ABCA1 EXPRESSION AND FUNCTION UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS - NOVEL LINKS BETWEEN APOPTOSIS, ATHEROSCLEROSIS AND HAEMOSTASIS

Christiane Albrecht

Chair of Physiology, Center of Life and Food Sciences Weihenstephan,
Technische Universität München

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PREFACE

This work reviews own research activities attempting to unravel physiological transport mechanisms within or out of cells which are based on the ATP-binding cassette transporter A1 (ABCA1). Herein I have focussed on ABCA1-mediated transport of the phospholipids cholesterol and phosphatidylserine both in rare hereditary diseases as well in disorders of the general population such as atherosclerosis or diabetes.

The scientific projects described in this work comprise both physiological and pathophysiological conditions and include expressional and functional studies investigating the cause and pathophysiological consequences of aberrant ABCA1 function. The projects including patients with various disorders were carried out at the Clinical Sciences Centre, the Hammersmith Hospital, the Charing Cross Hospital, Kings College Hospital, St. Thomas Hospital, Queen Charlotte's Hospital, the Royal Free Hospital and St. Mary's Hospital, London UK. The laboratory work was performed at the Clinical Sciences Centre, London, UK and at the Chair of Physiology, Technische Universität München, Germany.

Other scientific projects which are not outlined in the following chapters include studies on other ABC transporter proteins (see Appendix 2), drug targeting approaches or investigations on genetically modified organisms (see Appendix 3).

1 Introduction

The role of the ATP-binding cassette transporter A1 (ABCA1) in lipid homeostasis – a brief overview

The ATP-binding cassette (ABC) transporters are membrane proteins that transport a wide variety of compounds, including ions, peptides, sugars, and lipids, against concentration gradients at the cost of ATP energy (Klein et al., 1999). ABC transporter proteins form one of the largest and most diverse families known (Higgins, 1992), with more than 2000 distinct ABC genes present in various databases. The human genome contains 49 ABC genes, 16 of them with a known function, which have been classified in seven subfamilies named A to G according to a nomenclature working group.

Members of the ABC transporter superfamily play a pivotal role in the maintenance of various physiological processes. Their fundamental importance is underlined by the fact that several ABC transporters have been identified to be associated with human hereditary diseases. An overview about the implication of ABC transporters in genetic diseases and phenotypes is provided by M. Dean (www.ncbi.nlm.nih.gov) and shown in Table1.

Table 1: *Diseases and phenotypes caused by ABC genes (M. Dean, www.ncbi.nlm.nih.gov)*

Gene	Mandelian disorder	Complex disease	OMIM
ABCA1	Tangier disease, FHDLD[a]		600046
ABCA4	Stargardt/FFM, RP, CRD, CD	AMD	248200
ABCB1	Ivermectin, susceptibility	Digoxin uptake	171050
ABCB2	Immune deficiency		171026
ABCB3	Immune deficiency		170261
ABCB4	PFIC3	ICP	171060
ABCB7	XLSA/A		300135
ABCB11	PFIC2		603201
ABCC2	Dubin-Johnson Syndrome		601107
ABCC6	Pseudoxanthoma elasticum		603234
ABCC7	Cystic Fibrosis, CBAVD	Pancreatic, bronchiectasis	602421
ABCC8	FPHHI		600509
ABCD1	ALD		300100
ABCG5	Sitosterolemia		605459
ABCG8	Sitosterolemia		605460

[a]FHDLD, familial hypoapoproteinemia; FFM, fundus flavimasculatis; RP, retinitis pigmentosum 19; CRD, cone-rod dystrophy; AMD, age-related macular degeneration; PFIC, progressive familial intrahepatic cholestasis; ICP, intrahepatic cholestasis of pregnancy; XLSA/A, X-linked sideroblastosis and anemia; CBAVD, congential bilateral absence of the vas deferens; FPHHI, Familial persistent hyperinsulinemic hypoglycemia of infancy; ALD, adrenoleukodystrophy.

As the present summary of own research work does focus on ABCA1, this transporter shall be briefly introduced in the historical context.

In 1994, aiming at the identification of new members of the recently described ABC transporter family, Luciani et al. designed a PCR approach based on the high degree of homology within this family of proteins (Luciani et al., 1994). They identified two novel proteins, named ABC1 and ABC2, present on the human genome in very close locations of the same chromosome. Both proteins represented a full-transporter structure, leading to the formation of a new subfamily for their classification, the ABCA subfamily. This subfamily contains the largest ABC proteins known to date, some of them over 2100 amino acids long and 200 kDa of predicted molecular weight. Excluding ABCA14-A17 proteins, which have been only cloned in rodents (Chen et al., 2004; Ban et al., 2005), the human ABCA subfamily is composed of 13 members, which can be divided into two subgroups. One subgroup is formed by five genes (ABCA5, A6, A8, A9, and A10) that are located in a cluster in human chromosome 17 (Arnould et al., 2005).

Mutations in members of the A subfamily of ABC transporters have been described to be responsible for the development of severe hereditary diseases related with cholesterol efflux or phospholipid translocation (ABCA1, Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999), fatal surfactant deficiency (ABCA3, Shulenin et al., 2004) or harlequin ichthyosis (ABCA12, Kelsell et al., 2005). In addition, most of them are hypothesised to be responsible for the treatment resistance of several carcinoma cell lines, participating in the subcellular sequestration of drugs.

The clinical implications of ABCA1 deficiency were recognized when the *ABCA1* gene was discovered to be responsible for Tangier disease (TD; Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999) and familial high-density lipoprotein (HDL) deficiency (Marcil et al., 1999). TD is an autosomal recessive disorder and patients have defects in both alleles of ABCA1. Familial HDL deficiency is characterised by a less severe phenotype than that found in TD but has a dominant mode of inheritance. Both conditions are characterised by low levels of HDL, the deposition of lipid-laden macrophages in tissues and increased atherosclerotic disease in a proportion of patients. The pathophysiology of this process is thought to result from failure of ABCA1 to transport cholesterol and phospholipids out of cells to form complexes with apolipoprotein (apo) A1 in order to generate HDL (Oram et al., 2000).

For efficient apoA1 mediated transport, a functional ABCA1 protein and specific binding at the plasma membrane (Chambenoit et al., 2001) is crucial since nonfunctional ABCA1 as seen in TD can indirectly result in increased catabolism of apoA1 thus affecting HDL synthesis (Francis et al., 1995; Remaley et al., 1997). ApoA1 also plays a significant role in the stabilization of ABCA1 protein as *in vitro* experiments demonstrate that apoA1 binding to ABCA1 inhibits its degradation by calpain proteases (Arakawa and Yokoyama, 2002; Wang et al., 2003). The polar surface of the 9/10 helical segment of apoA1 has recently been found not only to play a major role in imparting cholesterol efflux capability but also in preventing degradation of ABCA1 (Natarajan et al., 2004). It has been recently shown in transgenic mice that in vivo modification of the phospholipid/apoA1 ratio by

various lipases could play a major role in directing cholesterol efflux through either the ABCA1 or the scavanger receptor B1 (SRB1) pathway (Yancey et al., 2004)

To date no clear consensus exists regarding the process by which apoA1 removes cholesterol and phospholipids from the plasma membrane. There are studies supporting the theory of membrane solubilization wherein apoA1 and ABCA1 simultaneously remove both phospholipids and cholesterol through a single step process (Gillotte et al., 1998; Denis et al., 2004). Another proposed mechanism includes a two step process in which ABCA1 lipidates apoA1 with phosphoplipids first to form phospholipid-rich nascent HDL particles which then remove cholesterol from cells by diffusion (Fielding et al., 2000; Wang et al., 2001). There is increasing evidence that apoA1 directly interacts with ABCA1 (Chroni et al., 2004; Fitzgerald et al., 2004; Wang et al., 2000). Further proof of this emerged from studies which showed that mutations in the extracellular loops of ABCA1 impaired both cross-linking with apoA1 and lipid efflux (Fitzgerald et al., 2002). Moreover, truncation mutations of apoA1 lacking helix 10 impaired cholesterol transport through the ABCA1 pathway (Panagotopulos et al., 2002).

The exact mechanism of ABCA1-mediated cholesterol efflux is not fully understood yet. Recently two models of efflux have been proposed. In the first model, the golgi apparatus processes excess intracellular free cholesterol into vesicles that are translocated to the plasma membrane by ABCA1 for exocytosis (Oram and Lawn, 2001). In this context it was reported that ABCA1 may take a part in enhancing vesicular trafficking of cholesterol from the golgi to the plasma membrane (Zha et al., 2003; Orso et al., 2000). In the other model, ABCA1 and its apolipoprotein vesicle endocytose and traffick to the excess intracellular collections of free cholesterol, where ABCA1 mediates its transport into the vesicle lumen and then exocytoses cholesterol (Takahashi and Smith, 1999). Recent literature suggests that ABCA1 mediates concurrent transport of cholesterol and phospholipids to apolipoproteins (Smith et al., 2004) though the availability of different lipids in the vicinity of ABCA1 may result in modification of the ratio of cholesterol/ phospholipid undergoing efflux (Oram and Yokoyama, 1996).

The pivotal role of ABCA1 in cholesterol homeostasis mandates a tight regulatory pathway. In addition to increased lipid efflux, a consequence of ABCA1 overexpression could also be the potential alteration of membrane structure with subsequent detrimental effects (Wang et al., 2000). Evidence for the tight regulation of ABCA1 is the short half life of the ABCA1 protein (Arakawa and Yokoyama, 2002; Oram et al., 2000) and its rapid turnover in macrophage cell lines (Wang and Oram, 2002). ABCA1 expression is regulated at transcriptional and post-transcriptional levels. Transcriptional regulation occurs mostly via sterols originating from foam cells and nuclear receptors such as the liver-X-receptor (LXR; Wagner et al., 2003; Repa et al., 2000; Venkateswaran et al.,2000) and peroxisome proliferator-activated receptor (PPAR; Chawla et al., 2001; Chinetti et al., 2001). In addition retinoid X receptors and retinoic acid receptors are involved in the regulation of ABCA1 (Costet et al., 2003). The observation that PPAR agonists and LXR ligands are ineffective in macrophages of TD patients (Venkateswaran et al., 2000; Chinetti et al., 2001) indicates that functional ABCA1 regulated by both LXRα and PPARγ is required for effective cholesterol efflux. LXRα has been shown to activate the sterol regulatory element binding protein-1 (SREBP-1; Yoshikawa et al., 2001) and stearoyl-CoA desaturases (SCD;

Wang et al., 2004) which results in increased unsaturated fatty acid (UFA) synthesis. UFAs serve as ligands for PPAR activation (Chawla, 2001) but have also been implicated in ABCA1 protein degradation (Wang and Oram, 2002; Uehara 2002). Therefore the enhanced ABCA1 transcription induced by LXR ligands may be counteracted by increased ABCA1 protein degradation implying that LXRα influences ABCA1 expression both on a transcriptional and post-transcriptional level. The influence of nuclear receptors on ABCA1 expresssion is shown in FIG 1.

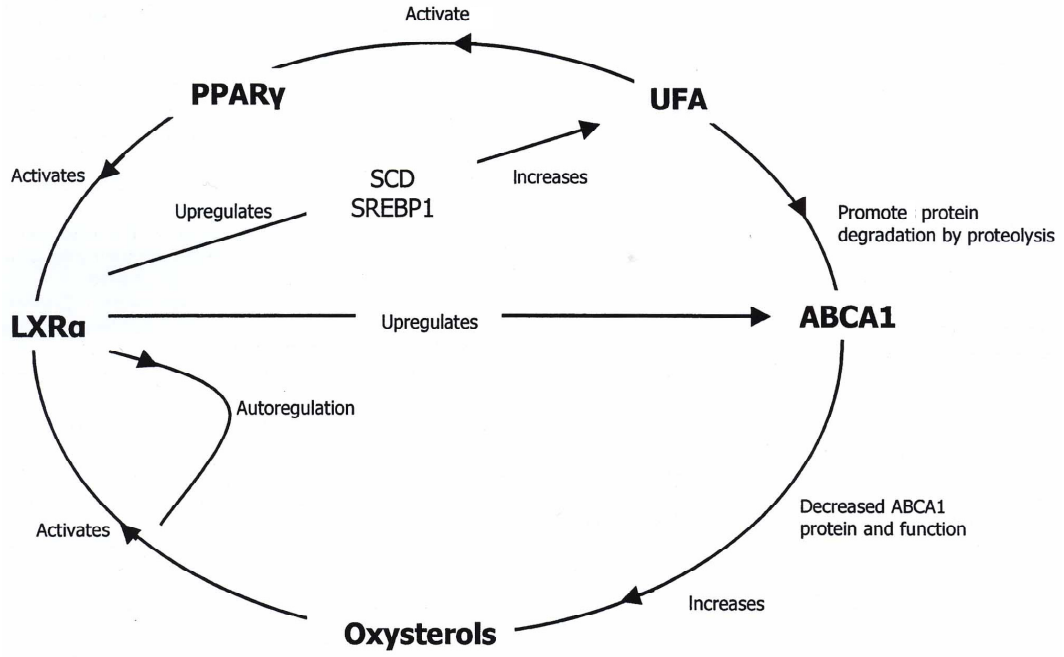

FIG 1: *Effects of nuclear receptors on ABCA1 expression. LXRα has been shown to activate the sterol regulatory element binding protein1 (SREBP1) and stearoyl-CoA desaturases (SCD) resulting in increased unsaturated fatty acid (UFA) synthesis. UFAs serve as ligands for PPARγ activation but are also implicated in ABCA1 protein degradation. Thus the enhanced ABCA1 transcription may be counteracted by increased ABCA1 protein degradation.*

The fact that post-transcriptional regulation plays a major role in ABCA1 protein expression is underlined by studies in mice which revealed a significant discordance between ABCA1 protein and mRNA levels (Wellington et al., 2002). It was suggested that the late endosomal shuttling of the ABCA1 to lysosomes could be a potential mechanism to regulate ABCA1 protein turnover (Neufeld et al., 2001). UFAs have been found to play a role in ABCA1 protein degradation (Wang and Oram, 2002; Uehara et al., 2002). This may be significant in disorders like type 2 diabetes and insulin resistance, conditions with increased levels of fatty acids where accelerated atherosclerosis is observed. UFAs have also been found to antagonize oxysterol dependent induction of LXRα thus affecting ABCA1 transcription (Ou et al., 2001).

Specific peptide motifs on short lived proteins play a major role in the degradation of ABCA1 by proteases. One of them is PEST, a sequence rich in proline (P), glutamate (E), serine (S) and threonine (T), which increases protein turnover by enhancing protein ubiquitination and proteosomal degradation (Rechsteiner and Rogers, 1996). This sequence was identified in ABCA1 (Wang et al., 2003) and appears significant in ABCA1

function as deletion of the PEST motif resulted in a 4-5 fold increase in ABCA1 protein, increased ABCA1 mediated efflux and enhanced apoA1 binding. It is proposed that PEST-dependent degradation of ABCA1 protein is mediated by calpain protease. *In vitro* experiments with peritoneal macrophages, transfected cells and mouse primary hepatocytes have shown that apoA1 binding increased ABCA1 protein without affecting mRNA levels (Wang et al., 2003). Interestingly this apoA1-mediated stabilization of ABCA1 protein is achieved by inhibition of PEST-mediated degradation by proteases (Arakawa and Yokoyama, 2002; Wang et al., 2003). ABCA1 phosphorylation, which is reported to be influenced by apoA1 (Martinez et al., 2003) and protein kinase C (Yamauchi et al., 2003), may also have a major effect on protein stability. There is also evidence that phospholipid transfer protein (PLTP) interacts with ABCA1 for its function in cholesterol efflux and also stabilizes ABCA1 protein (Oram et al., 2003). A summary of transcriptional and post-transcriptional processes regulating ABCA1 expression is shown in FIG 2.

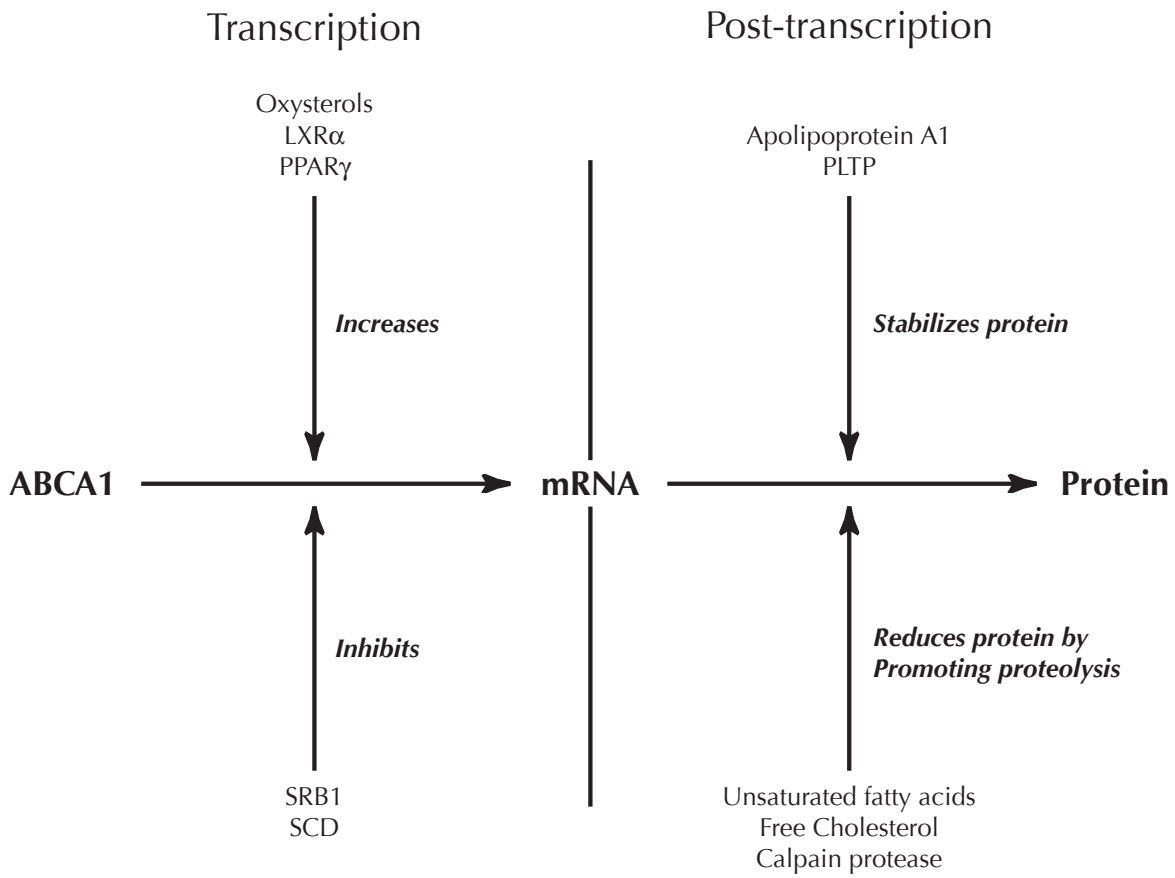

FIG 2: *Regulation of ABCA1 at transcriptional and post-transcriptional levels. PLTP: phospholipid transfer protein; SCD: stearoyl-CoA desaturases; SRB1: scavenger receptor B1. For details please see text.*

Apart from its role in lipid metabolism, ABCA1 has also been implicated in promoting engulfment of apoptotic cells (Hamon et al., 2000), low density lipoprotein (LDL) oxidation (Reddy et al., 2002), and the release of inflammatory mediators (Hamon et al., 1997; Zhou et al., 2002). These functions have been partly reproduced in ABCA1-deficient mice. Mice with a targeted inactivation of *Abca1* display morphologic abnormalities and perturbations in their lipoprotein metabolism concordant with TD (Orso et al., 2000). Interestingly, ABCA1-deficient mice do not only show severe abnormalities in their lipid

profile with substantial reductions in both apoB and apoA1 (Aiello et al., 2003), they also present with a marked reduction of pregnancies and placental malformations (McNeish et al., 2000; Christiansen-Weber et al., 2000). Moreover, compromised platelet function resulting in high bleeding tendency (Orso et al., 2000; Hamon et al., 2000) have been observed in ABCA1$^{-/-}$ mice. The latter is thought to result from a deficiency or reduced ability to expose phosphatidyserine (PS) from the inner leaflet of the cell membrane to the cell surface, which in platelets initiates the coagulation cascade. These findings suggest that ABCA1 is not only a cholesterol efflux protein but also a PS translocase (Hamon et al., 2000; Marguet et al., 1999) or a translocase regulator (Szakacs et al., 2001). The finding that PS also plays a key role as a recognition determinant for phagocytosis (Zwaal et al., 2005) proposes another link between ABCA1 and apoptosis.

In the following chapters (2.1-2.6), own research projects will be summarised where the diverse roles and distinct functions of ABCA1 were investigated and the implications of this gene in various diseases became apparent.

2 OWN RESEARCH PROJECTS

2.1 ABCA1 and cholesterol homeostasis/HDL deficiency

In own studies we investigated and characterised causes of severe HDL deficiency in a 42-year old female patient with progressive coronary artery disease. Our studies included the analysis of the patient's lipid profile, cholesterol efflux measurements, nucleotide sequence analysis and mRNA quantification of the *ABCA1* gene as well as immunoblotting of the ABCA1 protein in the patient's fibroblasts.

We found that apoA1 mediated cholesterol efflux from the patient's skin fibroblasts was significantly decreased (data not shown). ABCA1 mRNA expression, assessed by quantitative reverse transkription (RT) polymerase chain reaction (PCR), was approximately 3-fold higher in the proband's as compared to control cells (FIG 3a). Pre-incubation of cells with cholesterol increased ABCA1 mRNA approx. 5-fold in controls and 8-fold in the proband cells (FIG 3a), but similar amounts of ABCA1 protein were present in control and mutant cells (FIG 3b).

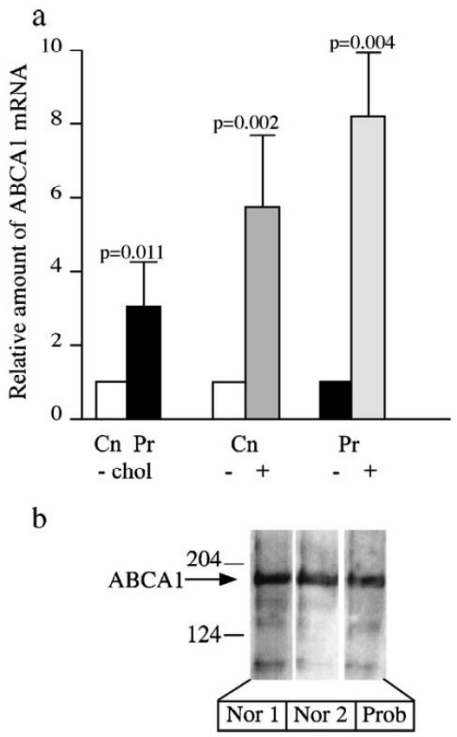

FIG 3: *Analysis of (A) ABCA1 mRNA expression by quantitative RT-PCR and (B) ABCA1 protein expression by immunoblotting in fibroblasts of the HDL deficient patient. Cn, Nor = control; Pr, Prob = proband; chol = cholesterol*

Nucleotide sequence analysis of the coding and promoter region revealed two novel autosomal recessive mutations in ABCA1, V1704D and L1379F. V1704D is predicted to lie within a membrane spanning segment (FIG 4). Thus it is not surprising that replacement of the hydrophobic valine residue with negatively charged aspartate in this position could disrupt ABCA1 function. The other novel missense mutation, L1379F, is predicted to lie in a large extracellular loop (FIG 4) and may constitute the binding site for apoA1.

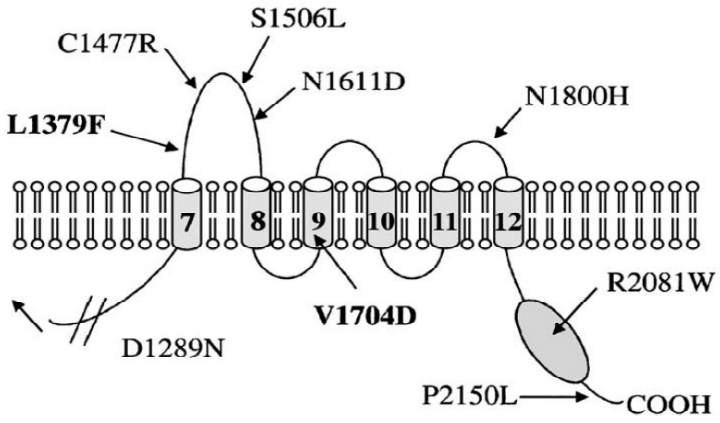

FIG 4: *Topology model of the carboxy-terminal half of ABCA1. Membrane spanning segments (7-12) and the nucleotide binding domain (oval) are shaded. Novel mutations identified in the patient are indicated in bold. Other known single amino acid substitutions in TD/HDL-deficient individuals are also shown.*

In order to prove a functional effect of the two novel mutations, wild-type and mutant ABCA1 were introduced into the full-length cDNA for ABCA1 and cloned in frame with enhanced green fluorescent protein (EGFP). Wild-type and mutant ABCA1-EGFP was expressed in transiently transfected HEK293 cells. While wild-type ABCA1 was mainly present at the plasma membrane (FIG 5, left panel), trafficking of both mutants to the plasma membrane was severely altered (FIG 5, middle and right panel).

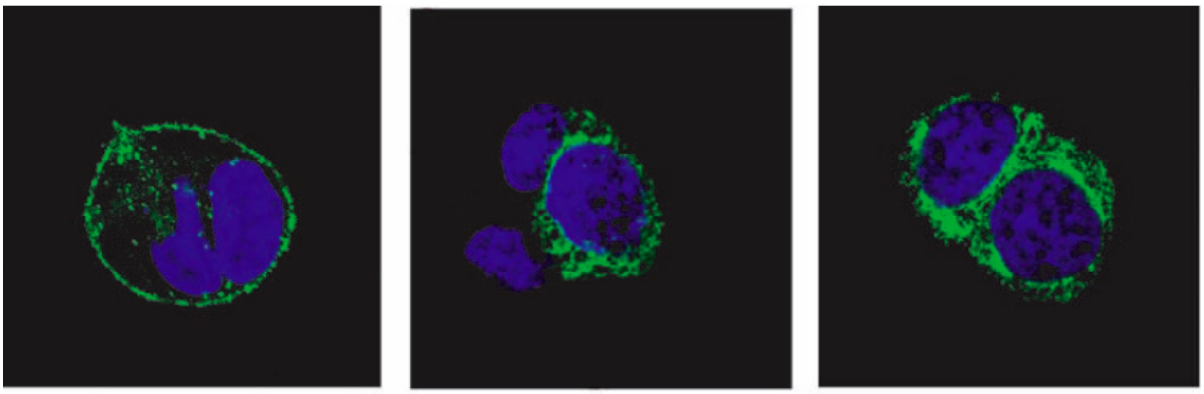

FIG 5: *Confocal microscopy in HEK 293 cells transiently transfected with wild-type (WT) and mutated (L1379F or V1704D) ABCA1 fused to EGFP; blue= nuclear staining with DAPI; green = ABCA1-EGFP*

In conclusion, the two novel missense mutations that we have identified in a patient with severe autosomal recessive HDL deficiency, prevent normal trafficking of ABCA1, thereby explaining their inability to mediate apo-A1-dependent lipid efflux.

This study was published in *Biochimica and Biophysica Acta*: C. Albrecht et al., Biochim Biophys Acta 1689 (2004) 47-57 (see Appendix 1).

2.2 ABCA1 and phosphatidylserine translocation/Scott syndrome

Scott syndrome (SS) is an extremely rare, moderately severe bleeding disorder. As worldwide only three patients have been identified, the pattern of inheritance is unknown (Toti et al., 1996; Weiss et al., 1997), and the rarity of affected individuals precludes conventional mapping approaches to identify the underlying genetic lesions. The defining characteristic of SS is the absence of Ca^{2+}-stimulated exposure of PS from the inner leaflet of the plasma membrane bilayer to the cell surface. This process normally provides an appropriate surface for the assembly of the tenase and prothrombinase complexes of the coagulation network. The failure to expose PS to the outer leaflet of the platelet plasma membrane is also observed in Epstein-Barr virus (EBV)-transformed lymphocytes of patients with SS.

Some candidate proteins such as phospholipid scramblases (Stout et al., 1997; Zhou et al., 1998; Zhou et al., 2002) or P-glycoprotein (Zhou et al., 1998; Toti et al., 1997) which are thought to be involved in PS translocation have already been investigated but appear to be normal in SS. As ABCA1 has been implicated in PS translocation both by genetic disruption in mice (Hamon et al., 2000) and chemical inhibition (Marguet et al., 1999), we assessed the expression and function of ABCA1 in a 59-year old female patient with SS that we have recently characterised (Elliott et al., 2004).

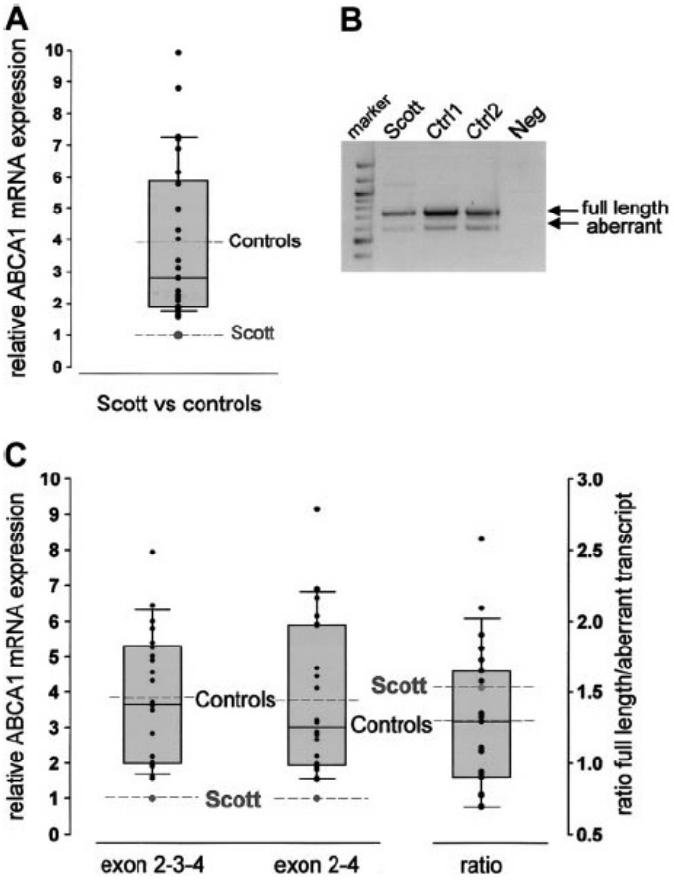

FIG 6: *Quantitative RT-PCR measurements of full-lenght (A, C; ex 2-3-4) and aberrant (C; ex 2-4) ABCA1 transcripts in control and SS leucocytes (B) RT-PCR and gel electrophoresis of the alternatively spliced ABCA1 transcript*

Our studies included analysis of the patient's lipid profile, nucleotide sequence analysis of the coding and proximal promoter region and mRNA quantification of the *ABCA1* gene. Furthermore we performed functional assays monitoring PS translocation in the patient's EBV-transformed lymphocytes with flow cytometric techniques as well as pharmacological experiments.

Using quantitative real-time PCR technique we detected substantially reduced levels of ABCA1 mRNA in the SS patient's leucocytes as compared to a healthy mixed control population (FIG 6A and C). This could not be explained by a change in the ratio of a recently described alternatively spliced aberrant transcript (Bellincampi et al., 2001; FIG 6B) as both transcripts (exon 2-3-4 and exon 2-4) were equally low in the SS patient (FIG 6C). Thus no preferential splicing occurred.

Nucleotide sequence analysis of the promoter region, exons and flanking intronic sequence revealed beside several known polymorphisms heterozygosity for a novel single base pair substitution c6064G>A in exon 42. This missense mutation is predicted to substitute R1925 with glutamin and was absent from unaffected family members and controls. R1925Q is predicted to lie 20 amino acids N-terminal of the Walker A motif in the second nucleotide binding domain and is conserved in all known mammalian ABCA1 transporters. (FIG 7)Substitution of R1925 with glutamin disrupts a stretch of 5 highly charged amino acids (RRKRK), which is strongly conserved between all members of the ABCA1 family of ABC transporters pointing to the functional importance of this residue (FIG 7).

