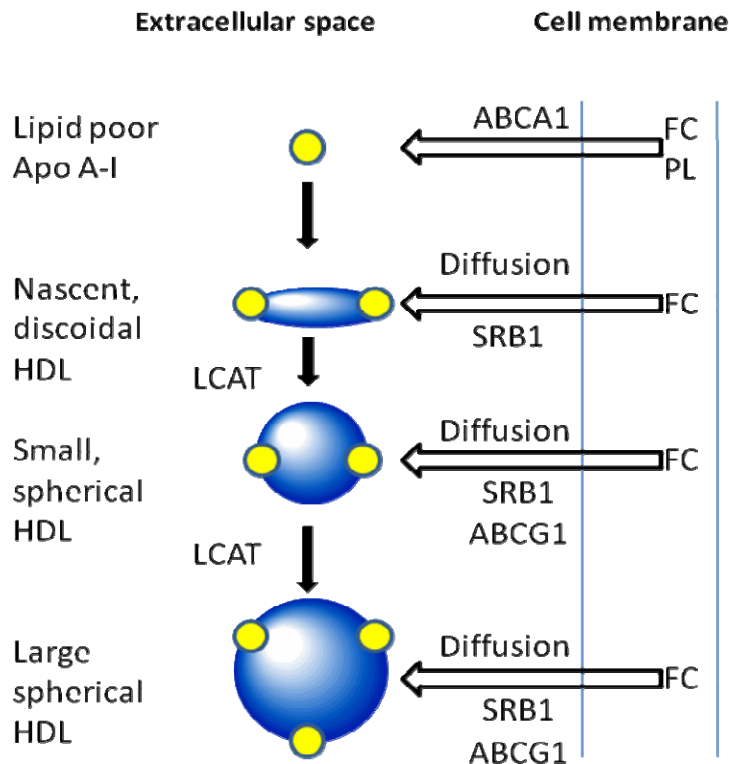


Figure 1.1 Cholesterol efflux from peripheral cells

Schematic diagram illustrating mechanisms on how HDL accepts membrane cholesterol via multiple mechanisms. Initial lipidation of lipid-poor ApoA-I takes place by the action of ABCA1, with subsequent contributions from ABCG1 and SR-B1 as well as diffusion, to generate large spherical particles. Free cholesterol (FC), phospholipid (PL), Lecithin-cholesterol acyl transferase (LCAT) (82). [Adapted from Barter & Kastelein JACC 2006].

1.43 The role of lecithin cholesterol acyl transferase (LCAT)

Esterification of cholesterol occurs on the surface of HDL particles. This occurs by ApoA-I activation of the enzyme Lecithin cholesterol acyl transferase (LCAT) (83). This catalyses the transfer of an acyl group from phosphatidyl choline to cholesterol forming esterified cholesterol and lysophatidylcholine (63;84). This reaction accounts for the formation of most of the esterified cholesterol in human plasma and serves to maintain a concentration gradient for unesterified (free) cholesterol between peripheral cells and HDL. This reaction transforms

HDL from a discoidal structure to a larger, spherical and more buoyant particle with cholesterol esters enclosed within a central lipid core.

Human LCAT deficiency states have been associated with premature atherosclerosis in some but not all patients (85). Although murine LCAT knockout models cause HDL deficiency, this has not consistently resulted in increased atherosclerosis, which may be partly due to the resulting low LDL-C. A more recent study measured faecal labelled cholesterol as a marker of RCT in LCAT deficient mice and provisionally report promotion of ABCA1 mediated cholesterol efflux in macrophages as well as high levels of lipid poor ApoA-I (86).

Transgenic LCAT overexpression predictably increased HDL levels in mouse and rabbit models, but was associated with unaffected or accelerated atherosclerosis in mice (87;88). Overexpression was considered anti-atherogenic in rabbits, possibly due to the presence of cholesterol ester transfer protein (CETP). The current evidence, therefore, does not indicate LCAT to be a crucial factor in preventing atherosclerosis.

Large, spherical HDL is transported in the circulation for uptake primarily by hepatic SR-B1, whereby esterified cholesterol is selectively removed and eliminated by conversion into bile (89). This is subsequently re-used in the entero-hepatic circulation. Recycling of HDL particles, result in smaller, lipid poor ApoA-I which are able to further circulate to attain more cholesterol from peripheral cells. The RCT cycle is illustrated in Figure 1.2.

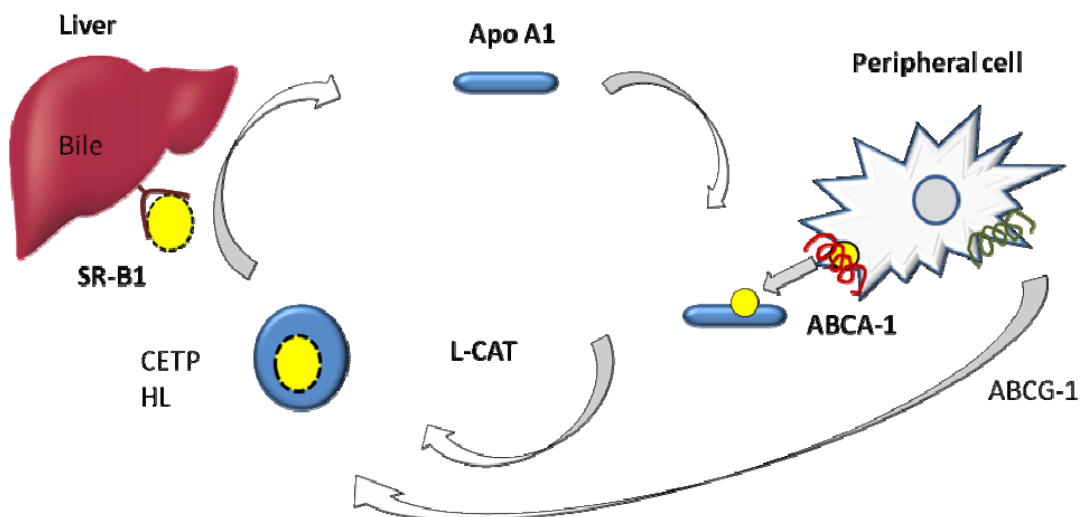


Figure 1.2 Reverse Cholesterol Transport

ApoA-I is mainly synthesised by the liver. Unidirectional export of cellular free cholesterol occurs via action of the transmembrane transporter, ABCA1. Subsequent cholesterol esterification is stimulated by LCAT action which results in a more buoyant particle conformation. Further cholesterol is exported by ABCG1 to mature HDL particles. Esterified cholesterol is selectively taken up at the liver via SR-B1 for removal into bile liberating lipid-free ApoA-I for further recirculation. Intravascular remodeling can occur predominantly by the action of cholesterol ester transfer protein (CETP) and hepatic lipase (HL).

1.44 The role of cholesterol ester transfer protein (CETP) in murine models of RCT

In RCT, cholesterol esters can be redistributed by the action CETP. This glycoprotein was originally isolated from humans in 1978. It is secreted from the liver and circulates in plasma, mainly bound to HDL (90;91). CETP mediates transfer of cholesterol esters from HDL to triglyceride rich lipoproteins and LDL. This is in equal exchange for triglyceride which is transferred to HDL. The net effect is the redistribution of cholesterol esters from HDL to LDL, for more efficient delivery to peripheral cells. Similar exchanges of cholesterol esters can also occur between LDL and VLDL. This protein is only present in certain species. Mice do not possess CETP and have lower plasma HDL-C levels

compared to humans. However, despite this, mice are resistant to developing atherosclerosis. Activity of CETP can be increased by a high fat diet. Studies in murine models have usually involved transgenic introduction of human CETP to mimic human cholesterol kinetics. Rabbits have relatively high CETP activity and have been shown to be prone to diet induced atherosclerosis (92). Conversely, CETP inhibition in rabbits was associated with a 97% rise in plasma HDL and reductions in atherosclerosis (93).

In pro-atherogenic animal models induced by LDL KO and ApoE KO, the introduction of human CETP has been shown to increase atherosclerotic progression. ApoA-I over expression in these experiments showed some protection against these effects (94). Measurements of *in-vivo* RCT in mice have been performed using a technique of labelling elicited peritoneal macrophages *in-vitro*, before injecting them into different transgenic mouse models. Hepatic and faecal cholesterol content is subsequently analysed after 48 hours as a measure of RCT. One such study showed no difference in hepatic or faecal delivery of labelled cholesterol in transgenic CETP mice (95). It seems CETP may be pro-atherogenic when ApoB lipoprotein transport is transgenically interrupted and conversely athero-protective when intact (96).

From an evolutionary perspective, the addition of CETP to existing transport mechanisms may be thought to be useful in an environment of relative lipid and caloric depletion. In this situation, CETP activity would provide more efficient peripheral redirection of cholesterol without the need for hepatic repackaging and secretion of HDL derived cholesterol.

1.45 The role of CETP in atherosclerosis in humans

It has been evident that CETP activity significantly affects plasma HDL-C concentration both in animal models and humans. It has therefore become an increasingly attractive therapeutic target to help prevent atherosclerosis. Concordant with animal data, increased CETP activity in human studies is associated with low plasma HDL-C concentration.

Human CETP deficiency has been described by a number of mutations, observed mainly in a Japanese population. Affected homozygote and heterozygote individuals have been reported to have an elevated plasma HDL-C concentration by 30% and 60% respectively. Analysis of these particles using electrophoresis, indicates HDL-C particles are large and have mainly α 1 or pre- α mobility. A concomitant reduction in catabolism of ApoA-I and ApoA-II is also observed (97). Plasma deficient of CETP was associated with increased cholesterol removal from macrophages which has been shown to be mediated by ABCG1 (98). HDL LCAT and ApoE levels were increased compared to control plasma contributing to enhanced cellular efflux (98). In homozygote subjects there is an additional reduction in LDL-C in keeping with an anti-atherogenic lipid profile (99).

Early reports suggested CETP deficiency was associated with higher CHD risk in Japanese-American men (100), although a subsequent analysis, in fact, showed a conflicting trend. A further study in Japanese subjects with a high HDL-C observed a significantly lower chance of developing CHD irrespective of any CETP mutations (101). Collectively, evidence suggests CETP deficiency combined with elevated HDL-C levels may protect against CVD.

With regard to human CETP polymorphisms, the Taq1B genotype has been extensively studied. B2/B2 homozygotes have been reported to have a 30% reduction in CVD risk compared with B1B1 in non-smokers (102). In both the Framingham and Veteran Affairs studies, carriers of the B2 allele had significantly lower CETP activity and higher HDL-C levels than B1 homozygotes (103;104). In contrast, Borggreve and colleagues reported -B2 and 1405VV alleles of *CETP* gene to be paradoxically associated with an increased risk of coronary disease, despite these variants being associated with higher HDL-C in the general population (105). A prospective study in healthy subjects demonstrated that future risk of CHD was positively associated with increasing CETP level, but this relationship was only observed in combination with elevated triglyceride levels (106).

These studies highlight the complex relationship between CETP, HDL-C and atherosclerosis and studies have not consistently assessed HDL-C function. CETP may have important effects on other important genes and proteins involved in atherosclerosis, independent of its effects on HDL-C. Indeed, a study in over 500 men concluded that variations in genes affecting RCT only accounted for approximately 12% of the variance in HDL-C levels (107).

1.46 The effect of drugs on RCT

RCT is a complex process with multiple steps, illustrated in figure 1.2. The effect of several drugs has been evaluated on this dynamic process using murine models. In humans, until recently PPAR α agonist clinical therapy has been the main treatment shown to improve HDL-C levels. Current clinical therapies and their effectiveness are discussed in section 1.54.

1.5 The role of HDL Cholesterol in cardiovascular disease

1.51 Epidemiology of HDL-C and atherosclerosis

There is ample evidence to imply higher HDL-C concentrations are protective against the development of atherosclerotic disease in humans. The Framingham heart study showed that people whose HDL was below 0.91mmol/l (35mg/dL) at baseline had a coronary risk eight times that of subjects whose HDL concentration was greater than 1.68mmol/l (108). Several studies have since confirmed these findings.

Indeed, this negative association is preserved at varying levels of LDL cholesterol as seen in figure 1.3. The intimate relationship between CHD, HDL-C and LDL-C was confirmed in a multinational study (Interheart) in almost 30,000 subjects, where the ratio of ApoB to ApoA ratio was analysed in quintiles. A linear relationship with CHD risk was evident with an odds ratio of 3.81 comparing the highest and lowest quintiles. It was concluded that smoking and dyslipidaemia accounted for two thirds of the attributable risk for myocardial infarction (109).

Recent analysis of two large prospective studies, however, indicate having a very high HDL-C concentration and very large particle size may actually increase risk of atherosclerosis. This was despite accounting for ApoA-I and ApoB levels. Higher ApoA-I concentration appeared to be associated with lower risk in both studies (110). A possible biological explanation is that very high HDL-C levels may represent accumulation of large, dysfunctional HDL-C molecules which are not cleared from plasma. These studies suggest studying HDL-C transport kinetics may be more informative to gauge CVD.

1.52 HDL particle heterogeneity

HDL molecules are a heterogeneous group of particles that can be subdivided on size by electrophoretic mobility or by apolipoprotein composition.

HDL₁, HDL₂ and HDL₃ represent spherical particles of α -mobility, in descending size and pre β -HDL represent small, discoidal particles. Pre β -HDL particles represent 5-7% of total plasma ApoA-I in humans and have been shown to conform in a stacking arrangement (111). HDL can be subdivided into particles that contain only ApoA-I (Lp AI) and particles that contain both ApoA-I and apolipoprotein AII (ApoA-II) (Lp AI:AI). Kinetic studies have discovered that concentrations of ApoA-I are predominantly determined by rates of catabolism and conversely, ApoA-II levels are governed mainly by rates of synthesis. *Schaefer* and colleagues, using a radio-iodination method, showed ApoA-I has a shorter residency time than ApoA-II and that this was an important determinant of ApoA-I concentration (112). Brinton confirmed these findings concluding the ApoA-I catabolic rate accounted for 66% of the variability of HDL-C (113).

Whether ApoA-II protects against atherosclerosis is currently uncertain, however, the presence of ApoA-II may have a secondary effect by influencing the distribution of ApoA-I in HDL particles. Newer proteomic techniques have revealed multiple additional proteins are present in HDL particles including apolipoproteins A-IV, M, CII and CIII. This has been recently reviewed by Davidsson and others (114). The function of many of these proteins and their isoforms are, as yet unknown. It is likely the combination of apolipoproteins will play a role in the various functions of HDL.

1.53 Anti-inflammatory and antioxidant roles of HDL-C

In addition to the primary role of cholesterol transport, HDL has been shown to have anti-inflammatory and antioxidant action.

1.531 Effect of inflammation on HDL and atherosclerosis

HDL may play a central role in regulating inflammation and is involved in innate immunity. During acute inflammation, HDL levels are reduced and this may represent a permissive state for inflammation to occur. Chronic inflammation is associated with a reduction in HDL-C concentrations. Serum Amyloid A (SAA) is an acute phase reactant that is increased up to one thousand fold during acute infection. During these times SAA has been shown to replace ApoA-I as the major protein component of HDL inhibiting its anti-inflammatory function (115). Key proteins involved in RCT have been shown to be down-regulated during periods of inflammation. Evidence from childhood studies propose an early atherogenic effect of inflammation, demonstrated by elevated antibody titres against oxidised LDL and increased CIMT (116).

DM and MS are recognised conditions associated with chronic low grade inflammation. This process may contribute to the altered lipid transport and low HDL seen in these conditions. Ridker and colleagues provided further evidence in a pivotal study showing elevated C-reactive protein (CRP) was a good predictor of CHD and provided additional predictive information compared to conventional risk factors alone (117).

1.532 Anti-inflammatory and antioxidant effects of HDL

An early step in atheroma plaque development is adhesion and migration of monocytes through endothelial cells. These subsequently differentiate into macrophages which can take up modified LDL-C via scavenger receptors. Activated or injured endothelial cells secrete adhesion molecules to promote monocyte binding which is thought to be an early signal in lesion development. Native and reconstituted HDL has been shown to inhibit cytokine-induced expression of adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in cultured human umbilical vein endothelial cells (HUVEC) in a dose dependent fashion with a preserved effect after HDL was removed (118). Unsurprisingly, HDL isolated from different humans exhibit differential inhibitory activities which may be partly explained by size and composition. Variation in particle size, cholesterol ester or triglyceride content did not affect this function, in contrast, varying the phospholipid composition significantly inhibited anti-inflammatory effects in endothelial cells (119). It is likely that ApoA-I exhibits an antioxidative effect that protects against phospholipid oxidation, absence of this effect would otherwise lead to endothelial cell activation. Wadham and co-workers have further demonstrated HDL inhibits CRP-induced expression of adhesion molecules (120).

HDL-induced release of NO has been shown to inhibit monocyte binding to endothelial cells. Inhibition of human LDL-C oxidation has been demonstrated *in-vitro* by Navab and others using ApoA-I and a synthetic ApoA-I mimetic (121;122). This occurs both by protecting LDL particles directly and guarding

arterial endothelial cells from the oxidising effects of LDL (122). Importantly, HDL-C taken from patients with CHD did not show equivalent inhibition of LDL oxidation nor LDL-induced endothelial adhesion molecule secretion (123;124). This implies HDL particle dysfunction in patients with established atherosclerosis.

Animal studies further associate HDL anti-inflammatory function directly to atherosclerotic lesion size (125). Bloeden and colleagues recently reported a study in which the majority of patients with CHD or equivalent had evidence of pro-inflammatory HDL-C (126). In this study there was evidence that administration of a novel ApoA-I mimetic molecule improved the *in-vitro* anti-inflammatory function of HDL without altering lipoprotein levels.

There is evidence to implicate dysfunctional antioxidant HDL function in patients with MS and IGH (127). De Souza and colleagues, who studied patients with MS, observed lower capacity of small HDL₃ species to prevent LDL oxidation and inhibit oxidised-LDL induced endothelial apoptosis. There was also diminished protection against free radical formation in human endothelial cells compared to similar HDL isoforms taken from control subjects (128). These particles were triglyceride enriched through mechanisms described in section 1.6. These data suggest increasing HDL function or increasing longevity in the circulation may prove a successful approach to effective HDL therapies in the future.

1.54 Current evidence from drug intervention trials

A number of pharmacological agents used to modify plasma lipoproteins have been studied in trials in an attempt to prevent CVD.

Several clinical trials have utilised fibrate therapy to investigate its effects on CHD. This class of agents activates a nuclear transcription factor, PPAR α and results in the synthesis of ApoA-I, the main protein component of HDL. ApoA-II and ABCA1 synthesis is also increased. This mechanism results in a net increase in blood HDL-C levels and delivers plasma triglyceride to the liver via HDL particles (129). Fibrates primarily lower circulating triglyceride concentrations by a number of mechanisms including reducing hepatic VLDL secretion, increasing lipolysis by activating lipoprotein lipase and increasing triglyceride disposal by increasing VLDL particle clearance (130;131).

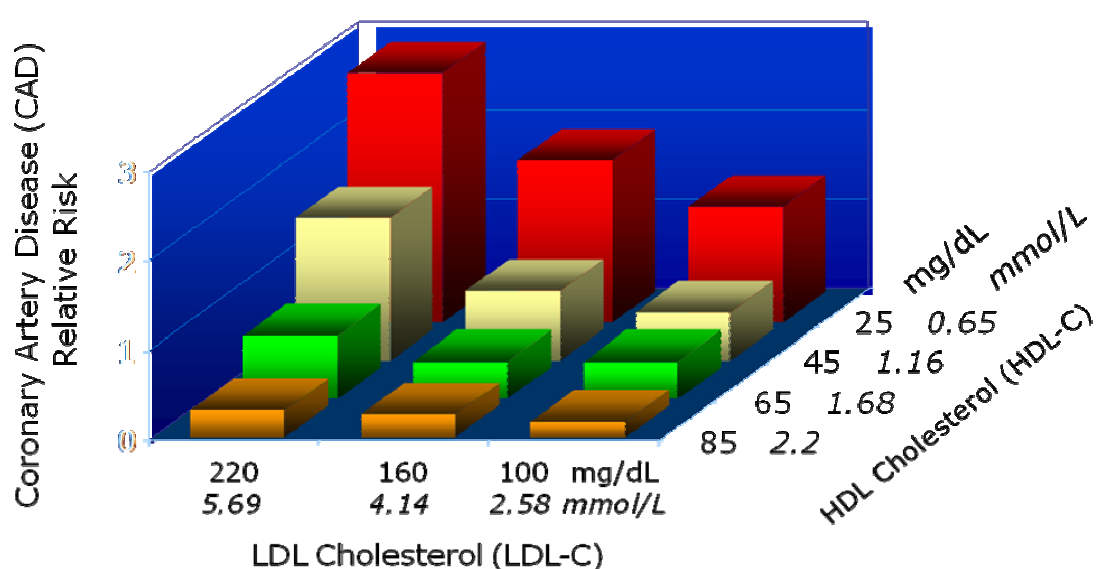


Figure 1.3 Risk of coronary heart disease and HDL-Cholesterol

Illustrates the independent relationship between coronary heart disease (CHD) risk and HDL-C concentration (quartiles) which is independent of LDL-C concentration (tertiles) in men aged 50-70. Reprinted from Castelli WP. Can J Cardiol. 1988 (132) with permission from Pulsus Group Inc.(lipids on line).

The Helsinki Heart Study investigated approximately 4000 men over 5 years. Subjects were middle aged, free of CVD with mean baseline HDL-C level of 1.22mmol/l. Gemfibrozil increased HDL-C levels by 11% and reduced plasma triglyceride and LDL-C by 35% and 11% respectively. A 34% reduction in total

coronary events was observed (133). Gemfibrozil was further tested in the Veterans Affairs HDL intervention trial in men with known CHD over an average of 5 years. HDL-C was raised by 6% and triglyceride concentrations were reduced by 31% without significantly altering LDL-C concentrations. Therapy was associated with a reduction in non-fatal MI and CHD death of 22% compared to placebo (134). The incidence of stroke was reduced in the treated group. Of interest, participants with insulin resistance seemed to experience the most risk reduction implying a possible specific benefit to this group of patients.

In contrast, a more recent large trial exclusively studied men with DM. The Fenofibrate intervention and event lowering in diabetes (FIELD) study was conducted in over nine thousand subjects. Fenofibrate raised mean HDL-C concentration by only 2% from a mean baseline of 1.10 mmol/l. Total CVD events were reduced, however, the primary endpoint of nonfatal MI and CHD death was not significantly altered (135). It has been proposed that introduction of statin therapy to the placebo group in this study may have diminished any potential beneficial effects of fibrate treatment as this was designed as a monotherapy study in statin-naïve patients. Clinical benefits were observed in patients with marked hypertriglyceridaemia and low HDL-C. There were also reductions in diabetes related microvascular disease in those assigned to Fenofibrate (136).

A further investigation of patients with DM observed changes in coronary angiographic findings in response to fenofibrate. The Diabetes atherosclerosis intervention study (DAIS) concluded a 40% reduction in progression of atherosclerosis using measurements of minimal luminal diameter (137).

Collectively, these studies indicate uncertainty to whether fibrate therapy reduces CVD.

Nicotinic acid has been used as a therapeutic agent to treat dyslipidaemia for many years. It lowers triglyceride by up to 50% and raises plasma HDL-C by up to 30%. Currently, this represents the most efficacious clinical treatment to raise plasma HDL-C concentration. However, its tolerability limits widespread usage. Flushing, dizziness and palpitations as well as hyperglycaemia are commonly reported adverse effects. A prolonged-release formulation has reduced this to some degree and future combination with prostaglandin D2 inhibition, could help resolve some of these inconvenient effects. The exact mechanism of action is still unknown, although a receptor for nicotinic acid has been discovered (138). Receptor-mediated inhibition of adenylyl-cyclase blocks lipolysis in adipose tissue, this has been shown to reduce free fatty acids and triglyceride levels (139). Most clinical trials investigating the effects of nicotinic acid treatment have shown cardiovascular benefit. The Coronary drug project in 1975 treated patients after a MI with Niacin and compared this with placebo. A 26% lower non-fatal CHD event rate was reported in the treatment group after 6 years. Despite these results, a mortality benefit was only apparent after 15 years (140). A smaller trial in one hundred and sixty patients with coronary artery disease showed angiographic improvements over 3 years as well as reduced clinical endpoints using niacin in combination with simvastatin (141). A small increment in blood glucose levels was observed as a result of therapy.

Surrogate endpoint studies using CIMT have investigated subjects with CVD established on statin therapy. These were randomised to Niacin or placebo and

although the treatment arm resulted in a 21% increase in HDL-C, there was only a trend towards lower CIMT progression (142). A follow up 12 month open label analysis of 130 participants did show significant reductions in CIMT compared to baseline (143). Results of ongoing trials should help to clarify whether nicotinic acid, in combination with simvastatin, prevents CVD more than statin therapy alone.

A contemporary trial using a novel CETP inhibitor, Torcetrapib has demonstrated up to a 60% increase in plasma HDL-C concentration. This degree of HDL elevation is beyond the current available therapy and was achieved in a dose dependent manner in combination with Atorvastatin. However, this trial was prematurely terminated due to an unexpected increase in cardiovascular events and observed mortality in the Torcetrapib treated cohort compared to those who were treated with statin alone (144;145). Furthermore, a separate study using Torcetrapib failed to demonstrate improvements in CIMT despite substantial increases in plasma HDL-C concentration in patients with familial hypercholesterolaemia or mixed hyperlipidaemia (146;147). It is thought that mineralocorticoid-mediated off-site toxicity may be responsible for these unexpected findings as hypertension and minor electrolyte differences were observed. There is now some evidence to support this drug related mechanism (148). In spite of this, CETP remains an attractive therapeutic target for prevention of CVD based on animal studies and naturally occurring mutations in humans.

Statin therapy remains the most effective pharmacological intervention and demonstrates consistent reductions in CVD events in multiple trials. The main

mechanism of action is inhibition of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, which is the rate limiting step for cholesterol synthesis. It is estimated 60-70% of plasma cholesterol is derived from hepatic synthesis. Reduced cholesterol synthesis results in increased cellular cholesterol uptake via the LDL receptor which results in the observed decrease in plasma LDL-C levels (149).

The UK collaborative Atorvastatin study investigated patients with DM and a additional risk factor for CHD. A mean reduction of LDL cholesterol by 40% along with a small (1%) increase in HDL was achieved. This resulted in a 37% reduction in major CVD events (150). A meta-analysis of over 18,000 patients with diabetes from fourteen randomised trials estimated a 20% risk reduction in CHD and stroke per mmol/L reduction in LDL-C over a 5 year period. This was irrespective of other risk factors or established CVD. It is estimated these benefits were observed when baseline LDL-C concentrations were above 2.6 mmol/l (151). Statin treatment has been observed to only mildly raise HDL-C levels by less than 10%. Mechanisms for this include indirect CETP inhibition and possibly ApoA-I synthesis (152). In a randomised study, Rosuvastatin was shown to increase HDL-C in patients with MS. This was in a dose dependent manner by reduction in particle catabolism resulting in increased HDL size (153).

There is evidence that statins may have beneficial effects beyond lipid lowering. This alternative mechanism was proposed as cardiovascular protection seemed to occur earlier than with previous treatments used to lower LDL-C. Possible pleiotropic mediators include guanosine triphosphatase proteins which are

involved in molecular signalling to control cell growth, cytokine release and molecular trafficking (154). Proven translational benefits from these mechanisms are still to be confirmed. Elevated plasma cholesterol levels are known to cause endothelial dysfunction by interrupting the NO biosynthesis pathway (155). Statins, therefore, have been shown to improve vascular endothelial function by reducing LDL-C similar to that seen with plasma LDL-C apheresis (156). Conversely, when statin-induced plasma LDL-C reduction was compared similar changes using Ezetimibe, which inhibits intestinal absorption of cholesterol, only statin treatment was associated with medium term improvements in endothelial function. These results indicate statin therapy may have pleotropic actions independent of their lipid lowering effect (157).

Trials have demonstrated an anti-inflammatory activity of statins which seems to have additional protective effects beyond lipid reduction. A primary prevention trial investigated subjects with relatively normal lipid levels (LDL-C <3.4mmol/L) and elevated high sensitivity CRP levels. Subjects were randomised to either Rosuvastatin or placebo. The study demonstrated CRP levels after treatment predicted cardiovascular events independent of lipid parameters (158). This provides some evidence that decreasing CRP may result in CVD benefits beyond LDL-C reduction.

Despite the proven efficacy of intensive statin therapy, a post-hoc analysis of patients with CHD treated with a high dose of statin demonstrated HDL-C remained an independent inverse risk factor to predict major cardiovascular events (159). A further analysis of patients undergoing coronary intervention showed similar effects. In this study, low baseline HDL-C was associated with

increased early events at 30 days and higher event rate and mortality at one year irrespective of LDL-C and statin therapy (160). 41% of patients with low HDL-C had DM and were more likely to have hypertriglyceridaemia. These findings indicate that patients with DM continue to be at risk of developing CVD despite statin therapy; it is, therefore, important to identify the specific abnormalities in lipoprotein metabolism that exist in such conditions.

1.6 Type 2 diabetes and alterations in reverse cholesterol transport

DM and states of insulin resistance are commonly accompanied by low plasma HDL-C and high triglyceride concentration, a phenomenon that is more widely known as diabetic dyslipidaemia and is primarily due to lack of normal insulin action. Insulin resistance and associated dyslipidaemia may therefore long precede, and even contribute to, the onset of DM (161). Hepatic insulin resistance leads to an elevated postprandial triglyceride concentration with delayed clearance (162). There is also evidence of increased hepatic VLDL production in DM and the MS (163). VLDL-triglyceride levels are estimated to be 1.5 to 3-fold higher in DM and excursions are largely due to obesity or poor glycaemic control (164).

Kinetic studies of ApoA-I are more difficult to perform than with ApoB as there is usually more than one ApoA-I particle per HDL molecule (see figure 1.1). ApoA-I can be shed by HDL particles during the remodelling process. Studies have demonstrated increased ApoA-I catabolism in DM contributing to low plasma HDL-C concentrations (165). Catabolism is further increased in the presence of excess triglyceride enrichment of HDL. Plasma lipid exchange by CETP between HDL and VLDL or LDL is elevated in hypertriglyceridaemic subjects

with DM (166;167). CETP activity correlates with plasma triglyceride levels in a process that seems to be substrate driven. Excess HDL triglyceride and phospholipid undergoes hydrolysis by hepatic lipase (HL) at the surface of hepatic endothelial cells (168). This process generates smaller spherical HDL and discoidal pre- β particles (168;169). High HL activity is associated with smaller particles as well as lower HDL-C concentrations (169). HL activity positively relates with insulin resistance which contributes to the formation of smaller, dense HDL particles. These are more susceptible to catabolic pathways and ApoA-I detachment, this mechanism is thought to mediate the observed lower plasma HDL concentrations (170;171).

There is little data to show consistent changes in the Apo A-I and All composition of HDL particles in DM, although there are reports that excess HDL remodelling occurs in DM increasing discoidal pre- β HDL levels. This has been reported mainly in patients with hypertriglyceridaemia and obesity (172). Similar mechanisms are likely to be responsible for low plasma HDL-C levels in MS as central adiposity has also been associated with increased ApoA-I and ApoA-II catabolism (173). Triglyceride enriched LDL particles are additionally subject to hydrolysis, mainly by lipoprotein lipase, resulting in small, dense particles in DM which are considered to have greater atherogenic potential (174).

1.7 Cellular cholesterol removal

It is estimated that approximately 60-80% of the total cellular cholesterol resides in the plasma membrane (175). It is likely that early endosomes have a higher cholesterol content than late endosomes or golgi apparatus. Although the ER is

able to synthesise cholesterol, its overall content is thought to be low (176). Cellular cholesterol can either be attained by receptor-mediated uptake of LDL-C, or *de-novo* synthesis by the ER. Given that cholesterol cannot be used as an energy source and few tissues are able to catabolise cholesterol directly, the export of cholesterol remains a vital mechanism through which excess cellular cholesterol can be removed.

ApoE in mice is usually carried by HDL (177). It has been shown that ApoE and HDL-C can independently promote cholesterol efflux (178). It is currently unclear whether ApoE plays a definitive role in RCT. Nevertheless, ApoE deficient mice spontaneously develop atherosclerosis and are commonly used as an experimental model to study atherosclerosis (179;180). Moreover, atherosclerotic lesions are found to contain both ApoA-I and ApoE proteins. The ApoE3 isoform in its free form has been shown to compete with ApoA-I binding to ABCA1 (181). All three isoforms, which differ by one amino acid, have been demonstrated to mediate cholesterol efflux in a dose-dependent manner. This effect was abolished in fibroblasts taken from Tangier disease (TD) patients which suggests ApoE selectively binds ABCA1 (181). Endogenous macrophage ApoE may have the added ability to mediate lipid efflux independent of ABCA1 (182). Macrophage ApoE secretion may, therefore, protect against atherosclerosis. Yamato et al., observed a positive relationship between ApoE secretion and plasma HDL-C in patients with DM (183). In this study ApoE secretion significantly improved after initiation of hypoglycaemic therapy. This proposes reduced HDL-C observed in DM may be associated with lower ApoE secretion which may predispose to atherosclerosis. However, the utility of this

study was limited by the use of a heterogeneous group of Japanese patients with no control group comparison (183).

Differences in cellular lipid removal, which is rate limiting in HDL assembly, has to date not been widely demonstrated in DM, nor is it known whether this contributes to low plasma HDL-C concentration. A study by Alenezi and colleagues aimed to address this question by studying patients with MS and low HDL-C with and without hyperglycaemia. Authors reported there was no defect in lipid efflux using skin fibroblasts from subjects and purified human ApoA-I (184). A pivotal study by Maulden et al., demonstrated lower ABCG1-mediated efflux in patients with DM and subsequent foam cell development (185). This group earlier demonstrated decreased ABCG1 gene expression and function in a mouse model of diabetes (84).

Using severely hyperglycaemic plasma as an acceptor for cholesterol, impaired cholesterol transport has been shown to occur from cultured fibroblasts. This reduction was subsequently reversed after insulin treatment (186). A decreased LpA-I/A-II ratio may also be implicated in lower cholesterol efflux (187). ApoA-II has been shown to bind both ABCA1 and SR-B1 but has variable effects on efflux depending on the cell type studied and conditions used (187;188).

The effect of HDL particle glycation has been examined in relation to excess glucose and diabetes. Hoang and colleagues examined this using ApoA-I incubated with either ribose and glucose (189). Modified ApoA-I resulted in lower efflux in both human monocyte-macrophage cell lines (THP-1) and human monocytes. This was observed at physiological concentrations of glycated ApoA-I similar to that seen in diabetic patients with complications. The effect

was attenuated by the use of drugs which inhibit AGE formation or disrupt cross-linking to glucose (189). Similar defects in efflux were observed with AGE-modified HDL. Another study showed AGE-modified albumin impaired membrane binding of HDL₃ particles in murine peritoneal macrophages. The reduction in HDL-mediated lipid export was consistently attenuated with inhibitors of AGE formation-Aminoguanidine and Metformin (190).