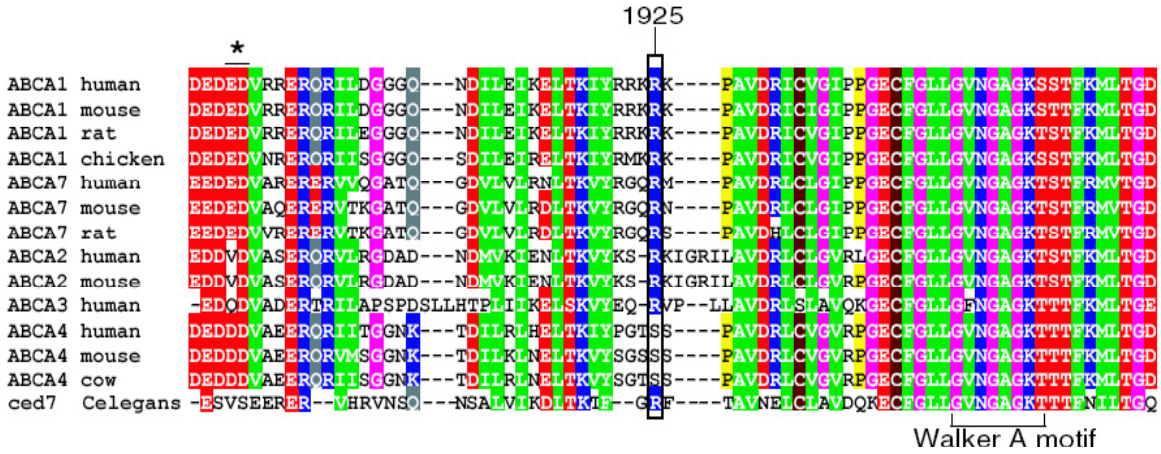

FIG 7: *Amino acid alignment of mammalian sequences encoding for ABC transporters of the A-subfamily and C elegans ced7 spanning the region mutated in the SS patient. The asterisk depicts a 2-amino acid deletion found in a patient with HDL deficiency.*

In order to assess whether the R1925Q mutation identified in the SS patient has an effect on ABCA1 function, in particular if it does affect PS translocation, two different approaches were chosen:

Firstly to test the impact on intracellular trafficking, we introduced the c6064G>A mutation into a full-length cDNA encoding ABCA1 fused to its carboxy-terminus to EGFP and expressed the fusion protein in HEK293 cells. In cells transfected with wild-type ABCA1-EGFP, fluorescence was present mainly at the cell surface, with punctuate intracellular vesicles visible in some cells and possible location in the Golghi in others (FIG 8A and 8B, left panels). In contrast, little or no fluorescence was detected on the surface of cells transfected with the ABCA1 R1925G-EGFP construct (FIG 8A, middle and right panel). Instead fluorescence was observed largely in the endoplasmic reticulum (ER), evidenced by co-localisation studies with sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2), a marker of the ER (FIG 8B, middle and right panels).

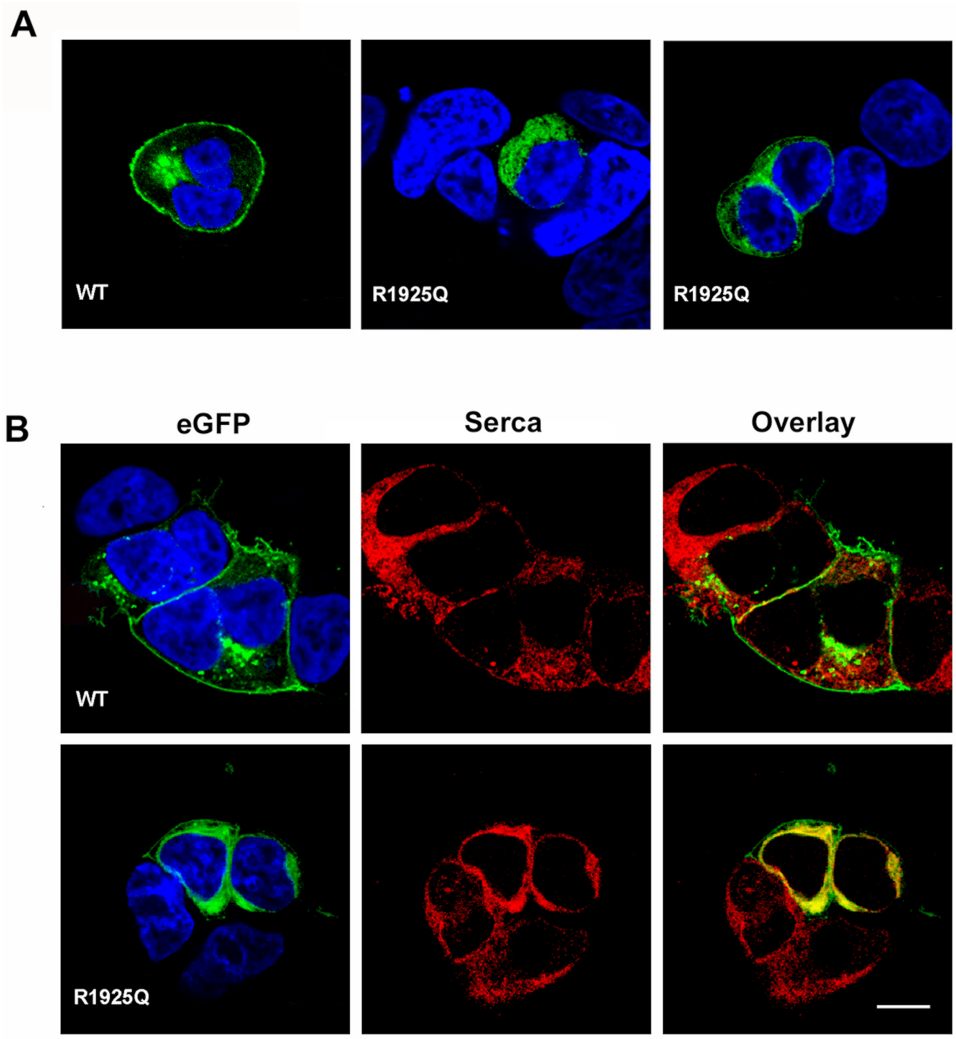

FIG 8: *(A) Confocal microscopy of wild-type (WT) and mutated (R1925Q) ABCA1 fused to EGFP in transiently transfected HEK 293 cells (B) co-localisation studies using SERCA, a marker of the ER; blue= nuclear staining with DAPI; green = ABCA1-EGFP; red = staining of the ER with SERCA; yellow = overlay between green and red indicating co-localisation*

Secondly, to test the role of ABCA1 in Ca^{2+}-stimulated PS translocation, we expressed wild-type ABCA1 in the EBV-transformed B-cell line from the SS patient using a retroviral expression system. ABCA1 expression did result in restoration of A23187-stimulated PS exposure similar to control EBV transformed B-cells (FIG 9A). Furthermore, PS translocation in SS-ABCA1^{+} cells was completely blocked by the ABCA1 inhibitor glyburide (FIG 9B). These results demonstrate that expression of wild-type ABCA1 complements the defective Ca^{2+}-activated PS translocation in the parental SS cells.

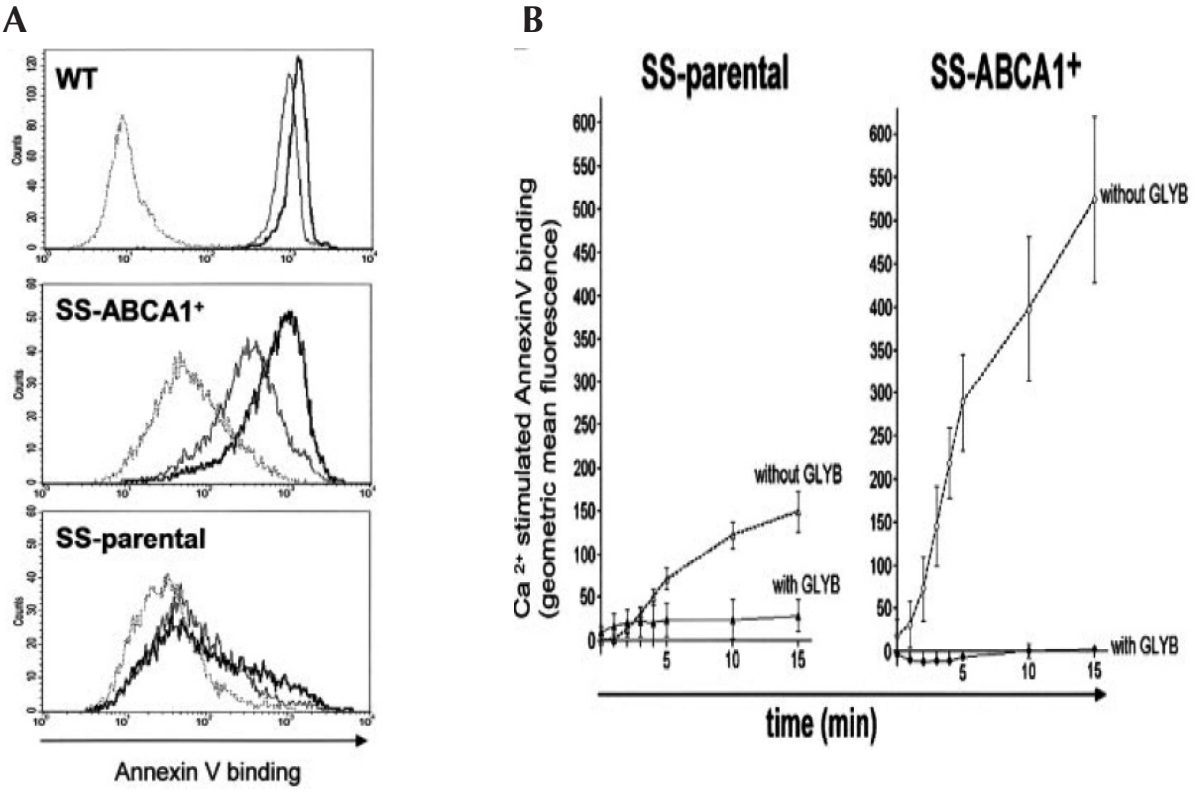

FIG 9: *Flow cytometry analysis of Ca^{2+}-induced PS translocation monitored by binding of fluorescent AnnexinV in parental SS cells (SS-parental), ABCA1 overexpressing SS cells (SS-ABCA1^{+}) and control EBV-transformed lymphocytes (WT). (A) AnnexinV binding at 0 (dotted lines), 3 (thin solid) and 10 min (bold solid) after stimulation with the calium ionophore A23187. (B) AnnexinV binding plus or minus preincubation with the ABCA1 inhibitor glyburide (GLYB; 1mM)*

However, despite demonstrating that ABCA1 plays a crucial role in the phenotype of this SS patient, nucleotide sequence analysis of the ABCA1 gene revealed no difference in the proximal promoter region, in intron-exon junctions or within the coding region that might explain the reduced *ABCA1* mRNA levels in the patient's leucocytes. Hence an additional unidentified mutation, potentially in a *trans*-acting regulatory gene, is hypothesised to account for reduced mRNA from both alleles.

Another intriguing characteristic of this SS patient is that her lipid profile lacks clinical features of TD or HDL deficiency which are phenotypes of other characterised mutations in ABCA1. This supports previous data (Singaraja et al., 2003; Kolovou et al., 2003; Haghpassand et al., 2001; Aiello et al., 2002) indicating that changes in ABCA1 activity can occur without alterations in the steady-state plasma lipid levels. Alternatively it is possible that ABCA1 might have a distinct role in blood cells compared with tissue-derived cells. However, as the SS patient's lipid profile was not typical for the phenotype of many ABCA1 mutations, identification of this mutation suggests separation of the function of ABCA1 in cholesterol transport from its role in PS translocation forging novel links between lipid homeostasis and haemostasis. These findings unravel a novel mechanism in platelet function and might identify a new drug target in haemostasis.

The studies mentioned above resulted in two publications: J.I. Elliott et al., Thromb Haemost 91 (2004) 412-415; C. Albrecht et al., Blood 106 (2005) 542-549 (see Appendix 1). The latter publication has received an Editorial comment (Blood 106 (2005) 396-397) and was selected for the cover page of Blood issue 106.

2.3 ABCA1 and diseases of immune hyperactivity

Whilst disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) are considered to result principally from lymphocyte hyperactivity leading to reactivity towards autoantigens (SLE, RA) and gut flora (IBD), they are also associated with activation of platelets with an accompanying increased risk of thrombosis (Joseph et al., 2001; Knijff-Dutmer et al., 2002; Danese et al., 2004). Thrombus formation is initiated by the cell surface exposure of PS which is normally confined to the inner leaflet of the plasma membrane of mammalian cells. Upon cellular activation and consequent increased levels of intracellular calcium, plasma membrane asymmetry is rapidly collapsed through the active transport of phospholipids by a scramblase protein and/or an outward aminophospholipid translocase (Sims et al., 2001). Through exposure of a negatively charged phospholipid surface the outer membrane is transformed into a catalytic surface for the assembly of active tenase and prothrombinase coagulation complexes.

Whilst platelets are presumed to be the cell type primarily responsible for thrombus formation, the mechanisms resulting in loss of membrane asymmetry are shared with other haematopoietic cells such as lymphocytes (Sims et al,, 2001). We therefore asked whether patient-derived lymphocytes exhibit an abnormal propensity towards loss of lipid asymmetry that might indicate a general defect of haematopoietic cells in the pathogenesis of SLE, RA and IBD. Furthermore, as we have demonstrated that ABCA1 plays a role in PS translocation and platelet activation in a patient with Scott syndrome (see chapter 2.2), we have tested a potential implication of ABCA1 by determining its expression in leucocytes of SLE and RA patients.

We have developed a rapid real-time flow cytometric assay of calcium ionophore-induced PS translocation and compared the rate of PS translocation on lymphocytes from patients with that by control cells. Twelve SLE patients (mean age, 49 years; range 32-66 years; all female), nine patients with RA and one with giant cell arteritis (GCA; 'RA group', mean age, 57 years; range, 32-72 years; seven female) and ten patients with IBD (four with Crohn's disease, six with ulcerative colitis: mean age 39 years; range 23-70 years; six female) were included in this study.

Lymphocytes, obtained by venepuncture from patients and control individuals were freshly isolated and stained with a fluorescent antibody ($CD4^{PE}$, $CD4^{CYCHROME}$, $CD4^{APC}$). By differential antibody-labelling of patient and control cells prior to mixing, we were able to compare the rate of PS translocation of two or three samples within a single tube. Appropriately labelled lymphocytes were equilibrated with AnnexinV and Ca^{2+}-induced PS exposure was monitored for 10 minutes (FIG 10A and B). For detailed description of

the density (false colour) plots depicted in FIG 10A, the validation of the flow cytometry assay and the evaluation of the data, please see the original paper in Appendix 1.

Evidence of PS exposure on $CD4^+$ lymphocytes was apparent within 5 minutes of stimulation with calcium ionophore (FIG 10A and B), closely followed by the appearance of bodies lacking cell surface markers such as CD4, CD8 and CD19 (data not shown). Such bodies neither bind AnnexinV nor take up PI (not shown) and are assumed to be the remnants of cells having shed $AnnexinV^+$ microparticles (Elliott et al., 2004).

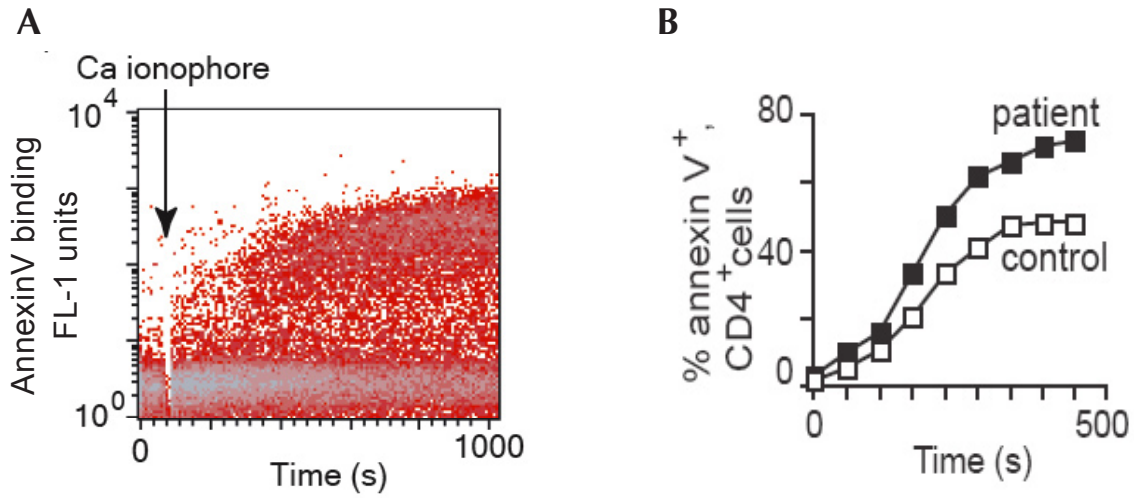

FIG 10: *Flow cytometric analysis of Ca^{2+}-induced PS translocation in freshly isolated lymphocytes from controls and patients. (A) Density plot of the response to stimulation with 4μM calcium ionophore (arrow). PS exposure is indicated by increased binding of fluorescently labelled AnnexinV. (B) Comparison of the rate of PS exposure on control and patient as a function of time*

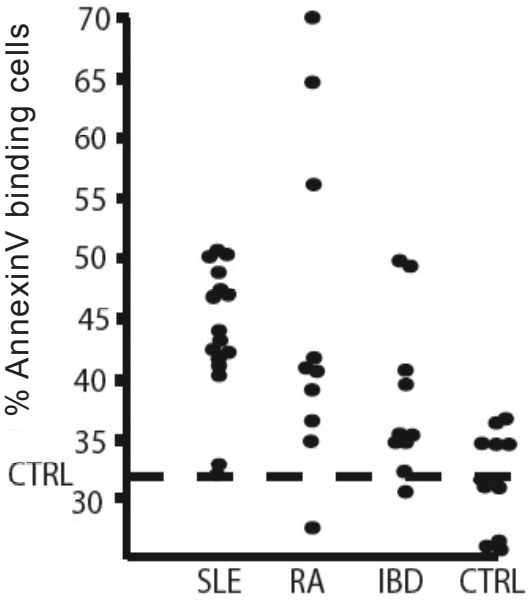

FIG 11: *Comparison of the rate of PS translocation on $CD4^+$ lymphocytes from control (CTRL) and patient groups*

We then compared the rate of calcium ionophore-stimulated PS exposure on $CD4^+$ peripheral blood lymphocytes from SLE, RA (this group included one patient with GCA) and IBD patients with that by control cells. For each patient group, the rate of PS translocation by $CD4^+$ T cells exceeded that by controls (mean ± S.D. SLE 44.8 ± 4.7, p = 2.9 x 10-6; RA/GCA 45.7 ± 14, p = 0.02; IBD 38.3 ± 6.4, p = 0.04; FIG 11).

As shown in FIG 11, the results from patient groups were distinct. In particular, whilst rates of PS translocation appeared significantly increased in the majority of SLE and RA/GCA patients, only a minority of those with IBD showed this phenotype. There was also a considerable heterogeneity amongst patient responses, particularly within the RA group.

Such variations might reflect the relatively small sample size or differences in the contribution that dysregulation of haematopoietic cell responses to intracellular calcium might play in disease. It may be relevant, for example, that rates of PS translocation were most significantly elevated in the SLE group and that antibodies to proteins bound to PS are more prevalent in SLE than in RA/GCA or IBD. That not all RA/GCA and IBD lymphocytes exhibit elevated PS exposure indicates that this phenotype may predispose towards, but not be essential for, disease. Moreover, it remains possible that those patients exhibiting accelerated PS exposure are at greatest risk of future thrombotic events.

No correlation was apparent between and rate of PS exposure and age, sex, or general inflammation as evidenced by levels of C-reactive protein (not shown), nor with drug regimen. In each disorder, the average rate of PS exposure was increased above control levels.

The accelerated rate of PS translocation we observed was accompanied by increased rates of cell breakdown (not shown). This is perhaps not surprising as PS exposure is usually associated with membrane blebbing. Such blebs are eventually shed as PS^+ microparticles that have a number of procoagulant properties, for example altering nitric oxide production in conductance and resistance arteries (Martin et al., 2004). Indeed, elevated levels of platelet, leucocyte and/or endothelial cell derived microparticles have been observed in RA and SLE (Knijff-Dutmer et al., 2002; Combes et al., 1999; Berckmans et al., 2002) and have been implicated in the thrombotic tendency of these disorders. It is interesting to note that in type 1 diabetes it has also been suggested that increased risk of microangiopathy may result from high rates of cell activation or apoptosis and concomitant shedding of microparticles (probably from several cell types) bearing exposed PS (Sabatier et al., 2002).

The cause(s) of the increased rates of PS exposure we have observed in patient cells remain to be elucidated. Indeed little is known about the mechanism of PS translocation (Sims et al., 2001). As it has been suggested that the protein ABCA1 acts as a PS floppase and rates of PS translocation are reduced in haematopoietic cells from ABCA1-deficient mice (Hamon et al., 2000) and we have demonstrated that ABCA1 plays a role in PS translocation and platelet activation in a patient with Scott syndrome (see chapter 2.2), we have tested a potential implication of ABCA1 by determining its expression in lymphocytes from SLE and RA patients.

However, in these disorders we did not gain evidence for direct interaction between ABCA1 and PS suggesting that ABCA1 may act upstream of any translocase. We did not find any significant difference in the level of leucocyte *ABCA1* mRNA between patients and controls (FIG 12).

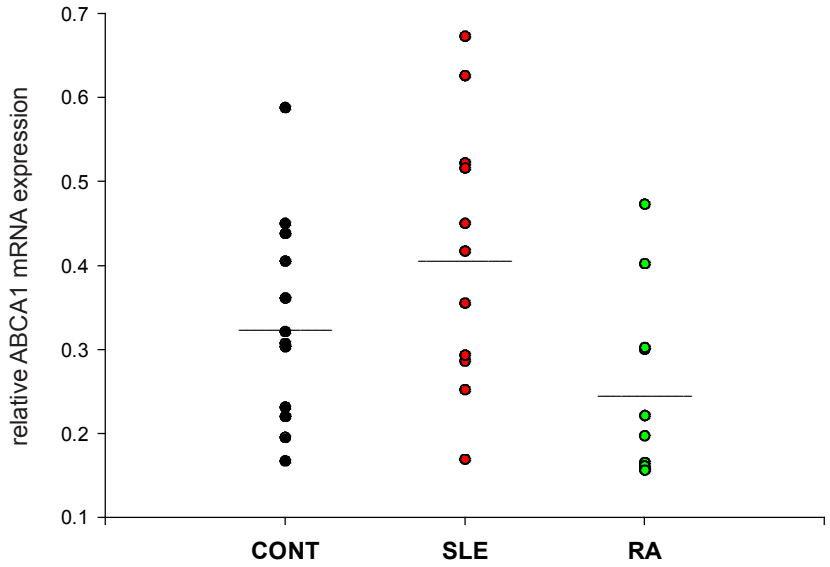

FIG 12: *Quantitative RT-PCR measurements of ABCA1 mRNA expression in leucocytes from controls (CONT) and patients with SLE and RA/GCA.*

To summarise this chapter, we have shown that lymphocytes from patients with SLE, RA/GCA and, to a lesser extent, IBD, exhibit heightened sensitivity to increased intracellular calcium, as evidenced by rapid exposure of PS to the cell surface and cell breakdown. These findings indicate that increased rates of PS exposure may contribute toward the elevated thrombotic risk in each of these disorders. It would be of interest to determine whether those SLE, RA and IBD patients exhibiting accelerated PS exposure are at greatest risk of thrombosis.

The results of this study were published in Thrombosis and Haemostasis: C. Albrecht et al., Thromb Haemost 93 (2005) 989-992 (see Appendix 1).

2.4 ABCA1 and vascular diseases/atherosclerosis

As mentioned earlier (see chapter 1 and 2.1) ABCA1 is a transmembrane protein involved in cholesterol and phospholipid transport from cells to lipid-poor apolipoproteins in the plasma. Mutations in the *ABCA1* gene were discovered to be responsible for TD (Bodzioch et al., 1999) and familial HDL deficiency (Marcil et al., 1999). These conditions are characterised by low levels of HDL, the deposition of lipid-laden macrophages in tissues and increased atherosclerotic disease in a proportion of patients. The failure of ABCA1 to transport cholesterol and phospholipids out of cells to form complexes with apolipoproteins in order to generate HDL (Oram et al., 2000) leads to intracellular sterol accumulation and the subsequent development of foam cells, a hallmark of the atheromatous plaque. Attention has focussed on ABCA1 because of its potential role in atherosclerosis, and the fact that therapeutic interventions could be targeted at the regulatory pathway controlling ABCA1 expression. Whilst the systemic effects of loss of function in ABCA1 deficient individuals were well characterised, less was known about the potential role of this

transporter in localised atheromatous disease in individuals without reported mutations in the gene.

In addition to act as a cholesterol efflux protein, ABCA1 is implicated in PS translocation between the leaflets of cell membranes (see chapter 1 and 2.2) and the engulfment of apoptotic cells (Hamon et al., 2000). Apoptosis, PS externalisation and lipid dysregulation play critical roles in the development and subsequent behaviour of atherosclerotic plaques making this transporter a promising candidate in the context of atherosclerosis. The key question of this study therefore was whether there is a localised, arterial loss of ABCA1 function in relation to atherosclerotic disease.

Our way to approach this question was to investigate both ABCA1 gene and protein expression in human carotid atherosclerotic disease. Therefore we analysed ABCA1 expression in atheromatous plaques taken from 18 patients undergoing carotid endarterectomy (CEA) and compared it to human arteries obtained from 10 controls with no phenotypic atherosclerotic disease. The relationship between *ABCA1* mRNA and protein expression was assessed by analysing the same specimen for both parameters. In order to gain insight into regulatory mechanisms involved in *ABCA1* gene expression, we also measured mRNA expression of one of its key regulators, the LXRα using real-time quantitative PCR. As leucocytes are known to be involved in the pathogenesis of the atheromatous plaque (Libby et al., 1996) and deficient leucocyte *ABCA1* expression being implicated in increased susceptibility to atherosclerosis in animal studies (Van Eck et al., 2002), we analysed *ABCA1* mRNA expression in peripheral leucocytes.

ABCA1 mRNA was significantly increased in plaques as compared to control arteries (FIG 13A, $p<0.0001$). In order to evaluate whether the up-regulation of *ABCA1* was restricted to the diseased artery or reflected in the systemic circulation, we compared *ABCA1* expression in leucocytes from patients undergoing CEA and age and sex-matched controls. In preliminary experiments *ABCA1* expression in leucocytes was comparable to monocytes and therefore seemed to reflect circulating monocyte *ABCA1* expression. No difference in ABCA1 expression levels was found ($p=0.6741$, FIG 13B) indicating a localised up-regulation of *ABCA1* mRNA levels in the plaque tissue.

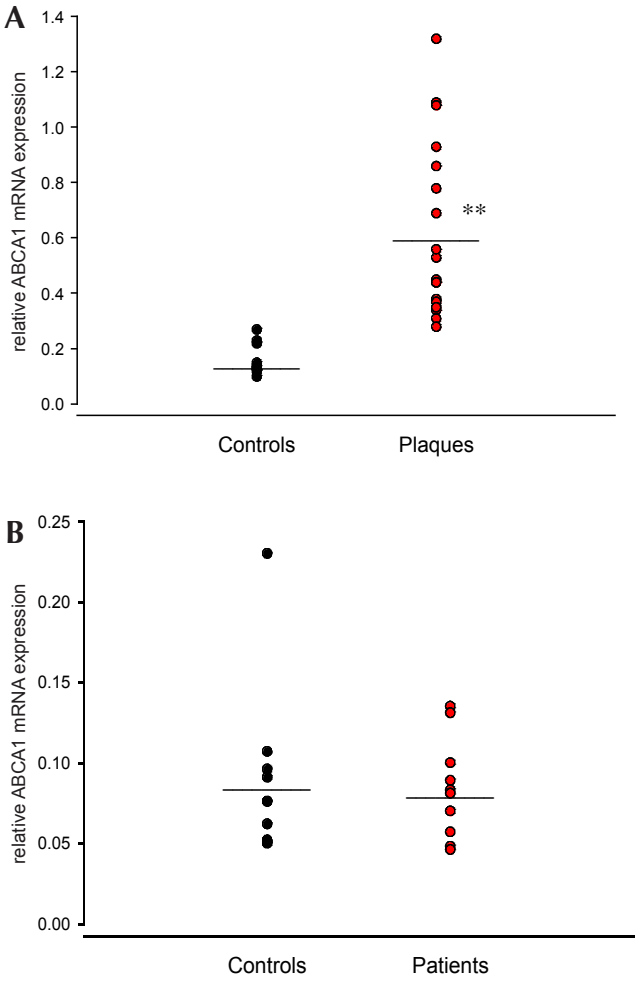

FIG 13: *Quantitative RT-PCR measurements of ABCA1 mRNA expression in (A) arteries and (B) leucocytes obtained from control subjects and patients undergoing CEA*

In order to assess ABCA1 protein expression, immunoblot analysis was carried out. Total membrane fractions of plaques and control arteries were tested with antibodies against ABCA1 (FIG 14A, upper bands) and Na^+/K^+-ATPase (FIG 14A, lower bands), a plasma membrane protein used to ascertain equal sample loading. In contrast to mRNA levels, ABCA1 protein expression was significantly lower in plaques than in control arteries. Semi-quantitative analysis using the OD of the bands confirmed the marked difference between controls and plaques ($p<0.0001$, Figure 12B) and remained highly significant after normalisation of ABCA1 expression to Na^+/K^+-ATPase ($p=0.0004$, FIG 14C). No difference with regard to the Na^+/K^+-ATPase loading control was found ($p=0.8316$, FIG 14B).

In control arteries, *ABCA1* gene expression was reflected by the presence of protein. Intriguingly, however, markedly lower levels of ABCA1 protein were present in advanced atherosclerotic lesions. Thus, despite increased transcription, a reduction in protein expression was observed.

There is evidence that the composition and microenvironment of the atherosclerotic plaque could be associated with ABCA1 protein degradation. In advanced atherosclerotic lesions, macrophages tend to accumulate large amounts of free cholesterol (Tabas, 1997). Increased intracellular cholesterol has been shown to accelerate the degradation of ABCA1 in macrophages (Feng and Tabas, 2002). Long chain fatty acids, present in the plaque (Felton et al., 1997), can promote macrophage ABCA1 protein degradation (Wang et al., 2004). Furthermore it has recently been demonstrated that ABCA1 contains a PEST sequence that appears to enhance protein degradation (Wang et al., 2003; see also chapter 1).

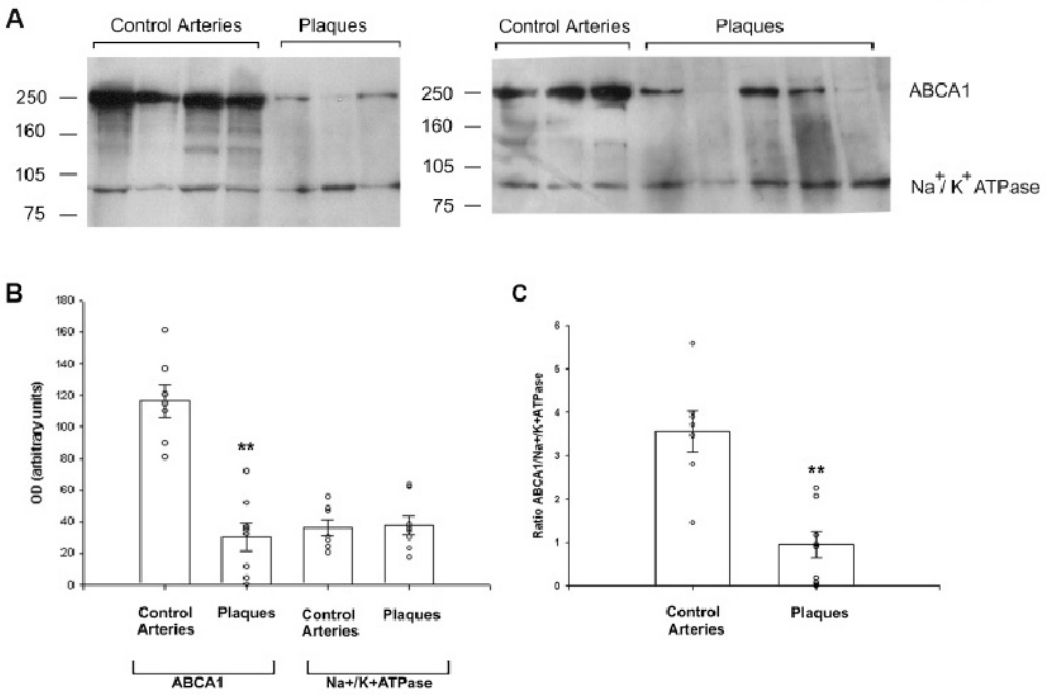

FIG 14: *Western blot analysis of ABCA1 in plaques and control arteries (A) Immunoblotting and (B) semi-quantitative analysis of ABCA1 and Na^+/K^+-ATPase protein expression using membrane fractions prepared from control and plaque specimens. (C) Ratio between ABCA1 and Na^+/K^+-ATPase protein expression according to results as presented in FIG 12B.*

In order to gain insight into potential underlying mechanisms regarding the regulation of ABCA1 expression, mRNA expression of *LXR*α was measured. The increase in *ABCA1* mRNA detected in plaques was paralleled by a significant more than two-fold increase in average *LXR*a mRNA levels (p=0.0287). Interestingly, in plaque tissue a significant correlation between *ABCA1* and *LXR*α mRNA expression levels was found (FIG 135A, r=0.85, p<0.0001) while in control arteries no association was detected (FIG 15B, r=0.24, p=0.5070).