The effect of modified plasma lipoproteins on cellular cholesterol removal in diabetes has been explored using standardised cellular models. In type I diabetes, it has been reported that cellular cholesterol removal may be increased (191). This observation was assessed using plasma taken from diabetic subjects which was diluted and subsequently added to cultured fibroblasts taken from healthy subjects. This model examined the effects of plasma lipoproteins on cholesterol efflux. Cellular cholesterol removal was unaltered after treating these patients with Simvastatin. Serum HDL levels in these patients appeared lower than is usually seen in patients with type I diabetes (191). As type I diabetes is associated with increased plasma AGE, these findings oppose results from that of *in-vitro* studies. Using a similar experimental design in patients with DM, de Vries and colleagues report increased cholesterol export to plasma taken from hypertriglyceridaemic DM patients. It was thought this was related to increased pre β -HDL formation (192). Other studies have since confirmed this finding in patients with hypertriglyceridaemia (172). No difference in cellular cholesterol removal was observed using plasma taken from normolipidaemic DM subjects compared to controls (192). These results would suggest diabetic status did not adversely modify the ability of plasma lipoproteins to remove cellular cholesterol.

Concordant with these findings, no difference in cellular efflux to plasma was observed in patients with the MS (193). Cholesterol export in this study was independently related to plasma cholesterol esterification, cholesterol ester transfer, phospholipid transfer protein (PLTP) activity and ApoE concentration. Of note, there were a moderate number of DM subjects included and a marginally higher lipid efflux was seen in DM patients with MS compared to DM without MS (193). These data would suggest any defect in cellular cholesterol removal in DM is more likely to be due to defects in a cellular transporter or transporter-acceptor interaction.

1.71 The role of hepatic and macrophage ABCA1 in murine models

In-vitro work using hepatocytes indicates HDL particles may be synthesised in an autocrine manner; As an HDL particle enlarges by the central accumulation of cholesterol ester, ApoA-I particles are able to dissociate. These are subsequently re-lipidated by hepatic ABCA1 to form further HDL particles (194).

Organ-specific mouse KO studies have implicated hepatic ABCA1 activity to be the main source for maintaining plasma HDL-C levels. It is estimated that in animals 80% of plasma HDL-C originates from hepatic ABCA1 lipidation of nascent apoA-I molecules (70). In hepatic-specific KO animals, there is a two to three-fold increase in ApoA-I clearance, which was predominantly renal, compared to wild-type animals. This was associated with a 2-fold higher turnover of ApoA-I but HDL tracer uptake at the liver was unchanged (195). These data indicate hepatic ABCA1 inactivation leads to decreased plasma HDL-C residency and accelerated renal clearance. A similar kinetic profile has

been demonstrated in naturally occurring human *ABCA1* mutations, with rapid turnover persisting when donor wild-type HDL particles were injected (196).

The contribution of macrophage *ABCA1* to maintaining plasma HDL-C has been investigated using transplanted macrophages taken from *ABCA1* KO animals. These were transplanted into irradiated wild-type mice, resulting in mice specifically deficient in macrophage *ABCA1*. This technique demonstrated macrophage *ABCA1* activity had little effect on plasma HDL-C concentration (197;198). Nevertheless, macrophage selective *ABCA1* depletion lead to accelerated atherosclerosis despite the little difference in plasma lipoproteins (197-199). Further evidence indicating the importance of peripheral blood cell *ABCA1* in atherosclerosis is demonstrated by a study of leukocyte *ABCA1* deficiency in a background of LDL-C receptor KO mice. These transgenic animals were observed to have a 60% increase in aortic atherosclerosis with histological features of advanced disease despite no change in plasma HDL-C levels. The study concluded this effect was mediated by altered macrophage recruitment (198). It is noteworthy that accelerated atherosclerosis was not observed in whole body *ABCA1* inactivation in mice (199).

1.8 The association of ABCA1 with cardiovascular disease in humans

1.81 Structure and function of ABCA1

This membrane transporter is part of the ATP-binding cassette (ABC) protein superfamily. This group of proteins transport a variety of organic and inorganic compounds. Higgins and Gottesmann initially observed that ABC transporters acted like a “flippase” which are able to transport lipids from the inner to the outer leaflet of the plasma membrane (200). The family now contains seven subclasses (ABCA to ABCG). Mutations in these genes have been shown to be responsible for several human diseases, including cystic fibrosis, adrenoleukodystrophy, sitosterolaemia and TD (201). The latter two diseases are due to abnormalities in lipid transport. ABC transporters are structurally configured either as homodimers or half transporter heterodimers. Structural motifs known as Walker A and Walker B are present in many transporters that use ATP, but a Walker C motif is unique to ABC transporters (202).

At least seven ABC transporters have demonstrated cholesterol as being a direct or indirect substrate for transport; ABCA1, G1, G5, and G8 have been shown to have a major role in cholesterol transport, significantly influencing lipoprotein metabolism (203).

ABCA1 is a 2261 amino acid plasma membrane protein comprising of two heterodimers (figure 1.4). Each contain Walker A, B and C motifs, the latter motif consists of two large extracellular loops that are usually glycosylated and connected by one or more cysteine bonds (204;205). ABCA1 has been shown to transport cholesterol, phospholipid (PL) and other lipophilic compounds such

as ApoE (178). Its homology to other transporters in the ABC family suggests transport may be facilitated by formation of a channel that helps lipids “flip” from the inner to the outer membrane bilayer, though the precise mechanism of efflux is not fully characterised.

The ‘flippase’ model implicates two separate conformations of the transporter, this allows binding of substrates on the cytoplasmic leaflet and release into the outer leaflet for subsequent access to extracellular acceptors. An alternative theory proposes a channel is formed within the membrane which is responsible for extracellular lipid translocation (206).

PL transport is thought to be another putative function of ABCA1. It is currently unclear whether ABCA1 transports both PL and cholesterol together or by two separate processes. It is possible to uncouple this process using a protein kinase inhibitor which has been shown to predominantly inhibit cholesterol efflux (207). Removal of cellular cholesterol by using an artificial acceptor-cyclodextrin did not affect PL transport but eliminated cholesterol efflux (208). These studies indicate PL transport may be an active function of ABCA1 with cholesterol efflux being a secondary passive process.

Phosphatidylserine (PS), a phospholipid, is largely confined to the inner membrane leaflet in healthy cells. Translocation, or “flop” of this phospholipid to the outer membrane leaflet is thought to trigger cellular apoptosis (209). Our group observed abnormal PS flop in B-lymphocytes in a patient with a rare bleeding disorder-Scott syndrome (210). Decreased ABCA1 expression in leukocytes taken from this individual was demonstrated and was caused by a novel missense mutation in *ABCA1* (210). Obvious lipid abnormalities in this

patient were not apparent (HDL-C concentration 1.3mmol/l) indicating ABCA1 may have a tissue specific role. In keeping with this study, defects in coagulation have been reported in TD patients (211). It is unclear whether ABCA1 promotes PS flop directly or regulates other permissive proteins (212). ABCA1 promotes calcium-induced microvesicular membrane protrusions which may promote PL transport physicochemically. Alterations of PS distribution is also thought to transduce non-apoptotic signals (213). Increased platelet PS flop has been observed in patients with type I diabetes and nephropathy. This may predispose to an excess risk of thrombosis (214). Indeed studies in humans have found an inverse relationship between HDL-C levels and risk of recurrent thrombosis (215). Large pulmonary emboli have been demonstrated to occur in patients with lower HDL-C concentrations (216). Van Leuven and others have, more directly, demonstrated that infusion of Apo-AI-phosphatidylcholine in humans abrogates the thrombotic effect induced by CRP (217). These studies implicate HDL-C to have direct anticoagulant action and this lipoprotein may play an important role in preventing arterial thrombosis.

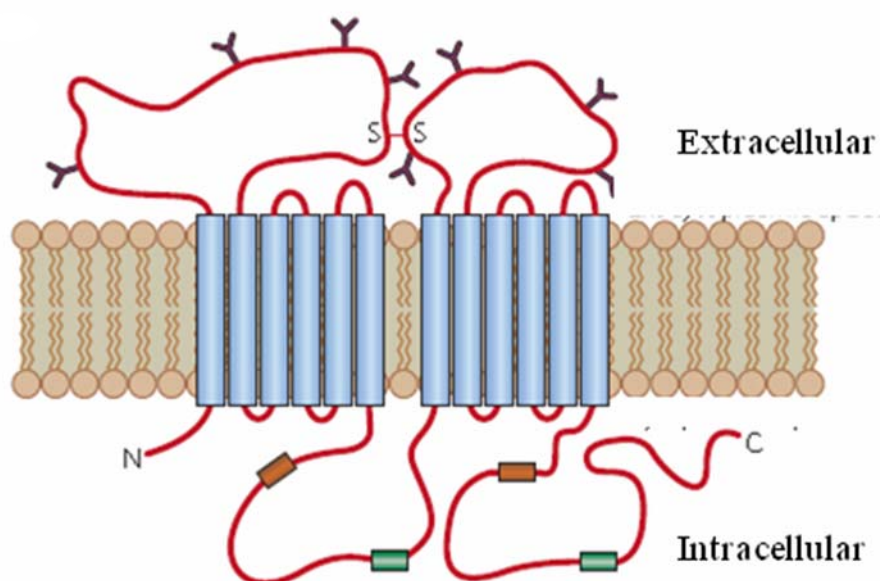


Figure 1.4 Structure of ABCA1 transmembrane spanning protein

ABCA1 consists of two transmembrane domains of six alpha helices (blue) and two intracellular nucleotide binding domains. The nucleotide binding folds are linked by a cytoplasmic peptide. The ATP-binding cassette on ABCA1 consists of Walker A and B motifs (coloured boxes) which are commonly found in proteins that utilise ATP (218). [Adapted from Maxfield & Tabas Nature 2005].

1.811 Intracellular cholesterol transport

ABCA1 targets membrane regions prone to accumulating cholesterol. The inward direction of movement of cholesterol from the plasma membrane toward intracellular storage occurs using the action of Acyl-Coenzyme A:cholesterol acyltransferase (ACAT). Excess free cholesterol is cytotoxic and this enzyme esterifies cholesterol in the ER which accumulates as lipid droplets in the cytoplasm (218). Efflux of cholesterol from the plasma membrane is associated with a rapid reduction of the ACAT sensitive cholesterol pool (219). Lipid esters in droplets can be hydrolysed by neutral cholesterol ester hydrolases to liberate free cholesterol for efflux. Some cells have an additional hormone sensitive lipase for triglyceride hydrolysis from fat cells (220). ACAT activity is positively regulated by cellular cholesterol levels acting as a homeostatic sensor.

The protein Caveolin-1 binds cholesterol and has been implicated in shuttling cellular cholesterol to and from the plasma membrane. This may transfer cholesterol to specific regions rich in cholesterol and sphingomyelin (221). Caveolin forms small membrane invaginations (caveolae) of 50-100nm diameter, these are considered a type of lipid raft (222). The interaction between these lipid rafts, ABCA1 and apolipoproteins may be an important determinant for transport.

1.812 Models of ABCA1 mediated intracellular cholesterol removal

There are currently two models which postulate how intracellular cholesterol is exported by ABCA1. The *exocytosis* model indicates excess intracellular cholesterol is packaged in vesicles or rafts and transported to ABCA1 within the plasma membrane. To support this, ABCA1 induction in the absence of apolipoprotein is associated with an increase in cell membrane cholesterol (223). In contrast the *retroendocytosis* model, proposes that a apolipoprotein-ABCA1 complex is initially endocytosed toward intracellular lipid stores which pumps cholesterol into this complex for transport to the plasma membrane and subsequent release by exocytosis. Studies in support of this have demonstrated intracellular trafficking of ABCA1 from the ER to the plasma membrane (224). A seminal paper by Neufeld demonstrated non-functional human ABCA1 accumulated at intracellular sites with apolipoproteins (225). Pharmacological inhibition of ABCA1 function has been reported with the use of Probucol. No changes in gene expression were observed by probucol in fibroblasts but binding of Apo-AI was abolished. This drug has been shown to cause accumulation of non-functional protein at the plasma membrane with resistance to degradation to calpain (226). This supports the functional requirement of ABCA1 to shuttle between intracellular sites and plasma membrane (227). A similar trapping mechanism has been reported to occur from Cyclosporin treatment (228). It is possible both mechanisms co-exist but the dominant mechanism by which efflux occurs is still subject to debate (229). In addition to specific transporter-mediated cholesterol removal, cholesterol can be non-specifically released, probably via diffusion, to a variety of extracellular

acceptors including lipoproteins (230). LCAT is considered to drive the rapid release of cholesterol by maintaining a free cholesterol concentration gradient. This occurs predominantly in HDL due to LCAT localisation and HDL particle capacity. The interaction of ApoA-I with ABCA1 stabilises and protects the transporter from proteolysis (231).

1.813 The binding of ApoA-I

ApoA-I is thought to bind with ABCA1 in its free form and lipidation of ApoA-I reduces further binding (232). In support of this Okuhira et al., demonstrated binding was inhibited by a monoclonal antibody against ApoA-I and this lead to a 70% reduction in efflux (233). Vedhachalam, using a competition assay with labelled ApoA-I, showed that ABCA1 creates two binding sites for ApoA-I which separately confer its regulatory and lipid transport function (234). Vedhachalam further proposed a three-step model for the efflux process; ApoA-I binding, phospholipid translocation and subsequent membrane bleb formation, this is followed by lipidation of ApoA-I (235).

Free ApoA-I is not the major form of HDL in plasma and although the dissociation constant of HDL surface is unknown, it is estimated this is less than 1% of plasma ApoA-I concentration so that only a small proportion of ApoA-I is free to bind to ABCA1 (232). There are several mechanisms that yield free ApoA-I particles from HDL, as explained above, including the action of CETP and PLTP (236;237). The latter remodels HDL into smaller particles releasing ApoA-I. This process is increased as triglycerides replace the cholesterol ester core (238). Furthermore, ApoA-I can be released from HDL particles to the cell

surface due to a higher affinity (239). This process is responsible for the formation of nascent HDL. In a landmark study, patients who presented with acute coronary syndrome were injected with a naturally occurring, mutated form of ApoA-I associated with longevity. Coronary atherosclerosis, as measured by intravascular ultrasound, showed evidence of regression after 5 weekly doses (240). There has been some subsequent debate whether this mutated form of ApoA-I, termed “ApoA-I Milano”, shows superior lipid removal properties compared to wild-type ApoA-I (241;242). These results indicate ApoA-I analogue or mimetic therapy may be an effective therapeutic option to augment cellular cholesterol removal.

1.82 Genetics of ABCA1 and cardiovascular disease

The human model of dysfunctional ABCA1 has been characterised by TD. This rare autosomal recessive disorder was first reported in 1960 where characteristic features of cholesterol ester accumulation were manifest in a variety of tissues leading to enlarged orange tonsils, hepatosplenomegaly, peripheral neuropathy and near absent HDL-C levels (243).

Homozygote individuals exhibit plasma HDL-C concentrations of less than 5% of normal. *ABCA1* is located on chromosome 9q21 and various mutations have been shown to be responsible for TD and familial HDL deficiency. This was first identified by multiple groups in 1999 (244-246;246;247). These studies confirmed *ABCA1* mutations were responsible for diminished ApoA-I mediated cellular cholesterol removal in skin fibroblasts taken from affected patients compared to healthy individuals. Currently over 70 mutations are known. Low ApoA-I levels are caused by accelerated catabolism of ApoA-I and ApoA-II with

excretion mainly via the kidneys. This condition is not associated with significant alteration in ApoA-I production (248;249). Defective endosomal cholesterol trafficking to the plasma membrane has been demonstrated to occur in fibroblasts taken from patients with TD and in murine models of this disease (225;250). A similar naturally occurring *ABCA1* mutation, has also been demonstrated to occur in chickens (251).

The incidence of premature coronary disease in TD is four to six fold higher (252). This is not as high as common genetic predispositions such as familial hypercholesterolaemia because concentrations of LDL-C are reduced, typically to 40% of normal in homozygotes. Heterozygotes have higher HDL-C levels-estimated to be 50% of normal, however, they still appear to have higher CHD risk despite normal LDL levels (253;254). Clee and colleagues, in a pivotal study, demonstrated ABCA1 activity in cultured skin fibroblasts taken from heterozygote individuals was inversely correlated to plasma HDL-C. There was also a relationship between ABCA1 activity and prevalence as well as severity of CVD. This crucial study provided the clearest evidence to link the degree of ABCA1 activity to the age of onset and severity of CVD. Van Dam et al., studied four different missense mutations in heterozygotes and showed ABCA1-mediated fibroblast efflux negatively correlated with the CIMT. An upper limit of normal, set at 0.8mm, was reached twenty five years earlier in affected individuals (255). Singaraja has since characterised the effect of several *ABCA1* mutations on function and concluded that complete loss of function mutations were associated with absent plasma membrane localisation and/or ApoA-I binding. However, other mutations such as the *W590S*, which was associated with preserved localisation and binding, remained unable to elicit lipid efflux.

Interestingly, this mutation is associated with reduced PL efflux as demonstrated by reduced annexin V binding. It is possible this plays a permissive role for cholesterol efflux to occur (256). This important work separated the severity of various *ABCA1* mutations by efflux function and degree of HDL reduction (257). Further work by this group have reported that several gene polymorphisms are associated with modified CVD risk without alterations in plasma HDL-C concentrations (254). Soro-Paarvonen explored cholesterol efflux in macrophages from subjects with familial low HDL-C without a known TD mutation. Results did not confirm defects in ApoA-I mediated efflux and no significant relationship between efflux and CIMT was observed. Patients however, exhibited decreased macrophage *ABCA1* gene expression in response to cholesterol loading, implying low HDL-C stemmed from an altered gene expression set-point (258). At a population level, the Copenhagen heart study has screened over 9000 individuals in the general population. 10% of individuals with the lowest HDL-C were found to be heterozygote for an *ABCA1* mutation (189). Specifically, heterozygosity for the common *K776N* mutation was showed to convey elevated risk for developing CHD with a hazard ratio of 2.4 (259). It is noteworthy that little functional information on this mutation was known at that time of the reporting. In contrast, current analysis of this cohort concluded heterozygote loss of function mutations were not associated with increased CHD (260). Frikke-Schmidt further reported common variants of *ABCA1* were associated with HDL-C levels in the Danish population and some of these variants predicted ischaemic heart disease (261;262). These findings were not supported by a smaller, population study (222). The common genetic polymorphism, *R219K*, is estimated to have a carrier frequency of 46% in

European people. Clee and colleagues report this variant to be independently associated with higher HDL-C and lower triglyceride levels. Carriers of this polymorphism were observed to have reduced CVD risk, and slower progression of CHD based on angiographic data (263). No obvious differences in cholesterol efflux were observed but it should be noted that a small number of individuals underwent detailed analysis yielding a relatively high inter-assay coefficient of variation (CV). In a separate investigation, common functional polymorphisms have been reported to influence the age of onset of symptoms resulting from CHD (264). It is likely common genetic variants have a minor to moderate phenotypic effect on HDL-C concentrations but predisposition to CVD is probably subject to interaction with other environmental factors.

With regard to glucose homeostasis, there have been reports that some genetic variants may occur more commonly in certain ethnic groups with DM compared to the background population. This has been reported by Brunham and colleagues (265-267). The R230C variant is associated with early onset DM in Mexicans with increased body weight and reduced fasting insulin levels (265). This variant is also seen in populations with a high prevalence of diabetes but is not seen in Caucasians (268). The interaction of several genes as well as environmental factors are more likely to be responsible for the development of the common form of DM, though monogenetic predisposition remains a possibility. Functional studies in these individuals are still lacking and altered ABCA1 gene expression or reduced transporter function has not been reported.

Physiological studies have established that Tangier *ABCA1* heterozygotes have evidence of endothelial dysfunction secondary to defective NO synthesis. This

was rapidly corrected after ApoA-I infusion confirming ABCA1 is likely to confer direct beneficial effects on blood vessels (269).

1.83 ABCA1 expression and regulation

Although widely expressed, high levels of ABCA1 messenger RNA (mRNA) (defined as greater than ten-fold compared to housekeeper genes) using polymerase chain reaction (PCR) are found in human macrophages, liver, placenta, small intestine and lung in decreasing order (270). Mouse studies have shown a similar pattern of tissue expression with high expression also noted in heart, brain and testicular tissue. However, importantly, a discordance between mRNA expression and protein abundance has been reported in mice in multiple organs except liver, kidney and testicles where a high level of protein is concordantly expressed (271). This suggests additional tissue specific regulation may occur at a post-transcriptional stage. Mouse adipose tissue was reported to have moderate expression but this was not localised using the *in-situ* hybridisation technique (271). It is estimated induction of ABCA1 in tissue macrophage cells represents the largest pool of ABCA1 in humans based from work on cultured cells (272).

ABCA1 protein levels are increased in the liver and in macrophages in response to a high fat diet (271;273). The specific gene transcripts induced in these tissues have been identified (274). Our group have reported ABCA1 gene expression is up regulated in human atherosclerotic plaques as well as aneurysmal arteries compared to non-atheromatous arteries and this was due to increased Liver X Receptor- α (LXR α) expression. ABCA1 protein levels were diminished in plaque tissue owing either to protein degradation or instability

(275). This study, importantly, demonstrated a positive relationship between peripheral blood leukocyte and monocyte ABCA1 gene expression.

In the absence of gene inducers, ABCA1 mRNA and protein are rapidly degraded with an estimated half life of 1-2 hours (276). This is due to phosphorylation of an amino acid sequence in the first intracellular loop known as PEST (Proline-Glutamate-Serine-Threonine) motif (277). Phosphorylation at these sites promotes proteolysis by a calpain protease but interaction with apolipoproteins promotes cell surface protein accumulation by inhibiting degradation (278). Protein stability has been demonstrated to increase ABCA1 levels by positive feedback (277). Human skin fibroblasts have historically been used as a model to study cellular cholesterol transport in humans. Using this model, cellular cholesterol loading did not stabilise ABCA1 gene expression but positively regulated expression via LXR (279). Fibroblasts also express *ABCG1*, another ABC cholesterol transporter which transports cholesterol to more mature HDL particles (280). Conversely, these cells do not express significant amounts of SR-B1 (281)

PLTP has also been shown to interact with and stabilise ABCA1 to enhance cholesterol removal in fibroblasts and macrophages by mimicking apolipoproteins (282). However, PLTP was not shown have a protective effect in LDL-KO mice when ApoA-I was overexpressed (283). Moreover, PLTP is highly expressed in atherosclerotic plaques which brings uncertainty to whether it represents an atheroprotective protein (284).

1.831 The role of nuclear transcription factors and drugs in the regulation of ABCA1

It is known that oxysterols, produced by intracellular cholesterol hydroxylation rather than cholesterol per se, upregulates *ABCA1* gene expression by binding to the nuclear transcription factor LXR α (285). In support of this, fibroblasts taken from patients with 27-hydroxylase deficiency did not increase *ABCA1* gene expression in response to cholesterol loading (285). This clinical disorder is characterised by cholesterol accumulation forming cerebrotendinous xanthomata and testifies to the dominant regulatory role played by 27-hydroxycholesterol in humans (286;287). 24-hydroxylation plays an important regulatory role to allow excess cholesterol to pass from the brain to circulation (288).

LXR α and retinoid-X receptor (RXR) join to form an obligate heterodimer which binds the DR4 response element in the *ABCA1* promoter region, inducing gene expression (289). Retinoic acid, a ligand of the orphan receptor RXR, can also induce expression. Together, these ligands have been shown to act in a synergistic fashion to upregulate expression (290). The LXR/RXR heterodimer confirmation can recruit further ligands to form co-activator and co-repressor complexes with opposing effects on subsequent target gene transcription (291). This activating mechanism is thought to result in the removal of excess cholesterol in cells expressing *ABCA1* and represents a dominant mechanism in macrophages (292). LXR α is mainly expressed in the liver, adipose tissue, intestine, kidney and macrophages. This transcription factor regulates multiple genes including *ABCG1*, *ABCG5* and *ABCG8* to increase efflux and aid biliary

cholesterol excretion (293;294). LXR α also increases fatty acid synthesis by actions on sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase and stearoyl-CoA desaturase (295).

Mitro and colleagues had demonstrated that glucose itself may positively regulate LXR α and LXR β . Glucose was shown to upregulate LXR α levels in human hepatoma cells (Hep G2) and in liver from mice fed with glucose. This effect seemed to be specific to glucose and was dose dependent at physiological concentrations (296). In this study, LXR target genes including *ABCA1* and *ABCG1* were upregulated in hepatocytes of glucose fed mice. The results of a subsequent study did not support these provisional findings and implicate carbohydrate response element binding protein (ChEBP) as a hepatic glucose sensor (297).

The transcription factor PPAR γ is a permissive binding partner of LXR and induces LXR α (298). PPAR γ activation is associated with a glucose lowering via peripheral insulin sensitisation and PPAR γ agonists are used as treatments for DM. PPAR γ agonists stimulate cholesterol efflux via LXR α with subsequent *ABCA1* induction and this has been shown to improve atherosclerosis in animal models (292;298). We have reported PPAR γ gene expression is lower in atheromatous plaques and ectatic arteries (299). *ABCA1* gene induction and subsequent cholesterol efflux has also been stimulated by PPAR α agonists in macrophage cell lines (292). In hepatocytes, *ABCA1* expression contributes to HDL synthesis which is shown to be PPAR-dependent (300). PPAR α agonists are currently used in clinical practice to lower triglyceride and raise HDL

cholesterol levels as outlined above and have been demonstrated to increase ABCA1 gene expression in monocytes (301).

A number of statins have been shown to inhibit induction of *ABCA1* using *in-vitro* models of murine and human macrophages. The effect was dose dependent and was inhibited by the addition of the post-reductase product mevalonate (302). Both Pitivastatin and Atorvastatin have been demonstrated to decrease *ABCA1* in a LXR α -dependent manner (303;304). Conversely, in a hamster model of insulin deficiency, macrophages treated with Atorvastatin in the presence of ApoA-I showed a disparate increase in *ABCA1* gene expression and efflux (305).

Pravastatin fed mice were associated with a decrease in ABCA1 but not LXR α expression in both hepatic cells and leukocytes (306). The decrease in LXR α and ABCA1 expression reported by most studies is probably secondary to reduced oxysterol generation secondarily to inhibition of cholesterol synthesis. Wong and colleagues further demonstrated the statin effect on ABCA1 expression is dependent on cellular cholesterol status, with cholesterol supplementation inhibiting, and cholesterol depletion augmenting the response (307). This mechanism is likely to be mediated through the cellular cholesterol sensor SREBP-2 (307;308). The additional effect of diabetes, similar to that seen in some animal models, may modify this interaction. Guan recently reported increased mononuclear cell gene expression in hyperlipidaemic, DM patients receiving simvastatin compared to untreated diabetic patients with either a normal lipid profile or hyperlipidaemia (309). In conclusion, the effect of

statin treatment on ABCA1 depends on the cholesterol status and diabetes status of the model used.

1.832 ABCA1 regulation in metabolic disease

It has been postulated that disorders associated with increased blood glucose and fatty acids may destabilise ABCA1. Both saturated and mono-unsaturated fatty acids exacerbate insulin resistance and free fatty acids have been directly shown to inhibit insulin signalling (310). Unsaturated fatty acids have been shown to inhibit cholesterol efflux from macrophages (311). Uehara and co-workers further identified that unsaturated fatty acids targeted a specific site in the DR4 promoter region which is a known regulatory site of LXR α . Fatty acid binding at this site resulted in an inhibition of *ABCA1* and *ABCG1* gene expression in cultured macrophages (312). Senanayake and colleagues report macrophages taken from patients with DM have elevated unsaturated fatty acid concentrations compared to cultured macrophages of healthy individuals. Despite this, in this study, there was no difference in macrophage ABCA1 expression observed whether macrophages were cultured in physiological or supraphysiological glucose concentrations (313).

1.9 The effect of glucose on the ABCA1 transporter

The direct effect of glucose on ABCA1 gene expression has been investigated with conflicting results. Human hepatoma (HepG2) cells grown in medium containing 5.5, 12.5 and 25mmol/l glucose increased gene expression with increasing glucose concentration (314). Uehara, in contrast, reported no such

dose dependent differences in gene expression in HepG2 cells or mouse macrophages lines (RAW246.7) (315). However, this study did demonstrate marked reduction in hepatic and macrophage ABCA1 gene expression in streptozotocin-induced diabetic mice (a model for type I diabetes) with insulin treatment correcting this defect (315).

AGE may have a negative effect on cholesterol export secondary to effects on ABCA1. Albumin represents 80% of proteins in plasma susceptible to glycation and oxidation. AGE formation is associated with ageing, enhanced in diabetes and is associated with diabetes related vascular complications (316). Moreover, serum AGE levels have been shown to increase with duration of DM (317). Post-mortem work indicates a greater number of receptors for AGE in coronary plaques taken from diabetic patients, which may influence plaque instability (318). Currently, there is little human *in-vivo* data directly examining the effect of AGE on this transporter. ABCA1-mediated cholesterol efflux is severely impaired when precursors of AGE are added to either cultured human fibroblasts or murine macrophages (319). Inhibition of ApoA-I binding and ABCA1 degradation have been reported mechanisms for this effect with no alterations in ABCA1 mRNA. Streptozotocin induction of diabetes in hyperlipidaemic swine was associated with increased coronary cholesterol plaque area with decreased ABCA1 staining in lesions compared to the non-diabetic animals (320). In contrast to these observations, Isoda and colleagues demonstrated the addition of glycated albumin was not associated with any changes in ABCA1 mRNA, protein or ApoA-I-mediated efflux in cultured macrophages (321). ABCG1 mRNA in these macrophages was, however, reduced with a corresponding decrease in protein and HDL-mediated efflux

(321). This indicates the possibility of differential gene regulation despite unaltered LXR α gene expression. Collectively, these studies do not indicate a clear mechanism of ABCA1 disruption by AGE formation.

A single study has reported reduced ABCA1 RNA levels in human peripheral monocytes taken from patients with DM and dyslipidaemia. ABCA1 levels were improved using a PPAR α agonist Fenofibrate (301). Subsequent studies have failed to show differences in peripheral blood cell ABCA1 expression in patients with DM (185;309;322;323). We have reported an inverse linear relationship between fasting blood glucose and ABCA1 expression in blood leukocytes taken from 32 healthy men (324). These results, in context of inconsistent findings from studies outlined above, have lead to the hypotheses in section 1.91.

1.91 Hypotheses and Aims

- We propose the inverse relationship observed between fasting plasma glucose (FPG) and ABCA1 mRNA extends over a wider range of glucose concentrations in patients with Impaired glucose homeostasis (IGH) (which includes impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) as per the American Diabetes Association classification 2005) (325). Gene expression will also be assessed in patients with DM who are naive of hypoglycaemic and lipid lowering therapies.
- We hypothesise this relationship with gene expression will lead to reduced ABCA1 function.
- We further postulate, changes in ABCA1 may mediate premature atherosclerosis in patients with impaired glucose regulation.

Aims

- To investigate whether acute changes in glucose levels within an individual during an oral glucose tolerance test (OGTT) result in differences in leukocyte ABCA1 mRNA and protein as well as function as measured by lymphocyte PL translocation.
- To measure and compare functional cholesterol efflux in primary dermal fibroblasts as well as PL transport in CD4⁺ T-lymphocytes between groups of patients with DM, IGH and healthy men.
- To investigate the relationship of *ABCA1* gene expression and function with CIMT, a surrogate measurement of atherosclerosis.

- Finally, to examine the effects of chronically lowering hyperglycaemia in drug naïve patients with DM. ABCA1 expression and protein concentrations will be measured before and after glucose lowering therapy.

Plan of investigation

For our power calculations, we wished to test the null hypothesis that the magnitude of the β coefficient (slope of the regression line) between ABCA1 expression and fasting plasma glucose is zero. Using our previous data, the standard deviation (SD) of log ABCA1 gene expression is 0.35, and of glucose 0.37 with a regression coefficient of -0.4556 (324). With 80% power and 5% significance, our hypothesis could be tested with 27 subjects in each glycaemic category. There are few robust data on any association between ABCA1 gene function and fasting plasma glucose. Assuming a 1:1 relationship between expression and function, our numbers should be sufficient to detect a significant relationship between function and FPG. Finally, we estimate a log SD of IMT of 0.198 (33), and a β coefficient between IMT and ABCA1 expression of -0.011 . Our sample size would be sufficient to demonstrate that this coefficient is significantly different from zero.

The following parameters will be assessed.

- Measurement of baseline leukocyte ABCA1 mRNA and protein.
- Assessment and measurement of CIMT
- Functional assessment of PS translocation in peripheral blood lymphocytes.

- A subset of subjects will undergo assessment of cholesterol efflux using skin fibroblasts from participants.
- Assessment of leukocyte ABCA1 expression after glucose lowering over six weeks using metformin therapy.

The study is limited to men in order to minimise variability in insulin sensitivity and lipid metabolism caused by changes in sex hormones during a menstrual cycle as well as to avoid differences caused by contraceptive use and menopausal status (326). This study is original as two tissue measurements of ABCA1 are planned in individuals and participants will be naive of medications known to affect glucose and lipid metabolism. A glucose lowering medication, Metformin, will be used to lower blood glucose over six weeks. Other commonly used treatments in DM have been shown to affect ABCA1. This work will complement another sub-study within the department (Rosiglitazone Plaque Study, GlaxoSmithKline) which investigates the effect of PPAR γ agonist treatment on leukocyte ABCA1 mRNA in high risk patients with DM.

2.0 METHODS AND MATERIALS

2.01 Patient recruitment

Men with a history of drug naïve DM or IGH were recruited from metabolic clinics at St Mary's hospital as well as from community practices. The local Diabetes Research Network (DRN) provided assistance in this process. Healthy volunteers were recruited through advertisement at St Mary's Hospital and via the DRN. Recruitment criteria are listed:

Patient inclusion criteria:

- Male subjects
- Age 18-70 years
- Ability to give written informed consent

Medical exclusion criteria:

- Known Type 1 diabetes.
- Significant cardiovascular disease, including atrial fibrillation, peripheral vascular disease, coronary heart disease, cerebrovascular disease or known carotid stenosis.
- Known microvascular complications from DM.
- Use of glucose altering or lipid lowering medications or drugs known to alter glucose or lipid metabolism.

After a 10 hour overnight fast, a clinical assessment was conducted using standard measurements of height and weight. Blood pressure was measured at the right brachial artery (Automated Omron sphygmomanometer). Waist circumference was measured mid-way between the costal margin and iliac crest. All clinical procedures were performed in the departmental metabolic

investigation unit. Written consent was sought from all participants and ethical approval for the study was obtained from Hammersmith, Queen Charlotte's and Chelsea Research Ethics Committee (*REC Reference 05/Q0406/33*). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

2.02 Blood specimens

Control participants and those known to have IGH underwent a 75g OGTT. Glucose measurements were taken at baseline, 1 hour and 2 hours. Fasting blood samples were taken from DM patients and these patients did not undergo an OGTT.

2.03 Skin biopsy

A cohort of participants underwent a 4mm dermal punch skin biopsy at their first or second visit. This was taken from the non-dominant forearm under sterile conditions using local anaesthesia (1% Xylocaine) and suture pack (Rochialle inHealth, Cambridge, UK). A non-absorbable 4/0 ethilon suture (Southern Syringe Ltd, Enfield, UK) was used to close the biopsy site with application of a sterile dressing. Arrangements were made for suture removal.

2.04 Carotid artery imaging

All subjects underwent B-mode ultrasound assessment of both carotid arteries. This was performed in the Irvine vascular studies department at St Mary's Hospital. Measurements were made by a single experienced observer who was blinded to the glucose status of the participant. CIMT was measured at the posterior wall of the common carotid artery, 1.5cm proximal to the bifurcation

(Phillips iU22). A qualitative assessment of the internal carotid arteries was also performed.

2.05 Glucose lowering treatment

After the first visit, patients with DM were offered pharmacological treatment to lower blood glucose using the recommended first line oral treatment for DM. Metformin tablets were administered at a maintenance dose of 500mg twice daily for a total of six weeks. Further fasting blood samples were taken upon completion of treatment. HbA1c, fasting glucose and standard clinical measures were repeated at this time point.

2.06 Demographic data

A total of 68 participants were recruited and underwent leukocyte gene expression studies. The demographic data is shown in Appendix III and biochemical data in Appendix IV. A summary of these data are shown in table 2.0.

Subjects were matched for age, there were no differences in age between groups ($p=0.55$). I also aimed to match study subjects for total cholesterol, smoking status and blood pressure as these parameters are independently associated with increased CIMT and CVD. There were no differences in these parameters between groups (see below). There was some overlap in HbA1c between subject groups in keeping with glycaemia being a continuous variable (figure 2.1). Patients with a known history of IGH were allocated to this group even if the study OGTT results did not show significant abnormalities. Patients

who were known to have glucose results borderline for diabetes were categorised to either IGH or DM according to results of the study OGTT.

As expected, patients with IGH and DM had higher BMI than healthy volunteers ($p<0.05$). BMI was not different between patients with DM and IGH. DM patients had higher waist measurements compared to controls ($P<0.05$).

There were no differences in systolic, diastolic and pulse pressure, between groups ($p=0.41$, $p=0.69$, $p=0.34$ respectively). As expected, patients with DM had higher fasting glucose concentrations compared to both other groups and had a higher Hba1c than controls ($p<0.001$) and IGH patients ($p<0.05$), illustrated in figure 2.1. Patients with IGH did not have higher fasting glucose levels compared to controls. Post oral glucose levels were higher both at 1 hours and 2 hour intervals in patients with IGH compared to controls (both $p<0.001$). Total cholesterol, LDL-C and triglyceride concentrations were not different across groups. HDL-C concentrations were lower in DM patients compared to controls ($p=0.001$). Proportionally more patients were receiving treatments for hypertension acting on the Renin-angiotensin-aldosterone axis compared to healthy men (21% versus 7%). More patients received inhaled medication for asthma compared to controls (13% versus 7%).

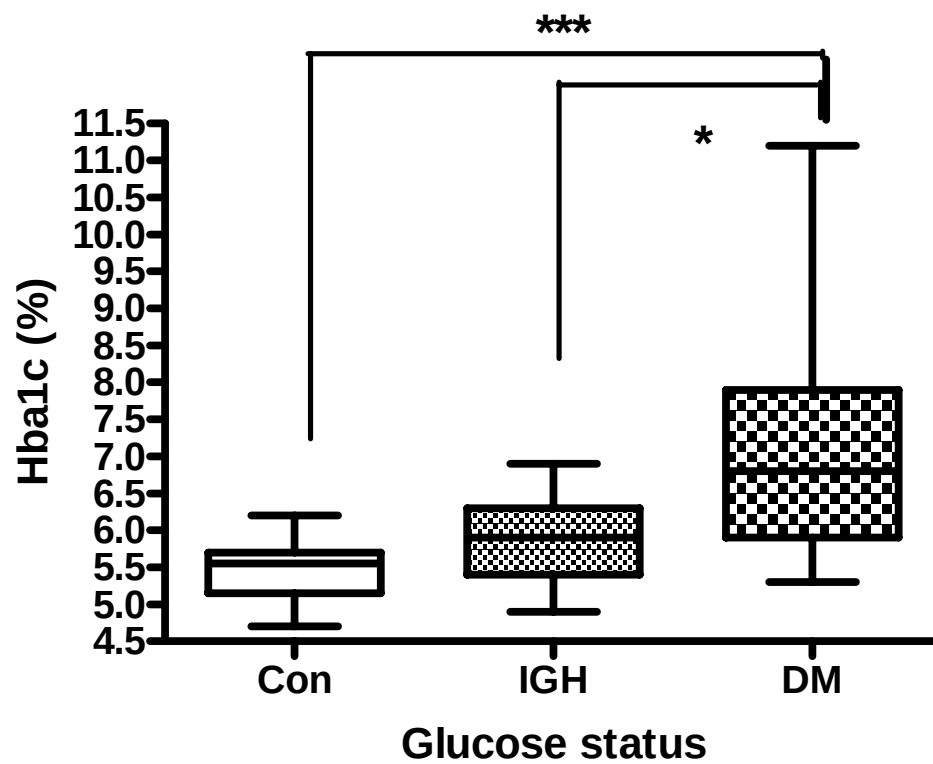


Figure 2.1

Hba1c according to glycaemic group in 63 individuals; DM vs Con $p < 0.001$, DM vs IGH $p < 0.05$. (Con=Control, IGH=Impaired glucose homeostasis, DM=Type 2 diabetes).

Table 2.0

*Demographic and biochemical parameters of participants. Normally distributed variables are represented as mean and standard deviation. Skewed data are displayed as median and interquartile range. * indicates significant differences compared to control subjects. # indicates significant differences compared to impaired glucose homeostasis (IGH) and control groups.*

	Control (n=30)	IGH (n=19)	Diabetes Mellitus (n=19)
Age /yrs	51.8 (15.9)	56.8 (8.2)	56.7 (10.8)
Weight /kg	77.7 (72.1,84.7)	77.9 (71.5,95.0)	90.1 (72.9,94.7)
BMI	24.8 (22.1,25.8)	26.9 (24.2,30.1)*	28.8 (26.6,30.5)*
Waist /cm	92 (85-96)	96 (87-106)	101 (97-104)*
Smoking (Active)	4 (13%)	4 (21%)	3 (16%)
Systolic Pressure /mmHg	133 (13.3)	136 (15)	130 (16)
Diastolic pressure /mmHg	82 (10.6)	82 (10.8)	83 (6.1)
Pulse pressure /mmHg	51 (9.3)	55 (11.8)	50 (10.3)
Fasting glucose mmol/l	5.1 (4.7-5.5)	5.7 (4.9-6.2)	6.7 (6.3-8.5) [#]
1 hour glucose mmol/l	6.0 (4.5-8.0)	9.5 (7.2-11.2)*	-
2 hour glucose mmol/l	5.0 (3.9-6.0)	6.8 (5.1-9.7)*	-
Mean glucose mmol/l	5.3 (4.4-6.3)	7.4 (5.9-8.6)	-
Hba1C (%)	5.55 (5.15,5.70)	5.90 (5.40-6.30)	6.80 [#] (5.90-7.90)
Cholesterol mmol/l	5.36 (0.86)	5.21 (0.97)	4.93 (1.1)
HDL-C mmol/l	1.54 (0.31)	1.34 (0.36)	1.19 (0.269)*
LDL-C mmol/l	3.27 (0.63)	3.30 (0.66)	3.05 (0.88)
Triglyceride mmol/l	1.17(0.75-1.58)	0.97 (0.77-1.47)	1.38 (0.99-2.1)

2.1 LABORATORY METHODS

2.11 Chemical pathology laboratory methods

Fasting blood samples were taken from all participants for measurement of glucose, total cholesterol, HDL-C, triglyceride. LDL-C was calculated using the Friedewald formula (327). Electrolytes, renal function, liver enzymes and thyroid function were measured using these samples. Participants who had clinically significant aberrations in these parameters were excluded from the study. Cholesterol, lipoprotein and triglyceride measurements were performed using an Olympus AU 2700 autoanalyser (Olympus Life & Material Science, Ireland). Thyroid stimulating hormone was measured using an Immulite 2500 autoanalyser (Siemens, Siemens Medical solutions & diagnostics LTD, Gwynedd, UK). Plasma free fatty acid concentrations were measured using an *in-vitro* colorimetric method (Wako Chemicals, Neuss, Germany) using a Cobas Mira (Hoffman-Laroche and company, Basel, Switzerland). Fasting glucose, creatinine and liver enzymes were measured using standardised laboratory techniques. HbA1c was measured by cation-exchange high performance liquid chromatography using a Tosoh Automated Glycohaemoglobin Analyser G7 (Tosoh Bioscience Inc., Tokyo, Japan).

2.2 Leukocyte isolation and RNA extraction

Peripheral blood leukocytes were used for gene expression studies in keeping with our original study and lymphocytes as well as skin fibroblasts were used for the additional functional studies.

12ml of blood was collected into tubes containing EDTA and placed on ice. Red cells were lysed from whole blood by the use of erythrocyte lysis buffer (Qiagen, Crawley, UK). The leukocyte pellet was washed in buffer and stored at -80 °C in 2ml of lysis (RLT) buffer (Qiagen), this contained 10% β -mercaptoethanol (Sigma-Aldrich Co, Poole, UK). Samples were homogenised using Qias shredder columns (Qiagen) by centrifugation at 16000 x g (Eppendorf, Cambridge, UK) for 2 minutes. RNA was extracted using RNeasy Midi kit (Qiagen) according to manufacturing instructions and mRNA absorbance was measured using a spectrophotometer (Ultrospec 2000, Amersham Biosciences, Buckinghamshire, UK). Samples were immediately stored at -80 °C.

2.21 Monocyte isolation

Monocytes can be identified by expression of the lipopolysaccharide receptor; CD14. This was used to isolate this particular subset from other peripheral blood mononuclear cells. After isolating leukocytes from whole blood, a cell count was determined using a haemocytometer. The pellet was resuspended in MACS buffer (Miltenyi Biotec Ltd, Surrey, UK) and centrifuged at 300 x g for 10 minutes. After removal of supernatant, the pellet was resuspended in 80 μ l of MACS buffer mixed with 20 μ l CD14 microbeads per 10 cells. (Miltenyi Biotec Ltd.). The CD14+ cells were magnetically labelled with microbeads during a 15 minute incubation at 4 °C. The cells were washed with 1-2ml MACS buffer and centrifuged at 300 x g for a further 10 minutes. After resuspending in 0.5ml of MACS buffer, the sample was applied to a LC column (Miltenyi Biotec Ltd.) which was prior magnetised (MidiMACS, Miltenyi Biotec Ltd.) and rinsed with 3ml MACS buffer. The CD14+ cells were subsequently retained in the column.

After four washes in 3ml MACS buffer, the column was removed, placed in a collecting tube and eluted with 5ml of MACS buffer. To detach the beads from the monocytes, cells were incubated on ice for 2 hours and washed with MACS buffer at 400x g for 10 minutes at 4°C and supernatant removed. RNA was extracted from monocytes as outlined in section 2.2.

2.22 Leukocyte protein extraction

The leukocyte pellet was isolated and washed as above. This was suspended in 1ml of Lysis buffer (Appendix I) containing a dissolved pellet of Protease inhibitor mixture (Complete protease inhibitor cocktail, Roche diagnostics, Mannheim, Germany). The lysates were stored at -80°C. Initial attempts in performing Western immunoblotting techniques using a monoclonal ABCA1-antibody (Abcam, Cambridge UK) were unsuccessful due to frequent formation of double protein bands. Western blot development work was undertaken by Dr Christiane Albrecht and colleagues with the aim of producing a reliable assay to accurately detect ABCA1 protein. A novel enzyme linked immunosorbent assay (ELISA) was subsequently developed by our group which has the advantage of being more sensitive and quantitative than immunoblotting techniques. This methodology was validated in a variety of human tissues including arterial tissue, placenta and skin fibroblasts (328). Total cell lysates were prepared by incubating the leukocyte cell pellets with 1 ml ice-cold lysis buffer (50 mM mannitol, 2 mM EDTA, 50 mM, pH 7.6) containing complete protease inhibitor (Roche, Germany) and 0.1% w/v Triton X-100 for 60 minutes on ice. This was followed by five homogenization cycles (20 s each and 10 minutes resting time on ice after each homogenization step) in a Fastprep homogenizer. The leukocyte homogenates were centrifuged at 15,000 g for 15 minutes at 4°C and

the supernatants containing clear cell lysates were collected. Protein concentrations were determined by a bicinchoninic acid assay (329). The clear leukocyte cell lysates were stored at -80°C until ABCA1 protein measurement was performed. Frozen leukocyte lysates were thawed and ABCA1 protein concentration was measured using an ELISA method as previously described (330). Briefly, either 50 µl ABCA1 peptide (ab14148, Abcam, UK) calibrators (range 8-1000ng) or 50 µl of unknown leukocyte lysates were incubated in a ABCA1 peptide pre-coated and BSA blocked micro titre plate along with 100 µl (2 µg/l diluted in PBST) anti-ABCA1 rabbit antibody (ab7360, Abcam, UK). The incubation was carried out for 5 hours at room temperature while shaking on an ELISA plate shaker. The plate was washed with PBST and further processed by using biotin labelled anti-rabbit IgG goat antibody, streptavidin-HRP conjugate and tetramethylbenzidine enzyme substrate system. The enzymatic reaction was stopped by addition of sulphuric acid and absorbance measured at 450 nm on an ELISA plate reader (Sunrise, Tecan, Austria). ABCA1 protein concentrations of unknown samples were calculated from a calibration curve (Appendix I) and results were expressed as ABCA1 protein ng/ µg total protein. The inter-assay and intra-assay CV were <15%.

2.23 Isolation of primary fibroblasts

There was initial difficulty experienced in establishing fibroblast populations from biopsy material. This delayed the attainment of cholesterol efflux results from participants. Initial attempts using divided biopsy material resulted in lack of cellular proliferation and subsequent fungal contamination. Expertise was sought and fibroblast liberation was provided by Dr C. Poueyron and Ms. R Hannen, *Tissue engineering group, Institute of Cutaneous Research, London.*

An additional step of filtering of biopsy material through a cell strainer helped achieve quicker cellular proliferation. The protocol used was as follows:

Primary fibroblast cell lines were obtained from human dermal biopsy using enzymatic digestion. The biopsy specimen was incubated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Ltd, Paisley, UK) containing 1% antibiotic-antimycotic solution (Sigma-Aldrich). This was stored at 4°C for up to 24 hr before processing. Tissue was subsequently transferred into a 60 x 15 mm Tissue Culture Disk (Falcon, Marathon Lab supplies, London, UK).

The epidermis was removed using forceps and the sample cut into small pieces and placed in 4 ml of DMEM with 1% antibiotic-antimycotic solution containing 0.5mg/ml (0.1265 U/ml) Collagenase D (Roche Diagnostics). The sample was incubated with Collagenase D overnight at 37°C to release fibroblasts from the collagen scaffold. On day two, the cell suspension was filtered through a 70 µm Cell Strainer (Becton Dickinson (BD) Oxfordshire, UK) and centrifuged at 220 x g for 5 min. The supernatant was carefully removed and the cells resuspended in 4 ml DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) with 1% antibiotic-antimycotic solution. Cultivation was initially performed in 60 x 15 mm tissue culture disks. To maximise yield of fibroblasts, the cells that remained in the filter were also cultivated in the original culture disk.

After two days, cells were washed with warmed phosphate buffered saline (PBS) and culture medium changed. When sufficient cells were visible, these were transferred into T-25 Culture Flasks (NUNC, Fisher Scientific Ltd, Loughborough, UK). The application of trypsin-EDTA solution (Sigma-Aldrich)

for less than 2 minutes allowed early detachment of fibroblasts and avoided contamination by keratinocytes which remained adherent.

2.24 Subculture of normal human fibroblasts

The culture media was changed twice weekly until cells were 80-100% confluent. In order to passage, the fibroblasts were detached with 20 µl Trypsin-EDTA solution per cm² culture dish for a period of 3- 5 min. They were subsequently transferred with DMEM to inactivate trypsin and centrifuged for 5 min at 1000rpm in a Multifuge 3S-R (Heraeus). The cellular pellet was then finally resuspended in fresh, culture medium.

Cells at high density (10^6 - 10^7 cells/ml) were frozen by resuspending the fibroblast pellet in 1.5 ml freezing medium, comprising of FBS and sterile filtered 10% Dimethylsulfoxide (DMSO) (Sigma-Aldrich). The suspension was transferred to a cryopreservation vial (Corning Incorporated, New York, USA) and cooled at a rate of 1°C/min, to -80°C. These vials were subsequently stored in liquid nitrogen or at -80 °C. Imaging of cell morphology was attained using a Kodak DC120 digital camera with Olympus CK2 microscope (Olympus UK Ltd, Middlesex, UK) and processed with Adobe Photoshop 5.0 LE (Appendix II).

2.25 Fibroblast lysis and RNA extraction

Fibroblasts from a fully confluent T75 culture flask were washed with PBS, lysed and removed using 1.5ml of RLT buffer (Qiagen) containing 10% β-mercaptoethanol (Sigma-Aldrich). This was stored at -80 °C. The RNA extraction process was identical to that used for leukocyte cells as outlined above.

2.26 Fibroblast protein extraction

Fibroblasts from a fully confluent T75 culture flask were washed with PBS and removed using a 23 cm cell scraper (NUNC, Fisher Scientific Ltd). Cells were stored in 1ml of lysis buffer (Appendix I) at -80 °C.

2.3 ABCA1 FUNCTION

2.31 Fibroblast ABCA1 function-cholesterol efflux

The initial cholesterol efflux assay did not lead to reliable results and resulted in low radioactive counts with high variability (see Appendix II). Assay modification and development was required using a protocol which was provided by our collaborating group Dr C Albrecht (Physiology department, Munich Technical University, Germany) (331), this work is shown in section 3.2. Assay optimisation was attempted and was conducted in normal dermal human fibroblasts (Promocell, Germany) in cells of passage number under passage 5. Modifications to the protocol did not improve the variability seen in the assay. It was noted the protocol included prolonged labelling and equilibration steps which resulted in reduced radioactive counts. After helpful discussions with Dr Albert Groen, (Academic Medical Centre, University of Amsterdam) an alternative published methodology was selected (outlined below) which employed shorter incubation times (191). This resulted in more reproducible results as shown in section 3.2.

Day 1 (Cultivation):

Fibroblasts were counted using a Neubauer chamber and seeded at a density of $1-2 \times 10^4$ per well in a 6-well plate. They were grown until 60-80%

confluency at 37°C in 5% CO₂, in media containing 10% FBS and 1% antimicrobial solution.

Day 2 (Cholesterol loading):

Cells were washed and incubated with DMEM containing 0.5 µCi/ml 1,2-[³H] cholesterol (Perkin Elmer, UK) in the presence of 30mg/l cholesterol (Sigma-Aldrich) and 0.2% bovine serum albumin (BSA) (Sigma-Aldrich) at 37°C for 24hours.

Day 3 (Equilibration and Efflux):

Cells were washed 3 times with PBS-0.2% BSA solution to remove residual extracellular cholesterol. 10µg/ml ApoA-I (supplied frozen in 10nM in ammonium chloride, pH 7.4) from Sigma (LOT 084k16791/025K1442, Cat A0722) was added to half the wells in triplicate and DMEM with 1% antibiotic-antimycotic was added to the remaining control wells.

Day 4 (Efflux discontinuation and counting):

After 20 hours, the medium was removed, centrifuged at 4°C to remove any cellular debris. Radioactivity was assayed liquid scintillation counting (LSC). Cells were washed with PBS, dissolved using 500µl-1ml 1M NaOH and assayed by LSC. (β-counter -LSC 6000SC, Beckman, USA).

Further efflux experiments were performed using 1ml hexane-isopropanol (3:2 v/v) to extract cellular cholesterol without disrupting cell membranes. Hexane-isopropanol was incubated for 30minutes prior to LSC. Counts per minute (cpm) were measured in triplicate samples. Cholesterol efflux was calculated with the

equation below. In initial experiments, efflux was compared using a number of cholesterol acceptors including commercial preparations of ApoA-I and serum samples taken from subjects. ApoB containing lipoproteins were removed from serum through precipitation. This was achieved by mixing and centrifuging serum samples with 10% Magnesium-dextran sulphate (3000rpm, 30min at 4 degrees °C).

$$\text{Cholesterol efflux \%} = \text{cpm in supernatant} / [\text{cpm (cells + supernatant)}]$$

2.4 Lymphocyte phosphatidylserine translocation

2.41 Isolation of lymphocytes from human blood

ABCA1 is thought to contribute to phospholipid transport as well as cholesterol transport as explained in section 1.81 (332). PS is usually confined to the inner leaflet of cellular membranes, transfer to the outer leaflet is an early signal of apoptosis and transport can be stimulated by calcium ionophore (333;334). Reduced PS transport in lymphocytes has been reported by our group in a patient known to have a *ABCA1* mutation (210). We therefore elected to investigate the effect of glycaemia on function of ABCA1, as measured by PS translocation in CD4 positive T-lymphocytes.

The protocol for assessing PS transport in T-lymphocytes was obtained from Dr James Elliott who was a member of the Membrane transport group (MRC Clinical Sciences Centre, London). Ms Julia Feldmeyer contributed to the validation of this technique.

Density gradient centrifugation with FicollTM-sodium diatrizoate solution (Ficoll-Paque) (Amersham Biosciences, Buckinghamshire, UK) was used for separation of lymphocytes and peripheral mononuclear cells (PBMC) in human whole blood. 4 ml of the diluted blood was layered on 4 ml of Ficoll-Paque solution and centrifuged for 15 min at 400 x g with brakes and low acceleration. Differential migration during centrifugation resulted in the formation of layers which contained the different cell types. Lymphocytes were found at the interface between the plasma and the Ficoll-Paque solution because of their lower density. Lymphocytes were removed using a pipette and washed twice with PBS to remove platelets, Ficoll-Paque and plasma.

2.42 Phosphatidylserine translocation analysis

Lymphocytes were transferred into a polystyrene fluorescence activated cell sorting (FACS) tube (BD Falcon, UK) and were washed and suspended in phenol-red-free Dulbecco's modified Eagle medium (DMEM/F12) (Invitrogen, UK). 100 µl of this solution was transferred and stained with 15 µl mouse anti-human monoclonal IgG₁ antibody specific for the CD4 T-lymphocyte marker. This antibody was conjugated to a fluorochrome and was incubated with the cell suspension for 10 min. Labelled T-lymphocytes were then washed with DMEM/F12 to remove excess antibody. To measure PS translocation, 25-50 µl cell suspension was equilibrated with 15 µl AnnexinV^{FITC} (AV) (Becton Dickinson) and diluted in 1 ml DMEM/F12. Baseline fluorescence was measured for 1 min prior to addition of 1 µM calcium ionophore; calcimycin (Sigma-Aldrich). Cells were analysed in real-time for PS externalisation using flow cytometry, measurements were acquired using FACS LSR machine (Becton Dickinson) and data analysis was performed using FACSCalibur

(Becton Dickinson). Identical instrument calibration settings were used for each experiment and Cell Quest software (Becton Dickinson) was used for data acquisition and analysis. Cumulative PS translocation was measured for a total of 8min after the addition of calcimycin. Using multicolour gating, external PS exposed cell events (PS+) were expressed as a percentage of all CD4+ cell events. Non-CD4+ cells were excluded from the analysis (Figure 2.2). Figure 2.2(a) shows distribution of all cellular events according to size and granularity. Figure 2.2(b) demonstrates differential staining of different lymphocyte populations. Figure 2.2(c) shows an example of back gating of cellular events, only events in lymphocytes are seen. Figure 2.2(d) demonstrates analysis of cumulative PS+ cells (FL1-H positive) before and after calcimycin administration. The sensitivity and throughput rate of this methodology allows detection of PS externalisation in CD4+ T-lymphocytes, but can also be applied to detect rarer cellular events.

2.43 Fluorochromes

The fluorochromes used and corresponding wavelengths of cytometer lasers are listed in Table 2.1.

A mouse anti-human monoclonal IgG₁ antibody specific for the T-cell marker CD4 was used. These clones were conjugated with R-Phycoerythrin (PE), Allophycocyanin (APC) or Phycoerythrin-Cychrom5 (PE-Cy5).

Table 2.1*Fluorescent activated cell sorting (FACS) fluorochromes and laser wavelengths.*

Fluorochrome	Excitation Peak [nm]	Emission Peak (nm)	Laser Wavelengths (nm)	Laser
FITC	495	520	488	Argon
Phycoerythrin (PE)	565	575	488, 514, 568	Argon
Phycoerythrin- Cychrom5 (PE-Cy5)	565	670	488, 514	Argon
Allophycocyanin (APC)	650	660	633, 635, 647	Helium- Neon

Fluorochromes APC and PE were selected for use after demonstrating differential binding properties of PE-Cy5 compared to PE which caused up to a 12% difference in AV positive events (figure 2.3). This change resulted in improved resolution between CD4⁺ cell populations. Two labelled CD4⁺ populations were analysed in a single FACS tube (Becton Dickinson) to minimise inter-assay variability. Control subjects and patients who had IGH underwent an OGGT with PS flop measurements at time points 0, 1 and 2 hrs. Samples were incubated at 37 °C and analysed simultaneously. Incubation of CD4⁺ cells at 37 °C for 2hrs had no significant difference in PS flop compared to freshly isolated cells (figure 2.4).

CD4⁺ PS externalisation rates from patients with DM were compared to control CD4⁺ cells by using fasting samples from patients with DM compared to a sample taken from a single healthy subject. Rates of PS exposure were

analysed using Cell Quest with measurements taken at 40 second intervals for 8 min.

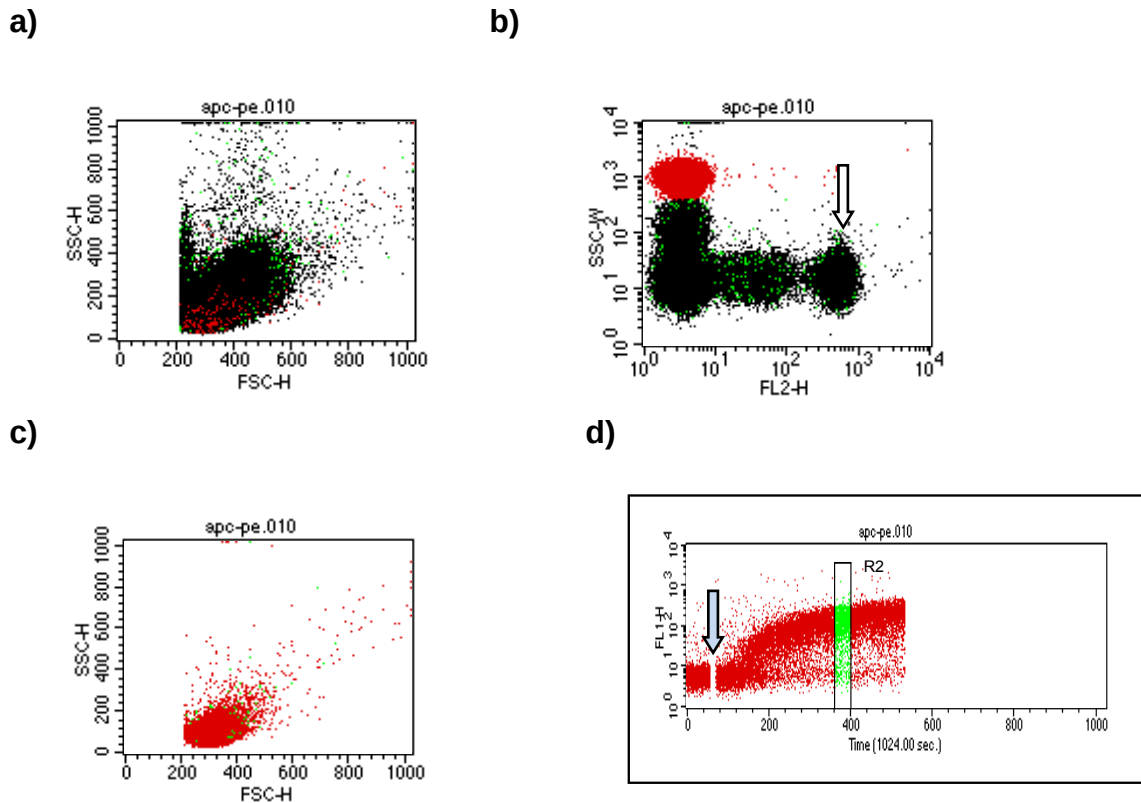


Figure 2.2 Flow cytometry results and gating.

(a) All cellular events are shown according to cell size (FSC-H) and granularity (SSC-H).

(b) Differential lymphocyte staining is illustrated; APC staining cell population (red) is plotted against PE staining cells (arrow).

(c) Using back gating only CD4⁺ cells are shown.

(d) The rate of PS translocation (AV positive events) is measured at baseline over 1 min and after calcium ionophore administration (blue arrow) for 8 mins. Measurement of AV positive cell events were taken for analysis at 40 s intervals (bar).

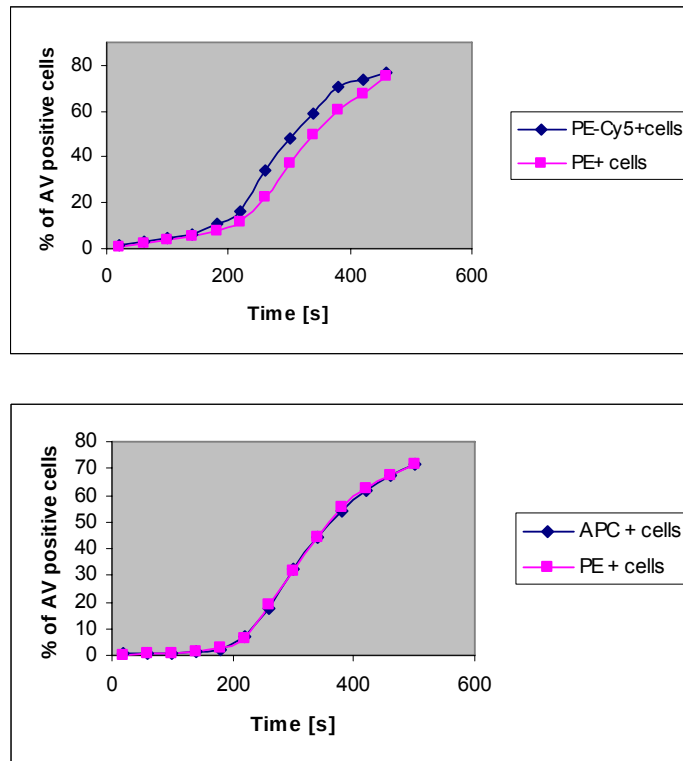


Figure 2.3 Comparison of conjugated fluorochrome pairs

Analysis of AV-positive events from single populations of lymphocytes from a control subject according to separate pairs of antibody-fluorochromes conjugates (PE-Cy 5 and PE versus APC and PE). Mean values taken from at least quadruplicate experiments.

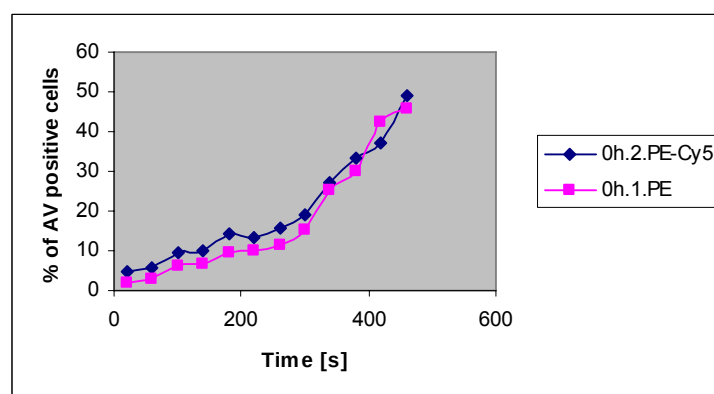


Figure 2.4 Timing of lymphocyte isolation and effect on PS flop

Shows A-V positive cells according to lymphocyte isolation timing.

0h.2 represents a control sample incubated at 37deg for 2 hours prior to lymphocyte isolation (blue). 0h.1 represents the control sample with immediate isolation of lymphocytes (pink). Mean values are shown from a duplicate experiment.

2.5 GENE EXPRESSION

2.51 cDNA synthesis

1 µg of RNA from each sample was diluted in 25 µL of PCR grade water. Reverse transcription to synthesise complementary DNA (cDNA) was performed using the ABI high capacity reverse transcription (RT) kit (Applied Biosciences, Warrington, UK). The composition of master mix (MM) is listed (Appendix I). A thermal cycler (Genius, Techne) was used to perform the RT reaction. Aerosol free filtered pipette tips were used and experiments performed in an ultraviolet light hood to help eliminate genomic DNA contamination. 25 µL sample was mixed with 20 µL 2xMM. Up to 40 samples were placed into the thermal cycler with pre-optimised settings according to RT kit instructions (Applied Biosystems) (Appendix I). cDNA samples were subsequently stored at -20 °C.

2.52 Quantitative Polymerase chain reaction

An adapted methodology was performed using SYBR green fluorescence. Primers were designed to be exon spanning to minimise non-specific amplification. PCR temperature settings are shown in table 2.2. Due to methodological difficulties which are outlined in section 3.0, a Taqman based method was also performed to measure gene expression using primer-probe sets (table 2.3).

A prior optimised laboratory protocol was used for PCR experiments using manufactured primer-probe sets (Applied Biosystems) (Appendix I for protocol). The sample was determined in duplicates each with a total reaction volume of 10µl. Amplification was performed using a Rotorgene 2000 real-time cycler

(Corbett Research, Cambridgeshire, UK). *β-Actin* was used as the housekeeper comparator gene.

Table 2.2

SYBR green Rotor Gene temperature settings used for PCR gene amplification

PCR Step	Temperature	Duration
Hold	50 °C	2 min
Initial denaturation	95 °C	10 min
Denaturation	95 °C	15 sec
Amplification	60 °C	10 sec
Annealing	72 °C	25 sec

Table 2.3

Applied Biosystems primer and probe set identification details.

FAM channel (470-510nm)	Applied Biosystems Set identification	Exon site	Amplicon length (base pairs)
<i>ABCA1</i>	(Hs01059122_m1)	34-35	103
<i>ABCG1</i>	(Hs00245154_m1)	5-6	58
<i>PPAR_γ</i>	(Hs00234592_m1)	7-8	107
<i>LXR_α</i>	(Hs00172885_m1)	6-7	78
VIC channel (530-555nm)			
<i>β-Actin</i>	(4310881E)	2-3	90
<i>GAPDH</i>	(4310884E)	3	118

All experiments were performed in duplicate with amplification of both gene of interest (GOI) and housekeeper in a single tube (multiplex) to minimise variation between experiments. A single PCR protocol was used for all gene measurements (Appendix I). The primer-probe set were premixed and consisted of 18mM primer and 5mM probe (Applied Biosystems). The GOI probe was conjugated to a reporter fluochrome (FAM) which is detectable once the probe is displaced by GOI primer amplification and cleaved by polymerase. The housekeeper probe had a VIC reporter flurochrome (Table 2.3). Primers were aliquoted to minimise disruption from repeated freeze-thaw cycles. Experiments with leukocyte ABCA1 and β -actin in subjects who underwent detailed functional analysis were repeated to yield mean gene expression data for analysis. Additional experiments in leukocytes were performed to validate findings using a separate housekeeper; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 2.3). Target gene quantity was calculated from a standard curve with four samples 1:1, 1:2, 1:4 and 1:8. To allow standardisation of cDNA quantity, gene expression was normalised to the housekeeper gene and expressed as a ratio. Analysis was conducted using the Rotorgene 6 comparative quantitation algorithm using the Rotorgene Software version 6 (Corbett Research, Cambridgeshire UK). To compare gene expression results from separate experiments the delta threshold cycle (dCt) measurements were compared.

2.53 DNA gel (Southern blot)

A 1% agarose gel was made to identify gene product by size using a standard protocol. DNA was stained using Ethidium Bromide (Sigma) Samples were mixed with 1µl of loading buffer (10x blue juice, Invitrogen) and applied to separate lanes within the gel. This was transferred to a tank (Bio-Rad laboratories LTD, Hertfordshire, UK) with application of 60 volt direct current. A DNA size marker (1kb-plus DNA ladder, Invitrogen) was added to the first lane to compare sizes of the resulting DNA products. The gel was inspected under ultraviolet light after 20 minutes using a Kodak DC290 digital camera and processed with Adobe Photoshop 5.0 LE.