The parallel increase in *LXR*α and *ABCA1* mRNA could be attributed to the oxysterol-rich environment inside the plaque potentially amplified by low ABCA1 protein levels. Increased degradation of ABCA1 protein could hypothetically diminish cellular cholesterol efflux, resulting in increased free cholesterol, enhanced intracellular oxysterol loading and stimulation of regulatory pathways involving LXRα (Schwartz et al., 2000). Nuclear receptors act as cholesterol sensors that respond to elevated sterol concentrations (Lu et al., 2001). It has been previously shown *in vitro* that *ABCA1* transcription is stimulated by LXRα and the retinoid X receptor (Costet et al., 2000) and the induction of *ABCA1* expression reflected that of *LXR*α (Laffitte et al., 2001).

In conclusion this study has shown that both the *ABCA1* gene and protein are expressed in mildly atherosclerotic arterial tissue *in vivo*. Advanced carotid atherosclerotic lesions are characterised by reduced ABCA1 protein levels despite significant upregulation of both *ABCA1* and *LXR*α mRNA. This finding has potentially important clinical consequences. The observation that upregulation of *ABCA1* mRNA fails to translate into ABCA1 protein implies that pharmacological targeting of the ABCA1 and LXRα pathways may not achieve the anticipated atheroprotective effect in advanced atherosclerotic lesions.

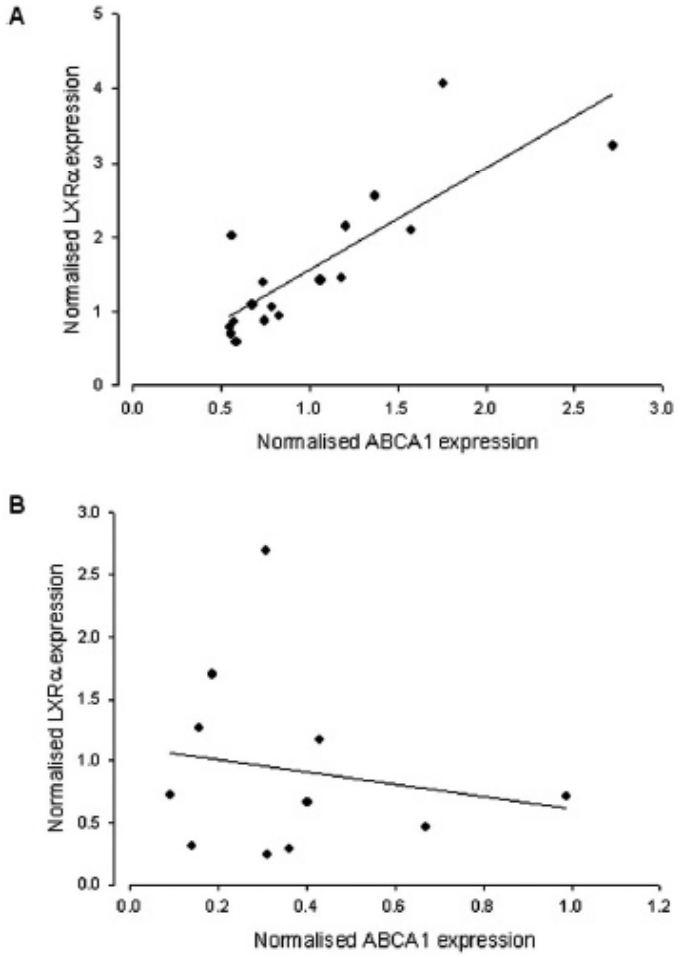

FIG 15: *Association between ABCA1 and LXRα mRNA expression in (A) plaque and (B) control tissues assessed by quantitative RT-PCR.*

In another closely related study we extended the analysis of nuclear receptors in atherosclerotic disorders from occlusive to ectatic atherosclerotic disease. Nuclear receptors like PPARγ and LXRα are known to modulate atherogenesis at various stages from cell recruitment, lipid accumulation and local inflammatory response (Castrillo et al., 2003; Joseph et al., 2003; Li et al., 2000; Marx et al., 1998). Moreover, as the atherosclerotic plaque behaviour is influenced by intraplaque inflammation (Plutzky et al., 1999; Cipollone et al., 2001; Martin-Ventura et al., 2004; MacGeer et al., 2002), matrix turnover (Putzky et al., 1999; Loftus et al., 2000) and the volume of the lipid core (Strary et al., 1994; Mathiesen et al., 2001) we aimed to evaluate the mRNA expression of the genes involved in lipid efflux (*ABCA1*, *LXRα*, *PPARγ*) pathways and to assess its relationship with parameters of inflammation (*COX-2*) and matrix turnover (*MMP-9*) in occlusive and ectatic atherosclerotic disease.

In this project CEA specimens from 16 patients (mean age: 72 years) and aneurysm tissue from 16 patients (mean age: 75 years) undergoing abdominal aortic aneurysm repair were collected. Inferior mesenteric arteries from colectomy specimens of 12 patients (mean age: 70 years) served as controls. Total RNA was extracted from pulverised tissue and reverse transcribed into cDNA. Quantitative real-time PCR was performed using fluorescently labelled probes for ABCA1, LXRα, PPARγ, COX-2 and MMP-9.

We found a reduced expression of *PPARγ* in human atherosclerotic tissues, both occlusive and ectatic (FIG 16A), when compared to normal arterial controls ($p<0.001$) while *LXRα* (FIG 16B) and *ABCA1* (FIG 16C) mRNA were significantly upregulated in both types of diseases ($p<0.01$).

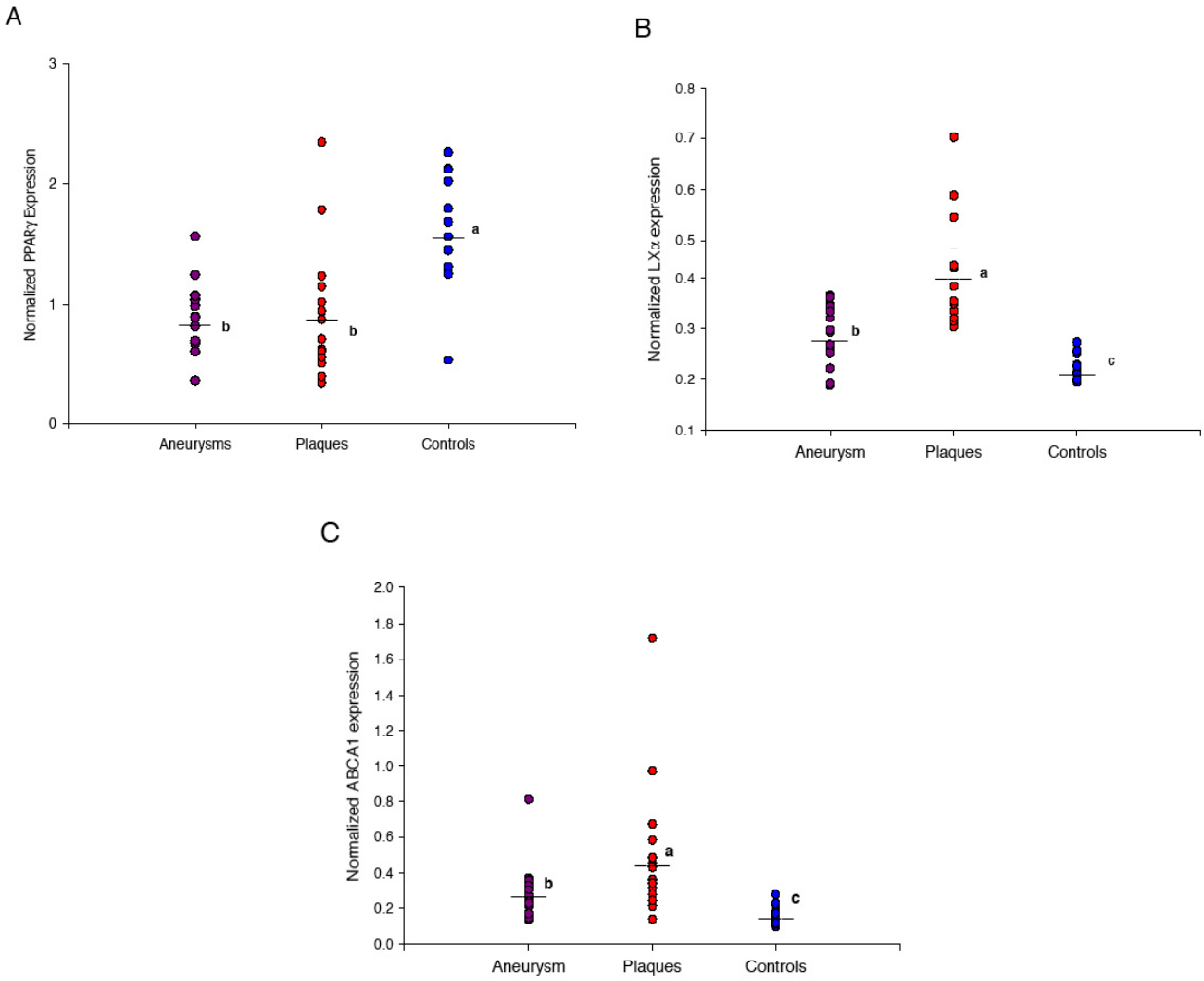

FIG 16: *(A) PPARγ (B) LXRα and (C) ABCA1 mRNA expression in control, aneurysm and plaque tissues assessed by quantitative RT-PCR.*

As shown in the study described above (Albrecht et al., 2004, see chapter 2.4 and Appendix 1) we have demonstrated that ABCA1 protein was low in carotid atherosclerotic plaques despite increased mRNA expression hypothesizing that reduced ABCA1 protein leads to an oxysterol rich plaque microenvironment which in turn stimulates LXRα with consequent upregulation of the *ABCA1* gene. This could explain the high mRNA levels of *LXRα* and *ABCA1* in these tissues. The variable levels of *ABCA1* and *LXRα* upregulation in both occlusive and aneurysmal disease could be attributed to the difference in the availability of ligands activating these genes. PPARγ, expressed by all major cells of the vasculature (Ricote et al., 1998), has attained remarkable interest in terms of atherosclerosis because of it's potential beneficial effects. This receptor can be therapeutically targeted using the thiazolidinediones, a group of drugs such as pioglitazone, rosiglitazone and troglitazone, agents that have been recently used in the treatment of type 2 diabetes and proved to be very effective in reducing insulin resistance (Chen et al., 2001; Fujiwara et al., 2000; Raji et al, 2002; Ruan, et al., 2003). The reduced expression of *PPARγ* in these tissues could potentially be due to the increased amount of cytokines in the plaque microenvironment (Zhang et al., 1996).

MMP-9 mRNA expression was significantly increased in diseased tissues ($p<0.0001$) though levels were markedly higher in occlusive disease ($p<0.01$, FIG 17A). *COX-2* expression was increased in ectatic but low in occlusive disease ($p<0.01$, FIG 17B).

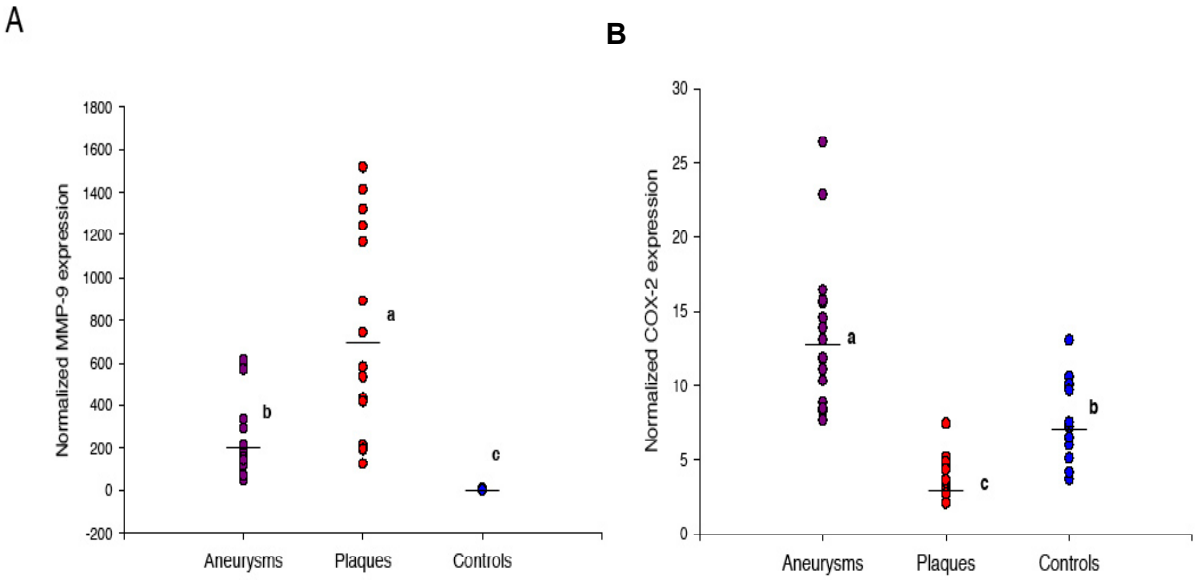

FIG 17: *(A) MMP-9 and (B) COX-2 mRNA expression in control, aneurysm and plaque tissues assessed by quantitative RT-PCR.*

MMP-9, implicated in degradation of the plaque fibrous cap, was previously found to be significantly elevated in both types of diseased tissues, more so in occlusive disease (Loftus et al., 2000; Orbe et al., 2003). Although its role in aneurysm formation is not quite clear, MMP-9 has been extensively studied in the context of plaque pathophysiology (Orbe et al., 2003; Papalambros et al., 2003). Surprisingly *COX-2* mRNA expression was decreased in atheromatous plaques in this study whereas the levels were significantly higher in aneurysms. Oxidised LDL has been reported to inhibit COX-2 in human macrophages in *in vitro* work (Eligini et al., 1999) suggesting that the impact of macrophage COX-2 may be attenuated in advanced atherosclerotic lesions (Linton et al., 2004). Moreover the levels of oxidised LDL may reach higher concentrations in the plaque than in the aneurysm wall. This also seems to reflect the recent findings that therapeutic COX-2 inhibition may not be beneficial in stabilising the plaque considering the fact that the majority of these plaques were symptomatic (Bea et al., 2003; Olesen et al., 2002).

The increased mRNA levels of *ABCA1* (FIG 18A) and *MMP-9* (FIG 18B) were significantly correlated in diseased tissues ($p<0.01$, $r=0.71$ and $r=0.78$).

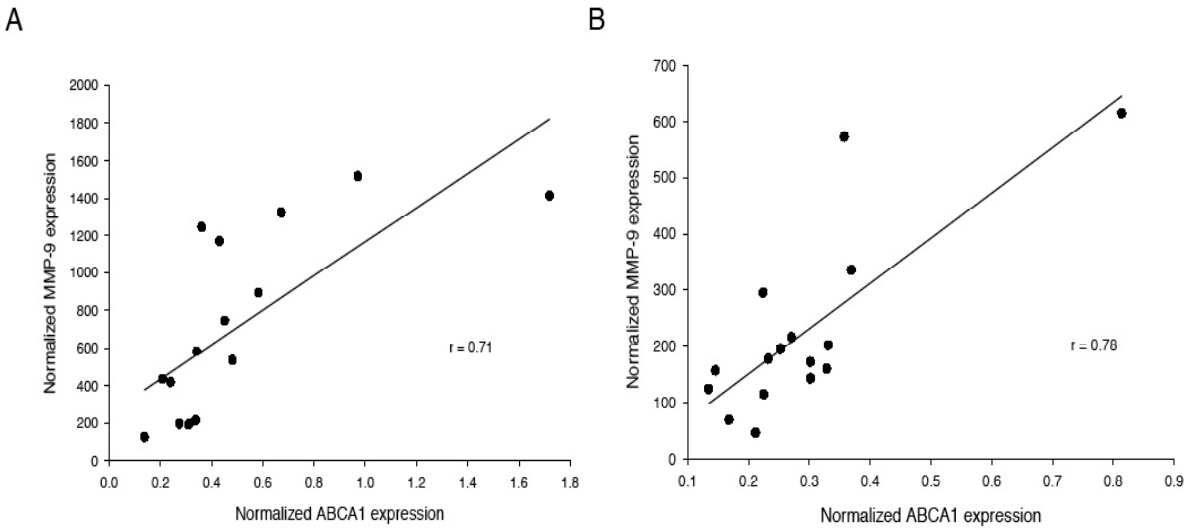

FIG 18: *Association between mRNA levels of ABCA1 and MMP-9 in (A) plaques and (B) aneurysm tissues assessed by quantitative RT-PCR.*

Low protein levels of ABCA1 in atherosclerotic plaques (Albrecht et al., 2004) could account for the increased *ABCA1* mRNA levels in these tissues. Proteases like calpain have been reported to be involved in ABCA1 protein degradation (Wang et al., 2003). MMP's are also known to degrade non-extracellular matrix proteins in addition to matrix proteins (Sternlicht et al., 2001) and were also found to be correlated to calpain in *in vitro* work (Popp et al., 2003; Postovit et al., 2002). This raises the possibility that common protein degradation pathways involving MMP-9 and calpain may be involved in ABCA1 protein degradation thus promoting matrix degradation whilst at the same time reducing lipid efflux.

PPARγ is known to suppress the synthesis of both COX-2 and MMP-9 (Hetzel et al., 2003, Mendez et al., 2003; Worley et al., 2003). Considering the low levels of *PPAR*γ in these specimens, it is tempting to speculate that PPARγ upregulation through pharmacological means using thiazolidinediones or synthetic ligands could potentially be beneficial in increasing lipid efflux through LXRα and ABCA1 and reducing inflammation through the inhibition of COX-2 and MMP-9 thus stabilising the atherosclerotic plaque.

In conclusion this observational study revealed low mRNA expression of *PPAR*γ in ectatic and occlusive disease and underlined the potential link between genes involved in lipid efflux and matrix degradation. The finding that reduced *PPAR*γ expression is observed in atherosclerotic tissues raises the possibility that upregulation of this pathway may be beneficial in the context of treating atherosclerosis.

The studies mentioned in this chapter resulted in two original publications and one review (see Appendix 1): C. Albrecht et al., Stroke 35 (2004) 2801-2806; S. Soumian et al., J Clin Pathol (2005, *in press*), S. Soumian et al., Vasc Med 10 (2005) 109-119 (review).

2.5 ABCA1 and glucose levels

In the studies mentioned above (see chapter 2.1 and 2.4) we were able to demonstrate an association between ABCA1 and cardiovascular disease. It has also been shown that patients with genetic ABCA1 variants, such as TD and familial HDL deficiency are at increased coronary heart disease (CHD) risk (Schaefer et al., 1980; Albrecht et al., 2004). More common variants in the *ABCA1* gene, such as R219K, also modify CHD risk. This is independent of HDL cholesterol and other plasma lipids. In high CHD-risk mouse models, targeted disruption of *ABCA1* in macrophages markedly increases atherogenesis and this is also independent of HDL cholesterol (Van Eck et al., 2002). This indicates that the association between ABCA1 and CHD is only partly explained by HDL-cholesterol concentrations.

On the other side there is a clear association between circulating glucose levels and cardiovascular disease. The risk of CHD is increased 2-fold in men with type 2 diabetes and 4-fold in women, after adjustment for other influences such as blood pressure, lipids, age and smoking. CHD risk is also increased with lesser degrees of glucose elevation (Eschwege et al., 1985). Even in the general population, there is a positive relationship between circulating glucose levels and CHD rates (Coutinho et al., 1999; Khaw et al., 2001). Type1 diabetes is also associated with increased CHD risk despite a favourable lipid profile, especially a relatively high HDL cholesterol concentration (Valabhji et al., 2001). In view of the continuous association of hyperglycaemia with CHD, which is observed in both type 1 and type 2 diabetes, glucose might be directly responsible for the vascular disease. Several mechanisms have been proposed, including free radical damage and protein glycosylation. A further potential mechanism is inhibition of ABCA1 transcription, with detrimental effects on reverse cholesterol transport and/or apoptosis. These observations in man and animals highlight the potential importance for atherogenesis of glucose-induced suppression of ABCA1.

At the start of this project no data were available about the expression and function of ABCA1 in apparently normal human populations. Due to the facts stated above we hypothesised that leucocyte *ABCA1* gene expression would be inversely associated with indices of glycaemia in normoglycaemic men.

In order to investigate this hypothesis we have studied *ABCA1* gene expression in leucocytes from healthy young and middle-aged men. Fasting blood was taken from 32 healthy non-smoking, normoglycaemic male subjects (age 23-46 years). *ABCA1*, *PPAR*γ, and *LXR*α gene expressions in circulating leucocytes were quantified using Taqman technology.

Significant inverse associations between *ABCA1* gene expression and both fasting glucose concentration ($r = -0.49$, $p = 0.008$) and age ($r = -0.39$, $p = 0.043$) were found (FIG 19 and FIG 20).

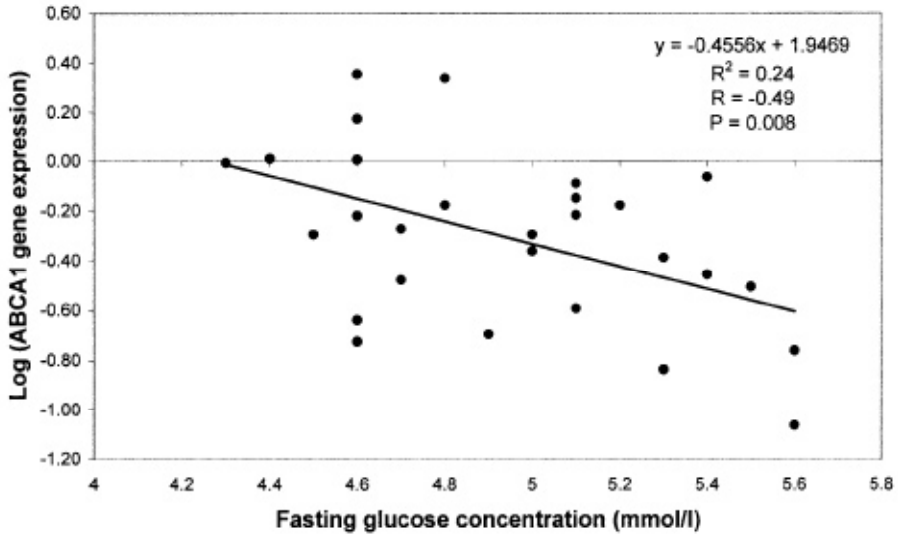

FIG 19: *Association between ABCA1 mRNA expression and fasting glucose concentrations in leucocytes of healthy men assessed by quantitative RT-PCR.*

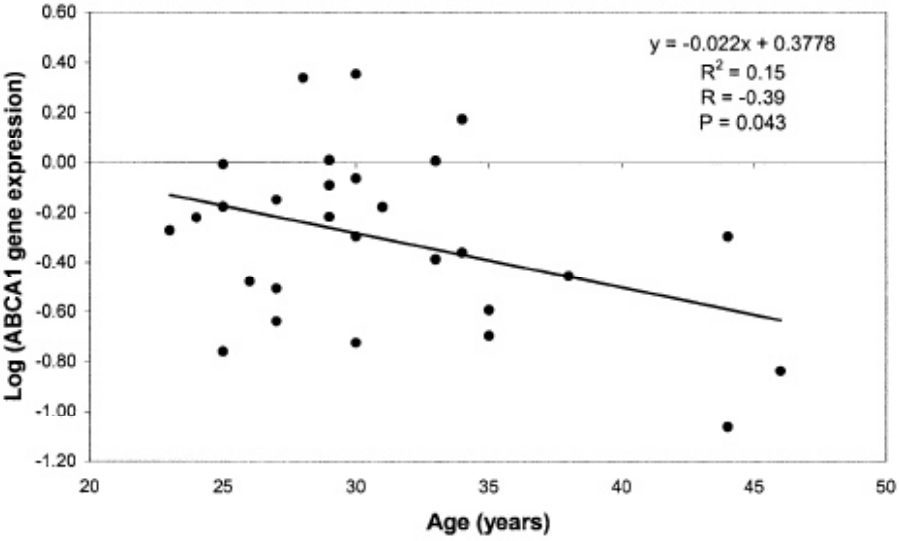

FIG 20: *Association between ABCA1 mRNA expression and age in leucocytes of healthy men assessed by quantitative RT-PCR.*

There was no association with HbA1c (r = -0.23, p = 0.238) or HDL cholesterol concentration (r = 0.02, p = 0.904, data not shown). In a multiple regression model, fasting glucose remained a significant independent predictor (p = 0.037) whereas age did not (p = 0.226). The fact that the significant association was with fasting glucose concentration, and not with HbA1c, may reflect the fact that the time-scale for changes in *ABCA1* gene expression more closely resembles that for glucose (minutes) than that for HbA1c (weeks).

Mechanisms underlying the association were explored; there were no significant associations between fasting glucose concentration and leucocyte *PPAR*γ gene expression, or between fasting glucose concentration and leucocyte *LXR*α gene expression.

This was the first demonstration of an association between *ABCA1* gene expression and fasting glucose concentration *in vivo*. The results have suggested a role for glucose in *ABCA1* gene regulation. They indicate that, if low *ABCA1* gene expression is involved in the higher risk of CHD for both type 1 and type 2 diabetes, this could be due through an association with glucose concentration, rather than HDL cholesterol.

This project is currently extended from healthy control subjects to type 2 diabetes patients and funded for three years by Diabetes UK. In this new study we aim to explore whether

- the inverse relationship between fasting plasma glucose and ABCA1 extends to function, in addition to gene expression.
- within an individual, an increase in blood glucose is associated with a decline in *ABCA1* gene expression and function.
- Carotid IMT, a surrogate for atherosclerosis, is associated positively with blood glucose concentrations and negatively with *ABCA1* gene expression and ABCA1 function.

This ongoing study will provide interesting insights into glycaemic influences on the function of ABCA1.

The results of the project with normoglycaemic men have been published in Metabolism: C. Albrecht et al., *Metabolism* 53 (2004) 17-21 (see Appendix 1).

2.6 ABCA1 and disorders in pregnancy

Placental cholesterol metabolism and lipid transport are crucial to the survival and development of the human fetus (Opitz, 1994; Tint et al., 1994). In addition to significant endogenous cholesterol production, (Belknap and Dietschy, 1988; Jurevics et al., 1997) the fetus may obtain cholesterol from exogenous sources including the placenta and maternal circulation. Cholesterol is also the substrate for human placental steroid hormone synthesis (Tuckey, 2005) which is central to maintenance of pregnancy through its promotion of Th2 cytokines (Piccinni et al., 2000; Szekeres-Bartho and Wegmann, 1996). The placenta is involved in the metabolism of lipoproteins (Grimes et al., 1996; Rindler et al., 1991) and recent studies have indicated that maternal cholesterol is a source for fetal cholesterol (McConihay et al., 2001; Napoli et al., 1997). We hypothesized that ABCA1 could play a major role in development of the placenta and normal growth of the fetus due to following reasons:

1. ABCA1 mediates cholesterol and phospholipid efflux and plays a major role in cholesterol metabolism (Oram and Lawn, 2001; see chapter 1, 2.1, 2.4)

2. ABCA1 is highly expressed in human tissues that are involved in cholesterol metabolism and steroidogenesis including the liver, placenta, adrenals and testis (Langmann et al., 1999)

3. Apart from its role in cellular cholesterol efflux, ABCA1 is also known to play a role in apoptosis (Hamon et al., 2000), inflammation (Reddy et al., 2002) and PS translocation which also can promote the coagulation cascade (Bevers, 1996; Hamon, 2000; Albrecht et al., 2005, see chapter 1 and 2.2). Apoptosis and PS translocation have critical roles in

placental development (Adler, 1995; Huppertz et al., 2004) and its regulation is imperative for a successful pregnancy.

4. The regulatory pathways of ABCA1 involving PPARγ (Chawla et al., 2001) have anti-inflammatory roles (Jiang, 1998) and are linked to the nuclear factor kappa B pathways (Li, 2000). PPARγ is pivotal to trophoblast differentiation and maturation to establish maternal-fetal transport and also to fetal tissue development (Asami-Miyagishi, 2004; Barak, 1999).

5. Loss of functional ABCA1 in null mice results in severe placental malformation with structural abnormalities, haemorrhage and cell debris in the spongiotrophoblast and labyrinthine trophoblast (Christiansen-Weber et al., 2000). This is associated with intrauterine growth restriction and increased rates of neonatal death.

In murine placentas *ABCA1* mRNA expression has been localised to the lining of decidual maternal blood vessels and the labyrinthine trophoblast layer (Christiansen-Weber et al., 2000). This localisation is consistent with ABCA1 playing a role in cholesterol transport. ABCA1 may also mediate apoptotic cell death in the placenta, as it does in the embryo (Luciani and Chimini, 1996). The localisation and role of ABCA1 in human placentas have not been established. In addition, there are no studies of ABCA1 expression in disorders associated with abnormal placentation, such as pre-eclampsia and antiphospholipid syndrome (APS).

APS is characterised by recurrent miscarriage, late fetal loss, thrombocytopenia and thrombosis in conjunction with positive antiphospholipid antibodies or lupus anticoagulant. Histological studies of APS placentas demonstrate necrosis, thrombus formation and acute or chronic inflammation (Salafia and Parke, 1997; Van Horn et al., 2004). Pre-eclampsia affects approximately 3% of primigravidae and is characterised by endothelial dysfunction and abnormal placentation with abnormal trophoblast invasion. Histological studies of placentas from affected pregnancies demonstrate parenchymal infarcts, decidual vessel atherosis and increased syncytial knot formation (Brosens and Renaer, 1972; Salafia, 1995; Salafia, 1998). There are no specific placental histological changes that distinguish between placentas from pregnancies complicated by primary APS or pre-eclampsia, and there is some overlap in the clinical features and the aetiology of the fetal complications of both conditions. APS and pre-eclampsia are both associated with placental apoptosis (Ishihara et al., 2002; Leung et al., 2001).

This study aimed to establish the localisation of ABCA1 in term placentas and to investigate whether *ABCA1* mRNA and protein expression is altered in placentas from pregnancies complicated by pre-eclampsia or APS.

Patients with pre-eclampsia (n=14) were recruited prospectively at Kings College Hospital, London. Pre-eclampsia was defined according to the International Society for the Study of Hypertensive Disorders in Pregnancy criteria. Patients with APS (n=8) were recruited prospectively from a specialist antenatal clinic at St Thomas' Hospital, London. The condition was diagnosed in women with the clinical features of the syndrome in addition to the following biochemical results: either anticardiolipin antibodies or lupus anticoagulant on ≥ 2 separate occasions > 6 weeks apart. Placentas from women with

uncomplicated pregnancies were obtained prospectively from women delivering at Queen Charlotte's Hospital, London.

ABCA1 mRNA localisation was tested using *in situ* hybridisation technique with a riboprobe specific to ABCA1. ABCA1 protein localisation in healthy placentas was assayed using immunohistochemistry. Furthermore *ABCA1* mRNA and protein expression in control, pre-eclampsia and APS samples was analysed using quantitative RT-PCR and immunoblotting techniques, respectively.

In situ hybridisation assays revealed that *ABCA1* mRNA was localised to the syncytium of placental villi and endothelia of fetal blood vessels within the villi (FIG 21 a,b,c). Stromal cells of placental villi did not seem to express mRNA for *ABCA1*. No staining could be seen with the sense control riboprobe (Figure 21d). Positive staining for mRNA for *ABCA1* was not homogeneous in syncytia or endothelia, but seemed to be present in all villi.

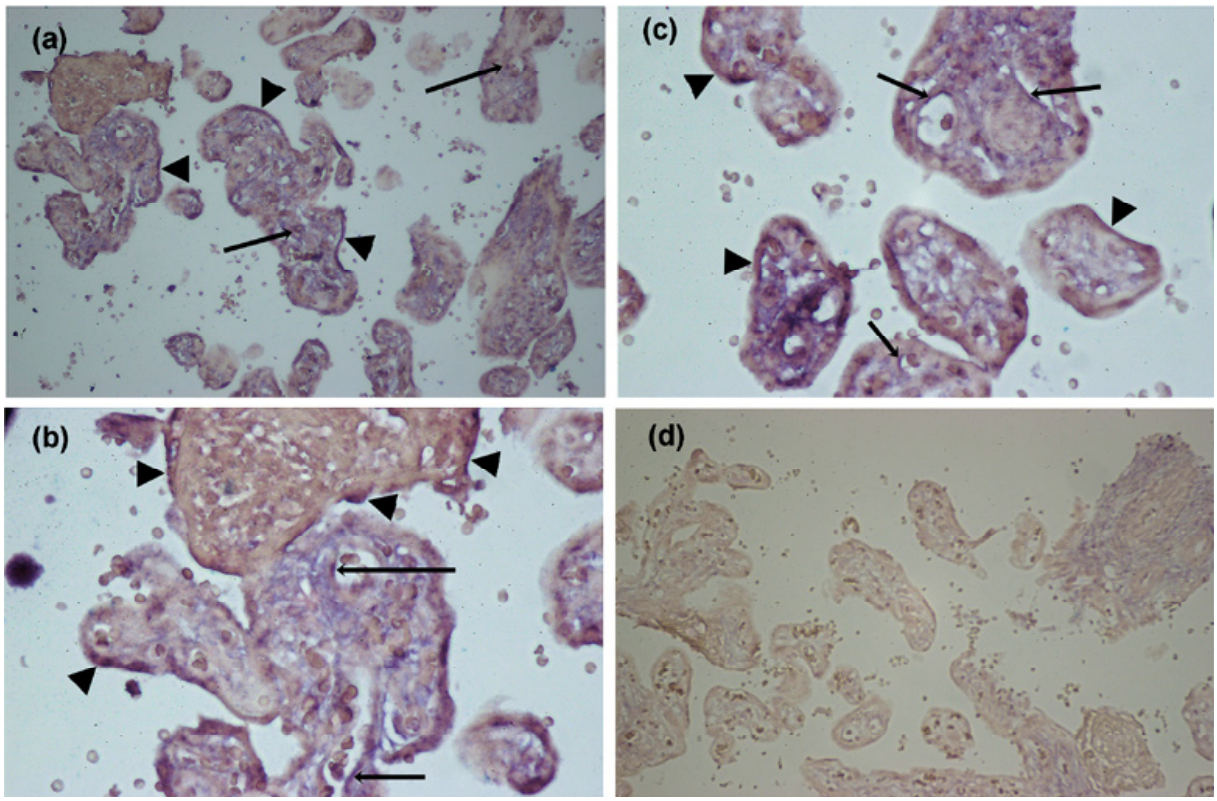

FIG 21: *Localisation of ABCA1 mRNA using in situ hybridisation. a-c: antisense riboprobe, d: sense ripoprobe. The top figure demonstrates that ABCA1 mRNA is localised in the syncytium and in endothelia.*

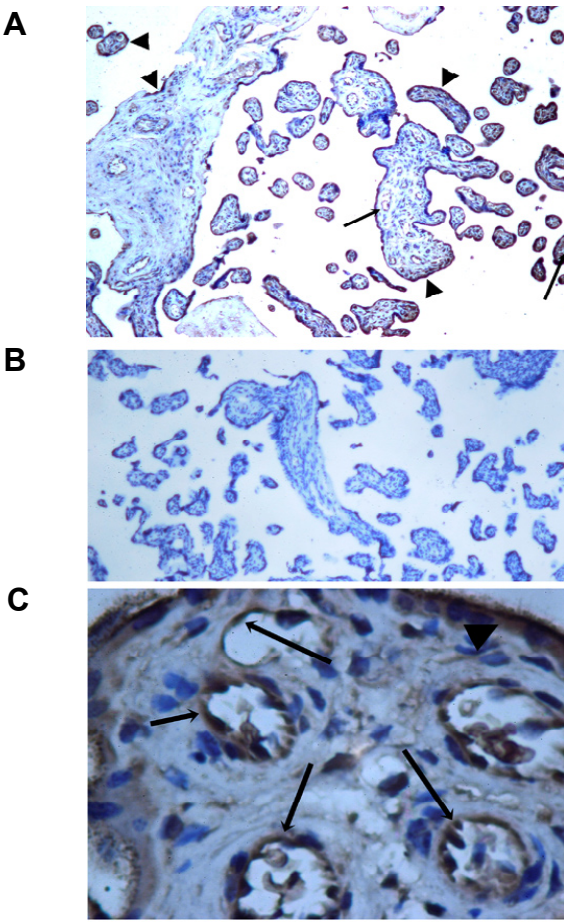

FIG 22: *Immunohistochemistry demonstrating ABCA1 expression in the syncytium and endothelia in a control placenta. (A) x100 magnification; (B) negative control (no antibody); (C) x750 magnification*

ABCA1 protein, investigated by immunohistochemistry, could be localised to the syncytium and vascular endothelial cells of placental villi (FIG 22A). No staining could be seen in controls in which the primary antibody was omitted (FIG 22B). At higher magnification the most intense staining seemed to be in the microvillous surface of the syncytium, as well as throughout the endothelial cells of the placental vessels (FIG 22C).

As the onset of labour has been shown to influence the placental mRNA expression of another ABC transporter, *MDR3* (Patel et al., 2003), we compared expression in pre-labour and labour placentas, but we did not identify any significant alterations in *ABCA1* mRNA expression with the onset of labour ($p>0.1$; FIG 23A and B, black circles). There was no difference in *ABCA1* mRNA expression between placentas from pregnancies complicated by pre-eclampsia (PEC) and 3rd trimester controls ($p>0.1$; FIG 23B). However, *ABCA1* mRNA expression was reduced in the placentas from women with APS when compared to controls ($p<0.0001$; FIG 23A).

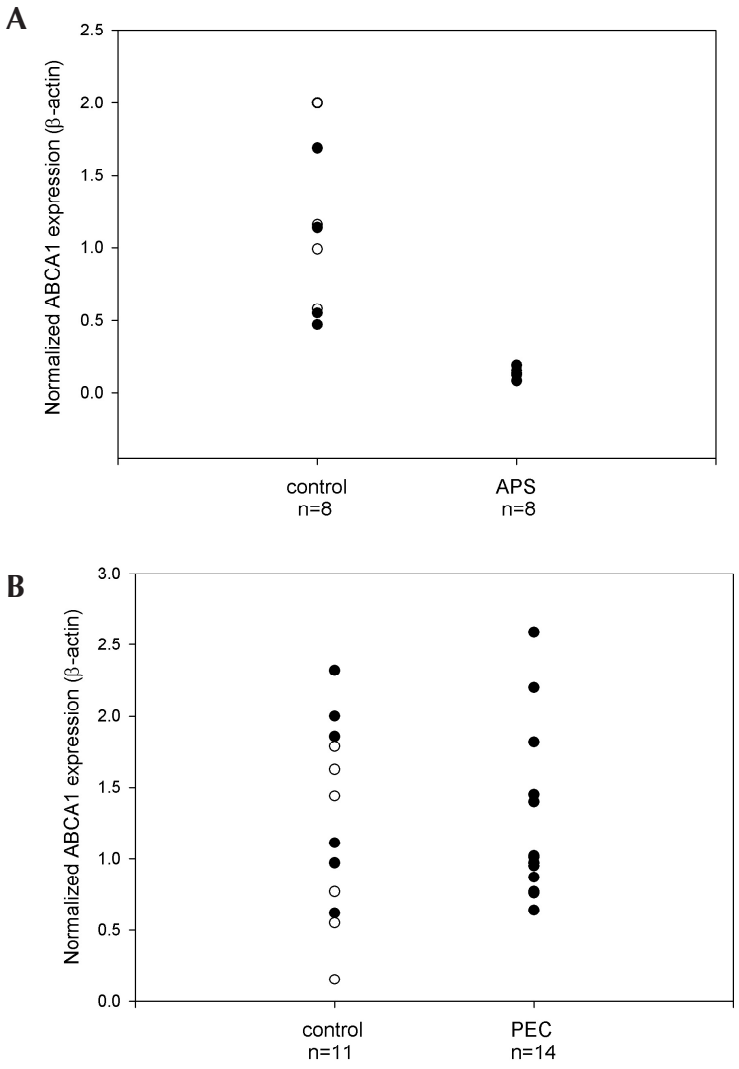

FIG 23: *Quantitative analysis of ABCA1 mRNA expression in different conditions of pregnancy. Controls: white and black circles denote pre-labour and labour specimens, respectively.*

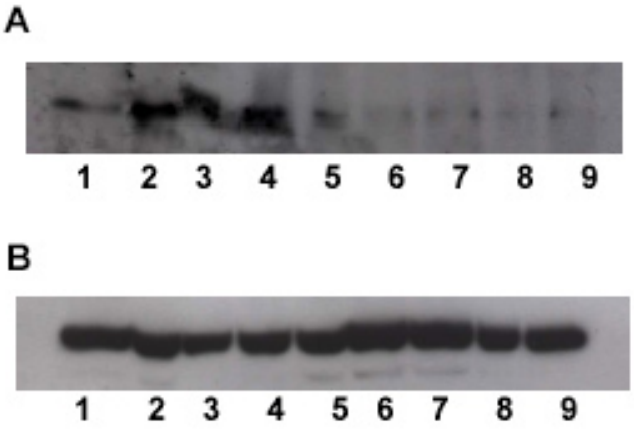

FIG 24: *Immunoblot analysis of (A) ABCA1 and (B) Na^+-K^+-ATPase in different conditions of pregnancy. Membrane proteins from placenta were separated by reducing SDS-PAGE and immunoblotted with anti-ABCA1/Na^+/K^+-ATPase antibodies.1-3: controls; 4-5: Pre-eclampsia; 6-9: APS.*

Immunoblot analysis on placental membrane fractions was carried out on samples from each subgroup in order to determine whether the decreased mRNA expression of *ABCA1* is paralleled by decreased protein expression. While there were no obvious differences in ABCA1 protein expression between placental samples from women with PE and controls, there was reduced expression in placentas from women with APS (FIG 24).

As shown above we have demonstrated that ABCA1 is expressed within the human placenta, and have localised *ABCA1* mRNA and protein to the syncytial membrane and endothelia in term placentas from uncomplicated pregnancies. The localisation of ABCA1 to the syncytia and vascular endothelia of human placenta is consistent with a role in cholesterol and phospholipid transport between the maternal and fetal circulations, and the high levels of protein in the microvillli of the syncytia imply that the main activity may be in the maternal to fetal direction.

Investigations in humans (Desoye et al., 1987) and baboons (Henson et al., 1997) have demonstrated the strong influence of maternal lipoproteins on placental production of progesterone thus confirming the importance of transplacental lipid transport. Studies in *ABCA1* null mice have clearly revealed the catastrophic effect ABCA1 deficiency has on the placenta, characterised by disrupted architecture, haemorrhage and ragged spongioblast inclusions (Christiansen-Weber et al., 2000). ABCA1 deficiency could thus potentially affect placental steroid hormone synthesis by its impact on lipid transport.

A decrease in ABCA1 protein may also influence the process of syncytialisation in human placenta as this is functionally similar to apoptosis (Adler et al., 1995) both involving condensation of nuclear chromatin, externalisation of annexin V, redistribution of PS, and the controlled loss of cell fragments. Reduced activity or loss of ABCA1 could decrease this process, thus leading to abnormal placentation. In this context a report of decreased annexin V in placentas from patients with APS (Rand et al., 1997) is consistent with this perspective, although other work showing no change in such factors (Lakasing et al., 1999) suggest that interpretation of these findings needs to be cautious.

We found decreased levels of mRNA and protein for ABCA1 in placentas from patients with APS, but not in women with pre-eclampsia. The pathophysiology of APS and pre-eclampsia share a number of similarities, including the involvement of prothrombotic and inflammatory processes, increased apoptosis and decreased cell growth. Therefore the difference in ABCA1 expression indicates that the apparent similarities result from the convergence of different processes.

In APS, antiphospholipid antibodies are specifically targeted to decidual tissue and can cause a rapid increase in decidual and systemic tumor necrosis factor (TNF)α levels (Berman et al., 2005) and this acts through the activation of the complement pathway which is a central mechanism in antiphospholipid antibody-induced pregnancy loss (Salmon et al., 2003; Salmon and Girardi, 2004). Moreover, the protective effects of TNFα deficiency and TNFα blockade seem to support these findings (Berman et al., 2005). The increased level of these cytokines may account for the downregulation of ABCA1 expression in APS placentas.

Th1 cytokines like TNFα have wide ranging effects on placental architecture, hormone synthesis and embryonic development (Hunt et al., 1996). Furthermore they are known to inhibit membrane transport proteins like ABCA1 and its regulatory pathways including PPARγ (Khovidhunkit et al., 2003; Zhang et al., 1996). The production of Th2 cytokines by the decidual T cells contributes to the maintenance of pregnancy while an exaggerated Th1 response seem harmful to the fetus (Mellor and Munn, 2000; Raghupathy, 1997).

Levels of Th1 cytokines like TNFα, IL-2 and IFNγ have been found to be higher in patients who had recurrent miscarriages (Chaouat et al., 1990; Raghupathy et al., 2001).

Although Th1/Th2 cytokine imbalances have a role in the aetiopathogenesis of pre-eclampsia and trophoblastic diseases, there is no clear understanding regarding the exact course of events (Dong et al., 2005; Wilczynski et al., 2002). The extent of the inflammatory process in the placenta could have an impact on ABCA1 expression levels. It was recently reported that TNFα levels, despite being elevated in the peripheral blood, are not increased in the placentas of pre-eclamptic patients (Dong et al., 2005; Hayashi et al., 2005). The degree of ABCA1 downregulation in the APS placentas may thus be a reflection of the extent of the inflammatory process in the placenta characterised by significantly higher number of inflammatory cells (Stone et al., 2005). The defined role of ABCA1 and its exact mechanism of function in normal human placentas or in disorders occurring during pregnancy remain to be elucidated.

The results of this project are summarised in a manuscript which will be submitted to *Placenta* in November 2005: C. Albrecht et al., manuscript in preparation (see Appendix 1).

3 Conclusions

In several studies we were able to show that rare hereditary diseases such as familial HDL deficiency or Scott syndrome are causally linked to aberrant ABCA1 function. Interestingly, and maybe even more important, we demonstrated that also common diseases such as atherosclerosis or disorders in pregnancy might be, at least in part, linked to defective ABCA1 expression and/or function. This is presumably associated with the fact that ABCA1 is involved in the regulation of various distinct physiological processes in the cell. Thus, apart from its role in lipid metabolism, ABCA1 has also been implicated in promoting engulfment of apoptotic cells, LDL oxidation and the release of inflammatory mediators (see chapter 1).

The critical role of ABCA1 at the macrophage and hepatic level regarding lipid efflux and HDL formation could have a major influence on the biogenesis and progression of atheromatous plaques. The aetiopathogenesis of an atheromatous plaque is influenced by lipid metabolism, disordered cell turnover and extracellular matrix turnover within its structure. ABCA1 plays a role in all of these events, thus potentially implicating this transporter in the initiation, progression and pathogenesis of atherosclerotic vascular disease (FIG 25).

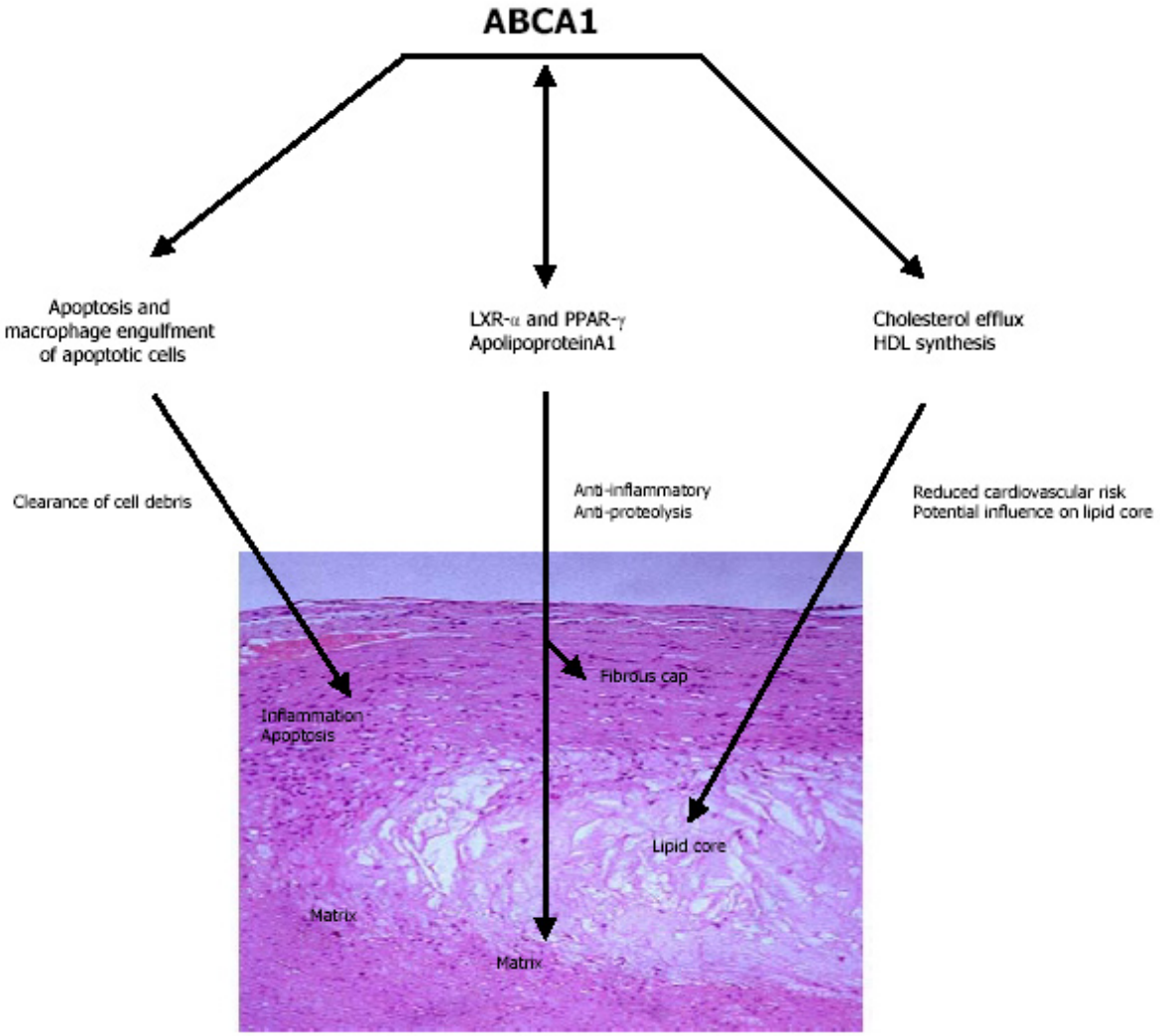

FIG 25: *Impact of ABCA1 at various critical processes in the development of atherosclerotic plaques.*

The PPAR-LXR-ABCA1 cascade has a substantial role in cholesterol homeostasis and inflammation and therefore has a promising potential for therapeutic manipulation. Further studies should be focused on apoA1 and ABCA1 interactions that seem to regulate each other and have a potent role in protection against atherosclerosis. The role of ABCA1 in glycaemic conditions is currently studied (see chapter 2.52.5) and will provide important information about the role of ABCA1 in diabetic disorders.

However, also principal questions regarding the mechanism of ABCA1 function remain to be elucidated. Whether ABCA1 represents structurally a multifunctional protein, i.e that it can act either as cholesterol efflux protein or as PS translocase, must be confirmed by studies using techniques such as site-directed mutagenesis. These investigations will also reveal whether a genotype-phenotype correlation for ABCA1 mutations exists. Moreover, as the results of our studies suggested that ABCA1 might have a different role in tissues than in the blood (see chapter 2.1 and 2.2), ABCA1 expression and localisation in blood cells should be investigated.

4 PERSPECTIVES

The clinical studies presented in this work suggest that ABCA1 is implicated, and thus might be therapeutically targeted, in atherosclerosis and disorders occurring during pregnancy. In this context it will be challenging to unravel other functions of ABCA1 and its pathways so that therapeutic strategies could be devised to prevent these diseases and their complications. In addition, as soon as the role of ABCA1 in diabetic disorders and its mechanism of action are being identified, an enormous potential in terms of therapeutic intervention for the treatment of diabetes could emerge.

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6 Summary

ABCA1, a member of the ATP-binding cassette transporter family, has been shown as to be a key regulator of lipid homeostasis. Our studies revealed that this gene is not only implicated in familial HDL deficiency, a hereditary disease characterised by low HDL and apolipoprotein concentrations, but that it is also involved in a rare bleeding disorder called Scott syndrome. We also demonstrated that ABCA1 is potentially involved in the initiation, progression and pathogenesis of atherosclerotic vascular disease. An association between ABCA1 and fasting glucose levels in healthy probands was demonstrated proposing that its expression may be regulated by glucose levels or even that ABCA1 could be implicated in diseases like type 2 diabetes. As cholesterol homeostasis and transfer is a critical step in the development of the embryo, we tested the role of ABCA1 in complicated pregnancies. In these studies we identified the localisation of ABCA1 mRNA and protein in human placenta and detected substanitially reduced levels of this gene in a disease called antiphospholipd syndrome. These studies open new options for therapeutical intervention as therapeutic strategies could be devised to prevent these diseases and their complications.

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APPENDIX

1 RELEVANT PUBLICATIONS

C. Albrecht, K. Baynes, A. Sardini, S. Schepelmann, E.R. Eden, S.W. Davies, C.F. Higgins, M. D. Feher, J.S. Owen and A.K. Soutar. Two novel missense mutations in ABCA1 result in altered trafficking and cause severe autosomal recessive HDL deficiency. Biochim Biophys Acta 2004; 1689: 47-57.

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Two novel missense mutations in ABCA1 result in altered trafficking and cause severe autosomal recessive HDL deficiency

Christiane Albrecht[a], Kevin Baynes[b], Alessandro Sardini[a], Silke Schepelmann[c], Emily R. Eden[a], Simon W. Davies[d], Christopher F. Higgins[a], Michael D. Feher[b], James S. Owen[c], Anne K. Soutar[a,*]

[a] *MRC Clinical Sciences Centre, Hammersmith Hospital, Faculty of Medicine, Imperial College, Du Cane Road, London W12 ONN, UK*
[b] *Lipid Clinic, Chelsea and Westminster Hospital, London SW10 9NH, UK*
[c] *Department of Medicine, Royal Free and University College Medical School, London NW3 2PF, UK*
[d] *Department of Invasive Cardiology, Royal Brompton Hospital SW3 6NP, UK*

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Abstract

Extremely low concentrations of high density lipoprotein (HDL)-cholesterol and apolipoprotein (apo) AI are features of Tangier disease caused by autosomal recessive mutations in ATP-binding cassette transporter A1 (*ABCA1*). Less deleterious, but dominantly inherited mutations cause HDL deficiency. We investigated causes of severe HDL deficiency in a 42-year-old female with progressive coronary disease.

ApoAI-mediated efflux of cholesterol from the proband's fibroblasts was less than 10% of normal and nucleotide sequencing revealed inheritance of two novel mutations in *ABCAI*, V1704D and L1379F. ABCA1 mRNA was approximately 3-fold higher in the proband's cells than in control cells; preincubation with cholesterol increased it 5-fold in control and 8-fold in the proband's cells, but similar amounts of ABCA1 protein were present in control and mutant cells. When transiently transfected into HEK293 cells, confocal microscopy revealed that both mutant proteins were retained in the endoplasmic reticulum, while wild-type ABCA1 was located at the plasma membrane.

Severe HDL deficiency in the proband was caused by two novel autosomal recessive mutations in *ABCA1*, one (V1704D) predicted to lie in a transmembrane segment and the other (L1379F) in a large extracellular loop. Both mutations prevent normal trafficking of ABCA1, thereby explaining their inability to mediate apoA1-dependent lipid efflux.

Keywords: ABCA1; Protein trafficking; HDL deficiency; Apolipoprotein AI; Cholesterol efflux

1. Introduction

Tangier disease [1–4] is a rare autosomal recessive disorder characterised by extremely low levels of high density lipoprotein (HDL)-cholesterol and of apolipoprotein (apo) AI in plasma, and by the accumulation of cholesterol in macrophage-rich tissues, notably the tonsils, which acquire a characteristic orange-yellow colour. Hepatosplenomegaly and peripheral neuropathy are also commonly associated with this disorder [5]. Tangier disease is caused by mutations in the ATP-binding cassette transporter A1 (*ABCA1*) gene that impair apoAI-mediated cholesterol efflux from cells [6–12], and these patients invariably have defects in both alleles of *ABCA1*. Some heterozygous relatives of Tangier patients display an intermediate phenotype of low to normal HDL-cholesterol and a 50% reduction in apoAI-mediated efflux of cholesterol [13]. In the absence of functional ABCA1 protein, newly secreted apoAI is rapidly degraded and cholesterol efflux deficiency ensues [14]. The disorder is often, but not always, associated with an increased risk of coronary artery disease [5].

Familial HDL deficiency is characterised by less severe HDL deficiency than that seen in Tangier disease, but with a dominant mode of inheritance. Nevertheless, it too results from mutations in *ABCA1* [12]. As yet, no clear distinction has emerged between the mutations in *ABCA1* that result in autosomal recessive Tangier disease and those that cause dominant HDL deficiency, or between those that do or do

* Corresponding author. Tel.: +44-208-383-2324; fax: +44-208-383-2077.
E-mail address: anne.soutar@csc.mrc.ac.uk (A.K. Soutar).

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not result in all the clinical manifestations of accumulation of cholesterol in the reticuloendothelial system. In this study, we describe a patient who has inherited two defective alleles of *ABCA1* from apparently unaffected parents, each encoding a previously undescribed single amino acid substitution (L1379F and V1704D). The patient has almost complete HDL deficiency and severe premature coronary artery disease, but does not have yellow tonsils or other overt signs of cholesterol deposition in macrophages.

2. Materials and methods

2.1. Patient and family studies

The proband was a 42-year-old pre-menopausal woman of English origin who was assessed in the lipid clinic at Chelsea and Westminster Hospital, London after diagnosis of coronary artery disease treated by coronary artery bypass graft. Family members were evaluated separately, with informed consent.

All blood samples for lipid and lipoprotein analysis were taken after an overnight fast of at least 10 h. Low density lipoprotein cholesterol (LDL)-cholesterol concentrations were calculated using the Friedwald formula [15]. ApoAI and apolipoprotein B were measured by nephelometry. A skin biopsy was taken aseptically under local anaesthesia with informed consent from the proband. Dermal fibroblasts were grown from 0.25 cm^2 explants of dermis cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mmol/l glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml.

2.2. Measurement of LCAT activity

Plasma LCAT activity was measured using the egg lecithin:human apoAI: [^{3}H]-cholesterol proteoliposome substrate method as described previously [16]. This assay largely reflects LCAT concentration and was carried out by incubating plasma (7.5 μl) with substrate (242.5 μl) at 37 °C for 1 h. Lipids were extracted by addition of chloroform–methanol and separated by thin-layer chromatography; radioactivity in the free cholesterol and cholesteryl ester bands was measured and the values used to calculate the percentage of cholesterol esterified per hour.

2.3. Agarose gel electrophoresis and immunoblotting

Lipoproteins in plasma (2 μl) were separated by electrophoresis on pre-cast agarose gels (Hydragel, Sebia) and either stained with Sudan black or transferred onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia) by pressure blotting. For immunostaining, monoclonal antibodies against human apoAI (Calbiochem) or LCAT (S. Schepelmann and J.S. Owen, unpublished data) were diluted 1:1000 in 3% (w/v) bovine serum albumin in phosphate-buffered saline (PBS). A goat anti-mouse Ig-HRP (Amersham Pharmacia) was used as a secondary antibody (1:5000 in 10% (w/v) milk powder/PBS-Tween), followed by detection by enhanced chemiluminescence (Amersham Pharmacia).

2.4. Measurement of cholesterol efflux

Control and proband skin fibroblasts were grown to 50–70% confluence in 12-well tissue culture plates. Cells were labeled by incubation with 1 μCi/ml [^{3}H]-cholesterol in DMEM containing 5% fetal bovine serum for 72 h. The monolayers were washed and incubated with BSA (1 mg/ml) in serum-free DMEM for 48 h to allow the labeled cholesterol to equilibrate with intracellular cholesterol pools. Cholesterol efflux was induced by the addition of 5 μg/ml human apoAI [17] in serum-free DMEM to quadruplicate wells; control wells received DMEM alone. Media were removed after 6 h, the cells washed once with PBS and then dissolved in 0.1 M NaOH. Radioactivity in media and cells were measured and the cholesterol efflux rate calculated as the % of dpm in medium/(dpm in medium + dpm in cells). The mean of values for patient's cells (apoAI-mediated efflux minus mean of unstimulated cells) was compared to the value for control cells (apoAI-mediated efflux minus mean of unstimulated cells) by an unpaired Student's *t*-test.

2.5. Nucleotide sequence analysis

DNA was extracted from venous whole blood [18]. Genotyping was as previously described [19] with markers from the ABI Prism linkage mapping set (PE Applied Biosystems). The coding regions and proximal promoter (starting from 395 bp upstream from the ATG of *ABCA1*) were amplified by PCR with primers located within 50–70 bp of each splice junction (primer details available on request: c.albrecht@csc.mrc.ac.uk). PCR reactions were carried out in a volume of 25 μl containing 100 ng of DNA, 250 nM of each primer, 200 μM of each dNTP, 1 × Thermopol buffer (New England Biolabs, Hitchen, Herts, UK) and 1 U of *Vent* polymerase (New England Biolabs). Amplification conditions were as follows: initial denaturation for 1 min at 97 °C, 35 cycles of 1 min at 96 °C, 1 min at 52–59 °C (depending on the primer pair) and 1 min at 72 °C, with a final extension at 72 °C for 10 min. Amplification products were analysed on 2% low melting agarose gels, purified from the gel with a gel extraction kit (Qiagen) and sequenced with both PCR primers using an ABI 3700 Prism automated sequencer. Sequences were analysed with Sequence Navigator software, and compared with the published sequence of ABCA1 cDNA (GenBank accession no: AF285167). ABCA1 mRNA was sequenced after amplification by RT-PCR from total RNA, isolated from cultured skin fibroblasts using RNA-Bee™ RNA isolation reagent according to the manufacturer's instructions (Biogenesis Ltd.). The RT reaction comprised 0.04 U of oligo-p$(dT)_{15}$ primer, 5 mM $MgCl_2$, 1 mM of each dNTP, 40 U RNAse inhibitor, 1 × reaction buffer (Roche)

and 20 U AMV reverse transcriptase (Roche). RT reaction mix (1–5 μl) was then used as template in a PCR reaction as described above, with primers that produced nine overlapping fragments of the coding regions (details available on request).

2.6. Analysis of polymorphisms in DNA by WAVE™ analysis

PCR fragments of *ABCA1* exons 28, 36 and 6 were amplified as described above with the following primers:

exon 28,	5′-TCTAACACTTGCCGTTTCCTGCTGTC and 5′-CCTCGTAAACATCTTTGGTCTGCTCG
exon 36,	5′-TTGTCTGTGTGTCCATGTCCTCACTG and 5′-GCTGGAACATTTCCTGATGATAGCCAG
exon 6,	5′-GGACCCAGCTTCCAATCTTCATAATCC and 5′-GCCTCACATTCCGAAAGCATTAGTGC

PCR products (8 μl of reaction mix) were denatured by heating to 94 °C, followed by cooling to 25 °C over 25 min to enhance heteroduplex formation, and analysed on a WAVE™ Nucleic Acid Fragment Analysis System (Transgenomic). The optimum temperatures for analysis of each fragment were predicted with Wavemaker software (Transgenomic) and confirmed empirically.

2.7. Quantification of mRNA

Skin fibroblasts were preincubated for 24 h in DMEM medium containing either 10% fetal bovine serum or cholesterol (30 μg/ml) complexed to fatty acid-free bovine serum albumin (2% w/v). Cell monolayers from two 9-cm dishes were washed twice with sterile Puck's saline A (Gibco BRL, Life Technologies), scraped into a sterile 50 ml tube in 5 ml of Puck's saline and pelleted by centrifugation for 10 min at 3000 rpm. Total cellular RNA was extracted as described above. Contaminating DNA was removed by incubation of total RNA with DNAse I (Promega). For cDNA synthesis, RNA (1 μg) was transcribed with a First Strand cDNA synthesis Kit for RT-PCR (Roche), according to the supplier's instructions. For Taqman analysis, the cDNA was diluted 20-fold with nuclease-free water.

Primers and probe for Taqman analysis of ABCA1 mRNA were designed with PrimerExpress software (PE Applied Biosystems) and the reaction optimised according to PE User bulletin number 2. The forward primer was 5′-GGGAGGCTCCCGGAGTT in exon 3, the reverse primer was 5′-GTATAAAAGAAGCCTCCGAGCATC in exon 4, and the FAM-labeled probe, spanning exons 3 and 4, was 5′-AACTTTAACAAATCCATTGTGGCTCGCCTGT. Single tube Taqman analysis was performed on an ABI prism 7700 sequence detection system with 300 nM of forward and reverse primers in the presence of 200 nM 5′ FAM-3 TAMRA-tagged probe. The internal standard was GAPDH mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were carried out in triplicate and contained 5 μl of diluted cDNA in a total volume of 25 μl. The amount of ABCA1 mRNA in cells was expressed relative to that of GAPDH, and was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{target} - Ct_{GAPDH}$. Fold induction (relative expression in patient/relative expression in control) was expressed as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct_{patient} - \Delta Ct_{control}$.

2.8. Immunoblotting of ABCA1 protein in cell membranes

Skin fibroblasts were preincubated in medium containing either 10% fetal bovine serum or 2% bovine serum albumin plus 30 μg/ml cholesterol for 24 h [11]. Cells in two 9-cm dishes were washed twice with PBS and harvested by scraping from the dish. A total membrane fraction was prepared as described by Rosenberg et al. [20]. Briefly, the cells were lysed by homogenisation in 1 ml ice-cold buffer A (50 mM maltose, 50 mM Tris–HCl pH 7.5, 2 mM EGTA, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine). Cell debris was removed by centrifugation at 500 × *g* for 10 min, the supernatant diluted 3-fold with buffer B (buffer A with 300 mM maltose), and the membrane fraction pelleted by centrifugation at 100,000 × *g* for 60 min. The membrane pellet was resuspended in 100 μl buffer B and protein content measured (Biorad protein assay). Membrane proteins were fractionated on reduced SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. ABCA1 protein was detected with a rabbit antipeptide antiserum, diluted 1:1500 (Abcam Ltd., Cambridge, UK) followed by peroxidase-labeled anti rabbit IgG, diluted 1:3000 (Dako).