Statistics

Baseline demographic data was analysed by one-way ANOVA and Kruskal Wallis tests with Holm Sidak and Dunn's methods for multiple pairwise comparison. A 2-tail t-test and Mann Whitney test were utilised to compare normally distributed and skewed data, respectively. Continuous variables were assessed by Spearman and Pearson correlation. In analyses of small sample number, Spearman correlation was the preferred test for correlation. A Kolmogorov-Smirnov test was used to assess normal distribution of variables. Variables which passed this test were also assessed using D'Agostino and Shapiro-Wilk criteria. Multiple OGTT time point gene measurements were analysed using a repeated measures one-way ANOVA. Multiple linear regression was used to assess independence. Sigmastat version 3.5 (Systat software, Inc. Germany) and GraphPad Prism, version 4.03 (GraphPad

software, San Diego, CA USA) software packages were used for analysis and generation of figures respectively. The threshold of significance in each test was set at $p < 0.05$.

3.0 VALIDATION OF METHODS

3.1 Validation of gene expression assay

Leukocytes were used for gene expression studies and there were initial problems with the assay. Amplification signals were seen in samples that contained only PCR grade water and primers (no template control). As these signals were detectable within a few amplification cycles (Ct) of DNA samples (figure 3.1) melt curve analyses were undertaken to identify these amplification products. An example of a melt curve is seen in figure 3.2. This demonstrates signal peaks at temperatures between 55-60°C and at 65°C in the no template control samples.

This occurred presumably due to either contamination or primer-dimer formation. Attempts to overcome these interfering signals were initially undertaken by cleaning the dedicated PCR hood and equipment and experiments were repeated using new primers to exclude DNA contamination. As this non-specific amplification persisted, I elected to alter the PCR settings initially by increasing the annealing temperature, followed by reducing primer concentrations to reduce the chance of dimerisation between primer pairs. These measures were not successful (figure 3.3) therefore alternative primer design was undertaken with the help of Dr Gaby deSilva (Imperial College, South Kensington Campus) to further reduce the probability of dimerisation. The basic local alignment search tool (BLAST), (National centre for biotechnology information, Bethesda, USA) was used to aid primer design to avoid complimentary and palindromic sequences. My standard curve DNA samples were amplified using a separate machine (ABI 7500 machine -Applied Biosystems) in Dr deSilva's laboratory. Results using this machine did not show

any evidence of non-specific amplification in the water samples. As non-specific amplification was observed only when amplifying certain genes, the most likely cause for this was therefore likely to be dimer formation between primers. I therefore elected to change to a more specific hydrolysis probe (Taqman) methodology to overcome this issue. This technique used pre-optimised primer-probe sets (Applied Biosystems) (Table 2.3). This change in methodology resolved non-specific amplification.

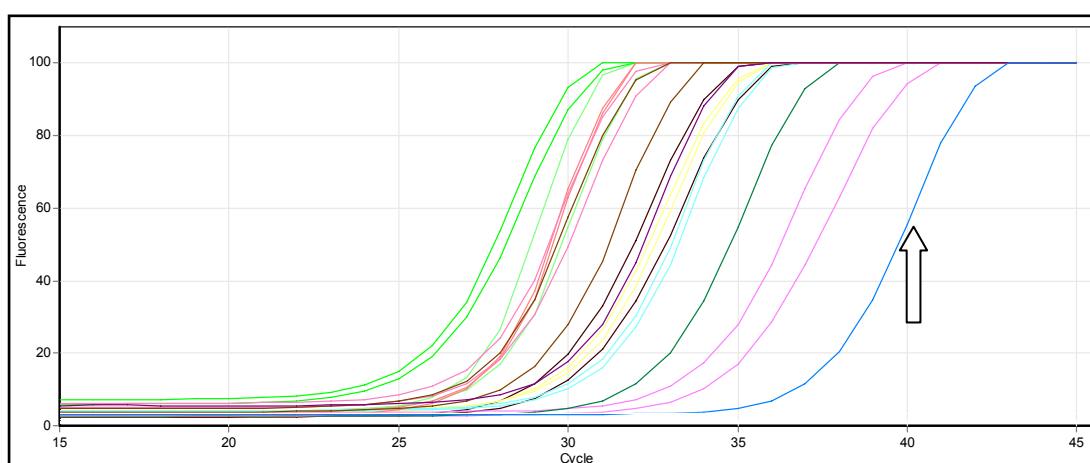


Figure 3.1 PCR amplification curves

DNA amplification curves from an ABCA1 PCR experiment using SYBR green fluorescence methodology. Amplification of multiple DNA samples are shown and a fluorescence signal can be seen in a no-template control sample (arrow, blue curve).

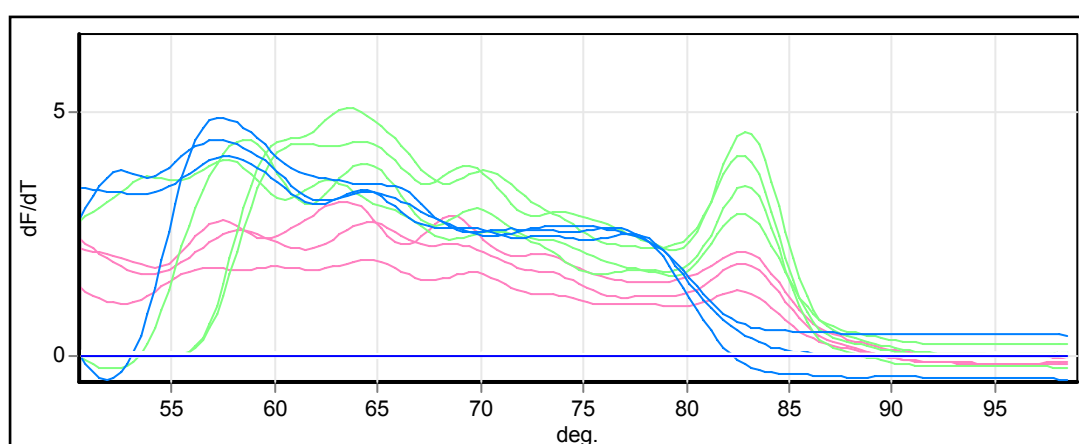


Figure 3.2 Non-specific amplification-Melt curve

A melt curve fluorescence analysis showing non-specific amplification in no-template control samples (blue) and DNA samples (green, pink). The Melting peak for the ABCA1 gene product is 82-83 °C seen in DNA containing samples. Earlier signal peaks between 55 and 70 °C are seen in samples including no-template control samples.

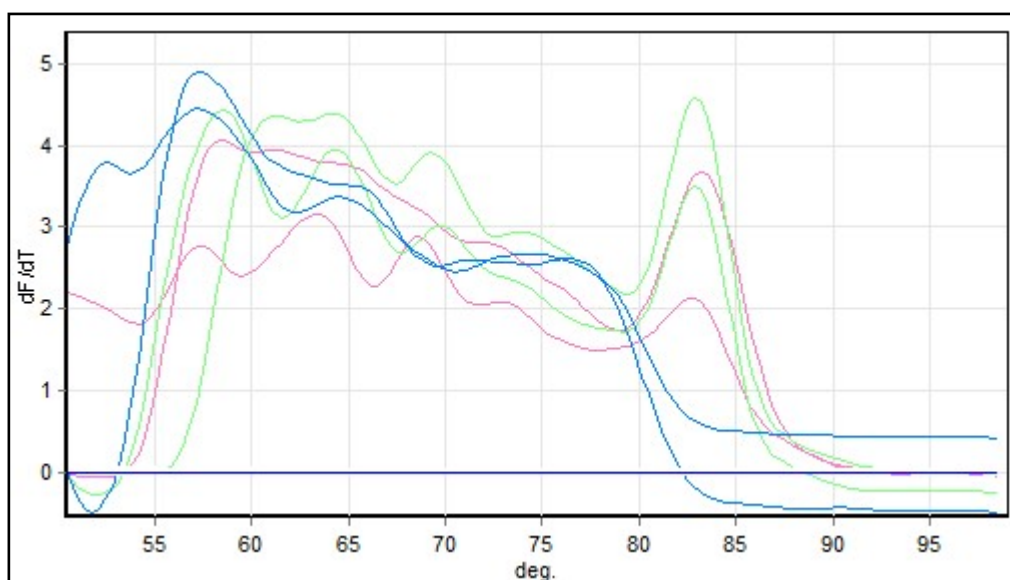


Figure 3.3 Melt Curve

Melt curve fluorescence analysis from a PCR experiment using reduced primer concentrations. ABCA1 gene expression was measured using SYBR green methodology. The gene product signal peak is seen at approximately 83°C. Additional fluorescence signal peaks are seen between 55 and 65°C in no template control samples (blue) and DNA samples (green and pink).

There was little difference in gene expression results whether the house keeper gene amplification was measured in a separate tube or in a single tube with the gene of interest (GOI). To minimise variability between multiple experiments, both GOI and housekeeper genes were amplified together in a single multiplex reaction. Gene expression of two common house keeper genes was compared in leukocytes from 23 subjects who also underwent a skin biopsy for functional ABCA1 assessment, which consisted of healthy men and patients. Gene expression of GAPDH and β -actin housekeeper genes were comparable in

these subjects, as seen in figure 3.4. Based on these results, β -actin was used as a single housekeeper comparator gene for future experiments.

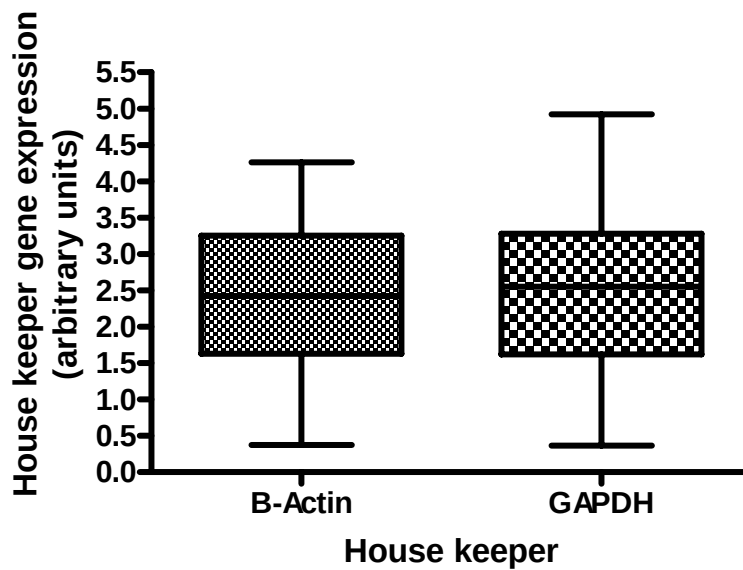


Figure 3.4 Gene expression of two housekeeper genes

Comparison of gene expression of two housekeeper genes in leukocytes using a hydrolysis probe (Taqman) PCR methodology. Sample measurements represent mean values of duplicate samples. (n=23) (arbitrary units).

The specificity of PCR amplification reaction was verified by gel electrophoresis (figure 3.5).

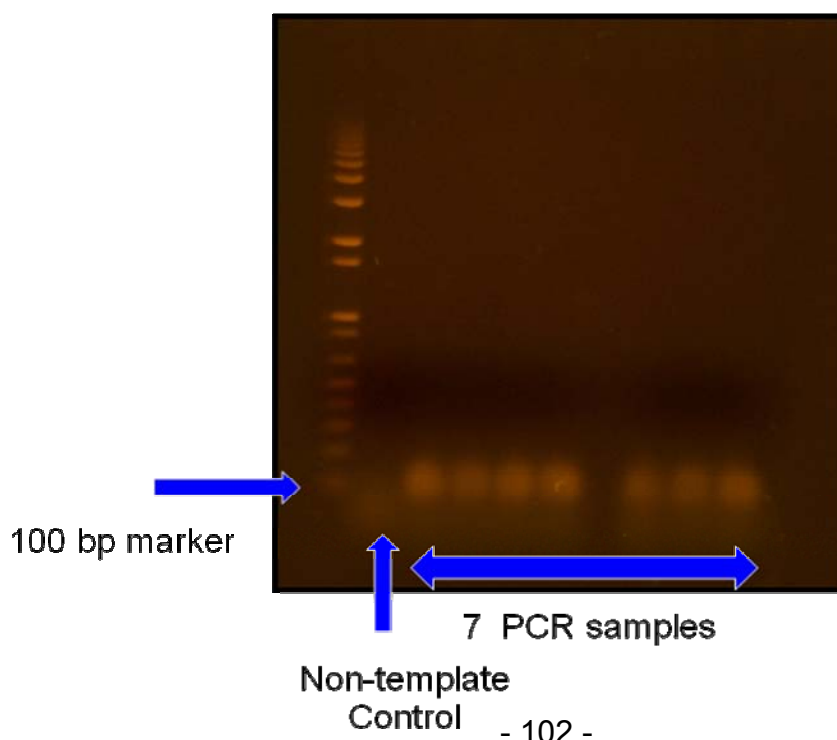


Figure 3.5 1% agarose gel

1% agarose gel, identifying ABCA1 DNA PCR amplification products in 7 fibroblast samples (ABCA1 gene amplicon length=103 basepairs).

The reproducibility of leukocyte ABCA1 gene expression (inter-assay CV) was assessed in a calibrator sample in each experiment. The mean CV of duplicate samples, measured in 7 different PCR experiments performed on different days, was 10% (Figure 3.6).

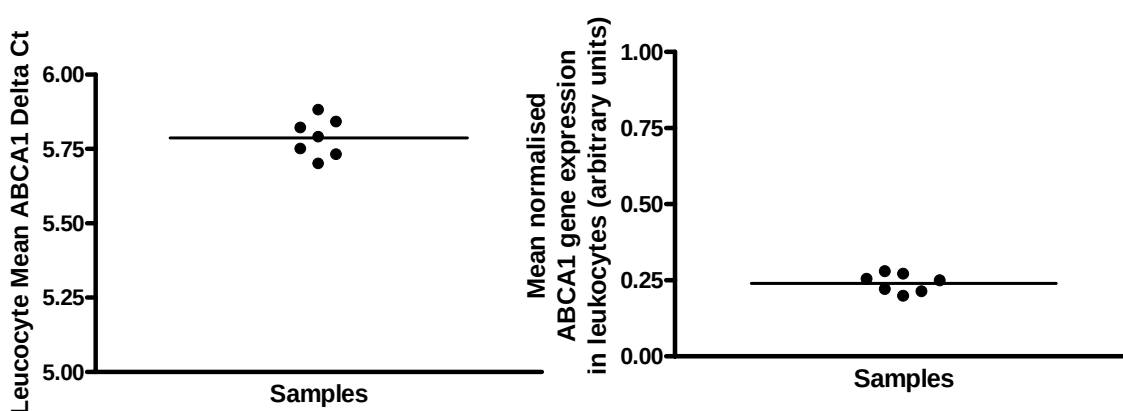


Figure 3.6 Reproducibility of ABCA1 gene expression

Mean ABCA1 Delta Ct PCR measurements and normalised ABCA1 gene expression measurements are plotted from duplicate samples taken from 7 separate PCR experiments performed on different days. The Mean CV for normalised gene expression was 10%.

Statistics

In participants who underwent detailed gene expression in leukocytes and functional studies using fibroblasts (n=25) cholesterol, HDL-C and LDL-C variables passed tests for normality. Despite this, in view of the relative small number of observations in this cohort, correlation was undertaken using a Spearman test.

3.2 Development and validation of the cholesterol efflux (ABCA1 function) assay

Skin fibroblasts were used to assess ABCA1 function (cholesterol efflux) but initial results using a provided laboratory methodology yielded low supernatant radioactive measurements, resulting in low efflux results with substantial count variability. A relatively high background efflux was also evident. This occurred despite repeating experiments using cells of differing passages. To examine this more closely, assay parameters from the original protocol provided by Dr Albrecht were altered (outlined below).

Original cholesterol efflux (ABCA1 function) methodology:

- Day 1:** Cultivate < 20,000 cells/well in wells with 1ml cultivation medium for 24h, 37°C, 5% CO₂ (until 40% -70% confluence).
- Day 2:** Remove cultivation medium, wash cells 1 x with 1ml /well medium A and incubate cells with 1ml/well labelling medium for 48 h, 37°C, 5% CO₂.
- Day 4:** Wash cells twice with 1ml/well medium A. Add 1ml/well equilibration medium and incubate for 24 h, 37°C, 5% CO₂.
- Day 5:** Wash cells 2 x with medium A
add 300 µl efflux medium and incubate for 2h, 37°C, 5% CO₂.
Collect the supernatant for LCS. Wash cells with PBS, dissolve in 300µl 1 M NaOH for LCS,
- LSC** Fill vial with 3ml scintillant
add 200µl sample and shake to mix, β-counter ³H for 2 min
(calculate cpm)

Medium A: DMEM + 1% Antibiotic-Antimycotic Solution

Labelling Medium: Medium A + 5% FBS + 1 µCi/ml 1, 2-[³H] Cholesterol

Equilibration Medium: Medium A + 1% BSA (7.5% solution)

Efflux Medium: Medium A + 20 µg/ml ApoA-I (2.15 µg/µl solution)

It was initially noted that radioactivity counts significantly diminished during the labelling and equilibration steps as shown in figure 3.7. Figure 3.8 illustrates the fundamental steps involved in a cholesterol efflux assay.

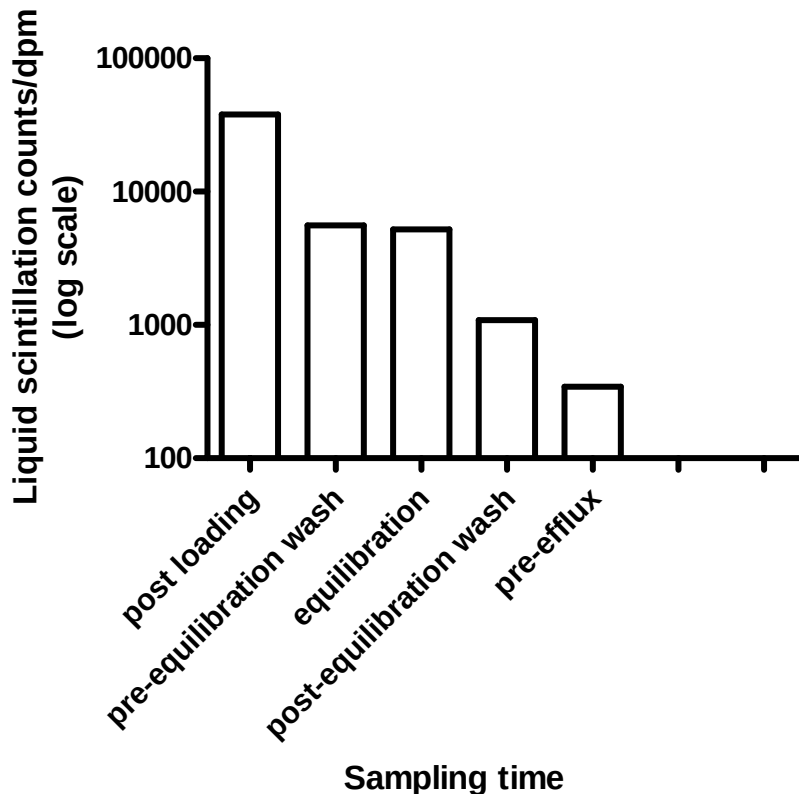


Figure 3.7 Supernatant ^3H counts during the efflux assay

Supernatant radioactivity counts (^3H) during the cholesterol efflux assay in primary dermal human fibroblasts (passage <5) (Promocell). Mean value of duplicate samples are shown.

The following parameters were altered to improve efflux supernatant counts and reduce variability:

- Labelling time
- Equilibration time
- Efflux duration
- Cholesterol acceptor (ApoA-I) concentration

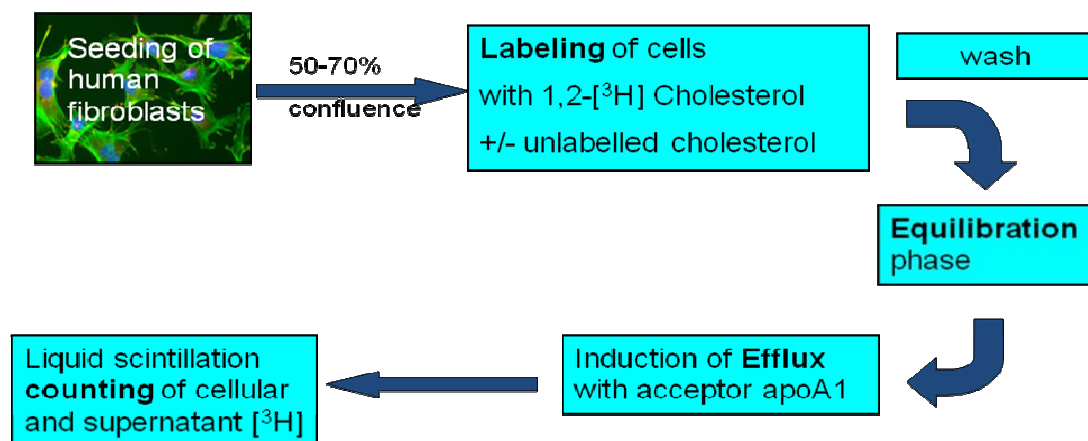


Figure 3.8 Cholesterol efflux assay

Fundamental steps involved in measuring cholesterol efflux from skin fibroblasts.

3.21 Labelling time

Labelling time of 48 hours was compared to 24 and 72 hours (figure 3.9).

Shortening the labelling time reduced cholesterol efflux measurements primarily due to low supernatant counts, typically <500 cpm. Labelling for 48 and 72 hours resulted in high background efflux. Net ApoA-I-mediated efflux was less than 5% in all experiments except when labelling for 72 hours. Variability of efflux was higher in cells that were labelled for 72 hours (mean 12.9%, standard deviation 4.5%, 3 hours after ApoA-I exposure) figure 3.9. In these experiments, specific ApoA-I-related efflux was not improved by increasing incubation time with ApoA-I.

3.22 Equilibration time

The next step was to examine whether altering equilibration times improved net efflux. Equilibration was undertaken for 24 and 48 hours and efflux was measured at 3 and 6 hours. Labelling time was kept constant at 48 hours as per protocol. High basal efflux was again observed in all experiments (figure 3.10) with little added specific efflux induced by ApoA-I (after 6 hours exposure with ApoA-I, cells equilibrated for 24 hours yielded a net efflux of 3.3%). Similarly, ApoA-I mediated efflux was 1.6% after equilibration for 48 hours.

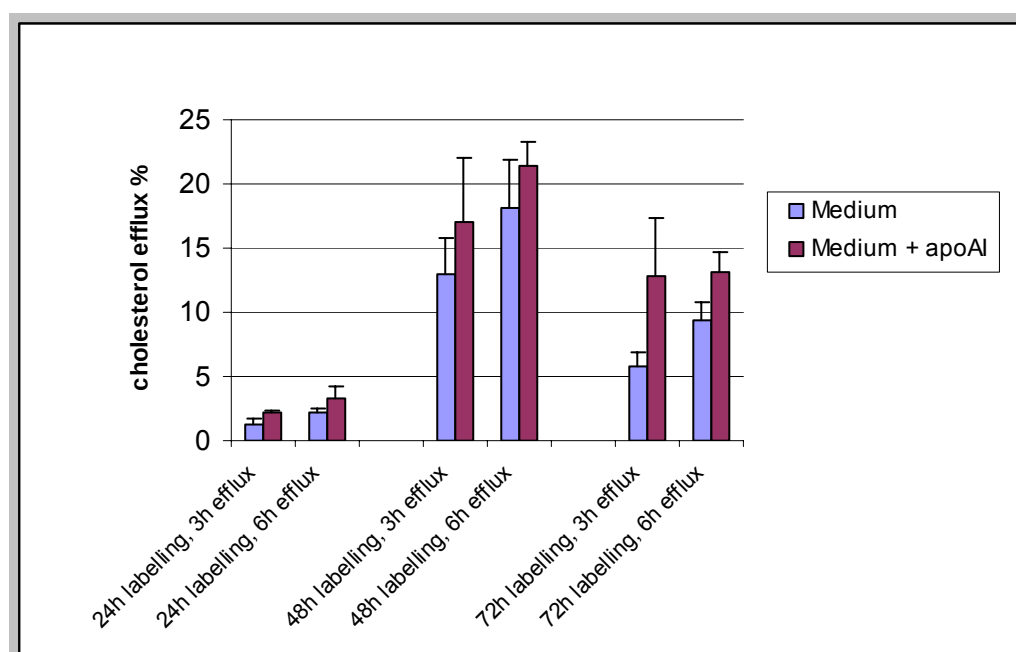


Figure 3.9 Effect of labelling time on cholesterol efflux

Effect of altering labelling times on cholesterol efflux in fibroblasts. Equilibration was for 18 hours, mean efflux and standard deviation from triplicate wells are shown. Efflux was measured after 3 and 6 hours of exposure to 10 μ g/ml ApoA-I (Sigma).

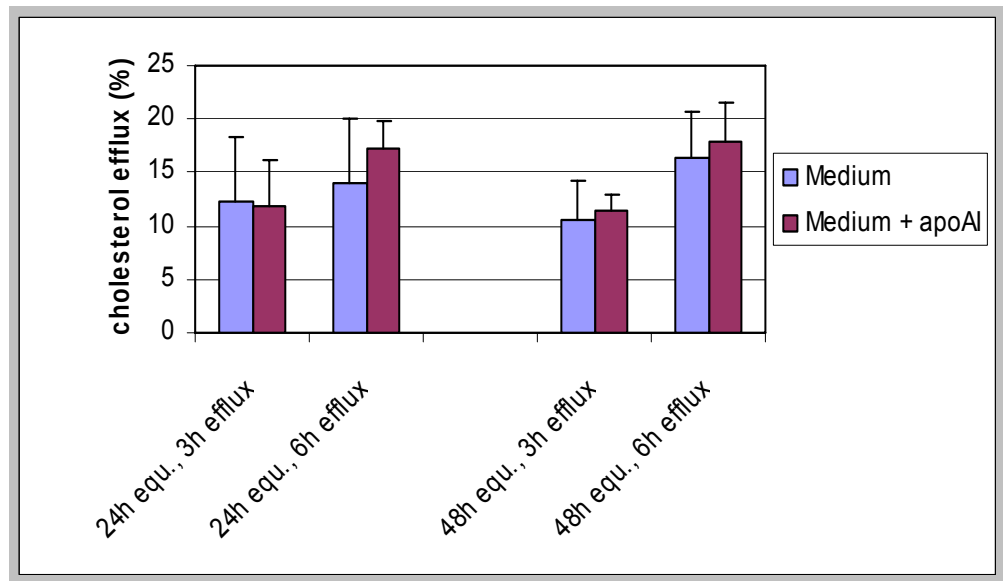


Figure 3.10 Effect of equilibration time on cholesterol efflux

Effect of equilibration times using Bovine serum albumin (BSA) on cholesterol efflux in fibroblasts (Promocell). Labelling time was 48 hours, mean efflux and standard deviation from triplicate wells are shown after 3 and 6 hours induction using 10 μ g/ml ApoA-I (Sigma).

3.23 Efflux duration

As changes in labelling and equilibration times did not substantially improve net efflux or variability, duration of exposure of fibroblasts and concentration of ApoA-I was investigated. Labelling and equilibration times were used as per protocol. Results are seen in figure 3.11. Background efflux was relatively constant up to 4 hours but appeared much higher after 6 hours of ApoA-I exposure. Non-specific efflux was comparable to that induced by ApoA-I after 1 hour. There was little difference in ApoA-I- mediated efflux at 4hours compared to 2 hours. The higher concentration of ApoA-I was not consistently associated with a higher efflux apart from at the 2 hour time point where it was 2-fold higher

than the background rate. A second source of commercially available ApoA-I (Calbiochem) did not increase net efflux results.

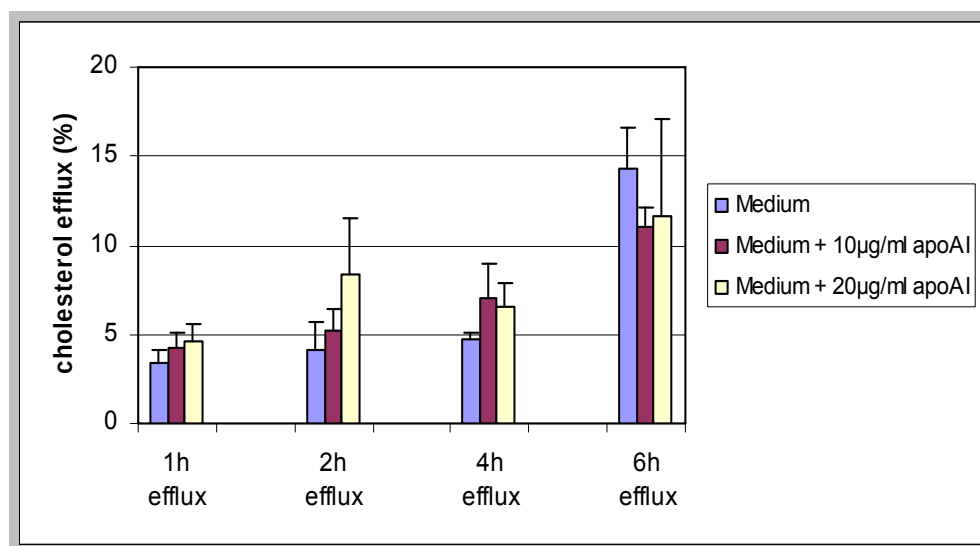


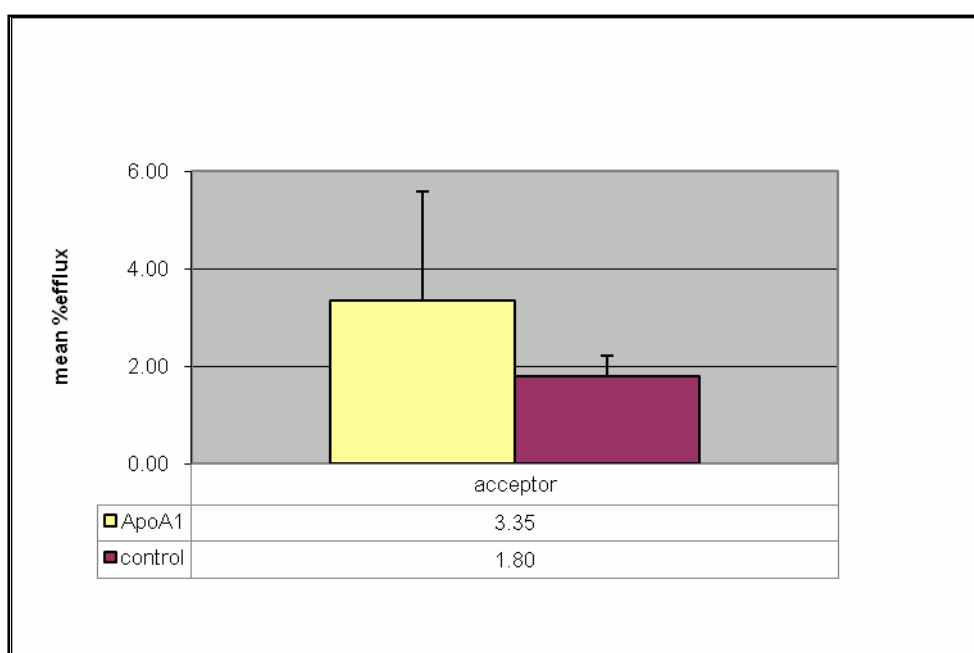
Figure 3.11. Effect of ApoA-I concentration and exposure on cholesterol efflux

Effect of ApoA-I concentration and duration of incubation on cholesterol efflux in fibroblasts. Labelling time 48 hours, equilibration time 24 hours. Mean and standard deviation are shown from quadruplicate wells.

The original assay was next run using freshly isolated primary fibroblasts taken from a study participant to see whether this affected net efflux. These results were compared to a second protocol which was established in the Academic Medical Centre, Amsterdam under the guidance of Dr Albert Groen. The main difference between protocols was that the second assay used shorter equilibration and labelling times (191). Furthermore, labelling was in the presence of unlabelled cholesterol instead of 5% FBS which was designed to induce cellular ABCA1 expression. Exposure to ApoA-I was continued for 20 hours which is considerably longer than the original protocol. Overall net efflux achieved was higher using the newer assay (4.2% versus 1.6%) but ApoA-I

produced a similar factor difference compared to background efflux (1.8 versus 1.9). However, the main advantage of the second protocol was it produced consistent results with lower count variability (figure 3.12). This second protocol was therefore adopted (see protocol below) for use for all further efflux experiments in this study (see results in section 5.0).

a)



b)

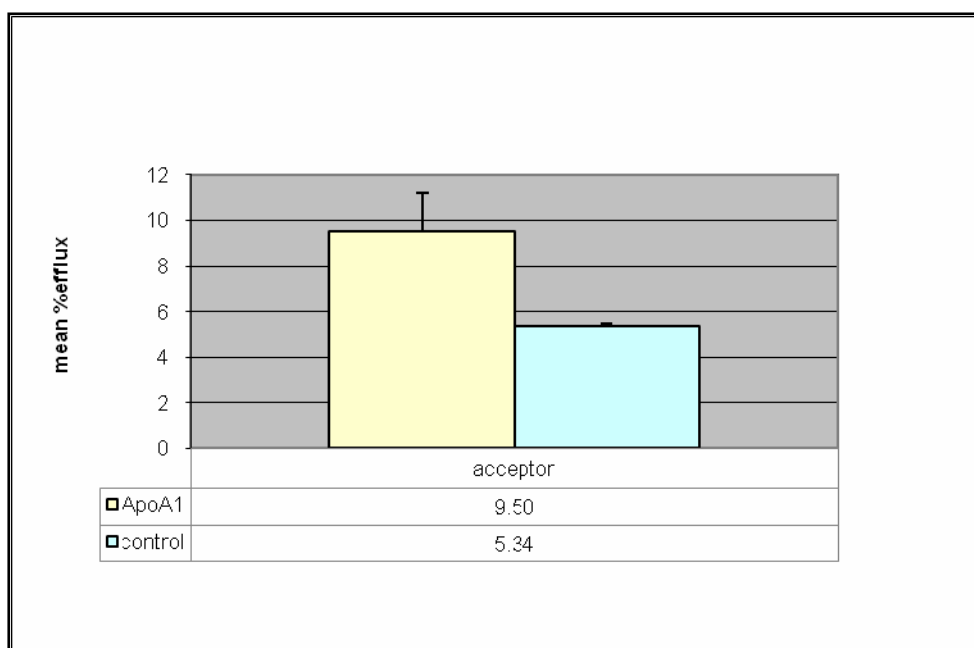


Figure 3.12 Comparison of results from two cholesterol efflux assays

Cholesterol efflux in cultured primary fibroblasts (passage number 4) comparing the original efflux protocol see section 3.2 (a) to a second adopted protocol (b) see section 2.31.

In summary problems with gene expression measurements were overcome by changing to a more specific methodology. Variability in results for the functional cholesterol efflux assay in skin fibroblasts was only improved by adopting a different published methodology. This assay was demonstrated to be specific to ABCA1 action by pharmacological inhibition.

3.3 Materials

Agarose	Sigma
Apolipoprotein-AI	Sigma
Annexin V	Becton Dickinson
Antibiotic-Antimycotic solution	Sigma
Bovine Serum Albumin	Sigma
Calcimycin	Sigma
Cell scraper	NUNC
Cell strainer 70µm	Becton Dickinson
Cholesterol	Sigma
1,2-[³ H] Cholesterol	Perkin Elmer
Collagenase D	Roche
Complete protease inhibitor cocktail	Roche
Cryopreservation vial	Corning
DMEM, DMEM/F12	Invitrogen
DMSO	Sigma
DNA ladder 1kb-plus	Invitrogen
Erythrocyte Lysis Buffer	Qiagen
Ethidium Bromide	Sigma
Fetal Bovine Serum	Sigma
Ficoll-Paque plus	Amersham
LC Column	Miltenyi Biotech
Loading Buffer (10xblue juice)	Invitrogen
MACS buffer	Miltenyi Biotech
β-Mercaptoethanol	Sigma

Microbeads CD14	Miltenyi Biotec
NaOH	Sigma
Normal human dermal fibroblasts	Promocell
PBS tablets	Sigma
Probucol	Sigma
Qias shredder columns	Qiagen
Scintillation fluid	Sigma
Suture pack	Rochialle inHealth
Tris-EDTA Borate	Sigma
Trypsin-EDTA solution	Sigma
<u>Conjugated FACS Antibodies</u>	Becton Dickinson
R-Phycoerythrin	
Phycoerythrin-Cychrom5	
Allophycocyanin & FITC	
<u>Other Antibodies</u>	
ABCA1 peptide ab14148	Abcam, Cambridge
Anti-ABCA1 rabbit antibody ab7360	Abcam, Cambridge
<u>Primer-Probe sets</u> (see Table 2.3)	Applied Biosciences
Primers (SYBR green method)	MWG Biotech, Munich
<u>KITS</u>	
cDNA high capacity RT kit	Applied Biosciences
First strand cDNA kit	Amersham Biosciences
NEFA kit	Wako chemicals
PCR light cycler fast start kit	Roche
RNeasy midi kit	Qiagen

Results

4.0 ABCA1 gene and protein expression

The effect of glucose on ABCA1 gene expression and related genes was investigated in both leukocytes and skin fibroblasts. Subjects varied according to glucose homeostasis. The immediate and short term effects of glucose were assessed in blood leukocytes during an OGTT.

4.0 The chronic effect of glucose on leukocyte ABCA1 gene expression

The chronic effect of glucose on ABCA1 gene expression was assessed in leukocytes. Gene expression data were available in 63 individuals; leukocyte extraction from 4 subjects did not yield RNA of adequate quality for PCR experiments. One healthy control (C12) was excluded as we discovered significant abnormalities in liver enzyme biochemistry which required further clinical investigation. Gene expression results were compared from multiple PCR experiments due to the sample capacity of the thermal rotor. Therefore, analysis was performed compared to a fixed sample. This calibrating sample was present in each PCR experiment. Variability in the measurement of the calibrator sample is shown in figure 3.6. Each experiment included samples taken from both patients as well as healthy men.

There was a significant negative relationship with glycaemia in 63 individuals, ABCA1 expression versus HbA1c ($\rho=-0.43$, $P<0.001$) figure 4.1; and versus glucose ($\rho=-0.31$, $p=0.01$). Verification was sought using comparative quantitation and delta-delta Ct methods of PCR analysis which yielded concordant results; Hba1c (both methods $p<0.01$) and fasting glucose ($p<0.01$ and $p=0.016$ respectively).

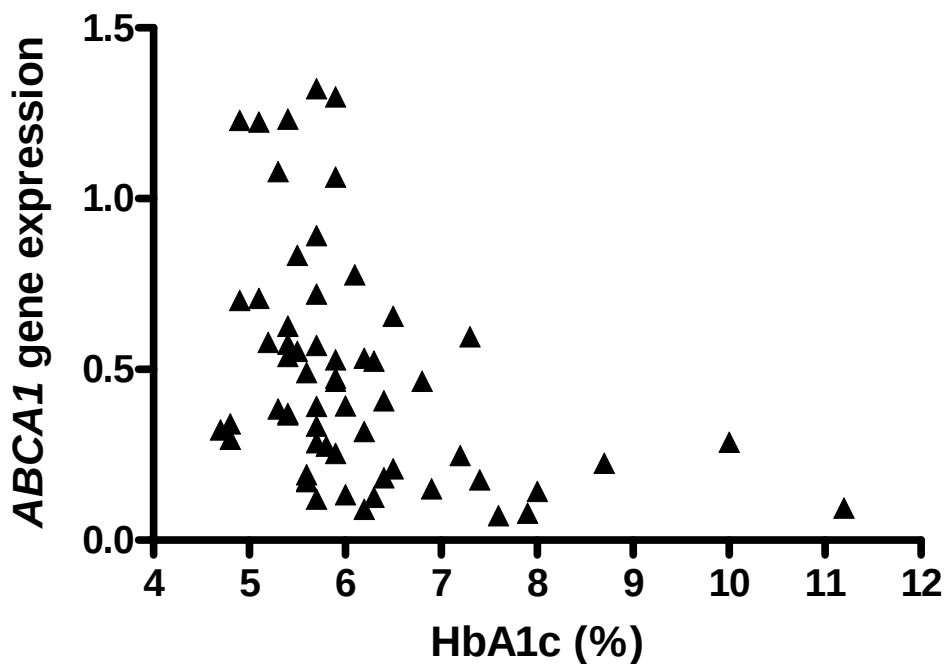


Figure 4.1

ABCA1 gene expression as measured by PCR. The relationship of HbA1c to ABCA1 gene expression (normalised to β -actin –arbitrary units and relative to a calibrating sample) in 63 participants (27 control, 18 IGH and 18 diabetes, $n=63$, $\rho=-0.41$, $p<0.001$).

A smaller cohort of 25 subjects (9 control, 6 IGH and 10 DM) were studied in more detail with measurements taken from circulating leukocytes and functional measurements in skin fibroblasts. Gene expression data for leukocyte ABCA1 in this cohort were analysed (using mean values from two experiments). Results confirmed a negative relationship with Hba1c ($\rho=-0.5$, $p=0.01$) and borderline significant relationship with fasting glucose ($\rho=-0.35$, $p=0.09$). These relationships persisted when ABCA1 expression was compared to GAPDH.

In DM patients, ABCA1 gene expression was lower than in healthy men ($n=63$ $p=0.03$) (figure 4.2). Gene expression in patients with impaired glucose homeostasis appeared lower than in healthy men but this did not reach statistical significance ($n=63$, $p=0.27$). There was no difference in gene expression between patients with IGH and diabetes ($p=0.33$). Analysis of the cohort who underwent more detailed measurements, also revealed significant

differences between groups with lower ABCA1 expression in patients with diabetes ($p=0.03$) and IGH ($p=0.05$) compared to that in healthy men.

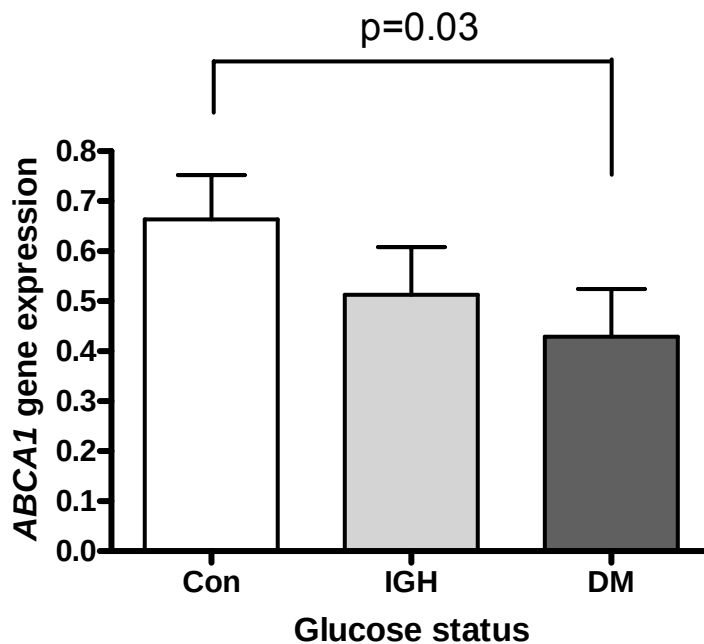


Figure 4.2

Leukocyte ABCA1 gene expression, expressed as normalised values (arbitrary units) from PCR experiments. Mean and standard error are shown according to subject glycaemic status in 63 participants Con=Controls ($n=27$), IGH=Impaired glucose homeostasis ($n=18$), DM=Type 2 Diabetes ($n=18$). Con vs. DM; $p=0.03$, Con vs. IGH; $p=0.27$, IGH vs. DM $p=0.33$.

4.1 The acute effect of glucose on leukocyte ABCA1 gene expression

The acute effect of glucose on ABCA1 gene expression was assessed during an OGTT where leukocyte samples were taken at baseline, 1 hour and 2 hours after 75g oral glucose administration. Gene expression in healthy subjects at 1 hour and 2 hour-post glucose time points was significantly higher than at baseline (repeated measures one-way ANOVA, $n=24$, $p<0.001$, $p<0.05$ respectively) (figure 4.3). Differences in gene expression during an OGTT were not seen in patients with IGH (figure 4.5).

There was a positive relationship between change in glucose (baseline versus 2 hours) and change in gene expression (baseline versus 2 hours) in samples taken from controls ($n=25$, $\rho = 0.45$, $p=0.023$) but this correlation between change in glucose and gene expression was not observed at 1 hour time points ($\rho=0.27$ $p=0.22$). Mean glucose concentration during the OGTT (a measure of glucose exposure) did not relate to the mean level of gene expression in healthy men ($p=0.82$). In patients with IGH, gene expression did not change during the OGTT ($n=16$, $p=0.12$) and did not associate with changes in glucose concentration. Taking both control and IGH subject groups together, one and two hour gene expression values were higher than baseline; $p<0.001$ baseline versus 1 hour, $p<0.05$ baseline versus 2 hour, but this did not relate directly to changes in glucose levels.

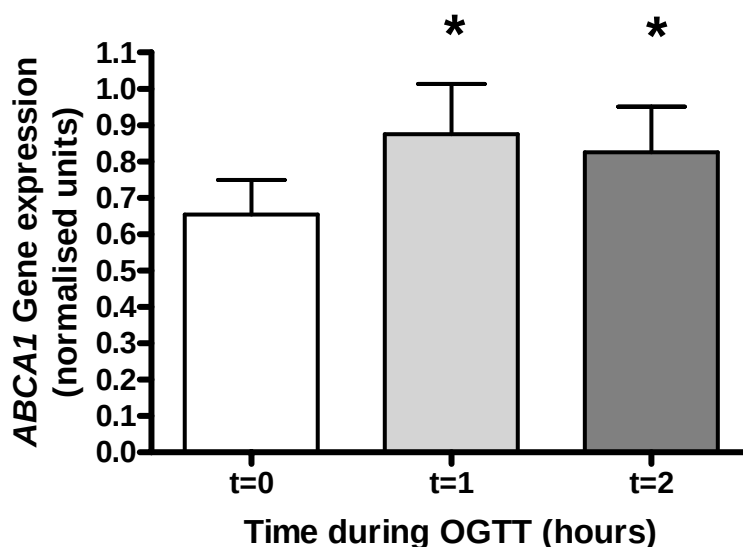


Figure 4.3

Leukocyte ABCA1 gene expression during an OGTT in healthy men. Results are at 3 timepoints during an oral glucose tolerance test (OGTT) (normalised relative units). ($n=24$ $t=0$ versus 1hr $p<0.01$; $t=0$ versus 2hr $p<0.05$).

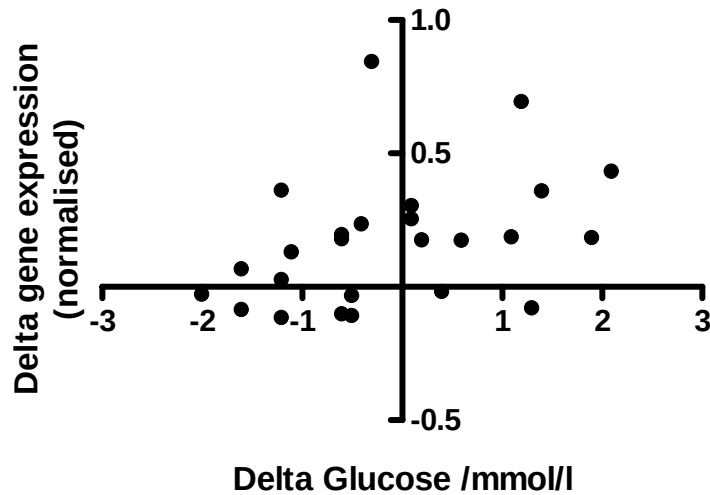


Figure 4.4

The relationship between change in glucose concentration (delta glucose mmol/l) and leukocyte ABCA1 gene expression in healthy subjects. Samples were taken before and after a oral glucose tolerance test (OGTT)-baseline versus 2 hours post-glucose administration (normalised relative units); n=25, rho=0.45, p=0.023.

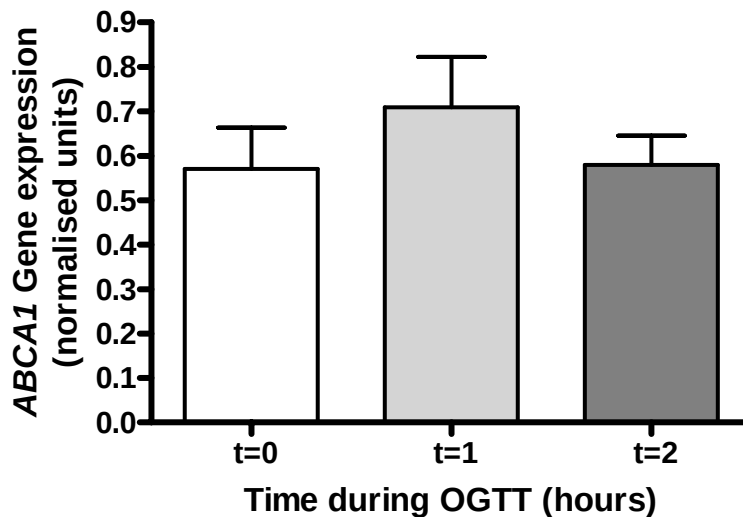


Figure 4.5

Leukocyte ABCA1 gene expression measurements during an oral glucose tolerance test (OGTT) in patients with impaired glucose homeostasis (IGH) (normalised relative units). (n=19 p=0.09, t=0 versus 1hr p=0.35; t=0 versus 2hr p=0.94).

4.2 Comparison of leukocyte and monocyte ABCA1 gene expression

Leukocyte and monocyte ABCA1 gene expression was measured in 7 healthy subjects to investigate whether gene expression was equivalent. This was conducted whilst establishing the SYBR green PCR methodology. Gene expression was measured before and in response to oral glucose (OGTT). There was a positive relationship between leukocyte and monocyte gene expression at baseline ($\rho=0.79$, $p=0.048$). The positive relationship between gene expression was not significant at 2 hours after oral glucose administration ($\rho=0.66$, $p=0.11$). Due to time limitations this work could not be validated using the Taqman hydrolysis probe method.

4.3 Relationship of $LXR\alpha$, $PPAR\gamma$ mRNA to ABCA1 and glycaemia

$LXR\alpha$ and $PPAR\gamma$ are nuclear transcription factors known to positively regulate ABCA1 gene expression. Gene expression of these transcription factors were measured to confirm this in our study cohort and to investigate whether glycaemia-mediated alterations in ABCA1 gene expression occurred through alteration of gene expression of these transcription factors. The inter-assay CV for $LXR\alpha$ gene expression using 11 leukocyte samples analysed in two PCR experiments varied from 0.25% to 3% (mean 1.1%). $LXR\alpha$ and $PPAR\gamma$ gene expression was measured in 25 participants (9 Control, 6 IGH and 10 DM). These subjects underwent more detailed gene expression studies in blood leukocytes. Results were obtained for 23 participants. There was no significant

relationship between either fasting plasma glucose or HbA1c levels and either leukocyte PPAR γ (versus HbA1c $p=0.13$) or LXR α (versus HbA1c $p=0.13$) expression. In this cohort leukocyte LXR α expression correlated significantly and positively with leukocyte ABCA1 ($\rho=0.52$, $p=0.01$) but there was no such relationship with PPAR γ expression. LXR α expression data was available in a total of 61 participants (26 control, 17 IGH, 18 DM), and a positive relationship with ABCA1 was confirmed ($n=61$, $r=0.41$, $p=0.001$), see figure 4.6 There was no difference in LXR gene expression between glycaemic groups ($p=0.89$) and gene expression did not vary significantly with age ($p=0.78$). Furthermore, there was no evidence of a continuous relationship between LXR α and glycaemia or other biochemical parameters ($p=0.56$ versus fasting glucose). Using bivariate linear regression, both fasting plasma glucose concentrations and leukocyte LXR α expression remained independent predictors of leukocyte ABCA1 expression (both $p<0.001$).

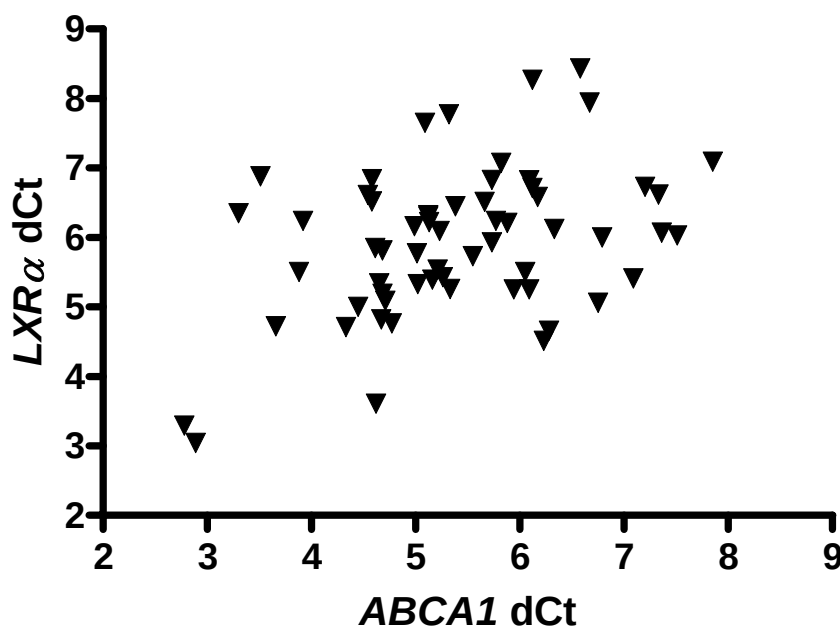


Figure 4.6

Positive relationship between leukocyte liver X receptor alpha (LXR α) gene expression and ABCA1 gene expression taken from subjects with varying glucose homeostasis, dCt=delta cycle number (arbitrary units); n=61, r=0.41, p=0.001.

4.4 The relationship of leukocyte ABCG1 gene expression to glycaemia

Expression of ABCG1 was not reduced in patients with IGH or DM compared to healthy men (n=25, p=0.24). Unlike with ABCA1, ABCG1 expression did not relate significantly to glycaemic indices (p=0.54, versus fasting glucose and p=0.94 versus HbA1c). However in a larger sample of 47 subjects there did appear to be a negative relationship to both mean glucose concentration during an OGTT (rho= -0.33, p=0.024) and HbA1c (rho= -0.31, p=0.033). Gene expression was not lower in all patients with hyperglycaemia (combined IGH and DM groups) compared to healthy men (p=0.16). ABCG1 strongly associated with ABCA1 expression (n=25, rho=0.53, p=0.008) and this was confirmed in the larger sample (19 control, 12 IGH, 16 DM rho=0.40, p=0.005). LXR α gene expression correlated positively with ABCG1 levels (n=25, rho=0.73, p<0.001).

Bivariate regression analysis, using LXR α and mean glucose as predictors of ABCG1 expression revealed neither to be significant independent contributors. When gene expression of ABCA1 and glycaemia (mean glucose or HbA1c) were analysed as independent predictors, only ABCA1 remained a significant predictor of ABCG1 RNA (mean glucose p=0.32; HbA1c p=0.65; ABCA1 p<0.05) confirming the relationship between glycaemia and ABCG1 was dependent on ABCA1 expression. There was no significant relationship between ABCG1 and PPAR γ expression (p=0.12).

4.50 The effect of glucose on ABCA1 protein

It has been suggested that diabetes has a direct effect on ABCA1 protein levels. The effect of glycaemia on ABCA1 protein concentrations was assessed in both leukocytes and fibroblasts. This was performed with the assistance of Dr Vijay Paul using a newly established methodology (335). Due to problems with leukocyte sample storage and transportation, protein data in leukocytes was only available in 50 participants (17 control, 17 IGH and 16 DM). Fibroblast protein measurements were obtained from all 25 subjects. ABCA1 protein measurements were expressed as concentration per total protein concentration.

4.51 Leukocyte ABCA1 protein concentration in subjects according to glycaemia

ABCA1 protein concentration was different between groups with borderline significance ($p=0.059$) see figure 4.7. Patients with diabetes had lower ABCA1 protein concentration compared to healthy men ($n=33$, $p=0.029$) and compared to IGH with borderline significance ($n=33$, $p=0.059$). There was no difference in protein levels between healthy and IGH groups ($n=34$ $p=1$).

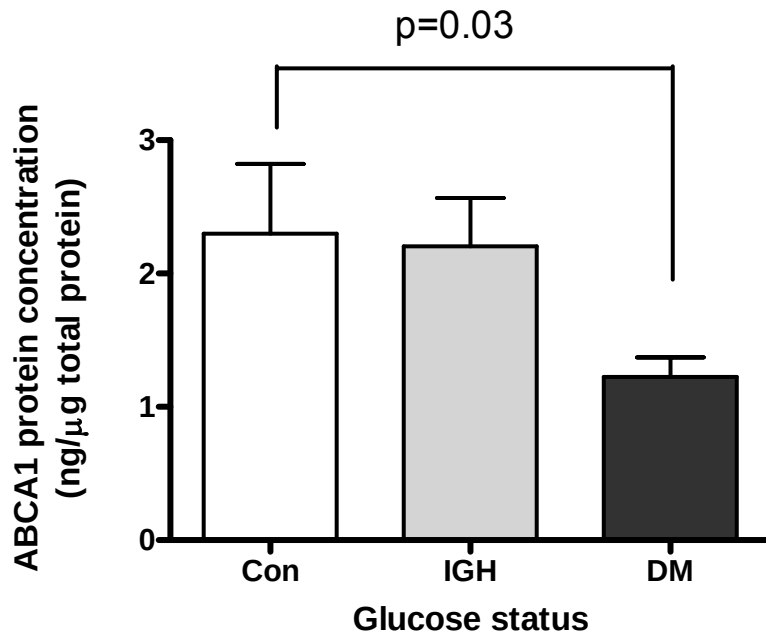


Figure 4.7

ABCA1 protein concentration according to glucose homeostasis (n=50, p=0.059). Protein concentration was measured using an enzyme immunoassay methodology(336). Con=control subjects, IGH=Impaired glucose regulation and DM=Type 2 diabetes. Con versus DM p=0.029, IGH versus DM p=0.059, Con versus IGH p=1.

There was no significant relationship between baseline protein concentration and fasting glucose ($\rho=-0.21$, $p=0.13$) and Hba1c ($\rho=-0.08$, $p=0.56$). Protein levels in leukocytes did not vary significantly during an OGTT ($p=0.67$). A negative relationship was evident between baseline ABCA1 protein and adiposity ($\rho=-0.39$, $p=0.005$ versus BMI and $\rho=-0.29$, $p=0.04$ versus waist circumference) see figure 4.8. When fasting glucose was entered into a bivariate model with BMI or waist circumference, glucose did not appear to be an independent determinant of protein levels ($p=0.6$) whereas markers of adiposity did independently predict protein concentrations (BMI $p=0.02$, Waist circumference $p=0.02$). Protein concentrations correlated positively with plasma HDL-C levels (n=50, $\rho=0.34$, $p=0.02$), see figure 4.9, but there was no

significant relationship observed between protein concentrations and gene expression in blood leukocytes ($p=0.68$).

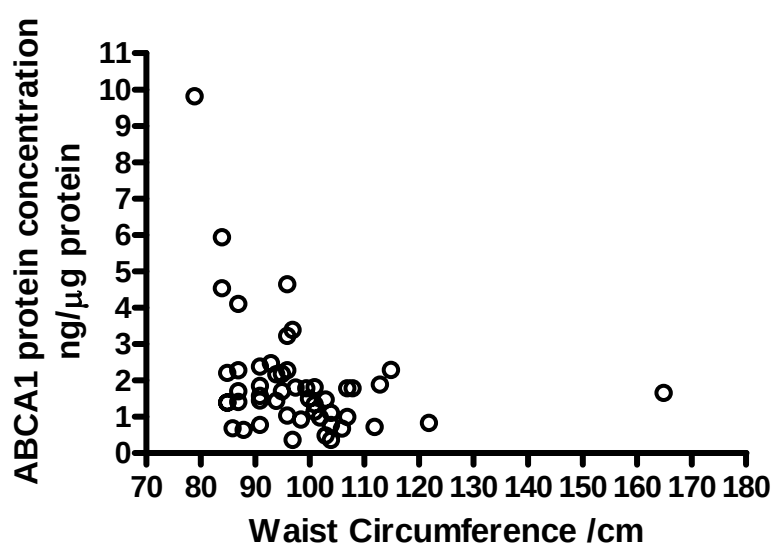


Figure 4.8

The relationship between subject waist circumference and ABCA1 protein concentration in leukocytes measured by an enzyme immunoassay ($n=50$, $\rho=-0.29$, $p=0.041$).

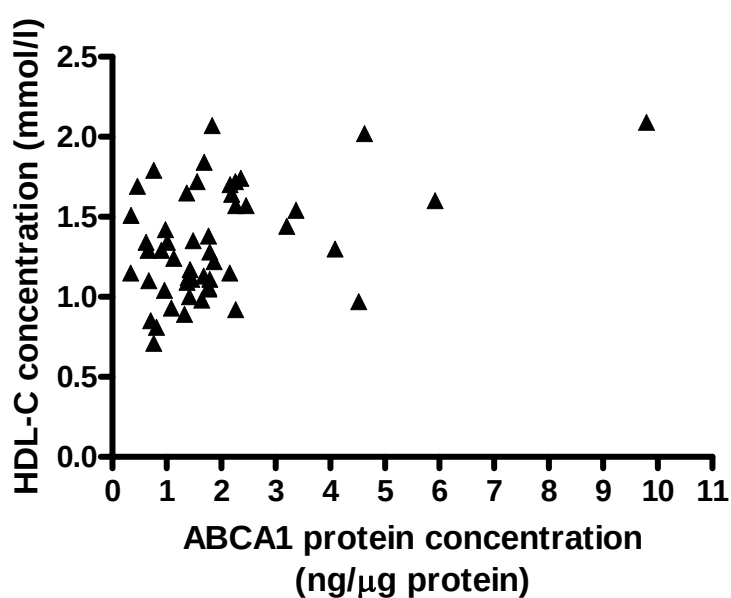


Figure 4.9

Positive relationship between leukocyte ABCA1 protein concentration (measured by an enzyme immunoassay) and plasma HDL-C concentration at the time of sampling in subjects with varying glucose homeostasis ($n=50$, $\rho=0.34$, $p=0.02$).

4.6 The relationship of fibroblast ABCA1 measurements with ABCG1 and glycaemia

Since undertaking this work it was discovered that a related transporter, ABCG1 contributes to cholesterol efflux in skin fibroblasts. Therefore, ABCG1 gene expression was measured in fibroblasts as well as leukocytes to compare to ABCA1 gene expression and indices of glycaemia.

Results of gene expression in fibroblasts were obtained for 21 of the 25 samples. (7 control, 6 IGH and 8 DM). Samples taken from 5 subjects (C6, C28, D3, D15 and D18) yielded very low expression of β -Actin. This skewed results of normalised ABCA1 expression. Similar low β -Actin gene expression levels were observed in these 5 fibroblast samples in PCR experiments involving LXR α and ABCG1 genes. There were no observed demographic nor cellular differences that could explain these results and cell passage number were equivalent in all fibroblast samples. Experiments were subsequently repeated with GAPDH and data are shown in figure 4.10. Based on these observations gene expression results normalised to GAPDH were used for analyses. Fibroblast ABCA1 mRNA level did not differ between glycaemic groups ($p=0.67$) and did not correlate with HbA1c nor fasting plasma glucose concentrations ($p=0.77$ versus fasting glucose).

Similar to observed measurements in blood leukocytes, ABCA1 mRNA level positively related to both ABCG1 gene expression ($n=21$, $\rho=0.8$, $p<0.001$) and LXR α gene expression ($n=21$, $\rho=0.78$, $p<0.001$).

There appeared to be a trend towards a difference in ABCA1 protein concentration between subject groups ($p=0.08$). Patients with diabetes had a

marginally higher protein concentrations than healthy controls (median concentration 0.38ng/μg versus 0.3ng/μg protein; $p=0.04$). There were no differences in protein content between other subject groups and no observed continuous relationship between fibroblast protein and glycaemia ($p=0.53$ versus fasting glucose, $p=0.73$ versus HbA1c). Protein expression in fibroblasts did not vary with adiposity.

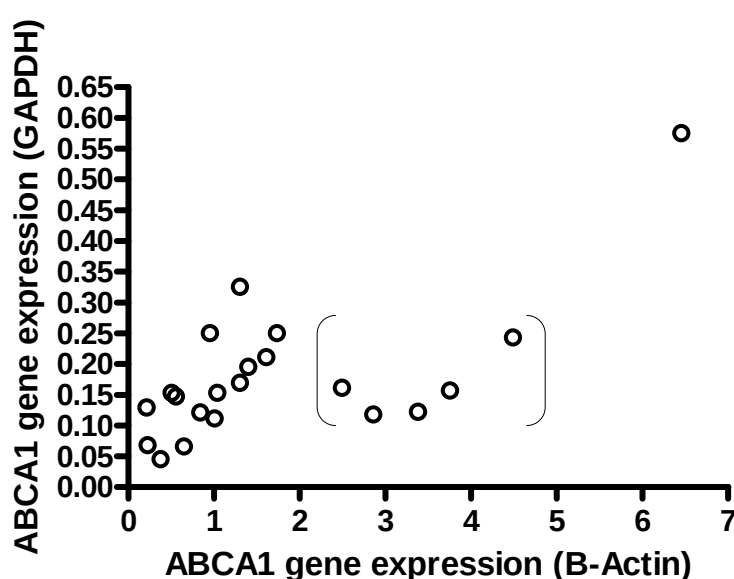


Figure 4.10

Comparison of gene expression of ABCA1 in skin fibroblasts using two house keeper genes, arbitrary units ($n=21$, $\rho=0.55$, $p=0.01$). 5 subjects (C6, C28, D3, D15 and D18) had disproportionately lower β -Actin gene expression measurements, yielding a higher ABCA1 normalised ratio relative to β -Actin (arc).

In summary, leukocyte ABCA1 gene expression was lower in patients with DM and associated negatively with glycated haemoglobin and fasting glucose in 63 subjects. This relationship was confirmed in a subset of 25 subjects whose samples were measured in a single PCR experiment which was performed twice. These experiments compared ABCA1 gene expression to two separate

housekeeper genes. The acute effect of glucose on leukocyte ABCA1 gene expression was assessed during an OGTT. ABCA1 gene expression increased at 1 hour and 2 hour time points compared to basal gene expression in healthy men. There was a positive correlation between ABCA1 and LXR α but not PPAR γ gene expression in leukocytes. Both LXR α and PPAR γ gene expression did not vary with glycaemia and did not confound the relationship observed between glycaemia and ABCA1 gene expression. ABCG1 gene expression in leukocytes did not vary with glucose homeostasis but did strongly associate with ABCA1 gene expression.

Leukocyte ABCA1 protein levels were lower in subjects with DM and this was assessed in 50 subjects. There was no significant continuous relationship between glycaemia and protein levels, but leukocyte protein concentrations did positively relate to plasma HDL-C and independently with markers of adiposity.

In fibroblasts, ABCA1 gene expression did not alter between groups of differing glucose homeostasis nor did gene expression correlate significantly with glycaemia in 21 subjects. Fibroblast ABCA1 gene expression positively related to both ABCG1 and LXR α gene expression. Fibroblast ABCA1 protein concentrations were slightly higher in patients with DM but did not vary with adiposity.

5.0 ABCA1 Function :

5A Cholesterol Efflux

5B PS translocation

ABCA1 function was measured in skin fibroblasts as cellular cholesterol export (cholesterol efflux) and in CD4-positive lymphocytes as trans-membrane phospholipid transport.

5.0 Cellular cholesterol efflux in skin fibroblasts

25 participants underwent a skin biopsy and adequate cell proliferation was achieved from all biopsies. At the time of undertaking these experiments, it was unknown whether human fibroblasts expressed *ABCG1* and therefore a number of acceptor particles were initially investigated to establish the range and variability of efflux in fibroblasts taken from healthy subjects. This is illustrated in figure 5.1. (HDL particles were isolated using ultracentrifugation from a healthy man, sample kindly provided by Dr M. Yee). The isolated HDL-3 particle (density range 1.20-1.21g/ml) produced the highest cholesterol efflux which is presumed to be the combined action of *ABCG1* and *ABCA1*. ApoB depleted serum produced equivalent cellular efflux compared to pooled Apo-AI. As serum without ApoB produced reduced levels of efflux compared HDL separated by ultracentrifugation, it is possible the reagents or process in which ApoB is removed may have had a negative effect on HDL particle function. Alternatively it is also plausible that HDL particles taken from two separate subjects had markedly differing HDL function. It was not possible to isolate HDL using both methods in a single subject.

To investigate whether observed efflux to ApoA-I was specific to *ABCA1* action, cellular cholesterol efflux was inhibited using a pharmacological inhibitor of *ABCA1* which interferes with *ABCA1* intracellular protein shuttling (explained in section 1.812). Probucol was applied to wells (20 μ M) for 2 hours prior to

commencing efflux with ApoA-I. This resulted in a 78% decrease in efflux to ApoA-I, illustrated in figure 5.2.

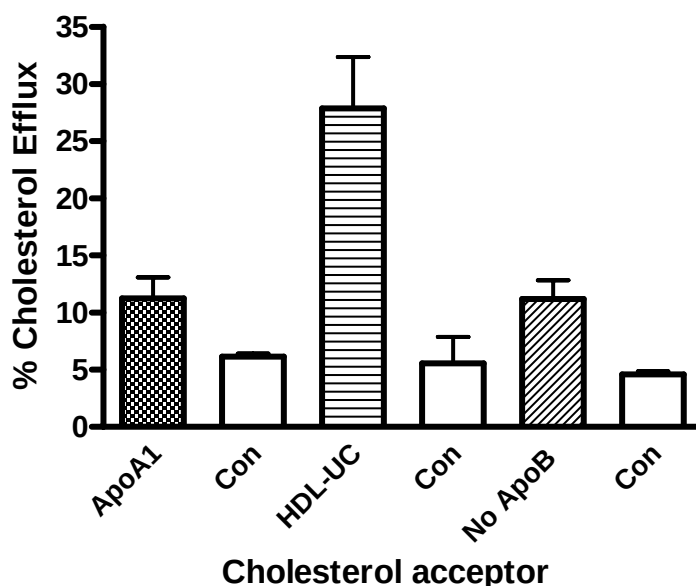


Figure 5.1

Cholesterol efflux in primary fibroblasts taken from a healthy subject. This compares various acceptors of cholesterol in equivalent concentration using an adopted methodology. Mean values and standard deviation taken from measurements in triplicate wells are shown. Cells were loaded with tritiated cholesterol containing 30mg/l unlabelled cholesterol for 24 hours. Cells were subsequently washed with PBS/BSA (0.2% v/v) and efflux was commenced using a particle acceptor for 20 hours at 37°C and 5% CO₂. ApoA-I (10µg/ml, Sigma), HDL-UC=HDL-3 particle (density 1.20-1.21g/ml), isolated by ultracentrifugation from a healthy donor. No ApoB=ApoB depleted serum taken from a healthy donor, con=control (background) efflux for each experiment in wells that contain only culture medium.