2.9. Plasmids/DNA construction

Full-length human ABCA1 cDNA was generated by reverse transcriptase PCR of mRNA obtained from skin fibroblasts of the patient and a healthy individual (see Nucleotide sequence analysis). Wild-type ABCA1 cDNA was cloned into pGEM®-11Zf vector (Promega) and replaced with the corresponding DNA fragment (*Asp*718–*Bam*HI) containing each mutation (4425 or 5401). Enhanced green fluorescent protein (eGFP) was fused in frame to the C-terminus of ABCA1 by overlapping PCR strategy as described previously [21] using the following primers: 5′-CCAGAGGAGATGCTTTCCTTAA/5′-CTTGCTCACCATTACATAGCTTTC for ABCA1 and 5′-GAAAGCTATGTAATGGTGAGCAAG/5′-TCTTTGTCGCGGCCGCTTTACTTGTACAGCTCGTCCATGCC (including a *Not*I restriction site) for eGFP. The PCR reactions were carried out with 100 ng DNA as described above (see Nucleotide sequence analysis) at an annealing temperature of 55 °C. The gel purified PCR products were pooled and 1 μl subjected to another PCR reaction with the outermost primers (annealing temperature 55 °C, extension 1.5 min at 72 °C, min). The PCR product was cloned into pCR® 4Blunt-TOPO vector (Invitrogen) and *Bam*HI–*Not*I ABCA1 cDNA fragments in the pGEM®-11Zf constructs replaced

with the ABCA1-eGFP hybrid. The resulting wild-type and mutant ABCA1-eGFP cDNAs were cloned with *Sal*I–*Not*I into pCI-neo mammalian expression vector (Promega) and all constructs verified by complete sequencing.

2.10. Analysis of expression of ABCA1 eGFP fusion proteins in HEK 293 cells

HEK 293 cells were plated on poly-L-lysine (Sigma) coated glass cover slips in six-well plates 12 h before transfection. Cells were transfected with plasmids containing wild-type or mutated ABCA1-eGFP cDNA according to the calcium phosphate transfection protocol (Clontech) with 2 μg of DNA/well, washed twice with PBS after 6 h, and fixed in 4% formaldehyde/4% sucrose 24 h after transfection.

Fixed cells were permeabilised with 0.1% Triton X-100 for 4 min and stained with DAPI (15 mg/ml; 1:10,000 dilution; Molecular Probes) for nuclear DNA. In order to identify the intracellular localisation of the wild-type and mutant ABCA1 proteins, cells were also stained with the mouse monoclonal antibody against sarco/endoplasmic reticulum calcium ATPase (SERCA 2; clone 11D8; Affinity Bioreagents Inc.) for 2 h at room temperature after blocking with 0.2% fish skin gelatin in PBS. The antibody against SERCA2 was detected by a goat anti mouse IgG secondary antibody conjugated to Alexa 568 (Molecular Probes, 1:400 dilution for 30 min, room temperature).

Cells were imaged by a Leica SP confocal microscope through a 63X 1.32 NA PlanApoChromat oil immersion objective. eGFP was excited by a 488 nm line of an Argon laser and Alexa 568 by a 568 nm line of a Krypton laser. In order to avoid bleedthrough, the fluorophores were excited sequentially. The emitted fluorescence was collected separately through a triple dichroic mirror 488/568/663. The emission filter bands for eGFP and Alexa 568 fluorescence were restricted to 500–552 nm and 594–620 nm, respectively. DAPI staining of nuclear DNA was excited by a 351 nm line of a UV laser and emission fluorescence collected by a 396–508 nm bandpass filter.

Stack of confocal sections separated by 1 μm increments were taken and images analysed by Metamorph software (Universal Imaging Corporation, USA).

3. Results

3.1. Clinical findings

The 42-year-old proband presented with progressive and premature coronary artery disease requiring coronary artery bypass surgery; at 1 year follow-up, angiography confirmed severe progressive coronary atheroma. The proband's fasting lipid profile revealed marked HDL deficiency, with HDL-cholesterol less than 0.1 mmol/l; total cholesterol was 3.8

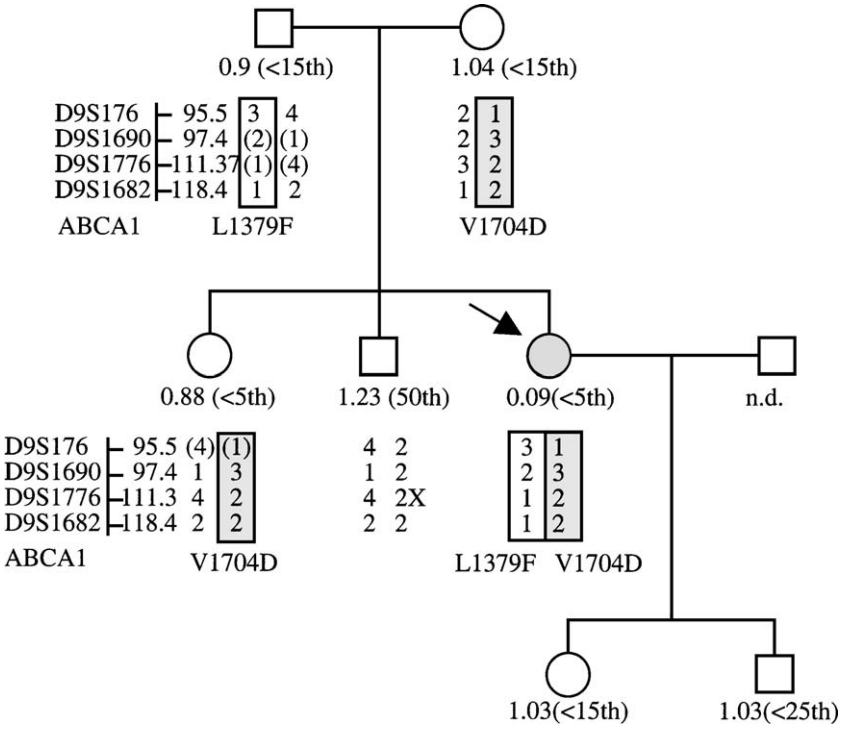

Fig. 1. Pedigree of the HDL-deficient proband. The proband is indicated with an arrow; values below each symbol indicate HDL-cholesterol concentration (mmol/l) and the centile in England according to age and gender [22]. Markers that flank ABCA1 on chromosome 9q31 [26] are shown with their position in bp $\times 10^{-6}$; ABCA1 is at ~ 101.15 (UCSC genome browser, http:// genome.ucsc.edu; April 2003 freeze). Genotypes are shown below each symbol (those in brackets were deduced). A recombination in the maternal allele of the proband's unaffected brother is indicated (×). In the proband, the paternal allele (clear box) carried the L1379F variant of *ABCA1*, and the maternal allele (shaded box) carried the V1704D variant.

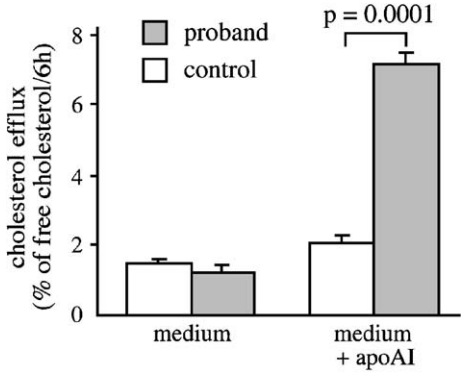

Fig. 2. Efflux of cholesterol from skin fibroblasts in culture. Skin fibroblasts were equilibrated with [^{3}H]-cholesterol and incubated in serum-free medium with or without human apoA1 (5 μg/ml). Cholesterol efflux was measured as the percentage of labeled cholesterol present in the medium after 6 h. The mean of values from three separate determinations is shown. The significance of the difference between proband and control was determined by unpaired Student's *t*-test.

mmol/l, triglycerides 2.52 mmol/l and the calculated LDL-cholesterol was 2.8 mmol/l. Lipoprotein analysis showed apoAI was < 0.3 g/l, apoB 0.83 g/l and Lp(a) 98 mg/l. Apart from HDL deficiency, no other risk factors for premature coronary disease were identified in the proband, except that she had been a modest cigarette smoker (10 per day). Although our study was focused on identifying the proband's clinical and biochemical phenotype, HDL-cholesterol levels were also measured in her immediate family. For both parents, her sister and her daughter aged 17 years these were below the 10th centile for their age and gender [22] (Fig. 1). The HDL-cholesterol of her 15-year-old son was just above the 10th centile for 16–24-year-olds, whereas that of her brother was close to the mean for his age and gender. The proband's father had coronary heart disease, but had only become symptomatic at age 70 years and her mother was well at age 79 years. On physical examination, the proband did not have enlarged yellow tonsils, hepatosplenomegaly or peripheral neuropathy; renal function was normal. Possible underlying causes of apoAI or HDL deficiency were investigated, including LCAT deficiency [23] and mutations in the genes encoding apoAI [23] or ABCA1 [9–12].

3.2. Investigation of inherited defects in LCAT or apoAI

Mutations in the genes for LCAT and apoAI were excluded as the cause of HDL deficiency. LCAT activity in the proband's plasma, as determined in a proteoliposomal assay [16], was at the lower end of the normal range, with an esterification rate of 8.5%/h, 67 nmol/ml/h (reference range of 7.7–12.8%/h; 60–101 nmol/ml/h) [24]; 74% of plasma cholesterol was esterified (reference range 68–75%) [24]. Immunoblotting of plasma proteins fractionated by agarose gel electrophoresis revealed normal amounts of LCAT protein, but undetectable apoAI in the proband's plasma. Analysis of the sequence of the apoAI gene in the proband's DNA [25] revealed no mutations in the coding sequence, intron/exon splice junctions or proximal promoter.

3.3. Investigation of inherited defects in ABCA1 as the cause of HDL deficiency

There was no difference in the basal rate of cholesterol efflux from the proband's skin fibroblasts cultured in serum-free medium compared to that in control fibroblasts (1.5 vs. 1.2% of cellular free cholesterol in 6 h) (Fig. 2). However, in the presence of apoAI, efflux from the proband's cells barely increased over the basal level, whereas efflux from control cells was increased by approx. 6-fold to 7.0% of cellular free cholesterol, a value consistent with other studies [11]. This marked reduction of apoAI-mediated cholesterol efflux from the proband's fibroblast (< 10% of normal) suggests defective ABCA1 activity in her cells. Analysis of markers flanking *ABCA1* on chromosome 9q31 [26] revealed that the proband had inherited two different alleles of *ABCA1*, and that neither of her unaffected siblings had inherited the same two alleles (Fig. 1).

Table 1
Genetic variants in *ABCA1* in the HDL-deficient proband

Exon	Base no.[a]	Codon[b]	Base substitution	Amino acid change	Comments
1	273		hmz C to G	none	common [27]
2	368–370	Leu 26	hmz TTA to CTG	none	CTG in genomic sequence [27]
5	764	Leu 158 (98)	htz A/G	none	common [29]
6	947	Arg 219 (159)	htz G/A	Arg to Lys	common [28]
14	2330	Ile 680 (620)	hmz C	none	common [28]
16	2763	Ile 825 (765)	hmz G	Ile to Val	common [28]
17	2930	Met 883 (823)	hmz A	Met to Ile	common [28]
28	4425	Leu 1379 (1319)	htz C/T	Leu to Phe	novel, rare
30	4571	Thr 1427 (1367)	htz G/A	none	common [28]
34	5050	Arg 1587 (1527)	htz G/A	Arg to Lys	common [28]
36	5401	Val 1704 (1644)	htz T/A	Val to Asp	novel, rare

[a] Based on published sequence for cDNA (GenBank accession AF285167).
[b] Based on ATG start codon = bp 291-3 in cDNA sequence above (previous codon no. in parentheses).

ABCA1 defects could not be excluded as the underlying cause of the apoAI deficiency, and hence the nucleotide sequence of the gene was determined. This revealed that the proband was heterozygous at a number of sites in *ABCA1*, as listed in Table 1. The majority of these were polymorphisms that have been observed in control populations [27–29]. However, two single base pair substitutions were novel, namely $C_{4425}T$ in exon 28 (based on GenBank accession no. AF28517), predicted to substitute L1379 with Phe, and $T_{5401}A$ in exon 36, predicted to substitute V1704 with Asp. Neither of these variants was found in 164 alleles of a control English population [30]. In comparison, the allele frequency of the R219K polymorphism was 0.22 (K allele), similar to that found in other European populations [31]. The proband's father was heterozygous for L1379F, and her mother and sister for the V1704D variant (Fig. 1).

3.4. Expression of normal and mutant ABCA1 in vitro

The L1379F and V1704D mutations were introduced into the full-length cDNA for ABCA1, fused at its carboxy-terminus to eGFP. The C-terminal eGFP tag does not alter either the expression and function of the ABCA1 protein or its localisation [32,33]. Both wild-type (WT) and mutant

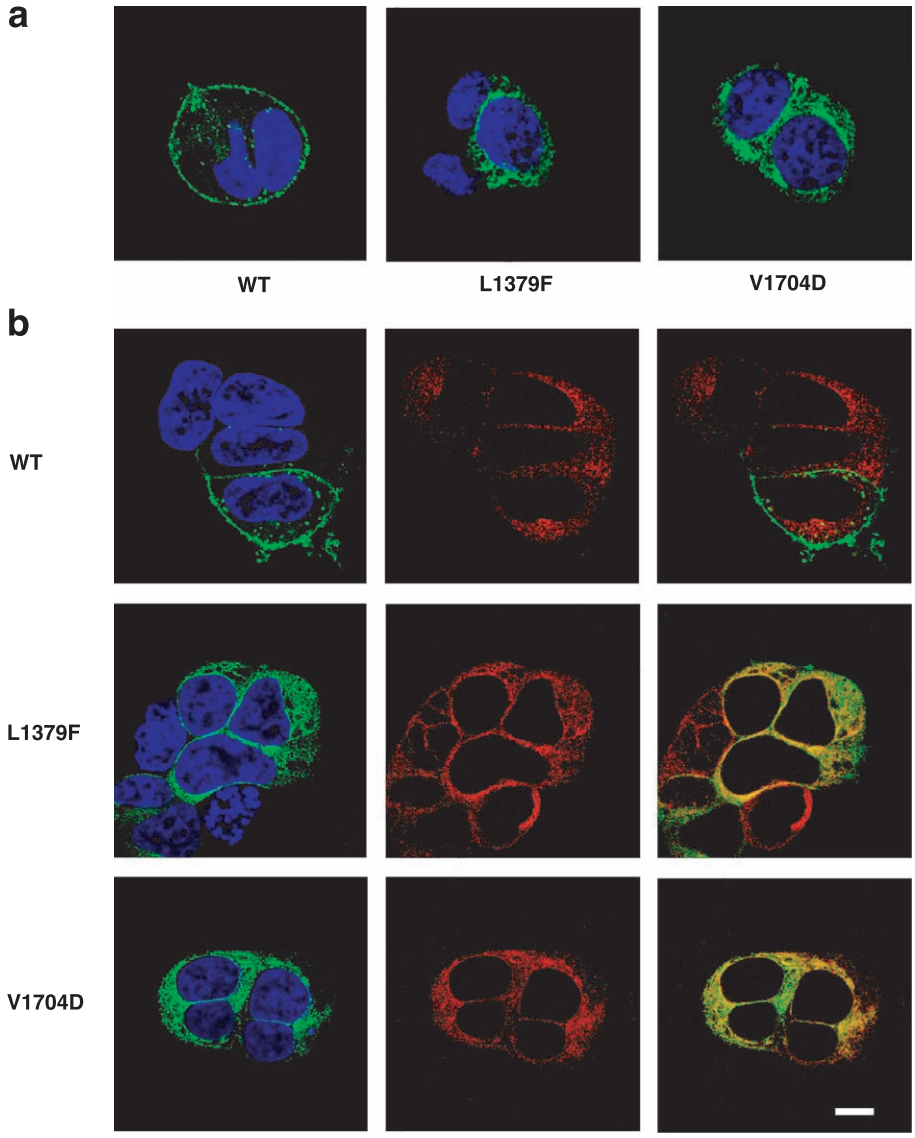

Fig. 3. Expression of ABCA1-eGFP fusion proteins in HEK 293 cells. (a) Confocal sections of transiently transfected HEK 293 cells expressing wild-type ABCA1-eGFP (left panel), ABCA1-eGFP L1379F (middle panel) and ABCA1-eGFP V1704D (right panel); eGFP fluorescence is shown in green and DAPI staining of nuclear DNA in blue. Both mutations caused severe alteration in ABCA1 trafficking to the plasma membrane as compared to the wild-type (WT). (b) HEK 293 cells expressing wild-type ABCA1-eGFP, ABCA1-eGFP L1379F and ABCA1-eGFP V1704D were stained for SERCA 2 as an ER marker. Confocal sections are shown for eGFP (green, left column), SERCA 2 (red, middle column) and the co-localisation of eGFP and SERCA 2 in the overlay (yellow, right column). A large extent of co-localisation was found for both mutants, suggesting retention of L1379F and V1704D in the ER. In contrast, wild-type ABCA1 localised predominantly to the plasma membrane and no co-localisation with the ER marker was apparent. Dimension bar indicates 10 μm.

ABCA1-eGFP proteins were found to be expressed in transiently transfected HEK293 cells. As expected [32,33], normal ABCA1-eGFP was present mainly at the cell surface (Fig. 3a, left panel) with punctate intracellular vesicles visible in some cells and possible accumulation in the Golgi in others (data not shown). In marked contrast to this, trafficking of both mutated proteins to the plasma membrane was severely altered in comparison to the wild-type (Fig. 3a, middle and right panels). Their intracellular localisation pattern suggested that both were present largely in the endoplasmic reticulum (ER). This was confirmed by the observation that both mutant eGFP fusion proteins, but not wild-type ABCA1-eGFP, co-localised extensively with sarco/endoplasmic reticulum calcium ATPase (SERCA 2), a marker of the ER [34] (Fig. 3b).

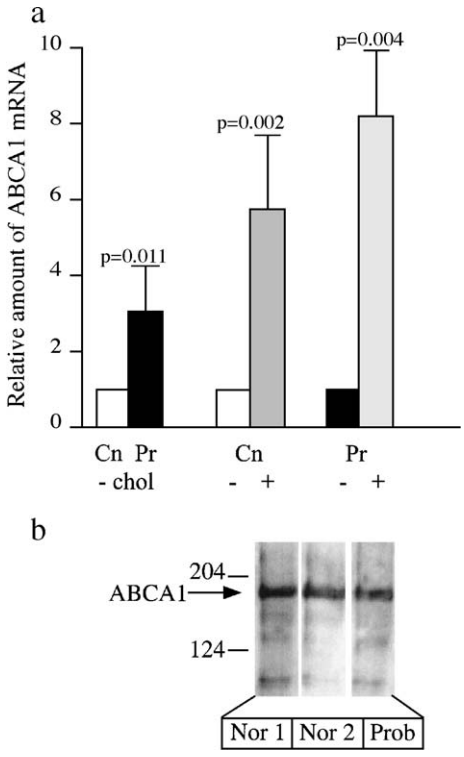

Fig. 4. Quantification of ABCA1 mRNA and protein in cultured skin fibroblasts. (a) Total RNA was isolated from skin fibroblasts incubated for 24 h in medium containing 10% serum or in serum-free medium containing 2% BSA and 30 μg/ml cholesterol. Basal ABCA1 mRNA levels in the proband's cells incubated with serum is expressed relative to the mean of values (normalized to 1.0) from cells from four different normolipaemic controls (left). Also shown is the effect of cholesterol loading on ABCA1 mRNA levels in control cells (mean of four, centre) and the proband's cells (right), relative to the value in cells incubated with serum. Each value represents the mean ± S.D. of two Taqman real-time PCR determinations of at least three different preparations of RNA. The variation between control cell lines was less than 10% of the mean for cells incubated in medium and 15% for cells incubated with cholesterol. Significant differences (*P* values) were determined by unpaired Student's *t*-test. (b) Membrane proteins from cultured fibroblasts (40 μg in approx. 25 μl of extract) were fractionated by reducing SDS-PAGE (12% acrylamide gels) and immunoblotted with rabbit polyclonal anti-ABCA1 peptide. Bound antibody was detected with peroxidase-conjugated anti-rabbit IgG by chemiluminescence (2 min exposure). The positions of marker proteins (kDa) on the blot are indicated.

3.5. ABCA1 expression in cultured skin fibroblasts

Cultured skin fibroblasts from the proband contained 3-fold more ABCA1 mRNA relative to GAPDH mRNA than control cells when assayed by real-time PCR (Fig. 4a). The variation in ABCA1 mRNA content relative to GAPDH mRNA in cultured skin fibroblasts from four different control subjects was less than 10% ($\Delta Ct = 6.84 \pm 0.15$). When the cells were preincubated with cholesterol to induce ABCA1 expression, the mean relative level of ABCA1 mRNA increased by approximately 6-fold in control cells ($\Delta Ct = 4.16 \pm 0.27$, $n = 4$), and more than 8-fold in the proband's cells (Fig. 4a). In contrast, there was no discernible difference between the amount of ABCA1 protein in membranes from the proband's and control cells when estimated by semi-quantitative immunoblotting (Fig. 4b).

4. Discussion

We describe a patient of English origin with severe HDL deficiency and premature coronary disease who is heterozygous for two rare alleles of *ABCA1* that are predicted to cause single amino acid substitutions, L1379F and V1704D. These mutant alleles have not been described previously and, as far as we are aware, this is the first individual of English origin known to have a disorder caused by mutations in *ABCA1*. There is strong evidence that these mutations in *ABCA1* are the underlying cause of the HDL deficiency. Firstly, the clinical and biochemical phenotype of the proband is consistent with defective *ABCA1*, and other possible causes of HDL deficiency were excluded. Secondly, the two *ABCA1* variants were not found in 164 alleles of English origin and, finally, both mutations were found to disrupt trafficking to the plasma membrane of ABCA1 protein when expressed in cultured cells in vitro. Thus, the data are consistent with a diagnosis of HDL deficiency caused by autosomal recessive defects in *ABCA1*.

Both L1379 and V1704 are conserved between human, mouse and chicken ABCA1 and the regions in which they lie are strongly conserved between ABCA1 and ABCA4 (formerly known as ABCR [35], Fig. 5). Simple Modular Architecture Research Tool (SMART; http://www.smart.embl-heidelberg.de) analysis predicts a secondary structure consistent with that known for other ABC transporters, for example P-glycoprotein, encoded by *MDR1* [36], and the bacterial lipid flippase MsbA [37]. ABCA1 is predicted to consist of two tandem repeats, each comprising a transmembrane domain with six membrane-spanning segments and an intracellular nucleotide binding domain. This topology is supported by observations that FLAG or HA peptides inserted in the extracellular loops of ABCA1 do not disrupt its function and are accessible at the cell surface [38,39].

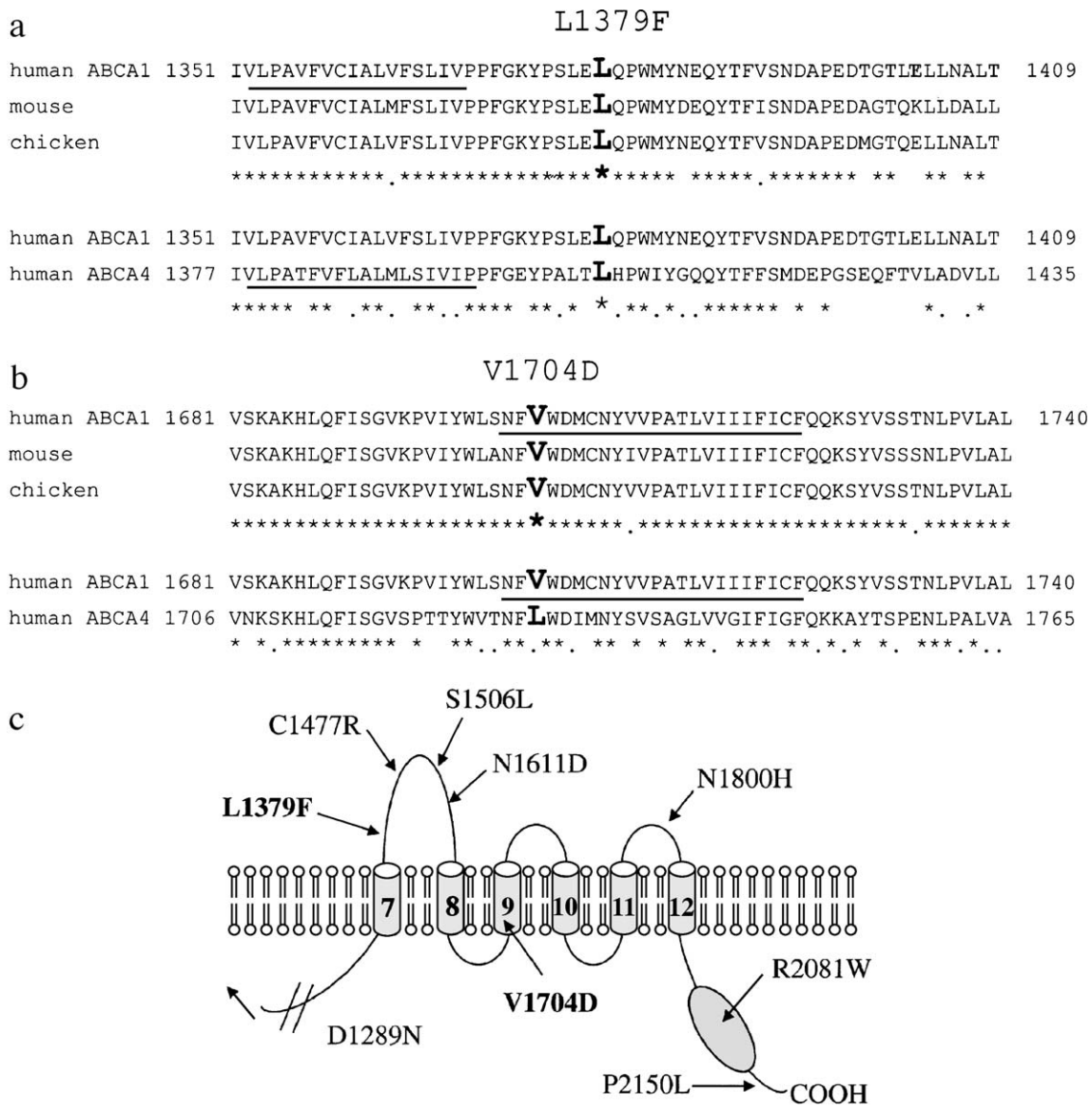

Fig. 5. Diagram showing alignment of human, murine and chicken ABCA1, and human ABCA1 and ABCA4. (a) Above, alignment of residues 1351–1409 of human ABCA1 (NM_005502) with residues 1291–1349 of mouse (GenBank accession: NM_013454) and chicken ABCA1 (AF363377). Below, alignment of the same residues of ABCA1 with residues 1377–1435 of human ABCA4 (NM_000350). The position of the L1379F substitution in ABCA1 is indicated in bold; the residues predicted by Simple Modular Architecture Research Tool (SMART; http://www.smart.embl-heidelberg.de) analysis of the amino acid sequence of ABCA1 to form membrane-spanning segment 7 in ABCA1 and ABCA4 are underlined. (b) Above, alignment of human, mouse and chicken ABCA1 and below, human ABCA1 with human ABCA4, showing the position of the V1704D mutation in ABCA1 (indicated in bold). Residues predicted by SMART to form membrane-spanning segment 9 in the carboxy-terminal half of ABCA1 and ABCA4 are underlined. (c) Diagram showing the predicted topology of the carboxy-terminal half of ABCA1; membrane-spanning segments and the nucleotide binding domain (shaded oval) were predicted by SMART. The two novel amino acid substitutions in the patient are indicated in bold; other single amino acid substitutions in the region in Tangier/HDL deficient individuals are also shown (reviewed by Singaraja et al. [31]).

Despite these insights into ABCA1 structure, the molecular mechanism for ABCA1-mediated cholesterol efflux is still poorly understood [40]. Various models are proposed, but each remains controversial, making it difficult to interpret the structure–function relationship of the two new mutations we describe. In our transfection studies both mutants found in the patient clearly disrupted the trafficking of the ABCA1 protein to the plasma membrane as visualised by confocal microscopy. However, V1704 is predicted to lie within membrane-spanning segment 9 and thus it is not surprising that replacement of the hydrophobic valine residue with negatively charged aspartate in this position disrupts trafficking of the protein. Interestingly, only one mutation in the first set of transmembrane segments of ABCA1 (residues 636–908) has been observed previously and none in the second set where V17104 is located. The known mutant protein has a deletion of L693 from transmembrane segment 3 (equivalent to transmembrane seg-

ment 9 in the carboxy-half of ABCA1), and also fails to be transported from the ER [39].

The other amino acid substitution, L1379F, is predicted to lie in the extracellular loop between transmembrane segments 7 and 8 that may constitute the binding site for apoAI [41]. This mutation predicts a relatively conservative substitution of one bulky hydrophobic residue for another and it is less clear why it should disrupt trafficking of ABCA1, but other amino acid substitutions in HDL deficient patients have been found to lie in the same predicted extracellular loop. Two of these, C1477R and S1506L, do not appear to disrupt transport of the protein to the cell surface, as judged by the accessibility of the protein in non-permeabilised cells, but the mutant proteins are unable to mediate cholesterol efflux [38]. However, the subcellular localisation of these proteins was not visualised by microscopy.

Skin fibroblasts from our proband contained approximately three times more ABCA1 mRNA than normal cells, showing that the defect in ABCA1-mediated cholesterol efflux resulted in up-regulation of the *ABCA1* gene. There was surprisingly little, less than 10%, variation in ABCA1 mRNA expression between different control cells. Preincubation with cholesterol increased ABCA1 mRNA levels in all cells, but more so in the patient's cells. Despite this difference in mRNA content, the amount of ABCA1 protein detected by Western blotting of membranes from the proband's or normal cells was similar. However, in localisation studies we were able to demonstrate that, although highly expressed, both mutant proteins display markedly disrupted trafficking to the plasma membrane suggesting severely impaired function. Failure of the mutant ABCA1 proteins to reach the surface and bind apoAI may also make them more susceptible to proteolytic degradation [40,42].

Both the proband's parents were alive and well at 80 years of age; clearly, the inheritance of both mutant alleles of ABCA1 was necessary for the almost total HDL deficiency and the unusually severe and progressive premature coronary disease seen in this female patient of 42 years. However, despite the almost complete absence from plasma of HDL, there was no evidence of the cholesterol accumulation in the liver, spleen or tonsils that is characteristic of Tangier disease. Although Tangier patients without all these clinical signs have been observed previously [5,43], there is currently no explanation for this phenotypic variation. The relationship between particular ABCA1 mutations, the reduction in HDL-cholesterol levels and defective lipid efflux has been discussed in a recent review [31], but why some Tangier disease patients have obvious cholesterol deposition while others do not was not raised. One possibility is suggested by emerging evidence that ABCA1 also mediates intracellular trafficking of substrate lipids, as well as of apoAI acceptor [44]. It is tempting to speculate that the ABCA1 proteins in our patient and that described by Bertolini et al. [43] retain some residual intracellular function that somehow reduces the cholesterol accumulation seen in macrophages in liver, spleen or tonsils from Tangier patients with null alleles.