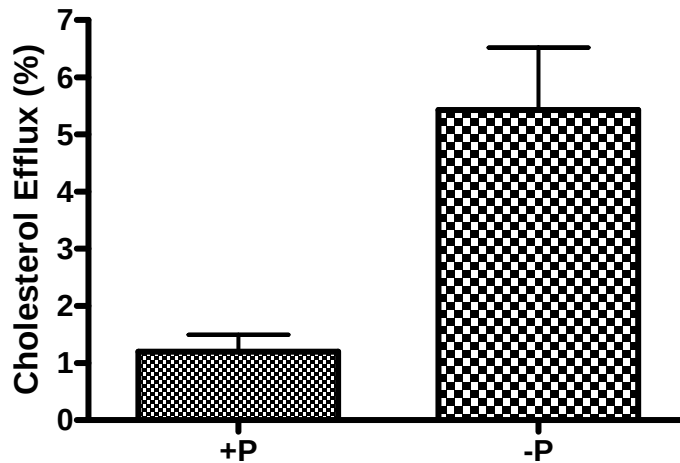


Figure 5.2

Pharmacological inhibition of cholesterol efflux to ApoA-I in human fibroblasts.

Probucol 20 μ M (Sigma) was added two hours prior to commencing efflux with ApoA1 (10 μ g/ml, Sigma). Cells were loaded with tritiated cholesterol containing 30mg/l unlabelled cholesterol for 24 hours. Cells were subsequently washed with PBS/BSA (0.2% v/v) and efflux was conducted for 20 hours at 37°C and 5% CO₂. Mean and standard deviation of triplicate wells. +P=with Probucol, -P=without Probucol.

The efflux assay was repeated in a number of experiments using primary cells to compare results using a cellular cholesterol extraction method (2,3 propanol) with results attained by cellular digestion (1M NaOH). This yielded comparable results ($\rho=0.70$) and is shown in figure 5.3.

The mean CV for efflux experiments in 25 subjects was 12%, see table 5.1.

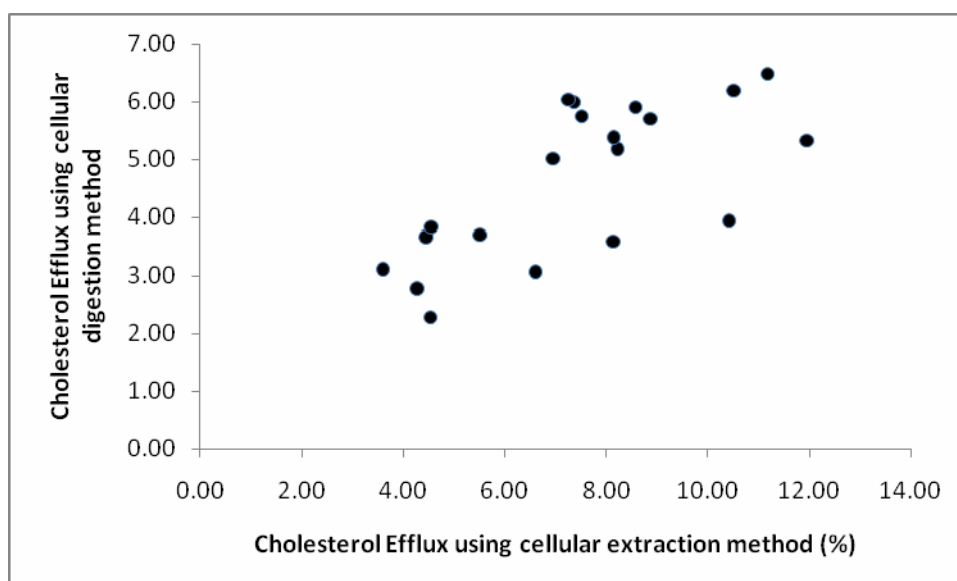


Figure 5.3 Measurement of labelled cellular cholesterol

Cellular efflux results comparing methods of cellular cholesterol extraction;cellular digestion using 1M NaOH for 1 minute versus cellular cholesterol extraction using 2,3 hexane-propanol for 30mins. Radioactivity was assayed by liquid scintillation counting (LSC 60000SC, Beckman, USA) for 2 minutes. n=20, rho=0.70,p<0.001.

Table 5.1

Cholesterol efflux data in participants (ABCA1 function). Experiments were performed in cultured skin fibroblasts taken from subjects.Total efflux refers to culture medium containing 10mg/ml ApoA-I compared to culture media without ApoA-I (control efflux). Delta efflux (Total minus control efflux) represents ApoA-I-mediated cholesterol efflux. Experiments were conducted in triplicate wells. Mean efflux CV in wells containing ApoA-I overall was 12.0%.

Subject Code	Total efflux (%)	Control efflux (%)	Delta efflux (%)	Factor efflux (Total/Control)	ApoAI efflux CV (%)	Mean Group CV (%)
C1	6.19	3.00	3.19	2.06	7.75	13.2
C4	6.48	1.66	4.82	3.90	13.29	
C6	5.75	2.50	3.25	2.30	22.35	
C7	5.99	2.82	3.17	2.12	12.80	
C8	5.32	1.47	3.85	3.62	13.16	
C9	3.07	1.52	1.55	2.02	10.40	
C21	2.50	1.45	1.05	1.72	8.78	
C28	5.70	3.50	2.20	1.63	23.12	
C29	4.98	2.93	2.05	1.70	6.91	
I1	3.70	1.22	2.48	3.03	4.67	
I6	3.94	1.75	2.19	2.25	16.72	
I10	5.02	2.36	2.66	2.13	5.11	
I12	6.03	1.15	4.88	5.24	18.43	
I15	3.11	0.7	2.41	4.44	4.88	

I16	4.55	3.5	1.05	1.30	22.04	12.0
D3	3.67	1.90	1.77	1.93	7.07	
D4	3.59	1.87	1.72	1.92	10.69	
D5	2.78	1.13	1.65	2.46	7.40	
D7	6.06	3.86	2.20	1.57	3.72	
D9	2.28	1.12	1.16	2.04	14.18	
D11	3.84	2.19	1.65	1.75	2.96	
D15	5.18	3.62	1.56	1.43	6.80	
D16	5.38	2.65	2.73	2.03	23.79	
D18	5.90	3.20	2.70	1.84	16.55	
D20	3.75	2.47	1.28	1.52	17.73	11.1

5.1 The effect of hyperglycaemia on cholesterol efflux

All efflux experiments were performed in cells of less than passage number 6. There were no differences in efflux according to passage number. When analysing individual groups, ApoA-I-mediated cholesterol efflux from fibroblasts was reduced in patients with diabetes compared to control subjects (1.8% versus 2.8%, $p=0.04$; figure 5.4) and correlated inversely with fasting plasma glucose levels ($\rho = -0.50$, $p=0.01$; figure 5.5) but not significantly with HbA1c ($\rho = -0.30$, $p=0.07$). After adjusting for protein concentration, efflux remained significantly lower in patients with diabetes compared to healthy subjects (4.5% versus 11.2%). There was no significant relationship between ApoA-I-mediated cholesterol efflux and age or body weight. ApoA-I-mediated cholesterol efflux from fibroblasts was negatively associated with waist circumference ($r= -0.53$, $p<0.01$) but this relationship was dependent on fasting plasma glucose level (bivariate analysis; waist circumference $p=0.3$, fasting plasma glucose concentration $p=0.06$).

5.2 Relationships between fibroblast ABCA1 and plasma HDL cholesterol

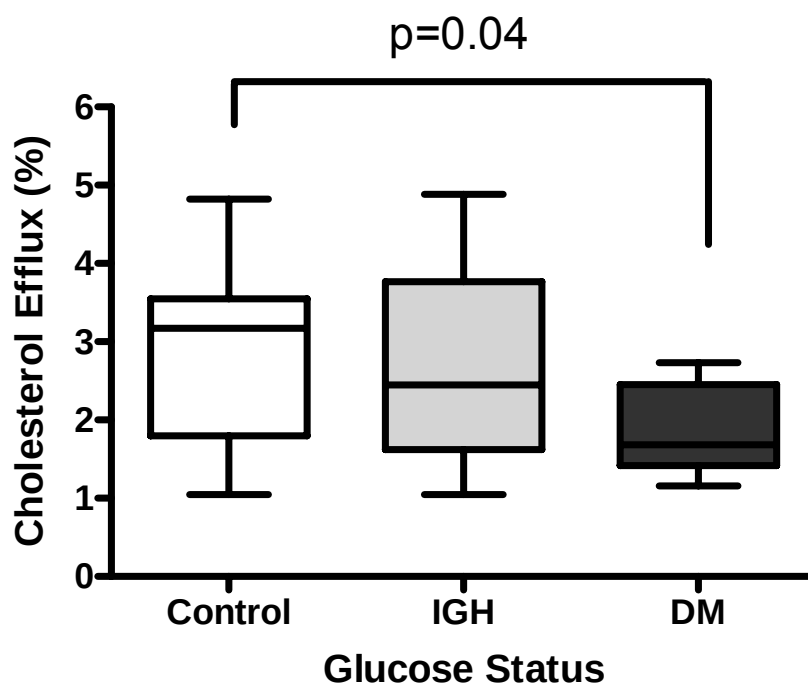
Overall there was no significant relationship between fibroblast ABCA1 expression and protein concentrations ($p=0.26$). ABCA1 gene expression did relate to plasma HDL-C concentrations with borderline significance ($n=21$, $\rho=0.41$, $p=0.06$). In fibroblasts taken from healthy individuals, there was a close correlation between ABCA1 expression and plasma HDL-C concentrations ($n=7$, $\rho=0.93$, $p<0.007$). ABCA1 expression was closely related to ABCG1 expression in fibroblasts ($n=21$, $\rho=0.80$, $p<0.001$) and which correlated with LXR α RNA ($\rho=0.78$, $p<0.001$). Multiple linear regression confirmed that LXR α predicted ABCA1 levels independent of ABCG1 (LXR α $p=0.004$, ABCG1 $p=0.21$).

Fibroblast ABCA1 expression positively related to total cholesterol efflux ($n=21$, $\rho=0.50$, $p=0.02$) but not significantly with ApoA-I-specific efflux ($n=21$, $\rho=0.38$, $p=0.09$). ABCG1 gene expression in skin fibroblasts did not correlate with cholesterol efflux ($\rho=-0.02$, $p=0.92$). ABCA1 protein concentration did not relate significantly to function ($\rho=-0.36$, $p=0.08$ versus ApoA-I specific efflux). Despite this, there was a significant and positive relationship between cholesterol efflux and plasma HDL-C concentrations ($\rho=0.41$, $p=0.02$; figure 5.6).

Table 5.2

Summary of Spearman correlation of ABCA1 parameters in fibroblasts. (FPG=Fasting plasma glucose).

	FPG	Hba1c	HDL-C	Protein	Efflux
Gene expression	0.07 P=0.77	- 0.24 P=0.30	0.41 P=0.06	-0.26 P=0.26	0.38 P=0.09
Protein Concentration	0.13 P=0.53	0.29 P=0.16	- 0.02 P=0.93		- 0.36 P=0.08
Cellular Efflux	- 0.5 P=0.01	- 0.30 P=0.07	0.41 P=0.02		

**Figure 5.4**

Cholesterol efflux in skin fibroblasts according to glycaemic status (mean and standard error). IGH=Impaired glucose homeostasis, Cells were loaded with tritiated cholesterol containing 30mg/l unlabelled cholesterol for 24 hours. Cells were subsequently washed with PBS/BSA (0.2% v/v) and efflux was commenced using 10 μ g/ml Apo-AI (Sigma) for 20 hours at 37°C and 5% CO₂. DM=Type 2 Diabetes. n=25, Control versus DM; p=0.04. Control vs. IGH; p=0.39.

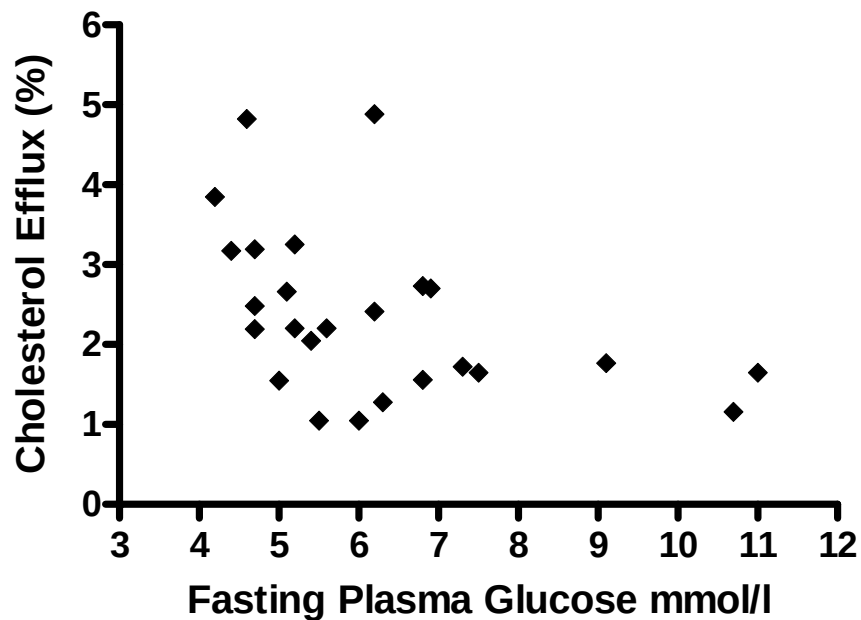


Figure 5.5

The relationship between fasting glucose and fibroblast ApoA-I-mediated cholesterol efflux Cells were loaded with tritiated cholesterol containing 30mg/l unlabelled cholesterol for 24 hours. Cells were subsequently washed with PBS/BSA (0.2% v/v) and efflux was commenced using 10 μ g/ml Apo-AI (Sigma) for 20 hours at 37°C and 5% CO₂. (n=25, rho= -0.50, p=0.01).

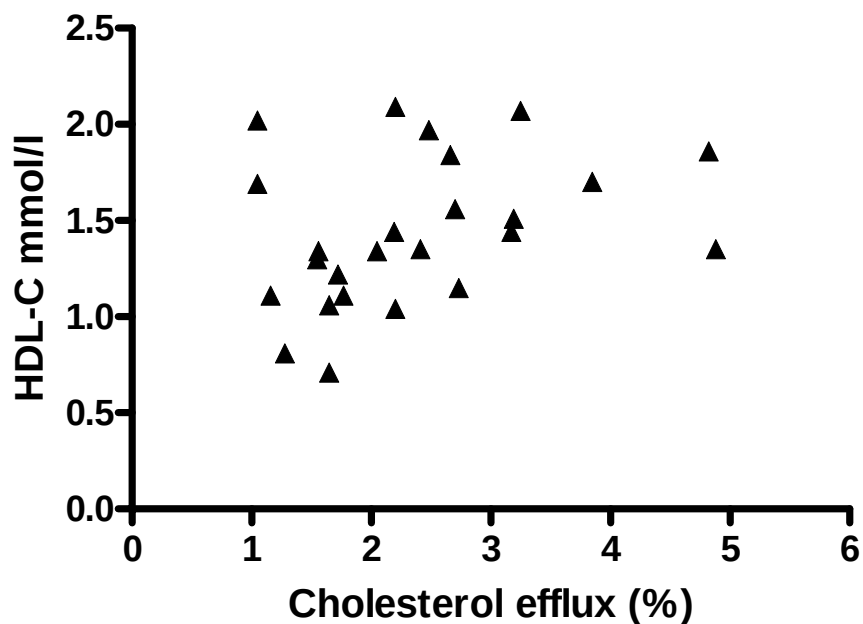


Figure 5.6

Positive relationship between ApoA-I mediated efflux in fibroblasts and plasma HDL-cholesterol concentration at the time of sampling. Cells were loaded with tritiated cholesterol containing 30mg/l unlabelled cholesterol for 24 hours. Cells were subsequently washed with PBS/BSA (0.2% v/v) and efflux was commenced using 10 μ g/ml Apo-AI (Sigma) for 20 hours at 37°C and 5% CO₂. n=25, rho=0.41, p=0.02.

5B Phospholipid translocation function in lymphocytes

5.3 The effect of chronic glycaemia on PS translocation in diabetic subjects

There was substantial inter-assay variability in this method. Fresh control samples assayed separately on 5 occasions yielded a CV of 31% (figure 5.7).

Apart from a marked difference in a single subject, there was no difference in calcimycin-induced PS translocation (flop) in 4 diabetic subjects versus a single healthy subject ($p=0.52$), see figure 5.7. It was not possible to repeat experiments with participant D5.

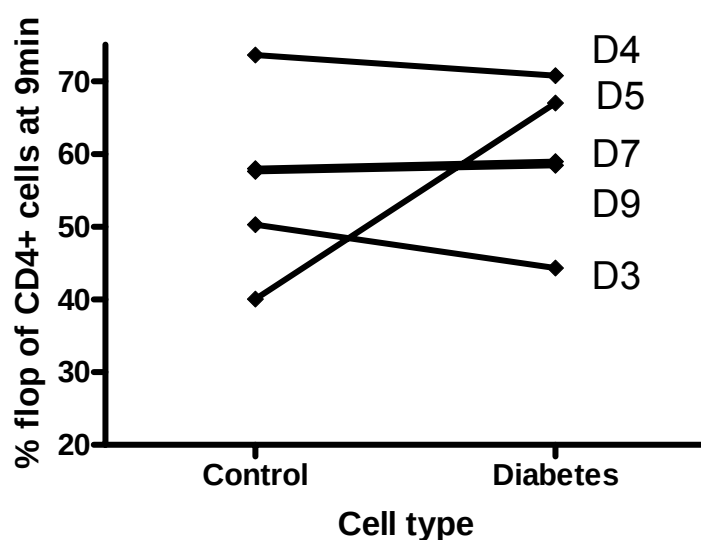


Figure 5.7

Comparison of phosphatidylserine (PS) membrane translocation in blood CD4 lymphocytes in response to calcimycin. Cumulative PS translocation was measured by flow cytometry for 9 minutes after addition of calcimycin. Measurements are shown in 5 patients with Type 2 diabetes (D3,4,5,7,9) and are compared to simultaneous measurements in a healthy male subject (control) ($p=0.52$).

5.4 The acute effect of glucose on phosphatidylserine translocation

This was assessed in CD4⁺ cells at 3 time points during an OGTT. Baseline PS flop and response to calcimycin was compared to 1hr and 2hr time points respectively. There were no consistent differences observed compared with baseline, illustrated in figure 5.8. Change in PS flop did not relate to changes in glucose concentrations at corresponding time points ($n=9$, $p=0.71$ at 1hr and $p=0.11$ at 2hr). A single control subject (C8) did exhibit a large reduction in PS flop from baseline at both time points.

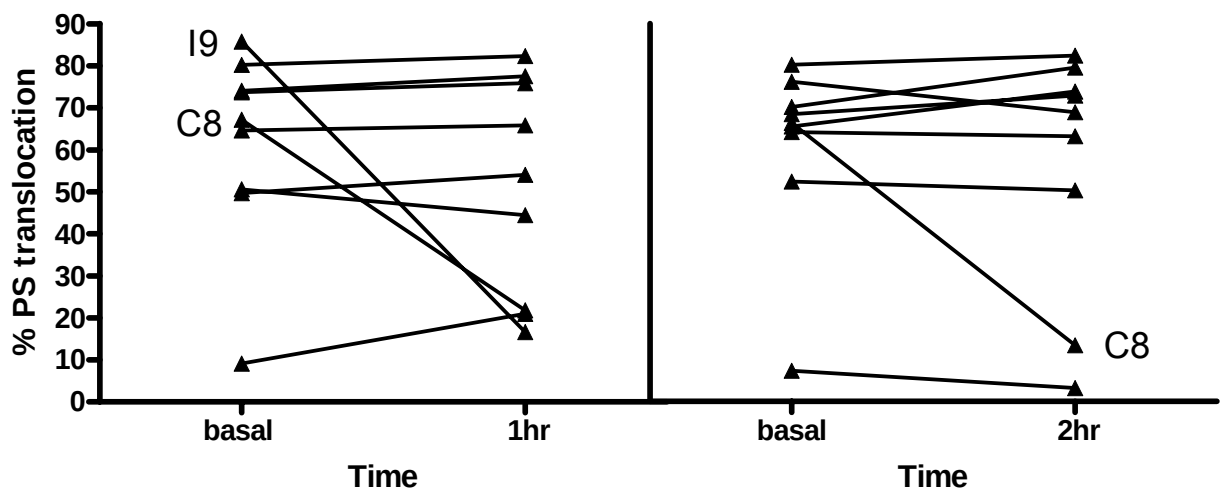


Figure 5.8

Phosphatidylserine (PS) translocation in blood CD4 lymphocytes during an oral glucose tolerance test. Cumulative PS translocation was measured by flow cytometry for 9 minutes after addition of calcimycin. This is compared to PS translocation performed 1 hour and 2 hours after oral glucose administration. (Participants who exhibited marked differences in response to glucose are labelled, C=Control participants, I=patients with impaired glucose homeostasis).

There were no significant relationships between phospholipid membrane translocation, as measured by PS flop, and cholesterol efflux, leukocyte gene expression or plasma HDL-C in participants ($p=0.61$, $p=0.78$, $p=0.27$ respectively). Based on these provisional results, together with practical

difficulties in simultaneously isolating leukocyte and lymphocyte populations for analysis, no further samples were taken for PS translocation measurements.

In summary, Apo-AI induced cholesterol efflux was reduced in skin fibroblasts taken from patients with DM. Furthermore, there appeared to be a negative association between cholesterol efflux and blood glucose levels at the time of initial skin sampling. Efflux did not independently vary with age nor adiposity but did positively correlate with plasma HDL-C concentrations in 25 subjects.

In skin fibroblasts, ABCA1 gene expression did not significantly relate to protein concentrations or function. ABCA1 gene expression did positively and significantly relate to total efflux (Apo-AI mediated and non-specific). There was a relationship between fibroblast ABCA1 gene expression and plasma HDL-C concentration of borderline significance. This relationship was significant in the subset of healthy men. These relationships are summarised in table 5.2.

In lymphocytes, variability in PS transport was observed between experiments and PS translocation did not vary with glucose homeostasis during an OGTT. PS transport did not significantly relate to other ABCA1 measurements in fibroblasts or leukocytes.

6.0 Carotid intima-media thickness

CIMT measurements were performed by B-mode ultrasound and were used as a quantitative measure of atherosclerosis. People with *ABCA1* mutation have been demonstrated to have increased CIMT. *ABCA1* gene expression was hypothesised to be inversely related to CIMT.

6.0 CIMT measurements

The reproducibility of measurements of IMT was assessed in the Irvine vascular laboratory, the CV for the measurement of IMT was 5.8%.

Measurements taken with current equipment were compared to that taken by the same radiographer using a previous scanner. The intra-observer variability in 67 measurements was 9.0% with a mean standard deviation of 0.08cm. Measurement methods are stated in an earlier section (2.04) and all measurements were performed by a single senior radiographer. Results are displayed in Table 6.1. Figure 6.1 illustrates ultrasound CIMT. The ultrasound image was taken from a diabetic participant (D12) and demonstrates a lipid rich plaque at the carotid bulb.

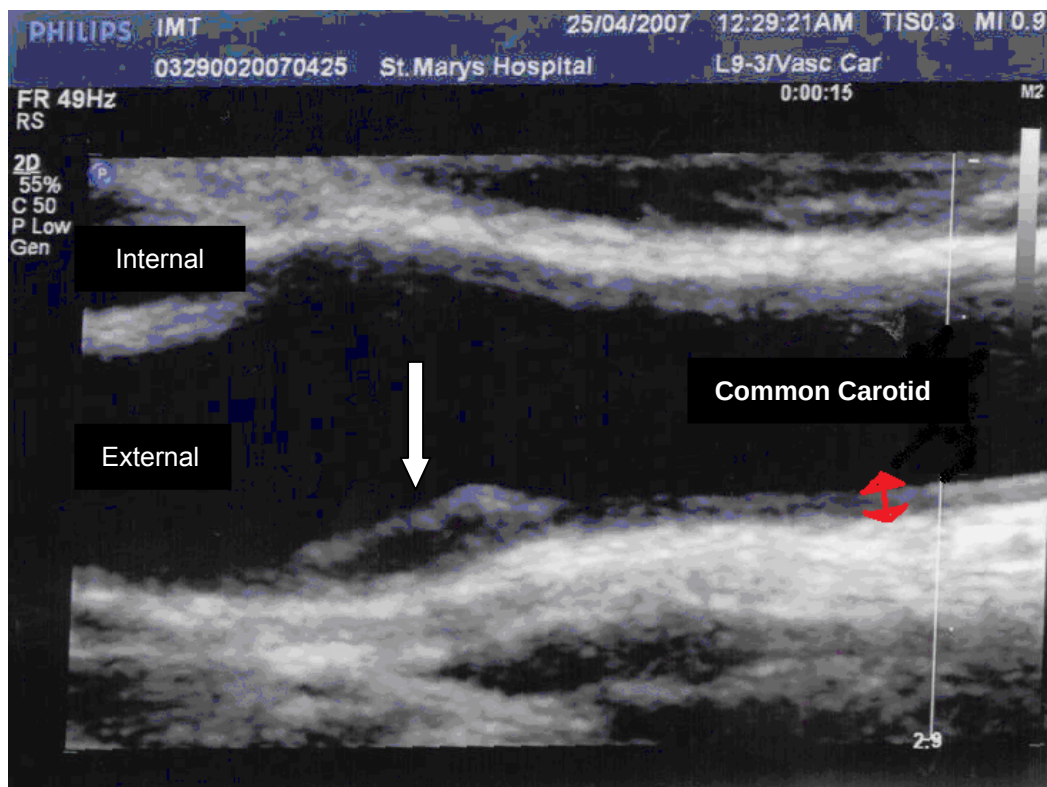


Figure 6.1

Longitudinal ultrasonographic view of the left common carotid artery taken from a participant with diabetes. A lipid rich atherosclerotic plaque can be seen at the bifurcation of the carotid artery (arrow). CIMT measurement was 0.072cm at the posterior wall as shown (double headed arrow).

Table 6.1

Carotid intima media thickness (CIMT) measurements (cm) in participants (n=68). Qualitative comments for images and measurements are included. (ICA=internal carotid artery, CC=common carotid artery).

Participant	LEFT	RIGHT	MEAN	Notes
C1	0.038	0.05	0.044	
C2	0.042	0.045	0.044	
C3	0.058	0.044	0.051	
C4	0.044	0.054	0.049	
C5	0.042	0.048	0.045	
C6	0.071	0.07	0.071	
C7	0.074	0.067	0.071	
C8	0.054	0.062	0.058	
C9	0.050	0.050	0.050	
C10	0.074	0.067	0.071	
C11	0.059	0.060	0.060	
C12	0.052	0.049	0.051	
C13	0.050	0.057	0.054	
C14	0.074	0.066	0.070	small R.bulb plaque
C15	0.100	0.074	0.087	bilateral ICA plaque <50%
C16	0.043	0.048	0.046	small calcified spec in R.CC
C17	0.048	0.046	0.047	Lean subject, checked
C18	0.058	0.052	0.055	L.IC -29% diameter reduction
C19	0.051	0.048	0.050	
C20	0.068	0.072	0.070	
C21	0.069	0.082	0.076	Generalised thickening
C22	0.083	0.077	0.080	
C23	0.089	0.079	0.084	
C24	0.051	0.064	0.058	
C26	0.070	0.070	0.070	
C27	0.089	0.073	0.081	L.bulbar plaque 42% reduction
C28	0.053	0.069	0.061	
C29	0.080	0.073	0.077	Ext. Carotid plaques only R>L
C31	0.053	0.063	0.058	
I-1	0.070	0.070	0.070	
I2	0.053	0.05	0.052	
I3	0.057	0.059	0.058	30-40% ICA stenosis
I4	0.068	0.063	0.066	L. Carotid bulb plaque
I5	0.08	0.079	0.080	
I6	0.072	0.07	0.071	minor plaque R.bulb
I7	0.094	0.088	0.091	

I8	0.072	0.065	0.069	
I9	0.071	0.067	0.069	
I10	0.077	0.058	0.068	R.ICA anomaly (kink)
I11	0.081	0.070	0.076	Bilateral bulb plaques<50%
I12	0.077	0.071	0.074	L.ICA plaque<50%
I13	0.058	0.061	0.060	R.ICA small plaque
I14	0.053	0.057	0.055	
I15	0.073	0.073	0.073	R.ICA small plaque
I16	0.052	0.078	0.065	
I19	0.071	0.053	0.062	
C25	0.080	0.058	0.069	L.small plaque at L.ICA
I20	0.058	0.042	0.050	
D13	0.072	0.08	0.076	R.bulb plaque of 0.135cm
D2	0.068	0.047	0.058	R.ant wall thicker
D3	0.069	0.069	0.069	Small calcium fleck L&R ICA
D4	0.073	0.064	0.069	L.small plaque
D5	0.081	0.081	0.081	
D6	0.055	0.058	0.057	
D7	0.103	0.075	0.089	
D9	0.068	0.074	0.071	
D10	0.062	0.078	0.070	L.distal CC small plaque
D11	0.086	0.065	0.076	
D12	0.072	0.058	0.065	L.large bulbar plaque (photo)
D14	0.094	0.067	0.081	Left diffuse thickened
D15	0.062	0.063	0.063	R.small ca fleck
D16	0.104	0.049	0.077	L.plaque, R.bulbar thickening
I17/D	0.066	0.052	0.059	
D17	0.072	0.063	0.068	R.ICA small ca fleck
D18	0.205	0.091	0.148	
D19	0.048	0.058	0.053	
I21	0.054	0.045	0.050	
D20	0.097	0.096	0.097	L double IMT, R.small plaque

6.01 Demographic and biochemical determinants of CIMT

There was a trend to increased CIMT measurements, with increasing glycaemia across the three glycaemic categories, see figure 6.2 ($p=0.09$). Patients with DM had higher CIMT than healthy men ($p=0.049$). There were no significant differences between other groups.

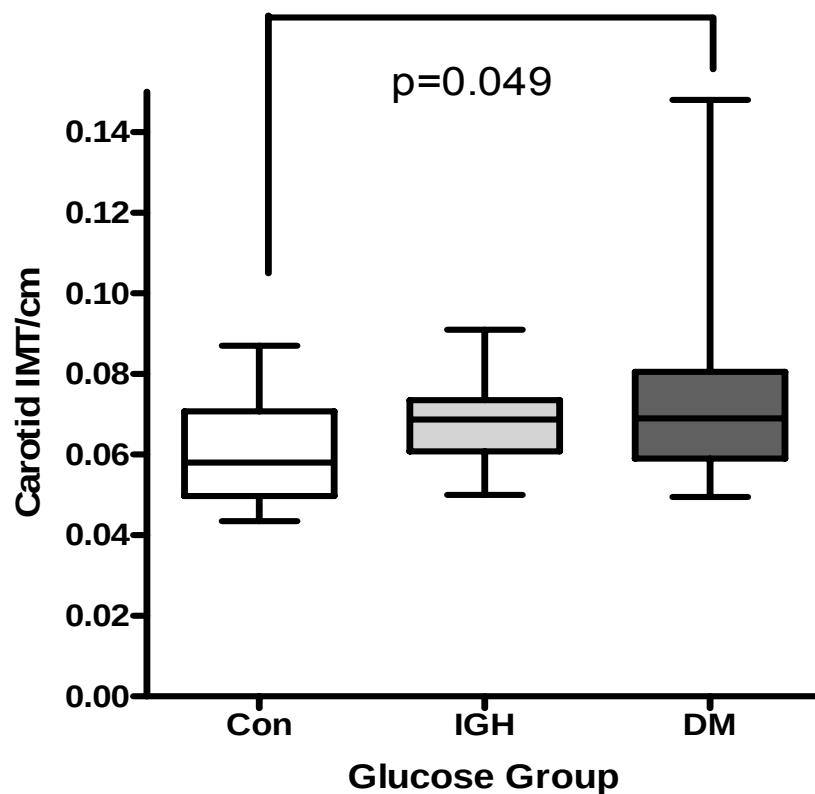


Figure 6.2

Ultrasound measurement of carotid intima-media thickness (CIMT) according to glycaemic status of subjects ($n=68$, $p=0.09$; Con vs DM $p=0.049$) (Con=control, IGH=impaired glucose homeostasis, DM=type 2 diabetes).

Spearman correlation demonstrated that several variables associated with CIMT. Age and measures of glycaemia and adiposity positively related to CIMT. Lipid and blood pressure parameters did not relate to CIMT (table 6.2).

Table 6.2

Correlations comparing demographic and biochemical characteristics to carotid intima-media thickness (CIMT) measurements (cm) (n=68). P-values are shown (indicates Mann Whitney test).*

PARAMETER	P VALUE
Age	0.003
BMI	0.005
Waist Circumference	0.003
Fasting glucose	0.005
Mean glucose (during OGTT)	0.0001
HbA1c	0.04
Mean Systolic BP	0.44
HDL-C	0.18
LDL-C	0.78
Triglyceride	0.11
Smoking Status	0.11*

When BMI, fasting glucose and age were entered in a multiple regression model, only age remained a significant independent predictor of CIMT ($p < 0.001$). Statistically, fasting glucose and BMI were inter-related and did not independently predict changes in CIMT ($p = 0.08$, $p = 0.16$ respectively). In contrast, mean glucose concentration (during an OGTT) did predict changes in CIMT independent of age and BMI ($p = 0.01$).

6.1 The relationship of ABCA1 to CIMT

CIMT associated significantly and negatively with ABCA1 gene expression ($p=0.046$ versus delta Ct). However, this relationship was confounded by the relationship of CIMT to glycaemia. Bivariate regression analysis demonstrates fasting glucose, but not ABCA1 expression independently predicted CIMT ($p=0.04$ and $p=0.69$ respectively). ABCG1 gene expression did not show a significant relationship with CIMT ($n=49$, $p=0.22$).

ABCA1 protein in blood leukocytes did not relate significantly with CIMT ($n=49$, $p=0.33$). Cellular cholesterol removal in skin fibroblasts, a measure of ABCA1 function, did not appear to associate with CIMT ($n=25$, $p=0.47$). However, a single patient had a cholesterol plaque at the site of CIMT measurement, which produced a skewed measurement, seen in figure 6.3. Excluding this skewed measurement, yielded results suggesting a trend between efflux and CIMT ($r = -0.35$, $p=0.095$), but this was not independent of fasting glucose on multivariate analysis ($p=0.29$). In the more detailed characterised cohort of twenty five participants, CIMT significantly associated with plasma triglyceride ($\rho = 0.63$, $p=0.009$) and negatively with HDL-C ($r = -0.50$, $p=0.012$). It is unclear why similar correlation was not seen with triglyceride and HDL-C in the entire study group.

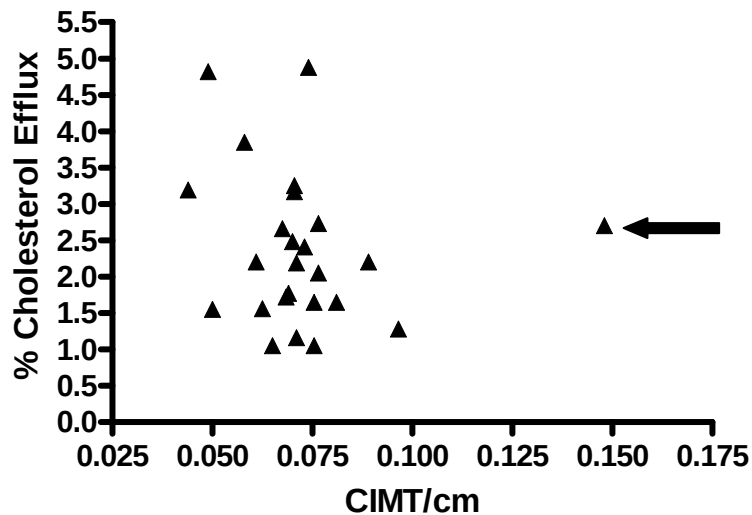


Figure 6.3

The relationship of Apo-AI mediated cholesterol efflux in skin fibroblasts to carotid intima-media thickness (CIMT), skewed data point (arrow) represents a patient who exhibited a carotid plaque at the site of measurement, $n=24$, $r=-0.35$, $p=0.095$.

In summary 68 subjects underwent CIMT measurements, patients with DM had higher CIMT measurements and CIMT positively associated with age, adiposity and glycaemia. The observed relationship between leukocyte ABCA1 gene expression and CIMT was confounded by the relationship between glycaemia and ABCA1 gene expression. Leukocyte ABCA1 protein levels did not significantly relate to CIMT. In fibroblasts, there was a trend towards a negative relationship between efflux and CIMT.

7.0 The effect of adiposity, age and glucose lowering treatment on ABCA1

It has been proposed that unsaturated fatty acids negatively regulate ABCA1 gene expression. Excess adiposity is associated with insulin resistance which is the cause for subsequent impaired glucose homeostasis. Therefore, measurements for both clinical and biochemical markers of adiposity were taken to investigate if these confounded the proposed relationship between glycaemia and ABCA1 gene expression and function. Our original findings suggested that ABCA1 gene expression may be related with age and this analysis in the present cohort is presented below. The effect of a glucose lowering treatment on leukocyte ABCA1 gene expression and protein concentrations was additionally undertaken.

7.0 The relationship of plasma fatty acids with ABCA1

Plasma NEFA concentrations were measured in fasting plasma samples taken from participants who underwent detailed gene expression and functional studies. Fasting plasma sample was unavailable in one subject. Fasting NEFA levels, positively related to both waist circumference (n=24, $\rho=0.42$, $p=0.043$) and HbA1c ($\rho=0.46$, $p=0.023$) and negatively with HDL-C ($\rho=-0.40$, $p=0.023$). Bivariate analysis (log transformed data) demonstrated waist circumference but not HbA1c independently predicted NEFA concentration ($p=0.03$ and $p=0.43$ respectively).

There was no significant relationship between ABCA1 mRNA and fasting NEFA concentration ($\rho=-0.17$, $p=0.42$). No significant relationships were observed between NEFA and either LXR α ($p=0.98$) or PPAR γ gene expression ($p=0.50$).

Cholesterol efflux nor CIMT significantly related to NEFA concentration ($p=0.53$ and $p=0.51$ respectively).

7.1 The relationship of age to ABCA1 expression and function

Leukocyte ABCA1 gene expression or ABCA1 protein concentrations did not alter with increasing age ($n=63$, $p=0.18$ and $n=49$, $p=0.22$ respectively). These findings contrast with results of gene expression our earlier study in younger men (324). Cholesterol efflux measured in skin fibroblasts (ABCA1 function) did not change with age ($n=25$, $p=0.37$).

7.2 The effect of glucose lowering on ABCA1 gene expression and protein content

Patients with DM were treated for six weeks with an AMP kinase activator, Metformin. Fasting samples were taken to measure ABCA1 expression and protein concentration before and after treatment. Twelve patients with DM agreed to participate with the treatment over a six week period. Over the period there was a mean reduction in Hba1c was 0.59% (range -1 to 2.7%) $p=0.003$ and non-significant reduction in fasting glucose was 0.55mmol/l (-1 to 2.4mmol/l) ($p=0.23$). Patients with the highest baseline Hba1c achieved the greatest reduction in glucose. There was a non-significant mean weight loss of 0.98 kg weight (-4.3 to 5.5 kg) observed ($p=0.15$). As expected, there was correlation between change in glucose and change in HbA1c ($r=0.72$, $p=0.009$). There was no significant relationship between change in weight and change in Hba1c ($\rho=0.42$, $p=0.18$).

Gene and protein data were available in 11 of the 12 subjects. Change in normalised gene expression in blood leukocytes did not significantly relate to change in Hba1c ($r=0.14$, $p=0.69$) or change in glucose ($r=0.36$, $p=0.27$), figure 7.1. Change in protein content did not appear to alter in accordance to change in glycaemia ($p=0.25$ versus delta Hba1c and $p=0.25$ versus delta glucose). Fasting NEFA concentrations did not vary significantly with change in glycaemia ($r=-0.19$, $p=0.59$) or change in weight ($p=0.15$). There was no relationship seen between delta NEFA and change in gene expression or protein expression ($p=0.95$ and $p=0.65$ respectively). There did appear to be a negative trend between change in weight and change in gene expression but this did not reach significance ($r=-0.48$, $p=0.14$) figure 7.2. Change in gene expression of ABCA1 and change in protein concentrations were positively related with borderline significance ($\rho=0.79$, $p=0.07$).

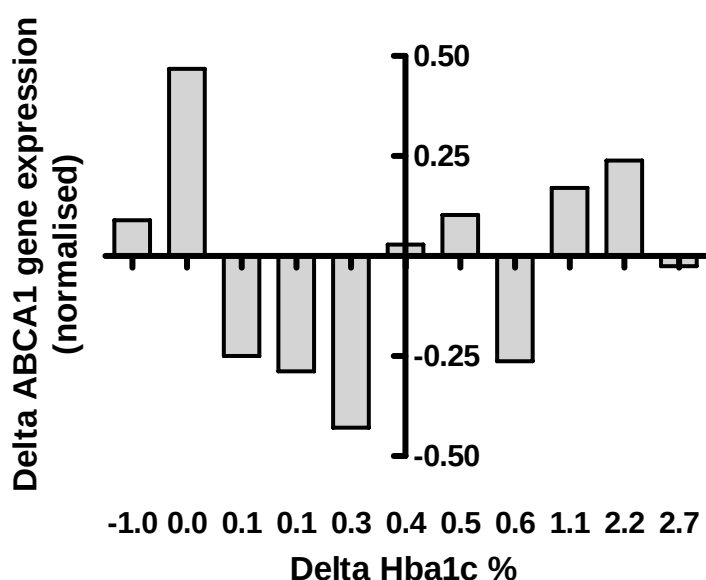


Figure 7.1

Relationship between change in Hba1c induced by glucose lowering treatment and change in leukocyte ABCA1 gene expression (measured by PCR and normalised to β -Actin) in patients with type 2 diabetes treated for six weeks with Metformin; $n=11$, $r=0.14$, $p=0.69$.

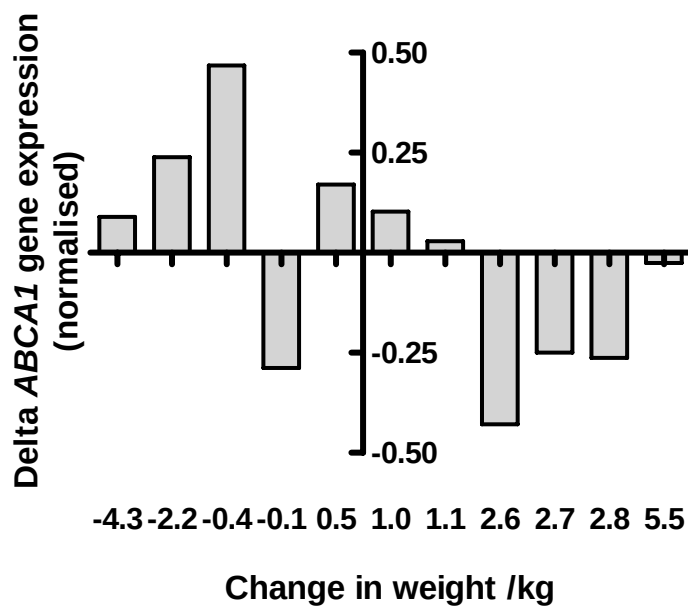


Figure 7.2

Relationship between change in weight and change in leukocyte ABCA1 gene expression (measured by PCR and normalised to β -Actin) in response to glucose lowering treatment with Metformin for six weeks in patients with type 2 diabetes; $n=11$, $r=-0.48$, $p=0.14$.

In summary, waist circumference independently predicted plasma NEFA concentrations. There were no significant relationships between measures of ABCA1 with plasma NEFA concentrations. Furthermore, measurements of ABCA1 gene expression, protein level or function did not vary significantly with age. The effect of glucose lowering treatment was assessed over six weeks in 12 subjects and reductions in glucose were not associated with significant changes in leukocyte ABCA1 gene expression or protein concentrations. Some subjects experienced weight loss during the treatment and a tentative inverse relationship between weight loss and leukocyte ABCA1 gene expression was observed. Changes in ABCA1 gene expression in leukocytes during treatment were concordant with changes in protein concentrations.

8.0 GENERAL DISCUSSION

8.1 ABCA1 and effects of glycaemia

Our pilot study demonstrated an association between fasting glucose and ABCA1 expression in blood leukocytes in normoglycaemic men (324). The basis of the current hypotheses and study was to investigate the effect of glycaemia on *ABCA1* expression and function in humans, by studying patients with impaired glucose homeostasis. These were age-matched, drug naive men with no differences in blood pressure or total cholesterol.

In blood leukocytes, patients with DM had lower ABCA1 expression in keeping with our original results and hypothesis. Moreover, a continuous negative relationship was observed between gene expression and glycaemia (both Hba1c and fasting glucose) in this larger group of individuals compatible with results from our original study. To my best knowledge, this is the first study to report this finding. A continuous relationship between ABCA1 expression and Hba1c was not seen in the original pilot study, this can possibly be explained by the narrow range of Hba1c values seen in healthy individuals.

Most of the decline in gene expression occurred up to an HbA1c level of 7.5%, with little further fall above this level. These results contrast with results in leukocytes from Hoang and colleagues (322) as that study did not find a difference in gene expression in patients with DM but did find a positive association with physical activity. One third of patients in this study were receiving oral hypoglycaemic medications for diabetes and patients had lower LDL-C levels than healthy subjects this may have influenced these findings.

My results appear also to conflict with that of Zhou and Mauldin et al., where monocytes and macrophages were studied. These studies showed that

expression and function of ABCG1 (but not ABCA1) was decreased in diabetic patients (185;323). Mauldin discussed a major draw-back of their study; patients were not well characterised, with little information available on demographics, clinical disease and medications (185). Disease related factors and medications could have influenced these findings as discussed below. Significant numbers of the patients studied by Zhou and colleagues, had evidence of microvascular complications; 40% with retinopathy and 43% had some evidence of nephropathy (323). Data from animal experiments indicate that complications such as nephropathy can reduce macrophage ABCA1 and increases cellular cholesterol content. Moreover, agents used in the treatment of diabetic nephropathy can restore macrophage ABCA1-mediated cholesterol efflux (337). More patients in my study were receiving these classes of medication for hypertension (either Angiotensin converting enzyme inhibitor or Angiotensin II receptor antagonist). However ABCA1 mRNA, protein and function remained lower in patients compared to healthy men with no difference in blood pressure across groups. It is therefore unlikely that these treatments had significant positive effects on ABCA1 expression and function in this study.

Concomitant medications used for these conditions are likely to have had bearing on results obtained by Zhou *and* colleagues. Patients were treated with hypoglycaemic medications and one-third were receiving insulin treatment (323). My study has the distinct advantage of investigating drug naive patients, who were not receiving either lipid lowering or glucose lowering medications and were clinically free of diabetes-related complications. It is feasible that hypoglycaemic treatments used by diabetic patients in the previous studies may have contributed to the discrepancies compared to our findings. For example,

insulin decreases human macrophage ABCA1 and ABCG1 expression *in-vitro* and the oral hypoglycaemic agent, glyburide has been shown to inhibit ABCA1 mediated cholesterol efflux (338;339). My results are compatible with observations made by Forcheron et al., in monocytes taken from patients with DM and dyslipidaemia (301). There was also a trend to reduced monocyte LXR α expression in that study, this was not observed in my experiments. ABCA1, ABCG1 and LXR gene expression were positively related in leukocytes as well as in fibroblasts, confirming a common regulatory pathway *in-vivo* in humans, similar to that *in-vitro*.

In the present study, skin fibroblasts were used to assess ABCA1 function, as a surrogate for arterial macrophages. Skin fibroblasts, have been employed for this purpose in previous studies where the functional consequences of genetic variation in *ABCA1* have been studied (246;247). My tritiated cholesterol-based technique for assessment of ApoA-I-mediated cholesterol efflux was derived from a validated methodology (192). Cellular cholesterol export was successfully inhibited by a pharmacological agent known to block the action of ABCA1 which confirmed specificity of the assay. I have demonstrated in healthy men, gene expression in fibroblasts was closely related to plasma HDL-C concentrations and there was a tentative relationship observed in patients with hyperglycaemia. Fibroblast ApoA-I-mediated cholesterol efflux was reduced in patients with DM and a continuous negative relationship was seen with glycaemia. To my knowledge, this is the first such observation in humans without a known *ABCA1* mutation. Fibroblasts have been used previously to investigate the effects of genetic abnormalities on ABCA1 function, subjects in the present study were from a diverse ethnic background and, therefore,

ethnicity related variants in *ABCA1* are unlikely to have contributed to observed differences in *ABCA1* gene expression and function. I have further demonstrated that *ABCA1*-mediated cholesterol efflux is positively related to plasma HDL cholesterol concentration, confirming its key role in HDL particle formation. These conclusions contrast with those from studies in patients with the metabolic syndrome (with and without hyperglycaemia) where cholesterol efflux was unrelated to HDL concentration (184). *ABCG1*-mediated cholesterol export is not thought to be a major contributor to maintaining plasma HDL cholesterol concentration but seems to be important in preventing atherosclerosis in animals (72). A positive relationship between *ABCA1* and circulating HDL-C in patients with diabetes has only been previously demonstrated in patients with common gene variants, who had evidence of proteinuria and renal dysfunction (340).

Reduced *ABCG1*-mediated cholesterol efflux in monocytes has been reported in diabetic patients by Zhou and colleagues and results for *ABCA1*-mediated efflux were not reported in this study (323). Mauldin and co-workers showed a 30% reduction in *ABCG1*-mediated efflux in macrophages taken from patients with DM with no difference in *ABCA1*-induced export. Macrophages, in this study, were cultured in autologous (diabetic) serum which appears physiological, but serum from diabetic patients is likely to contain altered concentrations of pre- β HDL compared to healthy people which could influence cellular efflux results (341). It is noteworthy that, unlike *ABCA1*, *ABCG1* transporter dysfunction has not been reported to be associated with a human clinical disease. Due to the synergistic relationship between *ABCA1* and *ABCG1*, an element of redundancy may exist for *ABCG1* as suggested from

results in animal knockout studies (74). However, ABCA1 seems to be crucial as it initiates cholesterol export and provides a lipidated ApoA-I substrate for subsequent ABCG1 action.

It should be noted that my gene expression measurements in leukocytes and cholesterol efflux and gene measurements in fibroblasts were made against very different backgrounds. Leukocyte gene expression likely relates to the conditions under which the leukocytes were taken at the time of sampling, whereas fibroblasts had undergone prolonged *in-vitro* culture before measurements were made. Consistent with this, I demonstrated both ABCA1 gene expression and protein concentrations were reduced in leukocytes taken from diabetic patients.

Prolonged *in-vitro* conditions may account for the lack of association between fibroblast RNA level and ApoA-I-mediated cholesterol efflux. Acidic environment can negatively influence efflux this finding may have relevance in advanced atherosclerotic plaques (342). Hoang and colleagues, similar to our findings in fibroblasts and leukocytes, did not find a relationship between human leukocyte and skeletal muscle ABCA1 gene expression (322). Alternatively, there could be discrepancies between ABCA1 gene expression, translation and protein activity and such discrepancies have been reported in animals (343). The observed lack of relationship between gene expression, protein concentrations and function in human skin fibroblasts should be further investigated. Importantly, however, despite prolonged *in-vitro* culture at constant hyperglycaemia (25mmol/l), a relationship was still apparent between fibroblast cholesterol efflux and the *in-vivo* glucose concentrations the fibroblasts had been exposed to in the subjects from whom they were taken. These findings

were in spite of DM patients having higher protein concentration in fibroblasts which indicates either altered protein localisation or protein modification due to a “diabetic milieu” leading to accumulation of dysfunctional protein. Future experiments should aim to explore whether DM patients have altered subcellular and plasma membrane localisation by interrupted shuttling of ABCA1. This could be assessed using biotinylation methods and would help explain the current findings in skin fibroblasts. Alternatively, DM patients may have physicochemical differences in cellular membranes that prevent Apo-AI interacting with ABCA1. Tang and Oram have reviewed the possible mechanisms by which diabetes could adversely affect ABCA1; AGEs have been shown to impair ABCA1 function in cultured cells and fatty acids enhances degradation of the protein (344). The effect of AGE could help explain our findings of reduced ABCA1 function in cultured fibroblasts but is not consistent with the observed increase in ABCA1 protein. Some of the proposed mechanisms by which diabetes negatively influences ABCA1 protein seem to be independent of effects on RNA. Comparing the effects of different concentrations of cholesterol on ABCA1 gene expression in fibroblasts could help establish if there are altered set points for gene expression in patients with diabetes.

The relationship of transporter function in fibroblasts to plasma glucose levels at the time of sampling implies the possible existence of mechanisms that can sustain a prolonged metabolic memory on the part of the cells. This is compatible with observations made by Senanayake and colleagues, where there was no difference in gene expression in monocytes, whether cultured in physiological or high glucose concentrations in either DM patients or healthy

individuals (313). In complete contrast, a study which investigated several ABC transporters in monocyte-derived macrophages from healthy individuals, demonstrated high glucose (25mmol/l) incubated for 72 hours specifically decreased gene expression of both ABCA1 and ABCG1. Protein levels were also decreased in response to high glucose incubation (345). If this is confirmed to occur *in-vivo*, as suggested by Mauldin et al.,(185) one can cautiously conclude glucose may compromise synergistic mechanisms involved in cellular cholesterol removal.

An alternative functional measure of ABCA1 was conducted in blood lymphocytes. This did not yield sufficient results due to practical limitations. There was no difference in phospholipid transport observed in hyperglycaemic patients compared to healthy men and no obvious differences in gene expression were seen during an OGTT. There was substantial inter-assay variability in this method and it is likely this was due to assay variability rather than for biological variation as samples from patients with DM yielded similar results to measurements taken from a single control subject (figure 5.3).

PS transport was considered to be a valid functional measure of ABCA1 and abnormalities in PS transport have been reported in a patient with a rare *ABCA1* mutation (210). However, in my study, the measure of exofacial PS membrane transport did not quantitatively relate to cholesterol efflux in fibroblasts, the latter was demonstrated to be specific to ABCA1. The measurement of PS flop, therefore, may not represent a reliable measure of ABCA1 activity. The effect of probucol on PS flop has not been studied and this could help clarify whether ABCA1 is required for this function as a number of mechanisms promoting phospholipid flop have been identified (346). PS flop

has been shown to occur independent of *ABCA1* and may be more dependent on changes in membrane organisation (334).

The acute effect of glucose on leukocyte *ABCA1* gene expression was investigated in my study using an OGTT in healthy men and patients with IGH. There appeared to be an increase in gene expression at 1 hour and 2 hour time points compared to baseline, with a concordant relationship between the change in glucose and change in gene expression at 2 hours. This may be related to an acute effect of insulin release, but the physiological relevance of this is unclear and would require further investigation, ideally employing a physiological method such as a euglycaemic-insulin clamp. This observation does indicate that gene expression of *ABCA1* in circulating blood leukocytes is dynamic and changes may occur in response to blood glucose concentrations. Whether these changes are due to acute hyperglycaemia or other related factors is unknown and could be further investigated by the administration of intravenous glucose. These results would be compatible with the view that *ABCA1* RNA is a rapidly turned over with an estimated half-life of 1-2hrs (276). Both leukocyte protein and efflux (*ABCA1* function) in skin fibroblasts positively related to plasma HDL-C concentration. This proposes leukocyte and fibroblast *ABCA1* activity may either reflect hepatic activity or alternatively peripheral cell *ABCA1* function could directly contribute to formation of HDL particles. Data from animal studies imply hepatic *ABCA1* is predominantly responsible for HDL formation with a minor contribution from intestinal and haemopoietic cells (69;70;197). The contribution of peripheral cell *ABCA1* to HDL formation in humans is unknown.

A specific atheroprotective effect of leukocyte specific ABCA1 activity has been previously seen in mice. Animals lacking leukocyte ABCA1 activity had more advanced atherosclerotic lesions due to increased recruitment of inflammatory cells (198). This suggests that leukocyte ABCA1 may have an important immune function contributing to its athero-protective effect, which may be distinct from its transporter function. This area of work warrants further investigation. The idea of dual ligand function has been explored in the related nuclear receptor, LXR. Joseph and colleagues report an anti-inflammatory effect of *LXR* activation in macrophages in response to pathogens both *in-vitro* and in mice (347). The reciprocal modulation of inflammation and lipid metabolism highlights a potential interaction of lipid metabolism and inflammation at a cellular (macrophage) level. This group further showed that *LXR* modulates innate immunity by inhibiting induction of key inflammatory genes (348). A report by Tang and Oram has suggested similar anti-inflammatory properties of ABCA1 in cultured macrophages (349). This area of work provides some evidence of cross-talk between lipid metabolism and inflammation in process of atherogenesis.

8.2 Independent effects of glucose, age and adiposity

The relationship of glucose to ABCA1 gene expression in leukocytes appeared to be independent of the amount of *LXR α* RNA. There was no direct relationship between *LXR* expression and glycaemia which contrasts with conclusions made by Mitro and colleagues (296). There was no observed association between *PPAR γ* and ABCA1 expression in my study but results

from animal studies implicate that PPAR γ agonist therapy reduces blood glucose levels through the action of ABCA1 (350). This pivotal study demonstrated mice devoid of pancreatic-specific *ABCA1* developed glucose intolerance in early life. This proposes the idea that pancreatic ABCA1 activity may influence insulin secretion and subsequent glucose lowering. Whether a similar mechanisms exist in humans is unknown, but studies investigating insulin secretion in Tangier patients carrying loss of function mutations, indicate mild hyperglycaemia (351). Reports have showed that Rosiglitazone may have variable effects on glycaemia and lipids in DM patients with certain *ABCA1* gene variants (352;353). It is unknown whether the amount of baseline ABCA1 RNA alters efficacy of PPAR γ agonist therapy or has any bearing on pancreatic secretory reserve. This area merits further investigation and implies ABCA1 may have some utility as a pharmacogenetic marker in the future. Both ABCA1 and ABCG1 have now been implicated in islet cell function in mice (350;354). Contemporary animal studies have demonstrated decreased ABCA1 protein with cellular cholesterol accumulation in macrophages in different mouse models of type I diabetes (355). These observations, along with our findings in humans, indicate a common glycaemia-mediated suppression of ABCA1. In support of this, *in-vitro* work indicates that hyperglycaemia specifically down regulates ABCA1 and ABCG1 mRNA and protein content in human macrophages (345). Supplementary work in vascular smooth muscle cells proposes hyperglycaemia leads to reductions in ABCA1 mRNA and protein content with concordant changes in *ABCA1* promoter activity (356). Further work is required to explore the mechanisms involved.

Dysglycaemia and atherosclerosis are known to be more prevalent with age, raising the question whether abnormalities in RCT are an age-related process. Our previous study showed age did not independently influence leukocyte ABCA1 expression levels in healthy men (324). In my study, subjects were age-match to help evaluate this. ABCA1 expression or activity did not decline with age. Berroughi and colleagues have demonstrated an age related reduction in function of HDL particles to remove cholesterol from macrophages and extrapolated that this may be principally mediated by differences in the ABCA1 pathway (357). These findings are not supported by my results in young and middle aged men. Pre- β -1 HDL concentrations seem to be important in determining efflux by ABCA1 in people with similar total plasma HDL-C concentrations (341).

Apart from glycaemic variables, markers of adiposity appeared to independently relate to measurements of ABCA1. Leukocyte ABCA1 protein was negatively related to BMI and waist circumference. It is unclear whether this effect is mediated by inflammation, as inflammatory markers were not measured, or due to excess fatty acids associated with metabolic syndrome. Total NEFA levels in my study were not different between subject groups but NEFA concentrations did correlate independently with waist circumference. Unsaturated fatty acids have been shown to destabilise ABCA1 and promote protein catabolism (276;358;359). Plasma NEFA levels may not represent an accurate measure of cellular unsaturated fatty acid burden. Consistent with this concept, work by Senanayake and others implicated monocyte-derived macrophages in patients with DM have higher unsaturated fatty acid levels, despite similar plasma levels compared to control subjects (313). Excess cellular fatty acids may lead to

lower availability of ABCA1. Moreover, there is now evidence to show fatty acids may differentially regulate ABCA1. Palmitic acid, a saturated fatty acid, was shown *in-vitro* to increase in both gene and protein expression in cultured monocyte-derived macrophages taken from healthy subjects (345). It is thought transporter activity is modified by altering protein localisation at the plasma membrane (360) In direct contrast, linoleic acid, an unsaturated fatty acid, showed a negative effect on ABCA1 expression (345).

Cholesterol efflux in skin fibroblasts negatively related to adiposity and efflux was lower in diabetic men despite an increased level of protein compared to healthy men. Possible underlying mechanisms may involve intracellular transporter trapping, altered protein turnover or transporter dysfunction and further work is required to clarify a mechanism. Elevated concentrations of AGE and inflammatory mediators have been shown to inhibit cholesterol efflux in similar models which may have contributed to my findings (320;361).

Medications which reduce glycation of HDL, such as Metformin, have been shown to improve cholesterol efflux via the ABCG1 pathway (362). The extracellular domains of the ABCA1 transporter are usually glycosylated (205) and it is currently unknown whether excess glycation occurs or can have any direct effect on transporter function. In the present study results from eleven patients treated with metformin over a six week period are reported. Although there was no direct relationship between change in glycaemic indices and changes in gene expression, metformin and lifestyle mediated weight loss appeared to have a positive effect on gene expression. As weight loss is generally associated with improvements in glycaemia through decreased insulin resistance, insulin concentrations are likely to be a confounding factor in these

observations. Increased gene expression in response to Metformin therapy was associated with concordant changes in protein expression and this indicates modulation gene expression results in a similar variation in protein translation. It is assumed an increase in protein levels would lead to increased function but this requires confirmation in people with DM.

Future approaches to measure the effect of hypoglycaemic therapy on gene expression, must account for changes in adiposity and should be adequately powered to differentiate between these inter-related factors. A physiological study; Pioglitazone on cardiac function and large arteries (PICCOLA) has a sub-study designed to assess the effect of PPAR γ agonist therapy on leukocyte ABCA1 gene expression in patients with diabetes at Imperial College.

8.3 Dysfunctional HDL and HDL kinetics

ApoA-I modification has been reported in DM and CHD and this may play a role in modifying cellular cholesterol export. Oxidation of HDL-C particles by myeloperoxidase, an enzyme released from phagocytes, has been shown to be more common in patients with established coronary artery disease. These modified proteins were shown to be less effective in inducing cholesterol efflux in cultured cells (363).

Dysfunctional HDL-C molecules have been reported in a number of inflammatory conditions which may contribute to the observed elevated risk of CHD in such patients. Navab et al., showed defective anti-inflammatory action of HDL on LDL-C particles in coronary disease patients compared to healthy participants (124). Animal models of atherosclerosis have further shown

correlation between pro-inflammatory HDL function and lesion size (125). Short term treatment with simvastatin has been demonstrated to improve the anti-inflammatory function of HDL particles in CHD or at risk patients (364). These studies highlight the importance of the antioxidant and anti-inflammatory function of HDL which may help prevent the CHD. De Souza and colleagues have demonstrated defective function in smaller HDL particles taken from patients with MS (128). Dodani further proposes HDL dysfunction may contribute to the increased CHD commonly risk seen in South Asian people (365).

Since the landmark ApoA-I “Milano” study (240), recombinant HDL and ApoA-I mimetic molecules have been developed with the to aim to increase whole body cholesterol removal. This therapeutic approach would involve direct infusion of ApoA-I or a structurally similar molecule. The amphipathic α -helix is the major structural determinant of ApoA-I required for interaction with ABCA1 and synthetic analogues containing this structure have been shown to remove cellular cholesterol and phospholipid .(366-368)

Mukhamedova reports that up regulation of ApoA-I and ABCA1-mediated efflux in mice, leads to increased whole body RCT *in-vivo* (369). A blinded, cross over study in humans has since investigated the effects of injecting reconstituted HDL into male patients with DM. This resulted in an improvement in the cholesterol removal capacity of plasma as early as 1 hour after injection, with up to a 60% increase in efflux by 72 hours. Improvements in inflammatory parameters were also noted (370).

Most interestingly, ApoA-I therapy has been associated with improvements in glucose metabolism (371). Recombinant HDL infusion in patients with DM has

reported to reduce plasma glucose through activation of skeletal muscle AMPkinase (372). It is noteworthy that an increase in insulin concentrations was also observed in this study. ApoA-I-mimetic therapy is biologically likely to overcome adversely modified ApoA-I particles in DM or CHD, but may not redress diminished transporter function. It is not known whether this type of treatment will have beneficial effects on atherosclerosis in humans, but this line of therapy remains a viable option for future drug development. It is entirely possible ApoA-I-based therapy may result in beneficial metabolic effects beyond lipid metabolism and raises questions whether low HDL-C is a cause or consequence of glucose intolerance.

A further explanation for the discordant results in different tissues is that mechanisms involved in regulation of ABCA1 in fibroblasts may be distinct from that in blood leukocytes. In addition the environment in which these cells function may affect cellular responses.

The decreased HDL-C seen in patients with DM and insulin resistance has been recognised, to date, to be due to increased particle catabolism secondarily to increased hydrolysis of triglyceride enriched particles via the action of HL and CETP (165;166). ApoA-I or HDL synthesis has not been reported to be compromised although others have postulated a decrease in RCT in patients with DM (309).

Our results, which show a positive relationship between fibroblast transporter function and plasma HDL concentration at the time of sampling, suggest a possible alternative view; decreased ABCA1 function may in-part contribute to the lower HDL-C levels observed in patients with impaired glucose regulation and insulin resistance. It has been recently discovered that common variants of

ABCA1 in diabetic patients had a moderate influence on HDL-C levels (373). This concept has obvious therapeutic implications. *ABCA1* is a potential pharmacological target which could increase plasma HDL-C levels which may lead to improvements in cholesterol excretion. Ultimately, this approach may achieve superior regression of atherosclerosis compared to currently available therapies. However, enhancing this initial step of HDL biosynthesis, may cause counter-regulatory changes in more distal steps of RCT, which may serve to preserve cholesterol homeostasis. An example of this is seen in *LCAT* knockout mice. These animals were shown to have relatively preserved macrophage RCT, despite *LCAT* being traditionally thought of as a critical component of RCT (86). In humans, Calabresi and colleagues have shown that *LCAT* is not required for cholesterol efflux in macrophages, mainly due to a secondary increase *ABCA1*-mediated efflux capacity (374).

A kinetic approach to investigation, is more likely to yield useful information about whole body cholesterol flux. Rader and colleagues have developed a RCT model in mice and have demonstrated that macrophage-mediated RCT better predicts atherosclerosis than steady state blood HDL-C levels (375). The effect of pharmacological agents was studied using this model and both *LXR α* and *PPAR α* agonist therapy were associated with increased RCT *in-vivo*, with decreased atherosclerosis (375). Although this is not reproducible in humans, patients with MS and low HDL-C have been studied using a kinetic approach. *PPAR α* agonist therapy in these individuals was associated with increased ApoA-I and ApoA-II production rate and increased catabolism with no net change in plasma HDL-C concentration (376). These results imply steady state HDL-C concentrations in blood yield only limited information about whole body

cholesterol transport and indicate that measurement of cholesterol flux is more likely to reliably predict changes in atherosclerosis. Other work has confirmed this impression in patients with MS and DM; Rosuvastatin increased HDL-C levels by decreasing catabolism of ApoA-I thereby increasing HDL particle transit time in the circulation (153;377). Current clinical practice is limited to the widespread use of measuring plasma lipoprotein concentrations to help stratify CV risk. The future development of standardised measures of HDL function and whole body RCT is likely to reflect whole body cholesterol removal more accurately but this will require verification in humans. This strategy may help development of more targeted therapies to help prevent atherosclerosis more effectively in the future.

8.4 Increasing ABCA1 availability and mechanisms by which glucose may affect ABCA1

A wealth of evidence currently proposes ABCA1 to be an atheroprotective protein. The effect of drugs that selectively induce ABCA1 expression merit further investigation. Both animal models and humans should be investigated despite differences in the RCT cycle between these species. Measuring RCT reliably in humans remains a practical challenge as measurement of hepatic cholesterol flux requires invasive hepato-biliary sampling which is not clinically appropriate.

Forcheron et al., have shown that PPAR α agonist therapy lead to an increase in monocyte ABCA1 RNA in patients with DM. However, the observed, concomitant lowering of glucose and free fatty acids could partially explain this effect (301). Several PPAR α and PPAR γ drugs have now been shown to

increase ABCA1 expression and HDL biosynthesis in the presence of *LXR α* in cultured macrophage and fibroblasts (378). Attempts to augment cellular cholesterol removal using LXR agonist therapy have been associated with liver steatosis and hypertriglyceridaemia. This occurs through fatty acid synthase and SREBP1c activation which induce enzymes involved in fatty acid synthesis (295;379). RXR-receptor activation, which increases ABCA1 expression, has not been as extensively explored because it has potential to form non-specific heterodimers with many other nuclear receptors.

Understanding the mechanism of how glucose may affect ABCA1 gene expression will be crucial to the development of any future therapeutic intervention. Until recently, it was thought insulin was a major signal to regulate gene expression, and conveyed information of glucose variation. However, it is now appreciated that glucose is directly able to modulate gene expression. This effect may be mediated by carbohydrate or fatty acid cellular sensors such as ChEBP or SREBP which can affect gene expression or through a direct effect of glucose. Glucose activates ChREBP by dephosphorylation, thereby allowing nuclear entry and DNA binding (380;381). ChREBP is highly abundant in lipogenic tissues such as liver and adipose tissue but is expressed ubiquitously (382). Target genes of ChREBP affect glycolysis, lipogenesis and gluconeogenesis (382). Inhibition of ChREBP in mouse models of obesity has been shown to produce metabolic improvements and therefore may be utilised to prevent MS, but its physiological role needs to be elucidated. In contrast, Mitro et al., demonstrated that glucose could directly bind and activate transcriptional activity of LXR, which subsequently controls various genes involved in cholesterol homeostasis including SREBP (296;296;383). In rat

pancreatic β -cells, incubation of glucose for 24 hours led to altered gene expression in two hundred genes suggesting direct effects (384). Investigation of gene promoter activity in cultured human cells could help identify underlying mechanisms to these observations. Present-day work in vascular smooth muscle cells proposes hyperglycaemia leads to reductions in ABCA1 mRNA through changes in *ABCA1* promoter activity with evidence this involves the p38-mitogen-activated protein kinase pathway (356).