Acknowledgements

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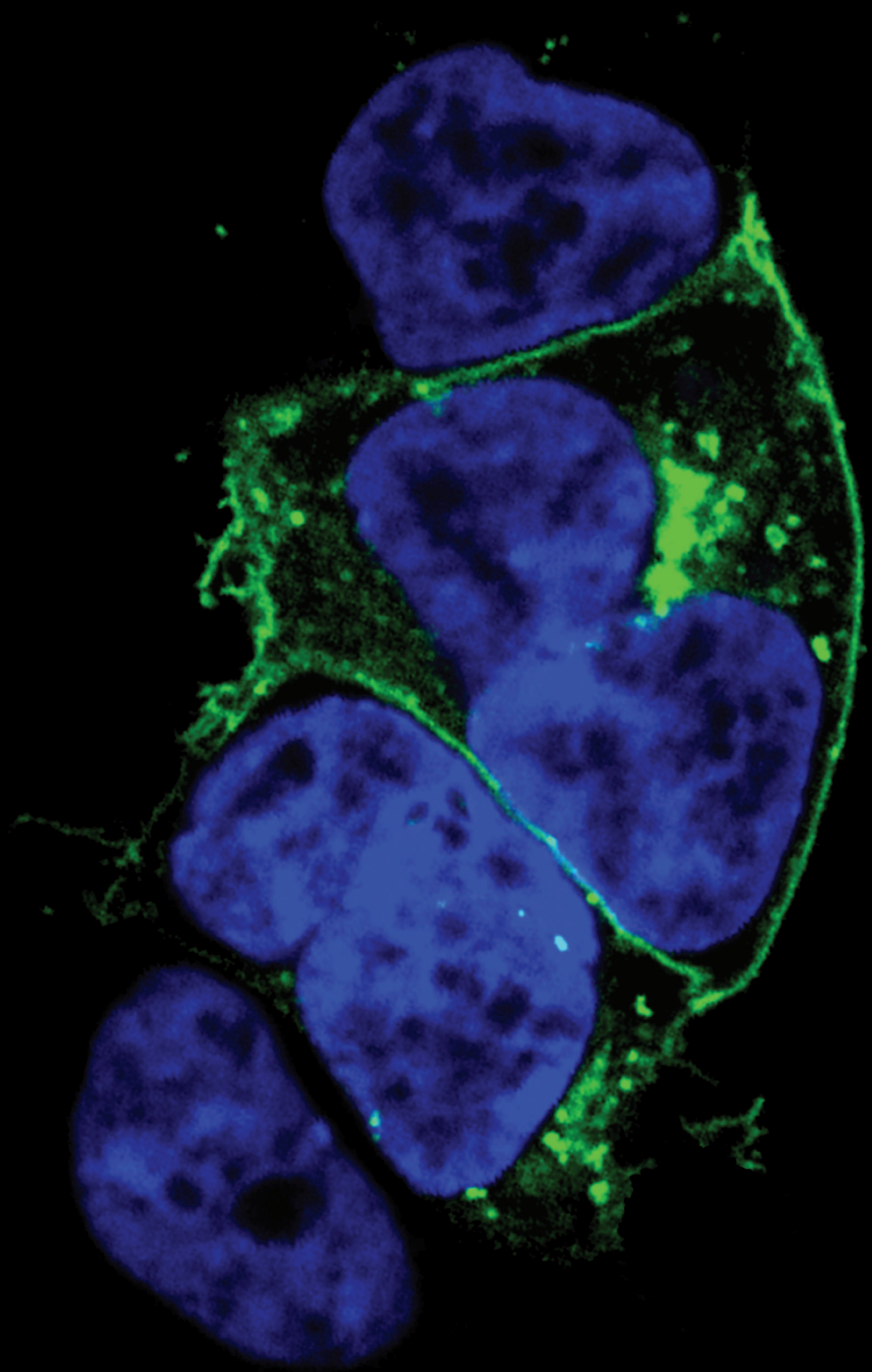

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ABCA1 and Scott syndrome

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A novel missense mutation in *ABCA1* results in altered protein trafficking and reduced phosphatidylserine translocation in a patient with Scott syndrome

Christiane Albrecht, John H. McVey, James I. Elliott, Alessandro Sardini, Ildiko Kasza, Andrew D. Mumford, Rossi P. Naoumova, Edward G. D. Tuddenham, Katalin Szabo, and Christopher F. Higgins

Scott syndrome (SS) is a bleeding disorder characterized by a failure to expose phosphatidylserine (PS) to the outer leaflet of the platelet plasma membrane. Because the adenosine triphosphate (ATP)–binding cassette transporter A1 (ABCA1) is implicated in the exofacial translocation of PS, we assessed its role in the pathophysiology of a patient with SS. Substantially reduced levels of *ABCA1* mRNA were found in the patient's leukocytes, compared with controls. The SS patient was heterozygous for a novel missense mutation c.6064G>A (ABCA1 R1925Q), absent from unaffected family members and controls. Both mutant and wild-type alleles were reduced in mRNA expression, and no causative mutation for this phenomenon was identified in the *ABCA1* gene or its proximal promoter, suggesting a putative second mutation in a *trans*-acting regulatory gene may also be involved in the disorder in this patient. In vitro expression studies showed impaired trafficking of ABCA1 R1925Q to the plasma membrane. Overexpression of wild-type ABCA1 in SS lymphocytes complemented the Ca^{2+}-dependent PS exposure at the cell surface. These data identify a mutation in *ABCA1* that contributes to the defective PS translocation phenotype in our patient with SS. (Blood. 2005;106:542-549)

Introduction

Scott syndrome (SS) is a rare, moderately severe bleeding disorder (Online Mendelian Inheritance in Man [OMIM] database: 262890). Because only 3 patients (one American, one French, and one British) have been identified, the inheritance pattern is unknown,[1,2] and the rarity of affected individuals precludes the use of conventional mapping approaches to identify the underlying genetic lesions. Hemostatic parameters of close relatives of patients with SS exhibit significant though clinically silent defects, indicating that any defective gene has substantial penetrance in the heterozygous state,[1,2] and do not preclude the possibility that the SS phenotype reflects the interaction of 2 (or more) defective genes.

The defining characteristic of SS is the absence of Ca^{2+}-stimulated exposure of phosphatidylserine (PS) from the inner leaflet of the plasma membrane bilayer to the cell surface, which in platelets normally provides an appropriate surface for the assembly of the tenase and prothrombinase complexes of the coagulation network. This defect is also observed in Epstein-Barr virus (EBV)–transformed lymphocytes of patients with SS. The mechanism by which PS is translocated to the cell surface following Ca^{2+} stimulation is controversial, and several proteins have been suggested as playing a role. However, both phospholipid scramblase 1[3-5] and P-glycoprotein[4,6] appear to be normal in patients with SS.

The adenosine triphosphate (ATP)–binding cassette transporter A1 (ABCA1) has been implicated in PS translocation both by genetic disruption in mice[7] and chemical inhibition.[8] It is not yet clear whether ABCA1 translocates PS directly or acts as a regulator of another protein.[9] Mutations in *ABCA1* underlie Tangier disease (TD) and familial high-density lipoprotein (HDL) deficiency,[10-13] conditions characterized by the absence of plasma HDL and greatly increased susceptibility to coronary heart disease. In addition, patients with TD have been shown to exhibit severely disrupted platelet function, consistent with the high platelet expression of this protein.[14,15] Further, in *abca1*-deficient mice aberrant PS translocation is reflected in a hemorrhagic diathesis characteristic of SS. We therefore hypothesized that defects in *ABCA1* underlie SS and assessed the expression and function of ABCA1 in a recently characterized patient with SS.[16,17]

Patients, materials, and methods

Patients

The SS proband is a 59-year-old white woman of British origin. Clinical features have been described previously.[16,17] Family members were evaluated separately. Controls for analysis of *ABCA1* mRNA expression were healthy volunteers between 23 and 71 years old. For mRNA analysis, 10 mLwhole blood was taken after an overnight fast. Approval was obtained from the Hammersmith Research Ethics Committee. Informed consent was provided in accordance with the Declaration of Helsinki.

From the Medical Research Council Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Hospital Campus, London, United Kingdom; National Medical Center, Institute of Haematology and Immunology, Research Group of the Hungarian Academy of Sciences, Budapest, Hungary; Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary; and Physiology Weihenstephan, Technical University Münich, Freising, Germany.

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Reprints: Christiane Albrecht, Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, 85354 Freising, Germany; e-mail: christiane.albrecht@wzw.tum.de.

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Lipid profile

Lipid parameters of the proband were assessed at the lipid clinic of the Hammersmith Hospital. Blood for lipid parameters was obtained after 12-hour overnight fast. Serum total cholesterol, triglyceride, and HDL cholesterol levels were determined by automated methods using commercial kits and interassay controls. Levels of serum apolipoprotein A-I, apolipoprotein B, and lipoprotein (a) were measured using an automated immunoturbidimetric assay (Beckman Coulter, Galway, Ireland). Low-density lipoprotein (LDL) cholesterol was calculated from the standard formula as follows: LDL cholesterol (mM) = Total cholesterol − (HDL cholesterol + triglyceride/2.2).[18] Apolipoprotein E phenotype was performed by immunoblotting.[19]

Nucleotide sequence analysis

DNA was extracted from whole blood using Nucleon DNA isolation kit (Tepnel Life Sciences, Manchester, United Kingdom) according to the manufacturer's instructions. The coding region and proximal promoter (395 base pair (bp) upstream from the ATG) of *ABCA1* were amplified from gDNA by polymerase chain reaction (PCR) with primers located 50 to 70 bp from each splice junction and from cDNA amplified by reverse transcription (RT) PCR from leukocyte RNA with primers that produced 9 overlapping fragments (primer and amplification details available on request). Amplification products were sequenced on both strands and the sequences compared with ABCA1 cDNA sequence (GenBank accession no. AF285167). The nucleotide substitution is designated as recommended by a working group on nomenclature.[20]

Analysis of polymorphisms in DNA by WAVE analysis

PCR fragments of *ABCA1* exons 6 and 42 were amplified with the following primers: exon 6, 5′-GGACCCAGCTTCCAATCTTCATAATCC-3′ and 5′-GCCTCACATTCCGAAAGCATTAGTGC-3′; exon 42, 5′-GTGGTTTAT-AGTCCTGCCTTCCAC-3′ and 5′-ACGAGCATCGTTGCTTGATTGGGT-3′.

PCR products were denatured at 94°C, followed by cooling to 25°C over 25 minutes to enhance heteroduplex formation, and analyzed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE).

ABCA1 gene expression in leukocytes

cDNA preparation. Leukocytes were isolated from 10 mL whole blood and RNA was extracted and reverse transcribed as described previously.[21] For *Taq*Man analysis, the cDNA was diluted 4-fold with nuclease-free water.

Real-time quantitative RT-PCR (*Taq*Man). Single-tube *Taq*Man analysis was performed on an ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, United Kingdom) with 300 nM forward and reverse primers in the presence of 200 nM 5′FAM-3′TAMRA–tagged probe located in exon 3 of the *ABCA1* gene as described recently.[21] The internal standard was glyceraldehyde-3-phosphate dehydrogenase (G3PDH), assayed with commercially supplied reagents (Applied Biosystems). Reactions were carried out in triplicate. The amount of *ABCA1* mRNA in leukocytes of the patient and diverse control populations was calculated according to the standard curve method described in Applied Biosystems User bulletin no. 2. *ABCA1* mRNA of all control subjects was expressed relative to that of G3PDH and calculated as relative (x-fold) expression as compared to the patient with SS.

Quantitative mRNA measurements of exon skipping (LightCycler). Previously, an alternative transcript of the *ABCA1* gene has been reported with an out-of-frame deletion of exon 3, which results in a truncated protein of 74 amino acids presumably devoid of function.[22] To assess the ratio of full-length to alternative transcript, a quantitative PCR method was developed using SYBR Green I and LightCycler technology (Roche, Penzberg, Germany). A reverse primer spanning exon boundaries 4 and 5 (5′-GCTTCAAGTTTGAGCTGGAT-3′) was combined either with a forward primer spanning exon junctions 2 and 3′, 5′-CTATGAACATGAAT-GCCATT-3′) to assess the 278-nucleotide (nt) full-length ABCA1 cDNA fragment including exon 3, or with a forward primer spanning the junction of exon 2 and 4 to measure only the 138-nt aberrant transcript. 18S RNA primers (forward 5′-AAGTCTTTGGGTTCCGGG-3′; reverse 5′-GGACAT-CTAAAGGGCATCACA-3′) amplifying a 365-bp fragment were used for normalization. PCRs contained 3 mM $MgCl_2$, 0.4 μM forward and reverse primer, and 1 μL LightCycler DNA Master SYBR Green I (10×, Roche). Before amplification, a preincubation step (60 seconds at 95°C) was performed to activate FastStart DNA polymer and to ensure complete denaturation of the cDNA. LightCycler PCR was performed with 40 cycles using following amplification conditions: denaturation 15 seconds at 95°C, annealing 10 seconds at 62°C, and elongation 25 seconds at 72°C. To each amplification cycle a fourth segment with an elevated temperature fluorescence acquisition point was added to remove unspecific signals before SYBR Green I quantification (3 seconds at 80°C, 84°C, and 85°C for the full-length product, the aberrant product and 18S, respectively). Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification.

Both transcripts of the SS proband were analyzed using the Fit Points and 2nd Derivative Maximum calculation described in the LightCycler Relative Quantification Software and compared to a leukocyte control population. *ABCA1* mRNA of all control subjects was expressed relative to that of 18S RNA and calculated as x-fold expression as compared to the SS patient. The relative distribution between the 2 transcripts is expressed as the full-length–aberrant transcript ratio.

Plasmids/DNA constructs

Full-length human ABCA1 cDNA was generated by RT-PCR of mRNA obtained from leukocytes of a healthy individual and the patient as described previously.[13] Wild-type cDNA was cloned into pGEM-11Zf vector (Promega, Madison, WI) and replaced with the corresponding cDNA fragment (*Asp*718-*Hpa*I) containing the patient's mutation (c.6064G>A). No other single nucleotide polymorphisms were introduced as verified by sequence analysis.

Enhanced green fluorescent protein (EGFP) was fused in frame to the C-terminus of ABCA1 by an overlapping PCR strategy as described previously.[13,23] The PCR product was cloned into pCR 4Blunt-TOPO vector (Invitrogen, Paisley, United Kingdom). *Bam*HI-*Not* I ABCA1 cDNA fragments in the pGEM-11Zf constructs were replaced with the ABCA1-EGFP hybrid. The resulting wild-type and mutant ABCA1-EGFP cDNAs were cloned with *Sal* I-*Not* I into pCI-neo mammalian expression vector (Promega) and all constructs verified by complete sequencing.

Expression and localization of ABCA1-EGFP fusion proteins in HEK 293 cells

Transfection, fixation, immunocytochemistry, and confocal imaging of HEK 293 cells was performed as previously described.[13] In brief, HEK 293 cells were plated on poly-L-lysine–coated (Sigma, St Louis, MO) glass cover slips 12 hours before transfection. Cells were transfected in 6-well plates according to the calcium phosphate transfection protocol (Clontech, Palo Alto, CA) with 2 μg DNA per well. Cells were washed twice with phosphate buffer saline solution (PBS) after six hours to remove plasmid DNA and fixed in 4% formaldehyde–4% sucrose 24 hours after transfection for localization studies. Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma, Saint Louis, MO) for 4 mintues and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 15 mg/mL; 1:10 000 dilution; Molecular Probes, Eugene, OR) for nuclear DNA. In order to identify the intracellular localization of the wild-type and mutant ABCA1 proteins, cells were also stained with the mouse monoclonal antibody against sarco/endoplasmic reticulum calcium adenosine trophosphatase (SERCA 2, clone IID8; Affinity Bioreagents, Golden, CO) for 2 hours at room temperature after blocking with 0.2% fish-skin gelatin (Sigma) in PBS. The antibody against SERCA 2 was detected by goat anti–mouse immunoglobulin G (IgG) secondary antibody conjugated to Alexa 568 (1:400 dilution for 30 minutes at room temperature; Molecular Probes). Cells were imaged with a Leica SP confocal microscope equipped with a 63×/1.32 PlanApoChromat oil-immersion objective lens (Leica, Wetzlar, Germany). EGFP was excited with the 488-nm line of an argon laser; Alexa 568, with the 568-nm line of a krypton laser. To avoid bleedthrough, the fluorophores were excited sequentially. The emitted fluorescence was

collected separately through a triple-dichroic mirror (488/568/663). The emission filter bands for EGFP and Alexa-568 fluorescence were restricted to 500-552 nm and 594-620 nm, respectively. DAPI staining of nuclear DNA was excited with the 351-nm line of an ultraviolet (UV) laser, and emission fluorescence collected with a 396-508–nm bandpass filter. Stacks of confocal sections separated by 1-μm increments were taken and images analyzed with Metamorph 5.0v1 software (Universal Imaging, Downington, PA). Figures were assembled for publication with Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA).

Analysis of allelic frequency

PCR amplification of leukocyte cDNA with primers (position 5702 and 6457) spanning the mutation was performed using standard PCR conditions and cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Direct DNA sequence analysis was performed on an ABI 3700 Prism automated sequencer.

Preparation of wild-type ABCA1 expressing SS EBV-transformed B cells

The full-length wild-type human ABCA1 cDNA[9] was cloned into a bicistronic retroviral vector SPsLdS,[24] modified to contain the neomycin-resistance cassette (kindly provided by Attila Ilias, Institute of Enzymology, Budapest, Hungary). The generation of retroviral particles and establishment of stable cell lines expressing the ABCA1 protein in SS EBV-transformed B cells (SS-ABCA1$^+$) were achieved using the method of Ujhelly et al[25] with minor modifications. Briefly, Phoenix-eco packaging cell line[26] was transfected by calcium phosphate coprecipitation (Gibco, Karlsruhe, Germany). The cell-free viral supernatant was collected and used immediately to transduce PG13 packaging cells.[24,25] The retroviruses produced by the G418-selected PG13 cell lines were used for SS EBV-transformed B-cell transduction. To prepare stable, ABCA1-expressing cell population, we used a sequential selection method as described by Kiffmeyer et al.[27]

RNA preparation and RT-PCR of cell lines

Total RNA was isolated by a single-step method using TriZol (Invitrogen). For each reaction 1 μg total RNA was reverse-transcribed into cDNA according to the manufacturer's protocol (Promega) using random hexamer oligonucleotides. The resulting cDNA was then subjected to PCR by using specific primers to detect human ABCA1 cDNA, but not the genomic *ABCA1* sequence. RT-PCR analysis for the housekeeping Abelson (ABL) gene transcript served as control for total RNA preparation and the RT reaction.[28] Oligonucleotides designed for PCR analysis of ABCA1 or ABL cDNAs were: 5′-ACAAGATGCTGAGGGCTGAT-3′ and 5′-CCCAAGACTATGCAGCAATG-3′ or 5′-GGGCTCATCACCACGCTCCA-3′ and 5′-CTGCCGGTTGCACTCCCTCA-3′, respectively.

Analysis of PS exposure by annexin V–binding assay

SS and control EBV-transformed B cells were harvested and resuspended in annexin-binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) prior to addition of propidium iodide (PI; 19 μg/mL) and Alexa Fluor 488-conjugated annexin V (1:80 vol/vol; Molecular Probes, Eugene, OR). Fluorescence was measured on a FACScalibur (Becton Dickinson, Heidelberg, Germany) flow cytometer; the simultaneous presence of PI in the samples monitored possible cell death. After the addition of either A23187 (1 μM; Boehringer Mannheim, Mannheim, Germany) or dimethyl sulfoxide (DMSO; as control), changes in annexin V–Alexa Fluor 488 binding over time were recorded at 1- or 5-minute intervals for 15 or 50 minutes.

In addition, effects of glyburide, cyclosporin A, verapamil, KO143, PSC833, and DMSO (as control) on the A23187-induced changes in the binding of annexin V–Alexa Fluor 488 were measured. Before addition of A23187 the cells were preincubated with annexin V–binding buffer for 10 minutes either with DMSO or with these agents at the concentrations indicated; changes in annexin V–Alexa Fluor 488 induced by A23187 binding over time were then recorded. Data from live cells, manually gated on the basis of excluding PI-stained cells, were analyzed with CellQuest software (Becton Dickinson). Ca^{2+}-dependent annexin V binding was calculated by subtracting the values measured in the presence of DMSO from the values detected in the presence of A23187. Each data point represents the mean value of at least 3 independent experiments. KO143 was a generous gift from Drs. J. Allen and G. Koomen (Division of Experimental Therapy, The Netherlands Cancer Institute, and Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands). PSC 833 was a gift from Novartis Pharma (Basel, Switzerland). Other chemicals were from Sigma. Inhibitors and A23187 were dissolved in DMSO.

Results

ABCA1 gene expression

Total RNA was prepared from leukocytes isolated from a recently reported SS proband[17] and a series of healthy controls (n = 30; aged 30-59). *ABCA1* mRNA levels were quantified by real-time RT-PCR relative to expression of *G3PDH* using a *Taq*Man probe located in exon 3 of the *ABCA1* gene. *ABCA1* mRNA was readily detectable in total leukocyte RNA from controls and the patient. The relative amount of *ABCA1* transcript detected including exon 3 varied within the control group (mean 3.90; Figure 1A). However, the relative *ABCA1* mRNA expression level in the SS patient was very low and lay outside the 10th percentile limits (1.76) of the controls.

An alternative *ABCA1* transcript with an out-of-frame deletion of exon 3, introducing a premature stop codon after 74 amino acids, has been reported in a number of cell lines.[22] We therefore investigated the expression of the 2 alternative transcripts in leukocytes of the SS patient and controls (n = 22, aged 23-71 years). Both transcripts were found in controls and in the proband (Figure 1B). Quantitative RT-PCR analysis using amplicons specific for the wild-type (exon 2-3-4) and aberrant (exon 2-4) transcripts revealed extremely low expression of the wild-type cDNA in the SS patient compared with controls (mean of controls, 3.85; Figure 1C). Analysis of the aberrant transcript demonstrated that the patient had similarly reduced levels of the alternative, nonfunctional transcript (mean of controls, 3.77; Figure 1C). Because the relative abundance of wild-type to aberrant transcript was similar for the controls (mean, 1.29) and the SS patient (mean, 1.53; Figure 1C), low *ABCA1* mRNA levels in the patient are not due to preferential alternative splicing.

Sequence analysis

Sequence analysis of the promoter region, exons, and flanking intronic sequence of the patient's *ABCA1* gene revealed no sequence changes that might explain the aberrant levels of *ABCA1* mRNA observed in the leukocytes of the SS patient. However, several known polymorphisms,[29-31] and heterozygosity for a novel single base pair substitution c.6064G>A in exon 42, predicted to substitute R1925 with glutamine, were identified (Table 1). The only available first-degree relatives of the patient, her mother and 2 children, are unaffected and homozygous wild-type for R1925 (Figure 2). The R1925Q variant was not found in 164 alleles of a control population of British origin,[32] evidenced by WAVE analysis. For comparison, the allele frequency of the polymorphism in exon 6 (R219K) in this population was 0.22, similar to that observed in other European populations (0.254).[33]

To investigate whether there was differential expression of the 2 alleles, a PCR product spanning the c.6064G>A mutation was generated by RT-PCR from total RNA isolated from leukocytes of

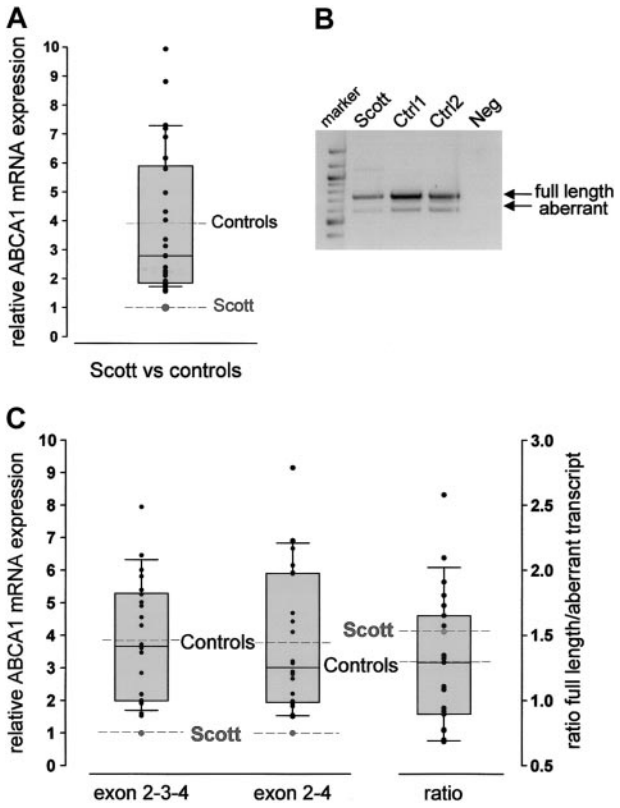

Figure 1. Relative expression of *ABCA1* mRNA in leukocytes. (A) Quantitative analysis of *ABCA1* mRNA using *Taq*Man technology and a fluorescent probe located in exon 3. Analysis was performed using the standard curve method. The control population is visualized by the box plots indicating the 10th and 90th percentile (error bars), the median (solid horizontal line), and the mean (dashed line). The relative amount of *ABCA1* mRNA in each control is represented as x-fold expression of the SS patient (set as 1, dashed line). Measurements were performed in triplicate. Because *ABCA1* mRNA expression did not differ between men and women, a mixed control population is shown. (B) Agarose gel electrophoresis (1.5% agarose gel) of RT-PCR products amplified from leukocyte RNA from the SS patient and 2 controls using primers spanning exon 1-6. The 2 bands correspond to the wild-type (746-bp) and the alternatively spliced (606-bp) transcript. Lane 1, 100-bp marker; lane 4: negative control. (C) Quantitative analysis of the 2 transcripts described in panel B using LightCycler technology. The SS proband showed reduced *ABCA1* mRNA levels for both the appropriately spliced (exon 2-3-4) and the alternatively spliced (exon 2-4) transcript. The ratio of the 2 transcripts did not differ between the patient and the controls (column 3). Analyses were performed using the FitPoints calculation method as described in "Patients, materials, and methods." The control population is visualized by the box plots (details are described in panel A).

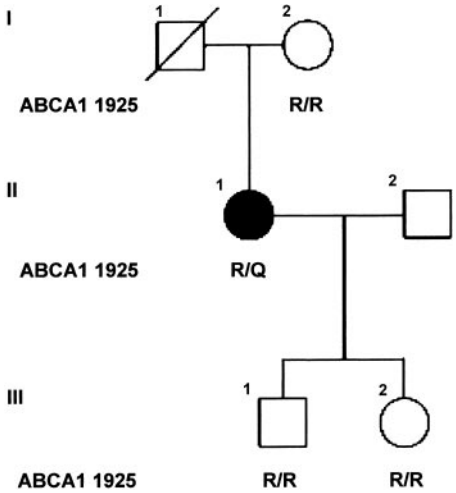

Figure 2. Family pedigree. The affected individual is indicated by the solid symbol and unaffected relatives by open symbols. The amino acids at codon 1925 of ABCA1 are shown for each individual tested (R/R, homozygote; R/Q heterozygote). Individual I.1 died following a myocardial infarct and previously had undergone amputation of his right arm following an accident without excessive bleeding, and thus is likely to be unaffected.

the SS patient. The PCR products were cloned and sequenced. Of 33 independent clones sequenced, 17 clones had the wild-type sequence, whereas 16 had the c.6064G>A mutation, indicating that both alleles were equally expressed in the *ABCA1* leukocyte mRNA population of the SS patient.

R1925 is predicted to lie 20 amino acids N-terminal of the Walker A motif in the second nucleotide-binding domain and is conserved in all known mammalian ABCA1 transporters (Figure 3). Substitution of R1925 with glutamine disrupts a stretch of 5 highly charged amino acids (RRKRK), which is strongly conserved between all members of the A family of ABC transporters. Interestingly, ABCA7, which is also believed to efflux cellular phospholipids but not cholesterol,[34] is identical to ABCA1 at position 1925 and the homologous region of the *Caenorhabditis elegans* protein ced7[35] shares significant sequence similarity with ABCA1 and ABCA3 (Figure 3), pointing to the functional importance of this residue.

Localization studies

To test the impact of the R1925Q mutation on intracellular trafficking of ABCA1, we introduced the c.6064G>A mutation into a full-length cDNA encoding ABCA1 fused at its carboxy-terminus to EGFP, and expressed the fusion protein in HEK 293

Table 1. Nucleotide substitutions in *ABCA1* in the SS proband

Exon	Nucleotide*	Codon†	Base substitution	Amino acid substitution	Comments	Reference (variant db SNP rsID)
1	273	5′ UTR	hmz G	None	Polymorphism	Pullinger et al[29]
2	368-70	Leu 26	hmz CTG	None	CTG in general sequence	Pullinger et al[29]
5	764	Leu 158	htz A/G	None	Polymorphism	Clee et al[30] (rs 2230805)
6	947	Arg 219	htz G/A	R→K	Polymorphism	Wang et al[31] (rs 2230806)
8	1226	Pro 312	htz C/T	None	Polymorphism	(rs 2274873)
14	2330	Ile 680	hmz C	None	Polymorphism	Wang et al[31] (rs 7031748)
16	2763	Ile 825	hmz G	I→V	Polymorphism	Wang et al[31] (rs 4149312)
17	2930	Met 883	hmz A	M→I	Polymorphism	Wang et al[31] (rs 4149313)
42	6064	Arg 1925	htz G/A	R→Q	Novel, rare	Present paper

Variant db SNP rsID: rsID is the dbSNP-assigned reference SNP ID.
UTR indicates untranslated region; hmz, homozygous; htz, heterozygous.
*Based on published sequence for cDNA (GenBank accession no. AF285167).
†Based on ATG start codon = bp 291-3 in cDNA sequence above.

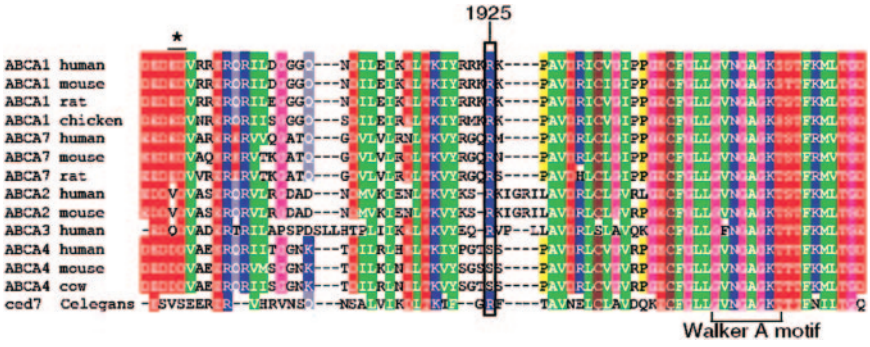

Figure 3. Amino acid sequence. Amino acid sequence alignment of mammalian sequences encoding ABCA1, ABCA2, ABCA3, ABCA4, ABCA7 and *C elegans* ced7, spanning the region mutated in the SS patient. The mutated residue (ABCA1 R1925Q) is indicated by a rectangle. The Walker A motif of the ATP-binding domain is marked. The asterisk depicts a 2-amino acid deletion found in a patient with HDL deficiency.

cells. The wild-type ABCA1-EGFP fusion protein is known to be expressed and to function normally in transfected cells.[7,36] As expected,[7,36] in cells transfected with wild-type ABCA1-EGFP, fluorescence was present mainly at the cell surface, with punctate intracellular vesicles visible in some cells and possible location in the Golgi in others (Figure 4A-B left panel). In contrast, little or no fluorescence was detected on the surface of cells transfected with the ABCA1 R1925Q-EGFP construct (Figure 4A middle and right panels; Figure 4B left panel). Instead fluorescence was observed largely in the endoplasmic reticulum (ER), evidenced by colocalization studies with sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2), a marker of the ER[37] (Figure 4B right panels).

Complementation of Ca^{2+}-stimulated PS exposure in SS lymphocytes by ABCA1

To test the role of ABCA1 in Ca^{2+}-stimulated PS translocation, we expressed wild-type ABCA1 in an EBV-transformed B-cell line obtained from the SS patient using a retroviral expression system. RT-PCR analysis showed that the stable, G418-selected SS-ABCA1$^+$ cell line has elevated levels of *ABCA1* mRNA expression as compared with the parental SS EBV-transformed B cells (Figure 5A). Expression of ABCA1 protein in EBV-transformed B cells (wild-type, SS-parental, and SS-ABCA1$^+$) was below the level of detection by 2 anti-ABCA1 antibodies (Novus Biologicals, Littleton, CO; and as described elsewhere[9]) in immunoblots or by immunofluorescent staining, though ABCA1 was apparent in transfected HEK cells (data not shown). The low-level expression of ABCA1 protein in transduced B cells despite high mRNA levels suggests posttranslational control; evidence that ABCA1 is posttranslationally regulated has been presented.[38-41] Nevertheless, the low level of ABCA1 expression was sufficient to restore A23187-stimulated PS exposure similar to wild-type levels (Figure 5B). Furthermore, PS translocation in SS-ABCA1$^+$ cells was completely blocked by the ABCA1 inhibitor

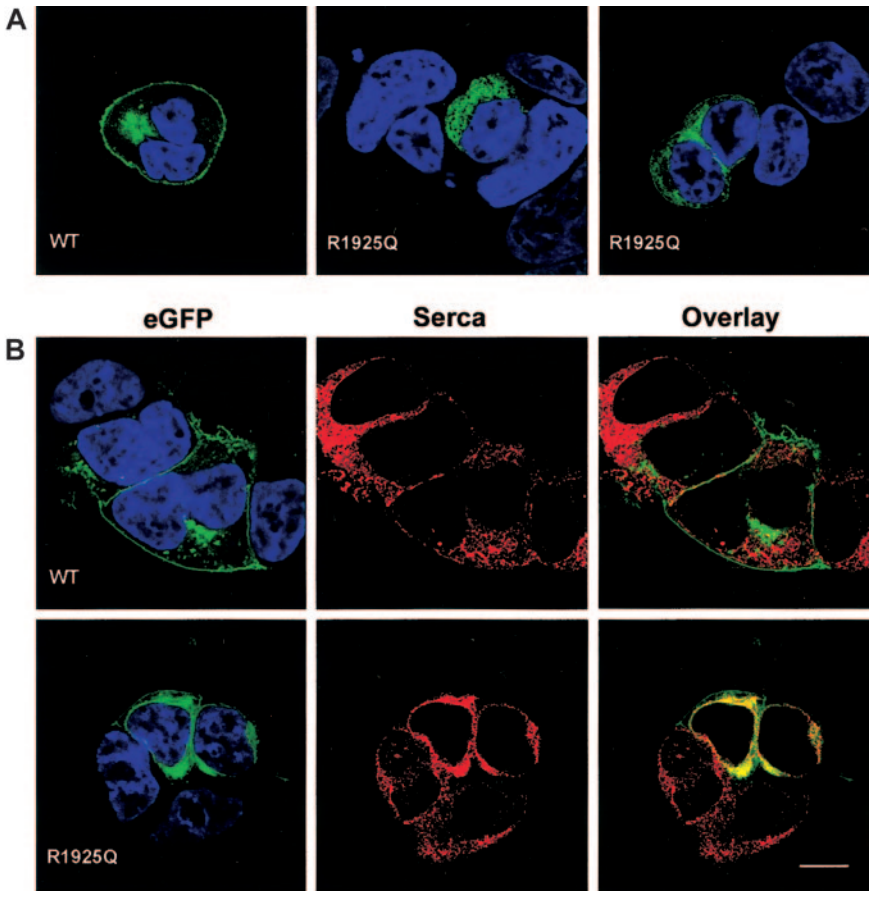

Figure 4. Expression of ABCA1-EGFP fusion proteins in HEK 293 cells. (A) Confocal sections of transiently transfected HEK 293 cells expressing wild-type ABCA1-EGFP (left) and ABCA1 R1925Q-EGFP (middle and right). EGFP fluorescence is shown in green and DAPI staining of nuclear DNA in blue. The mutation caused severe alteration in ABCA1 trafficking to the plasma membrane as compared to the wild type (WT). (B) HEK 293 cells expressing wild-type ABCA1-EGFP and ABCA1 R1925Q-EGFP were stained for SERCA 2 (red) as marker for the ER. The same confocal sections are shown for EGFP (green, left column), SERCA 2 (red, middle column) and colocalization of EGFP and SERCA 2 in the overlay (yellow, right column). Colocalization of ABCA1-SERCA2 was observed in cells transfected with mutant ABCA1, implying retention of R1925Q in the ER. In contrast, wild-type ABCA1-EGFP localized predominantly to the plasma membrane and no colocalization was detected. Scale bar indicates 10 μm.