The proposition that glucose lowering will lead to decreased CVD in DM has been subject to a lengthy debate and remains uncertain. Recent large studies investigating this have shown tight glycaemic control to be hazardous in older patients with long standing DM; An increase in cardiovascular mortality was shown despite a decrease in risk of MI (385;386). These deleterious results have not been replicated in other large studies but no cardiovascular benefits were seen (387). It is postulated that autonomic nervous system dysfunction may occur as a result of recurrent hypoglycaemia and this may help explain the excess mortality, but currently there is little evidence to substantiate this. In contrast, intensive treatment of glycaemia in both type I and DM in the early stages of disease has been associated with cardiovascular benefits many years later, even when glucose levels subsequently rise to that seen with less intensive treatments (19;19;23). The mechanisms underlying this early metabolic programming or glycaemia-mediated 'legacy effect' are yet to be unravelled and may involve early epigenetic changes. The preserved effect of glycaemia, at the time of sampling, on ABCA1 function in fibroblasts in my study may represent an example of such a phenomenon. Possible mechanisms underlying this include differences in cellular proliferation and cholesterol

trafficking which have been reported to modify efflux in fibroblasts (388). Alternatively, membrane modification, possibly through protein glycation, could result in adverse cell surface interaction leading to diminished efflux. This suggests altered physical properties may lead to transporter dysfunction. Finally, ER stress is associated with insulin resistance (389) and may result in reduced transporter function due to altered intracellular cholesterol trafficking.

8.5 The effect of ABCA1 on atherosclerosis

To investigate whether ABCA1 provides significant protection against CVD in patients with dysglycaemia, the relationship between ABCA1 and a marker of atherosclerosis was explored. A previous study suggested ABCA1 function negatively associated with CIMT in patients heterozygote for *ABCA1* mutations (255). For this reason, we measured CIMT in all study participants. DM patients had higher CIMT measurement compared to healthy men with borderline significance. There appeared to be a positive association of CIMT with age, glycaemia and adiposity. Using multiple regression, age and mean glucose independently predicted CIMT, this is consistent with other studies. However, there was no significant relationship observed with lipid parameters, blood pressure or smoking status and this is not consistent with previous findings from large studies. This inconsistency may be due the relatively small number of participants in the study. An independent relationship with ABCA1 expression was not observed and no significant relationships were observed with protein concentrations in leukocytes. ABCA1 function in fibroblasts negatively related to CIMT with borderline significance. ABCA1, therefore, did not show a strong influence on CIMT in our participants compared with the study which

investigated patients with a *ABCA1* mutation (255). A greater variation in cholesterol efflux was evident in individuals with mutated forms of *ABCA1* compared to individuals in my study and this may help explain why there was a lack of clear correlation between *ABCA1* function and CIMT in the present study. Consistent with my findings, Soro-Paavonen and colleagues did not find a significant relationship between efflux and CIMT in families with low HDL-C without a known *ABCA1* mutation (258). In this study, efflux was reduced in macrophages from affected individuals only when compared to protein and gene expression levels. The positive relationship between cholesterol efflux and HDL-C observed in my study is similar to that reported in fibroblasts in subjects with *ABCA1* mutation (255). This confirms the positive relationship between *ABCA1* activity and HDL-C concentration in humans with functional *ABCA1*.

Cellular cholesterol efflux was reduced by an average of 46% in patients with DM compared to healthy men. When correcting for *ABCA1* protein levels, efflux was reduced by 60% in the DM group, accentuating the difference. These results are comparable to the reduction in efflux seen in Tangier heterozygotes (390) and as such is likely to represent reduced protection against atherosclerosis.

Measurement of gene expression in fibroblast samples, in response to cholesterol loading, could investigate whether there is an underlying *LXR α* -mediated signalling defect in patients with DM which could account for diminished *ABCA1* expression. Although *LXR α* gene expression was not lower in fibroblasts taken from diabetic subjects, this would not account for differences in ligand activity. Work in macrophages has shown that *LXR* agonists can

overcome reduced cellular cholesterol export leading to reduced foam cell formation in patients with DM (185).

8.6 Limitations and future work

There are a number of limitations to this study; my data implies a novel glycaemia-mediated negative effect on gene expression and function of this transporter; this is based on observational association, with little evidence of causality. *In-vitro* investigation, as outlined above, could address this by measuring the maximum effect of cholesterol and glucose on gene expression and function by loading cultured cells with increasing concentrations. This work should also attempt to explore possible underlying mechanisms to help understand my findings. For example, the possibility of subcellular trapping of ABCA1 in fibroblasts could be investigated using membrane biotinylation techniques. However, the reported acute effect of oral glucose *in-vivo* indicates gene expression is dynamic and there is a suggestion that glucose may affect gene expression over a 2 hour period. Confirmation that mRNA levels from pooled leukocytes reflect gene expression in monocytes is required. The relationship between glucose lowering, AMP kinase activation and ABCA1 expression requires confirmation both *in-vitro* and in humans. Potential confounding variables, such as insulin concentration and physical activity were not accurately measured in my study. There was no difference in smoking status and no differences in alcohol consumption seemed apparent but the latter was not adequately quantified. Further analyses showed there was no difference in alcohol consumption between groups. Measurements of insulin secretion would be helpful to clarify any causal relationship with ABCA1. Indeed, the challenge of disentangling the effects of glycaemia from that of adiposity and associated low grade inflammation, should be considered in

future work. Ideally, patients and healthy participants should be matched for BMI to help control for changes due to adiposity. The study cohort should have been genetically characterised for common polymorphisms which may influence gene expression. Nonetheless, participants in my study were heterogeneous and recruited from a genetically diverse area, making it less likely that gene polymorphisms could account for the findings. The importance of the non-transport related function of HDL has been highlighted by various studies and should be incorporated in future investigation. A current population based study investigating HDL particle heterogeneity endorses the view that functional information remains vital to help characterise the effect of genetic variants of *ABCA1* on CVD (391). This warrants exploration to help understand these epidemiological findings with an approach to develop therapies designed to enhance cellular cholesterol removal. Future work in humans should be targeted to study monocytes and macrophages cultured for a short duration, as this is a more relevant cell type for atherosclerosis. There is also a possibility that glycaemic status may affect sub-populations of leukocytes (ie. granulocytes, lymphocytes, monocytes) differently. A greater number of participants would help clarify the numerous relationships of borderline significance observed in my work as this study was not powered to detect a modest effect. The potential relationship of transporter expression or function with surrogate markers of atherosclerosis could be further examined with use of specific imaging of regional circulation employing techniques such as coronary artery calcification. Developing molecular imaging techniques to target cholesterol would directly aid the measurement of cholesterol movement at a cellular and sub-cellular level.

Accomplishing such work in drug naive, dysglycaemic patients will be challenging due to clinical trends for early intervention with statin and hypoglycaemic therapies in such patients. Cholesterol export studies using participants own ApoA-I and HDL particles could further assess if there is a net pathophysiological defect in cellular efflux in these patients. This is under the assumption that frozen HDL lipoproteins isolated from plasma retain their full *in-vivo* functional properties. A comparison of different methods of separation would be required to validate this. Leukocyte ABCA1 may develop to be a useful biomarker for macrophage gene expression if a close relationship is confirmed by subsequent studies. This further assumes modulation of gene expression will be concordant in leukocytes and macrophages.

Other proteins are involved in HDL remodelling such as endothelial lipase may influence HDL-C levels in humans (392). These aspects should be considered in future studies investigating diabetes related CVD. There is some evidence that serum levels of this enzyme may be elevated in patients with DM (393).

Reverse cholesterol transport and excretion is slowly becoming unravelled and ABCA1 is still considered to be a critical component in this process. The present study concludes diabetes may adversely affect transporter availability and function. ABCA1 has the potential to be a therapeutic target for future drug development, the effect of enhancing transporter activity may have beneficial effects in patients prone to premature vascular disease.

PRESENTATIONS AND PUBLICATIONS

Oral Presentations

2007 RANK funds mini symposium on adiposity, UK.

2007 5th International Atherosclerosis Society (IAS), HDL Workshop, Santorini

2008 22nd Annual Conference, Hyperlipidaemia Education & Atherosclerosis Research Trust (HEART) UK

2008 13th International Congress of Endocrinology, Rio de Janeiro, Brazil

Poster presentations

2007 5th International Atherosclerosis Society, HDL Workshop, Santorini.

2008 Diabetes UK Annual Professional Conference, Glasgow.

2008 2nd Federation of European Biochemical Societies-ABC proteins, Innsbruck

2008 Arteriosclerosis Thrombosis and Vascular Biology Annual Conference, USA.

2008 77th Congress of the European Atherosclerosis Society, Istanbul, Turkey

Published Abstracts

Diabetic Medicine Volume 25, Suppl. 1, March 2008, Page. 34-162, Abstract 129

Atherosclerosis supplements 9(1) May 2008 Abstract P09-81

Arteriosclerosis Thrombosis Vascular Biology. 28(6) June 2008 e96 Abstract 347

Atherosclerosis 199(1), July 2008, Page 231-232

Endocrinologia & Metabologia 52; supplement 6 (II) November 2008; S769

APPENDICES

APPENDIX I

Buffers, solutions, protocols and results

Leukocyte lysis buffer for RNA extraction

RLT Buffer (guanidine thiocyanate 25-50%, Ph 7.0) (Qiagen)

10% β -mercaptoethanol (Sigma)

Leukocyte lysis buffer for protein extraction

50 mM mannitol (Sigma)

2 mM EDTA, 50 mM (Sigma) pH 7.6

Complete mini protease inhibitor cocktail (Roche)

Reverse transcription reaction mix (Applied Biosystems)

2 μ l 10xRT Buffer

0.8 μ l 25xdNTP (100nM)

2 μ l 10xRT RND primer

4.2 μ l PCR grade water

1 μ l Multiscribe RT-enzyme

MACS Buffer

PBS, 0.5% BSA, 1mM EDTA

Fibroblast Freezing medium

1.5 ml freezing medium comprising of FBS and sterile filtered 10% DMSO

Optimised thermal cycler settings for cDNA synthesis (Applied Biosystems)

Step 1	25°C	for	10min
Step 2	37°C		120min
Step 3	85°C		5seconds
Step 4	4°C		until samples removed and stored.

Optimised PCR reaction mix

1.25µl Primer 1 (Gene of interest)

1.25µl Primer 2 (Housekeeper gene)

12.5µl 2x PCR Master Mix (Applied Biosciences)

1µl (30ng) cDNA, 9µl PCR grade water

Taqman Hydrolysis probe PCR temperature program

PCR Step	Temperature	Duration
Hold	50 °C	2 min
Initial denaturation	95 °C	10 min
Denaturation	95 °C	15 sec
Amplification	60 °C	60 sec

Leukocyte ABCA1 Gene Expression Results :

(Mean values of duplicate measurements, normalised to housekeeper gene, arbitrary units. n=63). C=Control, healthy men, I=IGH, D=DM

<i>gene expression</i>			<i>gene expression</i>		
		<i>log delta Ct</i>			<i>log delta Ct</i>
C1.	0.286	1.692	I16.	0.254	1.898
C2.	0.334	1.651	I17.	1.628	1.522
C3.	0.276	1.807	I19.	0.393	1.772
C4.	0.708	1.482	I20.	0.533	1.061
C5.	0.340	1.633	D2.	0.079	2.016
C6.	0.296	1.673	D3.	0.072	2.061
C7.	0.172	1.812	D4.	0.408	1.633
C8.	1.666	1.194	D5.	0.095	1.996
C9.	1.692	1.256	D6.	0.226	1.807
C10.	1.229	1.022	D9.	0.287	1.761
C11.	0.553	1.562	D10.	1.590	1.543
C13.	1.224	1.356	D11.	0.177	1.845
C14.	1.079	1.635	D12.	0.385	1.782
C15.	0.192	1.829	D13.	1.063	1.466
C16.	0.834	1.611	D14.	0.143	1.910
C17.	1.322	1.541	D15.	0.209	1.820
C18.	0.720	1.550	D16.	0.248	1.746
C19.	0.323	1.812	D17.	0.125	1.974
C20.	0.475	1.660	D18.	0.466	1.641
C21.	0.580	1.513	D19.	0.656	1.493
C22.	0.575	1.654	D20.	0.892	1.366
C23.	0.528	1.683	I21/D.	0.596	1.607
C24.	0.392	1.753			
C25.	0.465	1.733			
C26.	0.090	1.959			
C27.	0.318	1.714			
C28.	0.490	1.543			
C29.	0.571	1.613			
C31.	1.233	1.233			
I1.	0.121	1.884			
I2.	0.183	1.800			
I3.	0.538	1.564			
I4.	0.525	1.528			
I5.	0.151	1.837			
I6.	0.370	1.627			
I7.	0.133	1.992			
I9.	0.175	1.915			
I10.	0.627	1.522			
I11.	1.298	1.297			
I12.	0.367	1.746			
I13.	0.702	1.530			
I15.	0.777	1.537			

Leukocyte LXR α Gene Expression Results :

(Mean values of duplicate measurements, normalised to housekeeper gene)

C=Control, healthy men, I=IGH, D=DM

Gene Expression (arbitrary units) n=61

	<i>gene expression</i>	<i>delta Ct</i>		<i>gene expression</i>	<i>delta Ct</i>
C1	0.410	7.77	I13	2.253	3.61
C2	0.627	5.54	I15	1.972	5.34
C3	0.284	6.82	I16	0.294	7.94
C5	0.419	6.32	I17	0.343	6.73
C6	2.271	5.26	I19	0.735	6.21
C7	0.278	8.27	I20	1.290	3.04
C8	1.125	6.35	D2	0.474	6.03
C9	0.973	6.88	D3	0.721	7.09
C10	2.998	3.29	D4	0.886	6.30
C11	1.037	4.76	D5	1.360	6.07
C13	0.627	5.5	D6	0.738	5.25
C14	0.411	6.22	D9	0.702	7.07
C15	1.239	4.51	D10	0.740	5.19
C16	0.539	5.77	D11	1.029	6.12
C17	0.951	4.82	D12	0.733	5.25
C18	0.895	5.09	D13	1.044	4.71
C19	0.481	6.71	D14	0.797	5.06
C20	1.232	5.43	D15	0.890	6.58
C21	1.296	6.61	D16	0.597	5.93
C22	0.702	6.09	D17	0.475	6.52
C23	0.529	6.45	D18	2.245	5.39
C24	0.630	6.24	D19	0.897	5.00
C25	0.515	6.51	I21/D	0.543	6.16
C26	0.907	5.41	D20	0.667	6.24
C27	0.755	5.73			
C28	0.525	5.81			
C29	1.292	5.33			
I1	0.238	8.43			
I2	0.606	5.5			
I4	0.512	5.85			
I5	1.052	4.66			
I6	0.428	7.65			
I7	0.318	6.62			
I9	0.466	6.00			
I10	0.763	6.84			
I11	1.000	4.72			
I12	0.893	6.83			

Leukocyte PPAR γ Gene Expression Results :

(Mean values of duplicate measurements, normalised to housekeeper gene)
C=Control, healthy men, I=IGH, D=DM

Normalised Gene Expression (arbitrary units) n=23

<i>gene expression</i>	
C1	0.60
C6	1.05
C7	0.18
C8	1.32
C9	0.68
C21	0.60
C28	0.64
C29	0.47
I1	0.68
I6	0.52
I10	1.04
I12	1.63
I15	1.22
I16	0.58
D3	0.79
D4	0.63
D5	1.01
D7	1.13
D9	1.58
D11	1.28
D15	1.01
D16	2.45
D18	0.50

Leukocyte ABCG1 Gene Expression Results :

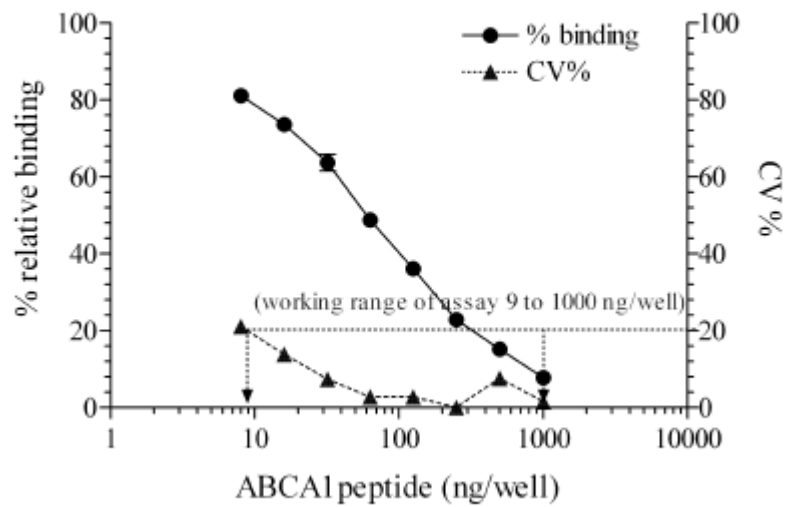
(Mean values of duplicate measurements, normalised to housekeeper gene, arbitrary units. n=47)

C=Control, healthy men, I=IGH, D=DM

<i>gene expression</i>		<i>gene expression</i>	
C1	0.657	D18	0.913
C2	0.566	D19	1.000
C3	0.643	D20	0.686
C4	1.048		
C5	0.682		
C6	1.166		
C7	0.375		
C8	1.056		
C9	1.257		
C10	0.695		
C11	0.836		
C13	0.947		
C14	0.988		
C15	0.411		
C16	1.067		
C17	1.417		
C18	0.990		
C21	0.707		
C28	0.987		
C29	1.158		
I1	0.444		
I2	0.535		
I4	1.061		
I6	0.570		
I9	0.847		
I10	0.922		
I11	0.800		
I12	0.679		
I13	0.528		
I15	0.725		
I16	0.411		
I17	0.575		
D13	1.317		
D2	0.466		
D3	0.369		
D4	1.276		
D5	0.460		
D6	0.638		
D7	0.620		
D9	0.511		
D10	1.153		
D11	0.655		
D12	1.001		
D14	0.642		
D15	0.586		
D16	0.803		

ELISA Calibration Curve

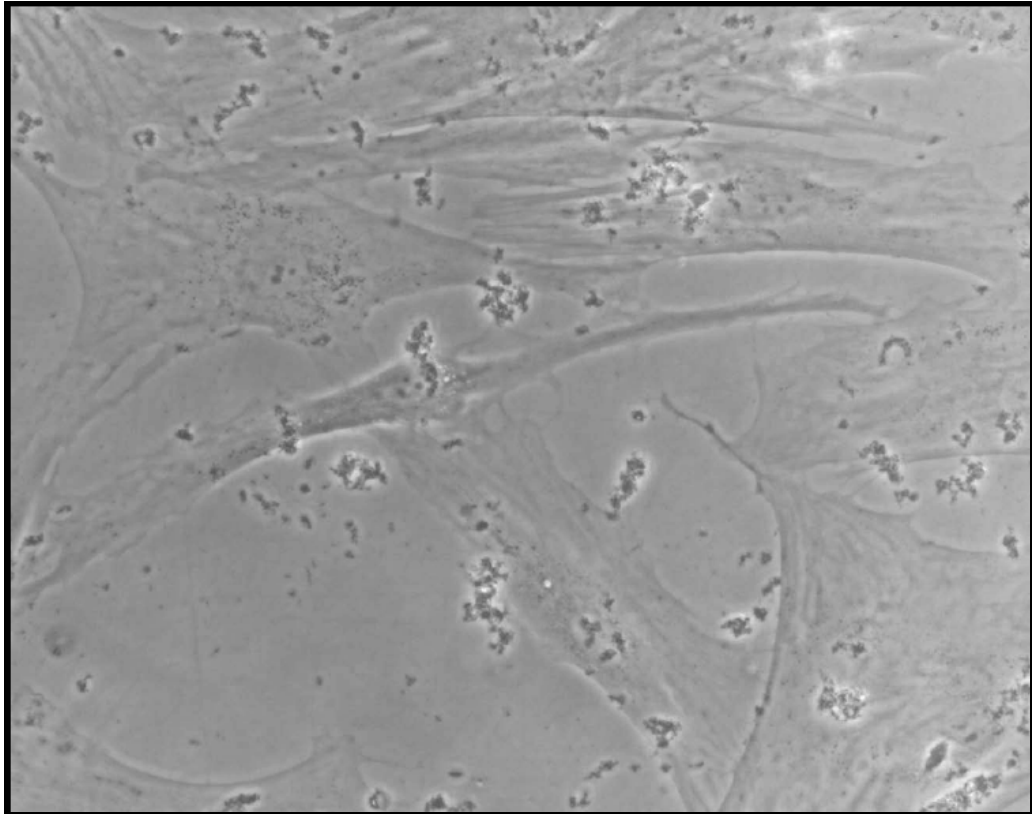
Calibration curve for ABCA1 quantification in human tissues. Taken from Paul V et al (394). A typical calibration curve from serial dilutions (8 to 1000ng/well) of ABCA1 peptide in assay buffer. The working range of (9 to 1000ng/well) of the calibration curve was derived on the basis of CV<20% between the replicates of ABCA1 calibrators.



APPENDIX II -Functional methodology in fibroblasts

Image of fibroblast cell morphology (high magnification):

Primary fibroblasts in culture, during cholesterol loading, a spindle shaped fibroblast is seen in the centre as well as surrounding cells undergoing mitosis. Cholesterol crystals are visible.



APPENDIX III Demographic data: Clinical examination measurements

wt=weight (kg), ht=height (m), WC=waist circumference (cm), sys=systolic blood pressure, dia=diastolic blood pressure, PP=pulse pressure.

	Ethnicity	Smoker	age	wt	ht	BMI	WC	Mean sys	Mean dia	Mean PP
Healthy Men										
C1	Chin	Y	32	66.0	1.72	22.3	74	112.0	76.0	36
C2	Chin/As	N	21	60.2	1.76	19.4	81	136.5	74.5	62
C3	As	N	49	60.2	1.66	21.8	81	137.5	89.0	49
C4	Cau	N	23	73.1	1.82	22.1	83	119.5	75.0	45
C5	Cau	N	22	75.8	1.79	23.7	88	143.5	103.0	41
C6	Cau	N	57	78.0	1.82	23.5	85	147.5	84.0	64
C7	Cau	Ex	49	73.0	1.80	22.5	85	118.5	74.0	45
C8	Cau	N	58	78.5	1.79	24.5	88	137.0	77.0	60
C9	Cau	N	38	81.5	1.85	23.8	87	117.5	70.0	48
C10	Cau	N	43	86.8	1.82	26.2	95	130.5	85.5	45
C11	Cau	Ex	35	72.5	1.75	23.7	85	124.0	68.0	56
C12	Afro	N	30	95.1	1.93	25.5	96	147.0	92.0	55
C13	As	N	51	83.0	1.82	25.1	100	120.0	89.0	31
C14	Cau	Y	55	77.3	1.74	25.5	94	136.0	90.0	46
C15	Cau	N	63	80.0	1.77	25.7	96	133.0	83.0	50
C16	Cau	N	44	80.4	1.77	25.7	91	133.5	81.0	53
C17	Cau	N	61	74.2	1.79	23.2	93	147.5	92.5	55
C18	Cau	N	60	87.7	1.79	27.4	97	165.0	111.0	54
C19	As	N	41	90.9	1.80	28.1	101	133.5	84.5	49
C20	As	N	52	95.7	1.84	28.3	107	123.0	80.0	43
C21	Cau	N	63	87.7	1.79	27.4	103	144.5	95.5	49

C22	Cau	Y	54	68.5	1.75	22.5	85	146.5	87.0	60
C23	Cau	N	70	86.1	1.78	27.2	107	123.3	77.3	46
C24	Cau	N	59	71.7	1.77	22.9	86	113.0	76.8	36
C26	Cau	N	75	72.8	1.70	25.3	95	134.3	74.0	60
C27	Cau	N	74	73.4	1.73	24.5	93	147.8	77.5	70
C28	Cau	N	66	67.3	1.78	21.4	79	124.8	72.5	52
C29	Cau	N	70	80.6	1.79	25.2	96	112.8	57.5	55
C31	Cau	N	74	68.1	1.62	25.9	91	146.5	83.5	63
Patients	Ethnicity	Smoker	age	wt	ht	BMI	WC	Mean sys	Mean Dia	Mean PP
I-1	Afro	Y	70	77.9	1.72	26.3	91	151.5	93.0	59
I-2	Afro	Ex	54	111.3	1.76	35.9	113	132.5	72.5	60
I-3	As	N	48	91.1	1.74	30.1	99	153.5	105.0	49
I-4	Cau	Y	58	71.5	1.68	25.3	91	140.5	86.0	55
I-5	Cau	Y	57	66.9	1.84	19.8	84	120.5	77.5	43
I-6	Cau	Ex	61	84.5	1.78	26.7	96	135.5	76.0	60
I-7	As	N	50	95.0	1.72	32.1	115	165.5	97.0	69
I-8	Cau	Ex	60	165.0	1.72	55.8	165	120.0	76.0	44
I-9	Cau	N	59	62.6	1.66	22.7	87	142.0	67.0	75
I-10	As	Y	59	71.4	1.63	26.9	95	145.0	88.5	57
I-11	Cau	Y	62	72.0	1.75	23.5	84	111.5	71.5	40
I-12	Cau	Ex	68	69.9	1.70	24.2	87	146.5	65.0	82
I-13	Cau	N	60	75.4	1.67	27.0	87	144.0	88.0	56
I-14	Cau	N	43	79.3	1.77	25.3	85	112.0	75.5	37
I-15	As	N	52	76.8	1.67	27.5	100	148.0	95.0	53
I-16	Cau	N	71	76.0	1.79	23.7	96	138.5	83.0	56
I19	Cau	Ex	49	100.1	1.91	27.4	104	128.0	73.5	55
C25//	Cau	Y	65	83.3	1.80	25.7	94	148.3	83.3	65
I20	Cau	Ex	42	111.7	1.88	31.6	106	138.8	84.5	54

D13//	Cau	N	53	102.8	1.74	34.0	112	146.3	93.5	53
D2	As	N	61	55.7	1.61	21.5	81	120.0	73.5	47
D3	Cau	N	71	88.8	1.80	27.4	103	154.0	86.0	68
D4	Cau	Ex	72	106.7	1.87	30.5	113	149.0	93.0	56
D5	Cau	N	42	87.3	1.74	28.8	100	126.0	88.5	38
D6	As	Y	34	71.9	1.74	23.9	91	106.5	74.5	32
D7	As	Y	54	89.4	1.74	29.5	102	131.0	76.5	55
D9	Cau	Y	41	96.5	1.81	29.5	98	130.0	88.5	42
D10	Cau	Ex	59	94.7	1.83	28.3	101	129.0	81.0	48
D11	Cau	Ex	49	90.4	1.68	32.0	104	125.5	77.5	48
D12	Cau	Ex	64	82.4	1.71	28.3	97	130.0	88.0	42
D14	Cau	N	61	92.5	1.87	26.6	99	147.8	85.8	62
D15	Cau	Ex	66	72.7	1.76	23.5	88	146.5	86.0	61
D16	As	Ex	53	91.7	1.79	28.8	104	127.8	81.8	46
I17/D	Arabic	Ex	57	90.5	1.80	27.9	108	115.0	77.3	38
D17	Cau	N	67	72.9	1.58	29.2	101	125.3	80.8	45
D18	As	Ex	67	90.1	1.72	30.5	106	157.3	88.0	69
D19	Cau	Ex	57	91.4	1.83	27.3	100	133.0	78.3	55
I21/D	As	N	44	64.3	1.64	23.9	91	120.0	76.8	43
D20	Cau	Ex	63	107.8	1.78	34.0	122	116.5	78.0	39

APPENDIX IV Participant biochemical measurements

Data in mmol/l unless otherwise stated. Serial glucose measurements were during an OGTT, Chol=total cholesterol, Trig=fasting triglycerides. Duration of diabetes stated (years), median 0.7 years.

	Fasting Glucose	1hr Glucose	2hr Glucose	Chol	HDL-C	LDL-C	Trig	HbA1c (%)
Healthy Men								
C1	4.7		3.1	4.87	1.51	2.89	1.04	5.7
C2	4.3	4.6	2.3	4.23	1.34	2.58	0.69	5.7
C3	5.6		5	3.58	1.42	1.81	0.77	5.8
C4	4.6	3.4	4.1	5.34	1.86	3.08	0.87	5.1
C5	4.7	3.2	3.5	5.55	1.35	3.49	1.56	4.8
C6	5.2	6.3	4.1	5.59	2.07	3.21	0.69	4.8
C7	4.4	4.7	3.9	6.72	1.44	4.26	2.23	5.6
C8	4.2	5.3	6.3	4.86	1.7	2.66	1.09	5.4
C9	5	3.7	4.7	3.85	1.3	2.21	0.74	5.4
C10	4.5	7.5	3.9	4.96	1.7	2.97	0.63	4.9
C11	5.1	4.2	4.5	5.33	1.64	3.35	0.75	5.5
C12	5.7	5.9	5.3	5.94	2.06	3.47	0.89	4.8
C13	4.8	4.4	6.1	4.35	1.1	2.72	1.17	5.1
C14	5.8	7	5.4	6.12	1.15	4.04	2.05	5.3
C15	5.5	6.6	6.9	7.48	1.72	4.73	2.26	5.6
C16	5	6	6.2	6.36	2.07	3.44	1.86	5.5
C17	5.1	3.7	3.9	6.03	1.57	3.54	2.01	5.7
C18	5	4.9	5.1	5.55	1.54	3.78	0.51	5.7
C19	4.2	4.2	3	4.61	1.28	2.80	1.16	4.7
C20	5.5	10.1	7.4	4.83	1.05	3.24	1.19	5.9
C21	5.5	9.7	4.6	5.18	1.69	2.97	1.15	5.2
C22	5.1	5.2	5.2	5.61	1.65	3.69	0.59	5.4
C23	5.6	7.1	5.8	6.15	1.42	4.08	1.42	5.9
C24	5.3	5.6	5.7	5.1	1.1	3.27	1.59	5.7
C26	5.6	9.3	6.7	6.37	1.56	4.08	1.6	6.2
C27	5.1	10	5.7	4.83	1.64	2.64	1.2	6.2
C28	5.2	10.5	3.6	5.65	2.09	3.23	0.72	5.6
C29	5.4	8.2	4.9	5.25	1.34	3.30	1.34	5.7
C31	5.1	7.8	4.3	6.11	1.79	3.72	1.32	5.4
Patients								
I-1	4.7	12	10.6	6.15	1.97	3.89	0.64	5.7
I-2	5.4	11.2	7.9	4.5	1.11	3.06	0.73	6.4
I-3	5.7	9.4	6.4	3.73	0.95	2.43	0.77	5.4
I-4	6.3	8.7	3.8	5.59	1.72	3.60	0.6	6.3
I-5	5.9	7.2	10.8	4.3	1.6	2.35	0.77	6.9
I-6	4.7	10.7	8.3	6.03	1.44	4.15	0.97	5.4

I-7	5.9	11.1	7.2	5.03	0.92	2.92	2.61	6.0
I-8	8.5	11.3	10.1	4.56	0.98	3.01	1.24	6.8
I-9	6.3	10.2	9.7	5.32	1.57	3.40	0.77	5.6
I-10	5.1	12.2	8.5	5.61	1.13	3.18	2.85	5.4
I-11	4.5	5	5.1	4.17	0.97	2.76	0.96	5.9
I-12	5.1	6.5	4.8	6.07	1.84	3.84	0.85	5.4
I-13	4.9	9.8	6.8	4.1	1.12	2.48	1.09	4.9
I-14	4.8	7.1	3.4	4.88	1.09	3.06	1.59	5.5
I-15	6.2	11.8	10.4	4.8	1.35	2.95	1.09	6.1
I-16	6	9.5	6.3	7.68	2.02	4.99	1.47	5.9
I19	5	6.4	6.4	5	0.93	3.48	1.29	6.0
C25/I	5.3	8.5	8.1	4.48	1	2.91	1.26	5.9
I20	5.9	7.5	4.2	6.4	1.29	3.81	2.84	6.2
D13/I	5.9	9.1	6.3	3.62	0.85	1.51	2.77	5.8
D2 (2)	8.5			6.73	1.29	4.85	1.29	7.9
D3 (3.1)	9.1			4.16	1.11	2.65	0.87	7.6
D4 (0.7)	6.4			3.91	1.22	2.24	0.99	6.4
D5 (0.1)	11			5.4	1.06	2.82	3.32	11.2
D6 (3.75)	6.3			5.18	1.74	2.99	0.99	8.7
D7 (0.3)	5.6			4.61	1.04	2.94	1.38	5.8
D9 (0.6)	10.7			5.94	1.11	3.84	2.17	10.0
D10 (0.2)	6.7			3.85	0.89	2.59	0.89	5.9
D11 (0.2)	7.5			4.01	0.71	2.56	1.62	7.4
D12 (6)	5.2			4.47	1.51	2.60	0.78	5.3
D14 (3)	11			5.23	1.29	3.23	1.56	8.0
D15 (0)	6.8			5.66	1.34	3.87	0.99	6.5
D16 (3.5)	6.8			4.53	1.15	2.60	1.72	7.2
I17/D (0)	6.3	9.4	6.5	5.09	1.38	3.34	0.82	6.4
D17 (3.75)	5.3			4.14	1.24	2.41	1.07	6.3
D18 (0.1)	6.9	14.3	11.4	7.44	1.56	4.90	2.16	6.8
D19 (3)	6.7	12.5	10.9	6.68	1.47	4.28	2.05	6.5
I21/D (0)	6.7	18.8	15.1	3.88	1.17	2.05	1.45	7.3
D20 (1)	6.3	12	13.4	4.26	0.81	2.16	2.82	5.7

Most subjects known to have DM did not undergo OGTT (grey box).

APPENDIX V Participant medication list

Healthy Men

**C1-5 ,C7-16,
C19, C22,
C24, C27,
C29, C31**

No medications

C6 Irbesartan

C17 Fexofenadine

C18 Loratidine, Budesonide nasal spray

C20 Irbesartan/hydrochlorothiazide

C21 Timolol eye drops

C23 Aspirin, Lansoprazole, Diclofenac

C26 Timolol eye drops

C28 Budesonide and Terbutaline inhalers

Patients

I-1 Nifedipine

I-2 Nil

I-3 Amlodipine

I-4 Nil

I-5 Nil

I-6 Tamsulosin

I-7 Ramipril, Doxazocin, Atenolol, Omeprazole

I-8 Aspirin, Ramipril, Indapamide, Felodipine

I-9 Bendroflumethazide, Ramipril, Alendronic acid, Symbicort* and Combivent# inhalers

I-10 Paroxetine

I-11 Desloratidine

I-12 Ramipril, Bendroflumethazide, Amlodipine

D13// Nil

I-13 Aspirin, Methotrexate

I-14 Nil

I-15 Becotide inhaler

I-16 Glucosamine

I19 Paroxetine

C25// Calcipotriol

I20 Salbutamol, Seretide (Budesonide/Formoterol) inhalers

D2 Nil

D3 Salbutamol, Symbicort* inhalers

D4 Allopurinol

D5 Fluoxetine

D6 Nil

D7 Ramipril, Tamsulosin, Omeprazole, Venlafaxine, Fluticasone, Diazepam and Naproxen PRN

D9 Cocodymol, Diazepam

D10 Nil

D11 Nil

D12	Enalapril
D14	Allopurinol
D15	Aspirin, Cannesten/Hydrocortisone cream
D16	Ramipril, Atenolol, Aspirin, Glucosamine
I17/D	Nil
D17	Allopurinol, Irbesartan, Tamsulosin, Testosterone, Aspirin, Cephalexin
D18	Allopurinol, Beclomethasone inhaler, Quinine Sulphate
D19	Tamsulosin
I21/D	Nil
D20	Allopurinol, Fish oil, Calcium +Vitamin D (AdCal), Vitamin C+E

*Symbicort =Budesonide/Formoterol
#Combivent = Salbutamol+Ipratropium bromide

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