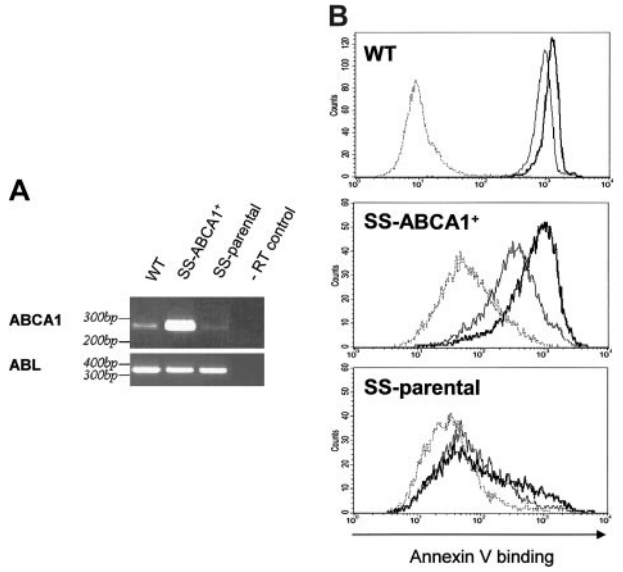

Figure 5. Expression of ABCA1 in SS-parental lymphocytes increased Ca^{2+}-stimulated PS exposure on the cell surface. (A) Verification of the elevated level of *ABCA1* mRNA in the transduced and G418-selected cell line by RT-PCR. Agarose gel electrophoresis of the PCR products using primers for ABCA1 or ABL and following templates: cDNAs obtained by reverse transcription of total RNA prepared from control EBV-transformed B cells (WT), from the stable SS-ABCA1$^+$ cell line, or from the parental EBV transformed SS B cells (SS-parental). The last lane shows a negative control using total RNA prepared from the SS-ABCA1$^+$ cells (-RT control) as template for the PCR. (B) Flow cytometry analysis of A23187-induced annexin V*488 binding for control wild-type (WT; top), ABCA1- expressing SS (SS-ABCA1$^+$; middle) or SS-parental cells (bottom). Representative experiments show annexin V–Alexa Fluor 488 binding in the absence of A23187 at t_0 (dotted lines), or annexin V–Alexa Fluor 488 binding induced by A23187 for 3 minutes (thin solid lines) or 10 minutes (bold solid lines). Ca^{2+}-dependent PS exposure of SS-ABCA1$^+$ cells was significantly increased compared to the SS-parental cell line.

glyburide[8,42] (Figure 6). Although basal binding of annexin V to SS-parental and SS-ABCA1$^+$ cells was somewhat higher than that to wild-type cells, this is likely to reflect biologic variation between transformed cells unrelated to disease because the phenotype was not apparent in fresh lymphocytes.[17] These results show that expression of wild-type ABCA1 complements the defective Ca^{2+}-activated PS translocation in the parental SS cells.

To confirm that the restoration of the ability of SS-ABCA1$^+$ cells to expose PS in response to stimulation with Ca^{2+} ionophore was due to increased (but low level) expression of ABCA1, we assessed its sensitivity to inhibitors. In preliminary experiments we found that glyburide, an inhibitor of ABCA1,[8,42] suppressed Ca^{2+}-stimulated PS exposure by control cells (Figure 6A). In contrast, inhibitors of other ABC transporters at concentrations that fully blocked the respective transporters (1-10 μM cyclosporine A (CsA), 30 μM verapamil (VRP), 1 μM PSC833, and 1 μM KO143[43-47]) had no effect on PS translocation. These results are consistent with other data indicating that ABCA1,[7,8] but not transporters such as MDR1,[4] regulates loss of lipid asymmetry. The data do not support, however, a role for ABCG2 as has been suggested elsewhere.[48] Glyburide completely blocked the remaining Ca^{2+} ionophore-stimulated PS exposure in SS-parental cells, consistent with residual expression of ABCA1. Further, because glyburide completely blocked Ca^{2+}-ionophore–stimulated PS exposure by SS-ABCA1$^+$ cells (Figure 6B), the data confirm that expression of wild-type ABCA1 reverses the defect in PS translocation of SS cells.

Discussion

SS is a mild bleeding disorder characterized by a defect in Ca^{2+}-stimulated exposure of PS at the outer leaflet of hematopoietic cell membranes. ABCA1 has been proposed as a PS translocase[7,8] or translocase regulator[9] and its potential role in the pathophysiology of SS was investigated. We identified a novel missense mutation in *ABCA1* (R1925Q) in the SS patient, which results in severely impaired trafficking and reduced expression of functional ABCA1 protein at the cell surface. We also demonstrated severely reduced steady-state levels of *ABCA1* mRNA in leukocytes of an SS patient. This could not be explained by a change in the ratio of an alternatively spliced aberrant transcript. Sequence analysis of the *ABCA1* gene of the SS patient revealed no difference in the proximal promoter region, in intron-exon junctions, or within the coding region that might explain the reduced *ABCA1* mRNA levels. Hence, an

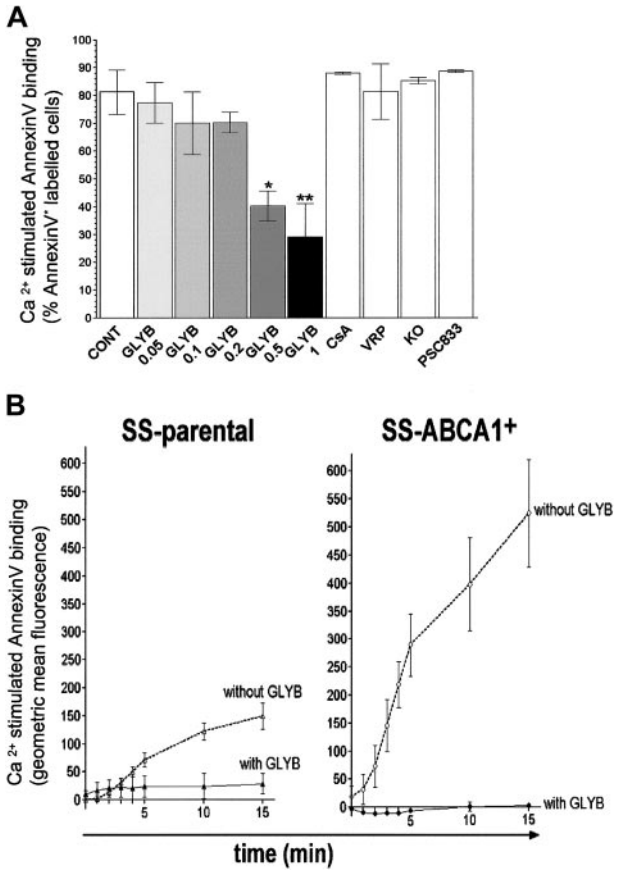

Figure 6. Glyburide inhibits Ca^{2+}-stimulated PS exposure in B lymphocytes. (A) Control wild-type EBV-transformed B cells were preincubated with the vehicle DMSO (CONT), or DMSO containing 0.05, 0.1, 0.2, 0.5, 1 mM glyburide (GLYB), 10 μM cyclosporine A (CsA), 30 μM verapamil (VRP), 1 μM KO143, or 1 μM PSC833 for 10 minutes. Ca^{2+}-activated PS exposure was measured after 10 minutes stimulation by A23187. Each data point represents the mean value of at least 3 independent experiments. $^{**}P < .005$; $^{*}P < .01$. (B) Glyburide inhibits Ca^{2+}-activated PS exposure by SS cells transduced with ABCA1. Ca^{2+}-stimulated PS translocation was analyzed by an annexin V binding assay. Cells were preincubated either with 1 mM glyburide or with DMSO only before addition of A23 187 at t_0, and changes in annexin V binding over time were recorded. Ca^{2+}-stimulated annexin V binding was calculated by subtracting the values measured in the presence of DMSO from the values detected in the presence of ionophore A23187 and is expressed as an increase in fluorescence (geometric mean of annexin V binding) by cells in the PI^- cell population. Each data point represents the mean value of at least 3 independent experiments; error bars represent SD. SS-parental indicates EBV-transformed B lymphocytes from the SS patient; SS-ABCA1$^+$ indicates SS-parental cells transduced with ABCA1.

Table 2. Lipid profile of the SS proband

Parameter	Baseline level	Desirable level or range
Total cholesterol, mM	6.5	<5.0
Triglyceride, mM	2.3	<1.7
HDL-cholesterol, mM	1.3	>1.3
LDL-cholesterol, mM	4.1	<3.0
Total cholesterol/HDL cholesterol	4.9	<5.0
Apolipoprotein B, mg/dL	122	<120
Apolipoprotein A-1, mg/dL	141	112-201
Lipoprotein (a), mg/dL	102	0–30
Apolipoprotein E phenotype	E2/E3	NA

NA indicates not applicable.

additional unidentified mutation, potentially in a *trans*-acting regulatory gene, is hypothesized to account for reduced *ABCA1* mRNA from both alleles. This putative second mutation and the incomplete loss of ABCA1 activity may account for the phenotypic differences observed between SS and TD.

The role of ABCA1 in this SS patient was confirmed by complementation with wild-type ABCA1 in transformed lymphocytes from the SS patient and further supported by pharmacologic data. Increased expression of wild-type ABCA1 in SS-parental cells restored the wild-type phenotype of Ca^{2+}-induced PS translocation. Although ABCA1 protein levels were low in EBV-transformed B-cell lines (wild-type, SS-parental, and SS-ABCA1$^+$), the restored ability of SS-ABCA1$^+$ cells to expose PS was blocked by glyburide, an inhibitor of ABCA1. These expression and pharmacologic studies strongly support the hypothesis that the SS phenotype observed in our proband is at least in part dependent on reduced ABCA1 activity.

Despite the severely reduced *ABCA1* mRNA levels in lymphocytes, the 59-year-old proband's lipid profile (Table 2) revealed only mild mixed (type IIb) hyperlipidemia characterized by elevations of both total cholesterol and triglyceride serum levels, and she did not show clinical features of TD or HDL deficiency, which are phenotypes of other characterized mutations in ABCA1.[10-12,33] Interestingly, a novel case of TD without symptoms of atherosclerotic disease has been reported,[49] despite harboring a null mutation that results in a nonfunctional ABCA1 protein. In addition, some patients heterozygous for ABCA1 mutations show greater than 50% HDL cholesterol levels, and single nucleotide polymorphisms (SNPs) in this gene associated with increased severity of atherosclerosis are not always associated with changes in lipid levels.[33] Bone marrow transplants in *ABCA1*$^{-/-}$ mice also suggest that blood-derived cellular expression of ABCA1 does not influence plasma lipid levels.[50,51] Taken together these data indicate that changes in ABCA1 activity can occur without alterations in the steady-state plasma lipid levels[33] as observed for this SS patient.

ABCA1 has been implicated in several cellular functions, namely, transport of lipids from the Golgi to the plasma membrane,[52] externalization of PS,[7] uptake of apoptotic cells,[8] secretion of interleukin 1β,[53] and formation of plasma HDL.[10-12,31,33] How ABCA1 is associated with these seemingly disparate processes and how its deficiency results in a variety of disease phenotypes is unclear. It is possible that the common feature linking these functions with both SS and TD relates to the formation of outward membrane protrusions such as microvilli, filopodia, lamellipodia, blebs, and spikes (echinocytes), consistent with our observation that defective microvesicle formation is the most pronounced phenotype in fresh SS lymphocytes.[17]

Alterations in hemostatic parameters and disrupted platelet function have been observed in TD patients,[15] and data on a patient with a prolonged bleeding time have been reported,[54] highlighting a potential role of ABCA1 in bleeding disorders. It has been clearly shown that ABCA1 is expressed on platelets and that lack of ABCA1 expression either in TD patients or ABCA1-deficient mice severely disrupts platelet function.[7,15,52] Stimulation of platelets from TD patients deficient in ABCA1 with either collagen or A23187 did not induce detectable differences in PS exposure as assayed by annexin V binding.[15] In contrast, we have demonstrated that our SS patient with severely reduced levels of functional ABCA1 has a defect in PS exposure in lymphocytes in response to A23187.[17] These differences may be due to methodologic differences because changes in PS translocation are more obvious at early stages after stimulation with A23187.[17] Alternatively, they may reflect differences due to the second unidentified mutation in our SS patient. It is also possible that ABCA1 might have a distinct role in blood cells compared with tissue-derived cells.

Acknowledgments

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Comment on Risueño et al, page 601

Antibody catches T-cell receptor in the act

Michael L. Dustin NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Risueño and colleagues report that APA1/1 monoclonal antibody can be used to detect TCRs that have interacted with antigenic MHC-peptide complexes in vivo.

A long-standing controversy in T-cell activation is whether the T-cell receptor (TCR) undergoes conformational changes upon engaging agonist major histocompatibility complex (MHC)–peptide complexes. In 2002, Gil et al[1] demonstrated that TCR ligation by agonist MHC-peptide complexes or activating antibodies induced binding of the cytoplasmic adapter protein Nck to a polyproline motif in the cytoplasmic domain of the CD3ϵ chain. This unmasking provided the best evidence for a ligand-induced conformational change in the TCR. While the functional necessity of this change for TCR signaling has not been proved, there is no question that this change is biochemically useful for identification of TCR complexes that have been engaged by agonist MHC-peptide complexes.

The utility of Nck recruitment to this site in situ is limited because Nck is also recruited to a downstream signaling complex including Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) and Vav, which, due perhaps to amplification, appear to account for most of the Nck recruited to the immunologic synapse.[2] Therefore, another way to detect this conformational change would greatly increase its accessibility in intact cells or in vivo.

In this issue of *Blood,* Risueño and colleagues show that the commercially available anti-CD3ϵ antibody APA1/1 detects the same conformational change in the TCR that is detected by Nck and can do so in fixed tissues. As with Nck binding, the APA1/1 epitope exposure did not require metabolic activity or signaling through the TCR, so this appears to be a direct response of the TCR machinery to agonist MHC-peptide complexes without intervening events. The APA1/1 epitope thus provides a new reporter for productive TCR engagement that is not subject to the interference issues that faced the biologic ligand Nck. The APA1/1 antibody detected TCR accumulated in the immunologic synapse formed by agonist MHC-peptide complexes, but not by antagonist MHC-peptide complexes. The APA1/1 antibody reacts with both mouse and human TCR complexes, indicating that it has broad potential applications for in vivo experimental immunology and perhaps clinical applications to detect concurrent T-cell activation in short-term cultures or in biopsy specimens. Thus, the use of APA1/1 should greatly extend the potential applications of the original finding by allowing detection of engaged TCR in vitro and in vivo and should speed a deeper understanding of the biologic importance of this conformational change. ■

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Comment on Albrecht et al, page 542

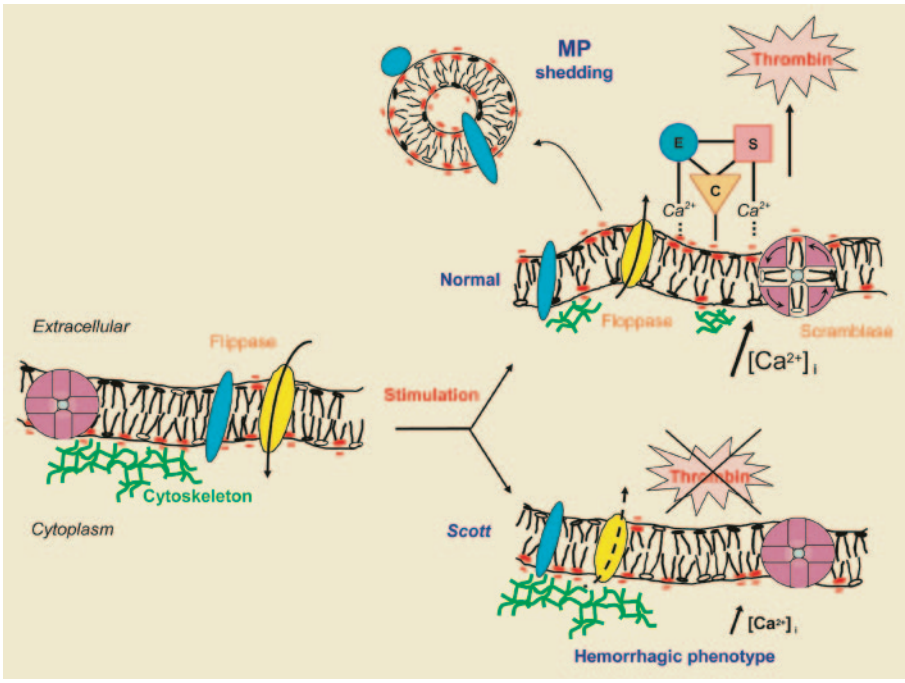
An ABC for Scott syndrome?

Florence Toti and Jean-Marie Freyssinet
U.143 INSERM UNIVERSITÉ PARIS XI; UNIVERSITÉ LOUIS PASTEUR STRASBOURG

Scott syndrome is an extremely rare inherited hemorrhagic disorder linked to the lack of exposure of procoagulant phosphatidylserine (PS) to the external leaflet of the plasma membrane of activated platelets and other hematologic lineages.[1-3] The loss of membrane lipid asymmetry, however, occurs in virtually all eukaryotic cells upon stimulation (including apoptosis)[3] and is associated with more ancestral functions than hemostasis. Hence, it is of prime interest to identify effectors of this basic process.

To date, only 3 documented cases of Scott syndrome have been reported, which is insufficient to allow the identification of positional candidate genes. It should be emphasized that none of these patients exhibited clinical symptoms other than bleeding, suggesting that the rapid PS egress for an efficient platelet hemostatic response and slower membrane remodeling occurring during apoptosis are under different control mechanisms. This does not, however, rule out that some or most of the actors can be shared. On the one hand, several studies have pointed to adenosine triphosphate (ATP)–binding cassette (ABC) transporters as possible phospholipid translocases (in particular, some that fulfill multidrug efflux function), but none has been confirmed as a true PS floppase. On the other hand, a nonspecific and energy-independent family of so-called scramblases has been proposed. But canonical phospholipid scramblase 1 (PLSCR1) is normally expressed in Scott cells, and other members or other candidate(s) remain to be characterized as effectors of bidirectional phospholipid transmembrane redistribution. Another ABC family member, ABC transporter A1 (ABCA1), involved in phagocytosis and cholesterol reverse transport and mutated in Tangier dyslipidemia, has been considered after the targeted deletion of the corresponding locus resulted in a phenotype evocative of partial Scott syndrome.[4] In this issue of *Blood,* Albrecht and colleagues precisely report 2 types of mutations that may contribute to the Scott phenotype.

The patient was found heterozygous for a novel missense substitution in *ABCA1* R1925Q, which has not been previously reported in the



Membrane remodeling in normal and Scott cells. In the plasma membrane, dynamic maintenance or loss of asymmetric phospholipid distribution results from opposite fluxes under the control of specific transporters governing inward (flip) or outward (flop) translocation. In the resting membrane, the flippase activity of aminophospholipid translocase is prominent and subtained (left, yellow ellipse). Thus, aminophospholipids, phosphatidylserine (PS; red negative polar heads), and phosphatidylethanolamine (PE; open polar heads) are sequestered into the inner leaflet. Following stimulation, specific vectorial floppase and/or nonspecific bidirectional scramblase activities are rapidly turned on, and PS and PE are moved to the outer leaflet (upper right). Flippase activity is shut down, leading to transient mass imbalance between the two leaflets. Membrane blebbing ultimately resolves into microvesicle or microparticle (MP) shedding after cytoskeleton degradation by calcium-dependent proteolysis. Stimulated cell membrane and MP therefore expose PS, which is particularly crucial in platelet procoagulant response for the calcium-dependent assembly of the cascade of clotting enzyme complexes. The assembly of these complexes, composed of an enzyme (E) and a cofactor (C) in the activation of a substrate (S), culminates in the generation of sufficient thrombin for efficient hemostasis. In resting Scott cells, flippase activity is indeed operational; stimulation remains without effect on the swift induction of floppase and/or scramblase and consecutive membrane shedding, explaining the resulting hemorrhagic phenotype (lower right). During the slower process of apoptotic cell death, however, PS egress is normal in Scott cells. As yet, floppase candidates have remained elusive.

relatively large cohort of Tangier patients described in the literature. In vitro expression experiments revealed an impaired traffic of ABCA1 R1925Q to the plasma membrane, and sequence comparisons pointed to a conserved functional importance of this residue. The second mutation, which remains to be identified, accounts for significant reduction of *ABCA1* mRNA levels and may affect a *trans*-acting regulatory gene. Using a retroviral expression system, complementation of the defect of PS externalization proved feasible in Epstein-Barr virus (EBV)–transformed Scott B cells. Unfortunately, no investigation of the protein expression levels was possible, most likely as a result of the lack of adequate antibody.

How these mutations relate to Scott syndrome may be debated, but this observation at least provides the first link between a defect in a transbilayer phospholipid transport pathway, that of ABCA1 here, and the bleeding phenotype. In light of other genetic disorders, other mutated or deleted elements may be considered, including other ABC true transporters or regulators such as ABCA1 itself, as already suggested for the latter.[5]

In Scott syndrome, bleeding episodes are moderate when compared with spontaneous hemorrhages in severe hemophilia, which affect a much greater number of patients. It has to be kept in mind that the Scott defect most likely can be traced back to a hematopoietic stem cell line or even earlier. Hence, the incapacity of PS externalization may turn lethal because of the prime role of PS as a recognition determinant for phagocytosis, for instance.[3] This could explain the surprising rarity of Scott syndrome patients who might have survived as a result of the intervention of an opportune rescue mechanism. ■

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Letter to the Editor

Characterisation of lymphocyte responses to Ca^{2+} in Scott syndrome

Dear Sir,

Scott syndrome (OMIM: 262890) (1) is a rare bleeding disorder characterised by a failure of phosphatidylserine (PS) exposure and microvesiculation by hematopoietic cells following stimulation with calcium ionophore or physiological agonists. As exposed PS acts as a binding site for the tenase and prothrombinase complexes of the blood coagulation cascade (2) its translocation to the cell surface is crucial in the regulation of haemostasis. Scott syndrome is generally believed to be inherited as an autosomal recessive trait (3, 4), however, its molecular basis is unknown. Whilst much early work focussed on potential defects in phospholipid translocation by scramblase, function and RNA levels for this protein appear normal in Scott cells (5, 6). It has also been suggested that the disorder reflects impaired Ca^{2+} uptake (7), though the generality of such a defect has been disputed (8).

Until recently, only two documented cases (one American, one French) have been reported (1, 3). However, a third (Welsh) patient has now been described (this report and (8)). The patient is a 59-year-old Caucasian female with a moderate bleeding phenotype characterised by post-partum haemorrhage and bleeding following dental extractions. Coagulation screening tests, platelet count, bleeding time, individual coagulation factor activity assays and platelet aggregation were all normal. Previous studies had shown an abnormal prothrombin consumption index (9), which has remained consistently abnormal over a 25-year period. EBV-transformed B lymphoblasts from Scott syndrome patients have previously been shown to have a defect in PS externalisation and this phenotype is diagnostic of Scott syndrome (10). We therefore compared the response of EBV transformed B cell lines from the Welsh patient and controls to stimulation

Correspondence to:
Dr. James Elliott
Membrane Transport Biology Group
MRC Clinical Sciences Centre
Faculty of Medicine
Imperial College Hammersmith Hospital Campus
Du Cane Rd
London W12 0NN, UK
Tel.: +44 20 8383 8269, Fax: +44 20 8383 8337
E-mail: james.elliott@csc.mrc.ac.uk

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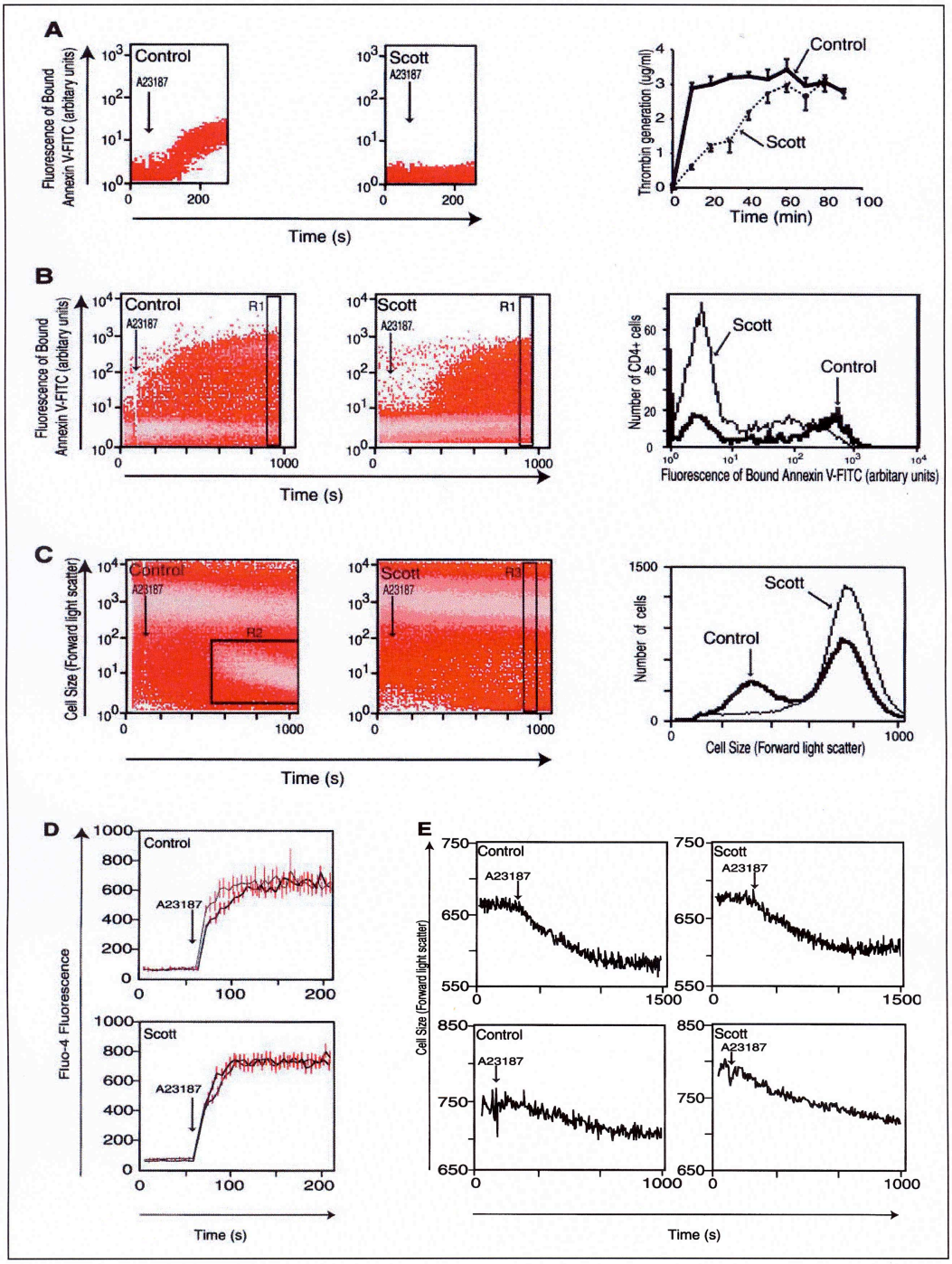
Figure 1: Responses of Scott and control lymphocytes to calcium ionophore A23187.
A) Scott and control EBV transformed B cell lines were equilibrated with annexin V^{FITC} and PI (Becton Dickinson, CA). The cells were analysed by flow cytometric analysis. Baseline fluorescence was established and then cells were stimulated with A23187. Plots depict the change in fluorescence of cell bound Annexin V^{FITC} which is a surrogate marker of PS exposure. To further demonstrate an increase in PS exposure EBV transformed B cells were washed and resuspended with 2 μM human prothrombin, 5 pM human factor Xa and 25 pM human factor V in a volume of 200 μl. Aliquots were withdrawn at various time points before and following the addition of A23187 and assayed for thrombin activity using the chromogenic substrate S-2238.
B) Freshly isolated lymphocytes obtained by venepuncture from the Scott patient and controls were washed and resuspended in phenol red-free DMEM (Sigma). Donors of control blood were healthy female volunteers aged between 24 and 60. Blood was collected from the Scott patient on three separate visits to Hammersmith Hospital. Lymphocytes were stained with anti-$CD4^{APC}$ and equilibrated with annexin V^{FITC} and PI prior to FACS analysis. After establishing baseline fluorescence, cells were stimulated with A23187. Density plots of annexin V^{FITC} fluorescence in PI^- cells staining positively for CD4 are shown and the number of $CD4^+$ cells and their annexin VFITC fluorescence is shown towards the end of the experiment (R1).
C) In the same experiment as represented in B, but without restricting analysis to $CD4^+$ lymphocytes, the appearance of small, (forward light scatter low) cells (marked as gate R2) is shown following stimulation of control lymphocytes with A23187. Cells within R2 were annexin V^{FITC}negative (not shown). In contrast Scott cells stimulated with A23187 showed no appearance of small cells. The relative sizes of control and Scott PI^- cells towards the end of the experiment (within gate R3 and similarly gated control cells). Results are representative of three separate experiments.
D) Ca^{2+}-uptake by Scott and control lymphocytes was measured by flow cytometric analysis. Lymphocytes labelled with anti-$CD4^{APC}$ antibody were incubated with the Ca^{2+}-indicator Fluo-4 AM, after baseline fluorescence was established; Ca^{2+} uptake was induced with A23187. Increased intracellular Ca^{2+} in the CD^{4+} population is indicated by raised Fluo-4 fluorescence (mean + S.E. fluorescence units. Two representative experiments are shown.
E) Cell shrinkage in Scott and control lymphocytes was analysed by flow cytometry. Lymphocytes were stained with anti-$CD4^{PE}$, anti-$CD4^{CYCHROME}$ antibodies in order to distinguish between Scott and control cells. Samples were mixed, baseline flow cytometric parameters established, and stimulated with A23187. Shrinkage of cells positively stained with CD4 antibodies was measured by reduction in forward light scatter. The results of two independent experiments are shown. The data are representative of three comparable experiments performed on separate occasions using three different control subjects.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Fluorescence of Bound Annexin V-FITC (arbitary units)
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Number of CD4+ cells
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Fluorescence of Bound Annexin V-FITC (arbitary units)
C
Cell Size (Forward light scatter)
Control
A23187
R2
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Time (s)
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with the calcium ionophore A23187. In contrast to the control cell line, EBV-transformed B cells from the patient exhibited a markedly delayed increase in prothrombinase activity, whilst binding to fluorescently labelled annexin V was severely diminished in multiple experiments (Fig 1A), confirming the diagnosis of Scott syndrome. Prothrombinase activity and (on some occasions) binding of annexin V was delayed rather than abolished and as this variability might reflect cell culture-dependent artefacts, we assessed the response of freshly isolated lymphocytes to A23187. Lymphocytes from the Scott syndrome patient and controls were isolated in parallel, and stained with anti-CD4 T-cell specific antibodies (Becton Dickinson, CA) isolating the response of a relatively homogeneous population of cells by flow cytometric analysis. Appropriately labelled lymphocytes were then either equilibrated with fluorescently labelled annexin V (AV; Becton Dickinson, CA) or mixed such that Scott and control lymphocytes could be distinguished on the basis of binding to differently labelled fluorescent antibodies, and then equilibrated with AV. Baseline fluorescence was established prior to addition of A23187 to initiate PS translocation. Multiparameter flow cytometric analysis permitted simultaneous analysis of cell membrane permeability (propidium iodide [PI] uptake, an indicator of cell death), cell volume (forward light scatter), and PS exposure to the cell surface (AV^{FITC} binding). As expected, control lymphocytes exhibited rapid exposure of PS, as evidenced by binding of AV (Fig. 1B). By comparison, PS translocation by Scott lymphocytes was significantly delayed, occurred in fewer cells, and then at a reduced level per cell. Interestingly, although the defect in PS translocation was partial, blebbing and shedding of bound AV (as evidenced by the appearance of residual small, granular annexin V-negative bodies) appeared severely impaired (Fig. 1C). Absence of this process, which appears to closely follow the initiation of PS translocation, appears the most marked characteristic of lymphocytes from this Scott syndrome patient.

In contrast to some (7), but in agreement with others (8) we were unable to find any consistent difference in Ca^{2+} uptake between EBV-transformed Scott lymphocytes and controls (not shown). To compare the response of freshly isolated lymphocytes Scott and control cells were stained with anti-CD4 antibody, washed and subsequently either treated separately or mixed. Lymphocytes were incubated with the Ca^{2+}-responsive fluorescent indicator Fluo-4 AM (0.3 µMol/L Molecular Probes) for 15 minutes, washed twice and resuspended in phenol red-free DMEM. Ionophore-induced Ca^{2+} uptake by freshly isolated lymphocytes from the Scott patient was equal to, or marginally greater than that by controls cells (Fig. 1D), even when incubated with Fluo-4 AM in a single tube to ensure equivalent loading. Similar results were found for $CD19^+$ B lymphocytes (data not shown). Although we found Ca^{2+} uptake to be normal in cells from the Scott patient, in agreement with a recent report (8), it remained possible that Scott lymphocytes are Ca^{2+}-unresponsive. To investigate this and to ascertain whether a Scott-specific lesion occurring prior to PS exposure could be identified, we compared the rate of ionophore-stimulated cell shrinkage in freshly isolated cells. Cell shrinkage, achieved by efflux of K^+ and Cl^- ions and the consequent loss of water, precedes PS translocation (11), and occurred rapidly following stimulation with A23187 (Fig. 1E). Though minor differences were apparent in initial cell volumes (which appears to reflect normal biological variation), as the rate and extent of volume decrease of patient and control cells was equivalent, lymphocytes from this Scott syndrome patient exhibit (at least some) normal responses to Ca^{2+} prior to PS translocation. Defective Ca^{2+} uptake by EBV-transformed B lymphocytes from the French patient may reflect natural biological variation, age-dependent decreased lymphocyte responsiveness, or differences in the underlying defects in lymphocytes from the French and other Scott patients.

To summarise, we have presented the first functional analysis of freshly isolated Scott lymphocytes and find no evidence that either Ca^{2+} uptake or the earliest responses to Ca^{2+} are defective. Though PS translocation was diminished in lymphocytes, complete absence of microvesiculation was the clearest phenotype, supporting studies suggesting that the two processes are intimately linked (12). In conjunction with previous findings, the data are consistent with a model in which PS translocation is potentiated by, but not entirely dependent on cell membrane structural changes required for blebbing, and that a defect in the latter process is the principal defect in cells from the new Scott patient.

Acknowledgement

We would like to thank the patient for her help in this study.

James I. Elliott, Andrew D. Mumford, Christiane Albrecht, Peter W. Collins, John C. Giddings, Christopher F. Higgins, Edward G. D. Tuddenham, John H. McVey

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Erratum

In the article by Kaneider, et al. entitled "Syndecan-4-dependent signaling in the inhibition of endotoxin-induced endothelial adherence of neutrophils by antithrombin" published in *Thrombosis and Haemostasis* December 2003 (Thromb Haemost 2003; 90: 1150-7) the following error was made:

Materials and methods

The preparation of antithrombin used for the study was virus-inactivated human plasmatic antithrombin III concentrate (Aventis Behring, Marburg, Germany).

Case Report

Accelerated exposure of phosphatidylserine on lymphocyte populations from patients with systemic lupus erythematosus or rheumatoid arthritis

Whilst disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) are thought to result from lymphocyte hyperactivity leading to reactivity towards autoantigens (SLE, RA) or gut flora (IBD), they are also associated with platelet activation and increased risk of thrombosis (1–3). It is unclear, however, whether the rate of thrombosis is a consequence of disease, or whether platelet activation reflects a defect in haematopoietic cells. For example, whilst antiphospholipid antibodies in SLE precede diagnosis (4), correlate with susceptibility to thrombosis (5–7), and may promote prothrombotic events (8), their presence might be a consequence of platelet activity.

Thrombus formation is initiated by the cell surface exposure of the phospholipid phosphatidylserine (PS) which is normally confined to the inner leaflet of the plasma membrane. Upon cellular activation and elevation of intracellular calcium, plasma membrane asymmetry collapses. Through the consequent exposure of a negatively charged phospholipid surface the outer membrane is transformed into a catalytic surface for the assembly of tenase and prothrombinase coagulation complexes.

Whilst platelets are presumed to be of primary importance in thrombus formation, mechanisms resulting in loss of membrane asymmetry are shared with other haematopoietic cells (9). We therefore asked whether patient-derived lymphocytes exhibit an abnormal propensity towards loss of lipid asymmetry that might indicate a general defect of haematopoietic cells in the pathogenesis of SLE, RA and IBD.

PS exposure at the cell surface (detected by binding of annexin V, AV) can be stimulated by calcium ionophore and occurs prior to membrane rupture and cell death as indicated by uptake of propidium iodide (PI). This model thus represents an acute form of apoptosis (10, 11). We have developed a rapid real-time flow cytometric assay of calcium ionophore-induced PS translocation (Fig. 1). The rate of PS exposure is dependent upon the concentration of calcium ionophore, but precedes uptake of PI at all concentrations tested (not shown). Evidence of PS exposure on $CD4^+$ lymphocytes was apparent within 5 minutes of stimulation (Fig. 1A), closely followed by the appearance of bodies lacking cell surface markers such as CD4, CD8 and CD19 (Fig. 1C and (12)). Such bodies neither bind AV nor take up PI and are assumed to be the remnants of cells having shed AV^+ microparticles (12). As expected, susceptibility to PS exposure and cell breakdown broadly correlated (not shown). However, as the latter phenotype is the less precise endpoint (for example as cell

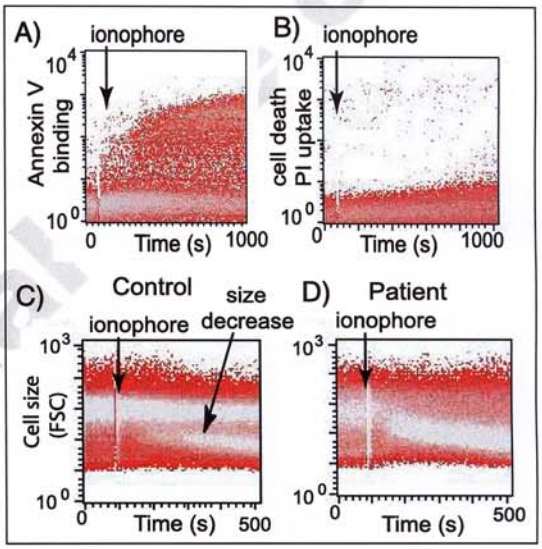

Figure 1: Translocation of PS and cell breakdown in freshly isolated lymphocytes. Lymphocytes obtained by venepuncture were stained with anti-$CD4^{APC}$. Lymphocytes were equilibrated with AV^{FITC} to measure PS translocation and propidium iodide (PI) as an indicator of cell death. Baseline fluorescence was established for approximately one minute prior to addition of calcium ionophore at the time indicated. Density (relative cell numbers) is depicted by a false colour plot, such that areas of white represent no cells, while increasing cell numbers are depicted as red speckling and red blocking, and with the highest cell density apparent as a grey/white core within areas of red block. (A, B) Density plots of the responses of $CD4^+$ T cells to stimulation with 4 μM calcium ionophore (A23187). (A) PS exposure as indicated by increased binding of AV^{FITC}. (B) Uptake of PI (indicating cell death). Little cell death was apparent within the time shown, but occurred gradually over longer periods (not shown). PS translocation prior to PI uptake is characteristic of apoptosis. (C, D) Density plots showing breakdown of lymphocytes as indicated by decrease in cell size (measured by forward light scatter, FSC). Cells were prepared as above, but stimulated with 6 μM calcium ionophore at time indicated by arrow. Shortly after stimulation, cell remnants appear (indicated by arrow). Cells in these plots are not gated on the basis of antibody-labelling. (C) control cells (D) cells from SLE patient.

Correspondence to:
James Elliott
Dept. of Membrane Transport Biology
ICSM, Hammersmith Hospital
Du Cane Road London
W12 0NN U.K.
Tel.: +44 20 8383 8269, Fax: +44 20 8383 8337
E-mail: james.elliott@csc.mrc.ac.uk
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*These authors contributed equally.

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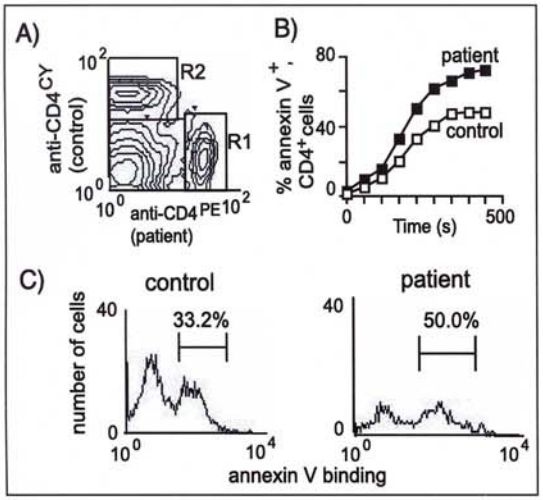

Figure 2: Comparison of the rate of PS exposure on patient and control lymphocytes. Lymphocytes from a patient and control were stained with anti-$CD4^{PE}$ and anti-$CD4^{CY}$ antibodies respectively to discriminate between $CD4^+$T cells from different donors. Cells were then mixed and then equilibrated with AV^{FITC} to permit analysis of PS exposure in a single tube. The rate of PS exposure stimulated by 4 μM calcium ionophore was then assessed by flow cytometry as in figure 1A. A) Contour plot showing mixed patient (R1) and control (R2) cells. (B) Histogram of PS exposure (percentage of cells binding AV^{FITC}) by patient (filled squares) and control (open squares) $CD4^+$ lymphocytes as a function of time. (C) Histograms comparing the frequency of control and patient $CD4^+$ lymphocytes having exposed PS. This is an alternative representation of data in figure 2B but at a single time-point. Histograms show the frequency of patient (right hand panel) and control (left hand panel) $CD4^+$ T cells bearing exposed PS at the time at which 33.2 % of the latter bound AV.

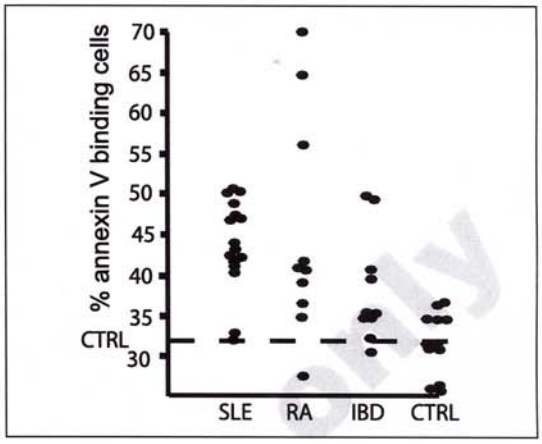

Figure 3: Comparison of the rate of PS translocation on $CD4^+$ lymphocytes from patient and control groups. In multiple experiments, the rate of PS exposure on $CD4^+$ cells derived from SLE, RA (including one GCA) and IBD patients and controls was directly compared in single tubes as outlined in panel B. To standardise results between days, the percentage of patient-derived $CD4^+$ cells binding AV was assessed at the time at which approximately 33% (± 0.5%) of control cells had bound AV (as in Fig. 2C). Though five control blood donors were used for comparison with SLE, RA/GCA and IBD patients, in several cases to standardise results, a single donor was used. To test whether this individual was representative of the normal population the rate of PS exposure by $CD4^+$ lymphocytes from this individual was compared in multiple experiments with that by cells from other normal donors (CTRL column, n=12; mean ± S.D. 32.3 ± 4.3).

phenotype is uncertain and samples must be run sequentially), it was not assessed in all individuals.

By differential antibody-labelling of patient and control cells, one can compare rates of PS translocation of two or three samples within a single tube. We therefore compared the rate of PS exposure on $CD4^+$ PBLs from SLE, RA (this group included one patient with giant cell arteritis) and IBD patients with that by control cells. Sample groups were as follows. Sixteen SLE patients fulfilling the American College of Rheumatology classification criteria for lupus were included in this study (mean age, 48 years; range 32–66 years; all female). Nine patients with RA and one with giant cell arteritis (GCA) ('RA group') (mean age, 57 years; range, 32–72 years; seven female). Ten patients with IBD (four with Crohn's disease, six with ulcerative colitis: mean age 39 years; range 23–70 years; six female). The majority of patients received prednisolone. Thirteen individuals, either healthy volunteers from the MRC-CSC, Hammersmith or patients receiving pre-operative assessment for unrelated conditions (mean age, 45 years; range, 24–84 years; eight female and five male) were studied in parallel as controls. The Riverside and Hammersmith ethics committees approved the study; patients and healthy volunteers were recruited after informed consent was obtained.

Lymphocytes, obtained by venepuncture from patients and control individuals were prepared by centrifugation of whole blood following layering on Histopaque (Sigma) in accordance with the manufacturers instructions. Freshly isolated lymphocytes were washed and resuspended in phenol red-free Dulbecco's modified Eagles medium (DMEM, Sigma) and stained with a fluorescent antibody as indicated ($CD4^{PE}$, $CD4^{CYCHROME}$, $CD4^{APC}$ – Becton Dickinson, CA). Appropriately labelled lymphocytes were then mixed such that patient and control lymphocytes could be distinguished on the basis of binding to differently labelled fluorescent antibodies (Fig. 2A), and equilibrated with 300 ng AV^{FITC} (Becton Dickinson, CA) for 3 min. In some experiments 0.75 μg/ml propidium iodide (PI – Becton Dickinson, CA) was also included as an indicator of cell death. Flow cytometric measurements were taken on a FACScalibur machine using CellQuest software (Becton Dickinson, CA). Baseline fluorescence was established for approximately one minute prior to addition of 4 μM calcium ionophore (A23187 – Sigma) to initiate PS translocation. Multiparameter FACS analysis permitted simultaneous analysis of PS exposure to the cell surface (AV^{FITC} binding, FL-1) in $CD4^+$ lymphocytes from patients or controls. Where indicated, cell membrane permeability (PI uptake, detected in channels FL-2 and FL-3), was measured as an indicator of cell death. Cell volume (forward scatter, FSC-H), is used routinely as a measure of the size of spherical cells, its sensitivity being greatest when light is collected over an angle of less

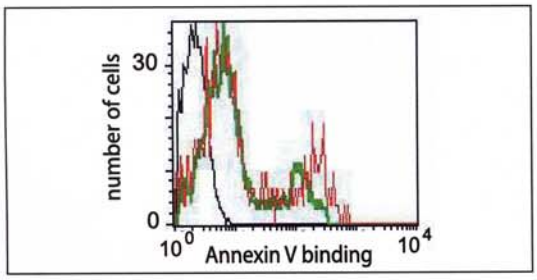

Figure 4: The rate of AV binding to cells with exposed PS. Lymphocytes from a normal individual were stained with anti-$CD4^{CY}$ and stimulated with 4 μM calcium ionophore. Five minutes later, PI was added and the cells analysed by real-time flow cytometry. After a further 40 sec, AV^{FITC} was added to the cell suspension. The histogram shows fluorescence (FL-1 channel) by PI-negative cells prior to addition of AV (black line); 30 sec after addition of AV^{FITC} (green line); 60 sec after addition of AV^{FITC} (red line).

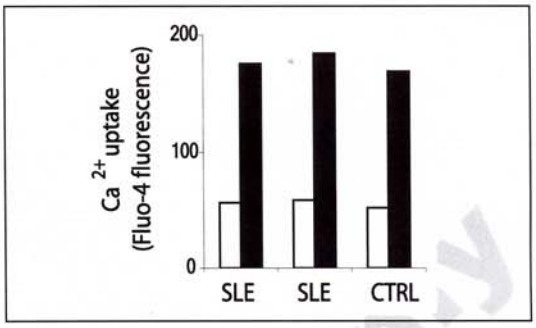

Figure 5: Calcium uptake by lymphocytes stimulated with calcium ionophore. Lymphocytes from control individuals and patients with SLE were stained with anti-$CD4^{PE}$, -$CD4^{CYCHROME}$, or -$CD4^{APC}$ such that patient and control lymphocytes could be distinguished on the basis of binding to differently labelled fluorescent antibodies. Lymphocytes were then mixed, incubated with 0.25 μM of the calcium indicator Fluo-4 AM for 10 min and washed. Calcium uptake (increased Fluo-4 AM fluorescence) was assessed by flow cytometry. Baseline fluorescence was established for 30 sec before 4 μM of calcium ionophore was added. The bar chart shows Fluo-4 AM fluorescence (FL-1 units) of indicated cell populations before (open bars) and one minute following stimulation (filled bars).

than 10^0 as in the FACScalibur. Debris was excluded from analysis on the basis of forward and side light scatter. Rates of PS translocation between groups were compared by two-tailed t-test.

We therefore compared the rate of PS exposure on $CD4^+$ PBLs from SLE, RA and IBD patients with that by control cells. Intra-experimental variability in such assays was low. However, to standardise results between days, in each experiment we determined the time at which approximately 33% of control $CD4^+$ cells had exposed PS (i.e. bound AV). This was then compared with the percentage of patient-derived $CD4^+$ cells having exposed PS at the same time-point. At this time-point, for each patient group the frequency of $CD4^+$ T cells that had exposed PS exceeded that by controls (mean ± S.D. SLE (n=16) 44.3 ± 4.9, p = 1.9 x 10^{-7}; RA/GCA (n=10) 45.7 ± 14, p= 0.02; IBD (n=10) 38.3 ± 6.4, p = 0.04; Fig. 3). No correlation was apparent between the rate of PS exposure and age, sex, or general inflammation as evidenced by levels of C-reactive protein (not shown), nor with drug regimen (the majority of patients receiving steroids, excepting one SLE, three of the RA and four of the IBD groups). Indeed in SLE the acute phase CRP response is known to be poor, despite tissue inflammation (13). Though a relatively small number of normal donors provided blood used in the experiments above, rates of PS translocation by lymphocytes were similar to those of other control donors, and therefore constituted a representative population (Fig. 3). Next, to demonstrate that the rate of binding of AV to stimulated cells reflects the rate of PS exposure *per se* and is not limited by the rate at which AV binds to PS, we stimulated lymphocytes with calcium ionophore and added AV only after six minutes, by which time we expected a proportion of cells to have exposed PS. A significant PS-exposed binding population was detectable within thirty seconds of adding AV, and AV binding per cell did not increase significantly after sixty seconds (Fig. 4). Thus AV binding to PS is rapid and not rate-limiting. Finally, as patient lymphocytes do not exhibit a difference in ionophore stimulated uptake of calcium (Fig. 5), the increased rate of PS exposure appears to reflect downstream events.

To summarise, we have shown that lymphocyte populations from patients with SLE, RA and, to a lesser extent, IBD, exhibit an increased rate of calcium ionophore-stimulated exposure of PS to the cell surface, as evidenced by the increased proportion of responding cells. It is not possible from these experiments, however, to assess whether the rate at which PS is translocated from the inner leaflet on individual cells differs between patients and controls. The cause(s) of the increased rates of PS exposure we have observed remain to be elucidated. Indeed, little is known about the mechanism of PS translocation. Whilst it has been suggested that the protein ABCA1 acts as a PS floppase and rates of PS translocation are reduced in haematopoietic cells from ABCA1-deficient mice (14), as there is no evidence for direct interaction between ABCA1 and PS this protein may act upstream of any translocase. We did not find any significant difference in the level of leukocyte *ABCA1* mRNA between patients and controls (not shown). These findings indicate that increased rates of exposure of PS in SLE, RA and IBD may contribute toward the elevated thrombotic risk in each disorder.

Christiane Albrecht[1,], Soni Soumian[1,*], Raymond Playford[2], Christopher F. Higgins[1], Timothy Vyse[3], James I. Elliott[1]*
[1]MRC-CSC, Membrane Transport Biology, London, U.K.
[2]Imperial College, Gastroenterology Section,
[3]Rheumatology Section, London, U.K.

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ABCA1 Expression in Carotid Atherosclerotic Plaques

C. Albrecht, PhD; S. Soumian, FRCS; J.S. Amey, MSc; A. Sardini, PhD; C.F. Higgins, PhD; A.H. Davies, FRCS; R.G.J. Gibbs, FRCS

Background and Purpose—The ATP-binding cassette transporter A1 (ABCA1) facilitates cholesterol efflux from cells, a key process in reverse cholesterol transport. Whereas previous investigations focused on mutations causing impaired ABCA1 function, we assessed the role of ABCA1 in human carotid atherosclerotic disease.

Methods—We compared the mRNA and protein levels of ABCA1, and one of its key regulators, the liver X receptor α (LXRα), between minimally and grossly atherosclerotic arterial tissue. We established *ABCA1* and *LXRα* gene expression by real-time quantitative polymerase chain reaction in 10 control and 18 atherosclerotic specimens. Presence of ABCA1 protein was assessed by immunoblotting. To determine whether differences observed at a local level were reflected in the systemic circulation, we measured ABCA1 mRNA in leukocytes of 10 patients undergoing carotid endarterectomy and 10 controls without phenotypic atherosclerosis.

Results—*ABCA1* and *LXRα* gene expression were significantly elevated in atherosclerotic plaques ($P<0.0001$ and 0.03, respectively). The increased mRNA levels of ABCA1 and LXRα were correlated in atherosclerotic tissue ($r=0.85$; $P<0.0001$). ABCA1 protein expression was significantly reduced in plaques compared with control tissues ($P<0.0001$). There were no differences in leukocyte ABCA1 mRNA expression ($P=0.67$).

Conclusions—ABCA1 gene and protein are expressed in minimally atherosclerotic human arteries. Despite significant upregulation of ABCA1 mRNA, possibly mediated via LXRα, ABCA1 protein is markedly reduced in advanced carotid atherosclerotic lesions. No differences in leukocyte ABCA1 expression were found, suggesting the plaque microenvironment may contribute to the differential ABCA1 expression. We propose that the decreased level of ABCA1 protein is a key factor in the development of atherosclerotic lesions. **(*Stroke*. 2004;35:2801-2806.)**

Key Words: atherosclerosis ■ ATP binding cassette transporter 1 ■ carotid artery plaque ■ carotid arteries ■ polymerase chain reaction

Cerebral atherosclerotic disease is the commonest etiological factor in stroke, a major cause of morbidity and mortality. Increasing attention is being given to the possible role of the ATP-binding cassette transporter A1 (ABCA1) in the development and behavior of atherosclerotic plaques. ABCA1 is a transmembrane protein involved in cholesterol and phospholipid transport from cells to lipid-poor apolipoproteins in the plasma. The clinical implications of ABCA1 deficiency were recognized when the *ABCA1* gene was discovered to be responsible for Tangier disease[1] and familial high-density lipoprotein (HDL) deficiency.[2] These conditions are characterized by low levels of HDL, the deposition of lipid-laden macrophages in tissues, and increased atherosclerotic disease in a proportion of patients. The pathophysiology of this process is thought to result from failure of ABCA1 to transport cholesterol and phospholipids out of cells to form complexes with apolipoproteins to generate HDL.[3] This, in turn, leads to intracellular sterol accumulation and the subsequent development of foam cells, a hallmark of the atheromatous plaque. ABCA1 mRNA is highly expressed in leukocytes and macrophages,[4] and in a wide range of human tissues, including placenta, liver, lung, and adrenal gland.[5] Attention has focused on ABCA1 because of its potential role in atherosclerosis, and the fact that therapeutic interventions could be targeted at the regulatory pathway controlling ABCA1 expression. Likely pharmacological targets include the liver X receptor-α (LXRα)[6] and peroxisome proliferator-activated receptor-γ.[7]

Insights into ABCA1 function and roles in disease have been elucidated by overexpression[8] and *ABCA1* gene inactivation studies in mice,[9] as well as observational studies in individuals deficient in ABCA1 because of gene mutations.[10] Although the systemic effects of loss of function in ABCA1-

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From the MRC Clinical Sciences Centre (C.A., J.S.A., A.S., C.F.H.), Faculty of Medicine, Imperial College, Hammersmith Hospital, Du Cane Road, London, UK; the Department of Vascular Surgery (S.S., A.H.D., R.G.J.G.), Faculty of Medicine, Imperial College, Charing Cross Hospital, Fulham Palace Road, London, UK. Current affiliation for C.A. is Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, Freising, Germany.

C.A. and S.S. contributed equally to the work.

Correspondence to Dr Richard Gibbs, Department of Vascular Surgery, Imperial College, Charing Cross Hospital, Fulham Palace Road, London W6 8RF. E-mail r.gibbs@ic.ac.uk

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deficient individuals is well-characterized, less is known about the potential role of the transporter in localized atheromatous disease in individuals without reported mutations in the gene.

Apart from its role in cholesterol efflux, ABCA1 is implicated in phosphatidylserine translocation between the leaflets of cell membranes[11] and the engulfment of apoptotic cells.[11] Apoptosis, phosphatidylserine externalization and lipid dysregulation play critical roles in the development and subsequent behavior of atherosclerotic plaques. ABCA1 is implicated in these processes, making the transporter a promising candidate in the context of atherosclerosis. The key question, therefore, is whether there is a localized arterial loss of ABCA1 function in relation to atherosclerotic disease.

To investigate both ABCA1 gene and protein expression in human carotid atherosclerotic disease, we analyzed ABCA1 mRNA and protein in atheromatous plaques taken from patients undergoing carotid endarterectomy (CEA) and compared it to human arteries obtained from controls with no phenotypic atherosclerotic disease. To gain insight into regulatory mechanisms involved in *ABCA1* gene expression, we also measured mRNA expression of one of its key regulators, LXRα. As leukocytes are known to be involved in the pathogenesis of the atheromatous plaque,[12] and because deficient leukocyte ABCA1 expression is implicated in increased susceptibility to atherosclerosis in animal studies,[4] we analyzed ABCA1 mRNA expression in peripheral leukocytes.

Methods

Subjects and Specimens

Carotid plaques were collected from 18 consecutive subjects with internal carotid artery stenoses of >70% undergoing CEA. Symptomatic carotid disease was diagnosed in 16 patients with a history of transient ischemic attacks, strokes, or amaurosis fugax, whereas 2 patients were asymptomatic. Ten inferior mesenteric arteries dissected from colectomy specimens of subjects having elective operation served as controls. These patients were phenotypically free of symptomatic atherosclerotic disease by history, examination, and a normal electrocardiogram tracing. Sections of the control arteries were stained with hematoxylin and eosin and assessed by a histopathologist for evidence of age-related atheromatous change. They were found to be within histological grading (types I to III) of the initial lesions of atherosclerosis according to the American Heart Association classification.[13]

The endarterectomy specimens consisted of the atheromatous plaque, together with adjacent intima and medial layers. The inferior mesenteric artery specimens were full-thickness and included the adventitial layer. Therefore, a greater preponderance of connective tissue would be expected in the control samples. Previous work indicates that ABCA1 is expressed primarily in macrophages within the atheromatous lesion,[14] therefore ABCA1 expression levels are expected to be higher in CEA (with advanced atherosclerosis) than inferior mesenteric artery specimens (with mild atherosclerosis).

To assess leukocyte ABCA1 expression, peripheral venous blood was collected pre-operatively from a subgroup of 10 patients undergoing elective CEA for symptomatic carotid disease and 10 age- and sex-matched controls, phenotypically free of symptomatic atherosclerosis. There were no statistically significant differences between subjects and controls with respect to age and sex. The study had ethical approval from the Riverside Research Committee and informed consent was obtained from subjects. Demographic details of patient and control groups are listed in Tables 1 and 2.

TABLE 1. Demographic Details of Patients and Controls (Arterial Specimens)

Characteristics	Patients (n=18)	Controls (n=10)
Age (mean)	72	66
Males	10	4
Females	8	6
Smoking	1	2
Diabetes	4	0
Hypertension	13	2
Coronary disease	7	0
Peripheral arterial disease (other than carotid)	2	0
Drugs		
Aspirin	13	1
Clopidogrel	2	0
Warfarin	2	0
Statins	11	1

ABCA1 Gene Expression

RNA Isolation and cDNA Preparation

Plaques and control arteries were immediately snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from approximately 30 mg of pulverized frozen tissue with the RNeasy Mini or Lipid Tissue Mini kit (Qiagen) according to the manufacturer's instructions.

Leukocytes were isolated from 8 mL whole blood and total RNA was extracted using RNeasy Midi columns (Qiagen). Monocytes were isolated from whole blood of 11 individuals using magnetic beads technique (Dynal, product no. 113.09) according to the manufacturer's instructions.

For cDNA synthesis, total RNA (100 ng for plaques/control arteries, 1 μg for leukocytes) was transcribed with a first strand cDNA synthesis kit for reverse-transcription polymerase chain reaction (Roche, UK), according to the supplier's instructions.

TABLE 2. Demographic Details of Patients and Controls (Blood Specimens)

Characteristics	Patients (n=10)	Controls (n=10)
Age (mean)	71.8	60.4
Males	5	5
Females	5	5
Smoking	1	0
Diabetes	4	0
Hypertension	6	2
Coronary disease	3	0
Peripheral arterial disease (other than carotid)	2	0
Drugs		
Aspirin	8	0
Clopidogrel	3	0
Warfarin	2	0
Statins	7	0

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction

Primers and probe for Taqman analysis of ABCA1 mRNA were designed to span 2 adjacent exons with PrimerExpress software (PE Applied Biosystems). The forward primer was GGGAGGCTCCCGGAGTT (exon 3), the reverse primer was GTATAAAAGAAGCCTCCGAGCATC (exon 4), and the FAM-labeled probe, spanning exons 3 and 4, was AACTTTAACAAATCCATTGTGGCTCGCCTGT. 5′-3′-sequences for *LXRα* primers and probe were: CAAGTGTTTGCACTGCGTCT, CAGGAATGTTTGCCCTTCTC, and CACTTCTAGGAGGCAGCCAC. Single-tube Taqman analysis was performed on an ABI Prism 7700 sequence detection system with 300 nM of forward and reverse primers in the presence of 200 nM 5′FAM-3′TAMRA-tagged probe for *ABCA1*, and 900 nM of forward and reverse primers in the presence of 300 nM 5′FAM-3′TAMRA-tagged probe for *LXRα*.

The internal standard was β-actin mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were performed in duplicate and contained 5 μL of 4-fold diluted (leukocytes) or undiluted (plaques and control arteries) cDNA in a total volume of 25 μL.

Quantitation

The amount of ABCA1 mRNA in cells was calculated according to the relative standard curve method described in the PE User bulletin number 2. Target quantity was calculated from the standard curve and normalized to β-actin (arterial specimens, leukocytes) or GAPDH (leukocyte-monocyte correlation). Relative ABCA1 and LXRα levels were evaluated for at least 2 different RT reactions.

Total Membrane Preparation and Western Blotting

Samples were snap-frozen in liquid nitrogen and stored at −80°C. Total membrane fractions were prepared using a protocol based on an established method published by Rosenberg et al.[15] Approximately 100 mg of pulverised sample was homogenized in lysis buffer (50 mmol/L mannitol, 2 mmol/L EDTA, 50 mmol/L Tris HCl pH 7.6, complete mini protease inhibitors; Roche). Nuclei and debris were pelleted by centrifugation at 500 × g for 10 min, and the supernatant loaded onto 600 μl of a 300 mmol/L mannitol, 2 mmol/L EDTA, 50 mmol/L Tris HCl pH 7.6 cushion. The total membrane fraction was pelleted by centrifugation at 100 000 × g for 45 minutes. The membrane pellet was resuspended in 40 μl of lysis buffer and incubated for one hour on ice with 50 U of benzonuclease (Sigma), then SDS added to a final concentration of 1%. Protein content was measured by Biorad Dc protein assay kit. 50 μg of membrane protein was loaded on a 7% SDS-PAGE gel and transferred onto polyvinylidine difluoride membranes (Immobilon-P; Millipore). Proteins were detected with a polyclonal antibody against ABCA1 (1:1500; Abcam, Cambridge, UK) and a monoclonal antibody against Na^+/K^+-ATPase (1:1000; Research Diagnostics, Flanders, NJ) followed by anti-rabbit or anti-mouse HRP secondary antibodies (1:1000; Dako Cytomation, Cambridgeshire, UK). Signal was visualised by chemiluminescence (Amersham ECL system).

Protein Quantification

The optical density (OD) of ABCA1 and Na^+/K^+-ATPase bands was determined using Metamorph software (Universal Imaging Corporation). The average digital signal per band was measured after subtraction of the appropriate background. The OD for each ABCA1 band was normalized to the corresponding average signal of the Na^+/K^+-ATPase band, estimated for the same sample as loading control. Data are shown as average±SEM.

Statistics

For statistical evaluations, the SAS 8.1 program package was used.[16] Samples were tested for normality by using the UNIVARIATE procedure and the Shapiro–Wilk W test. To test differences between the patient and control groups and plaques and control arteries for significance, an analysis of variance based on least-square means was calculated by using the General Linear Model procedure. The model used was: Y=group+residual error. For statistical analysis of ABCA1 and LXRα mRNA expression, the logarithmic delta cycle threshold (ct) values (ct target gene−ct β-actin) were used. Sex differences between the patient and control groups were assessed using the χ^2 test (FREQ procedure). To evaluate the relationships between LXRα and ABCA1 in arterial specimens, Pearson correlations were calculated by using the CORR procedure. $P<0.05$ was considered significant.

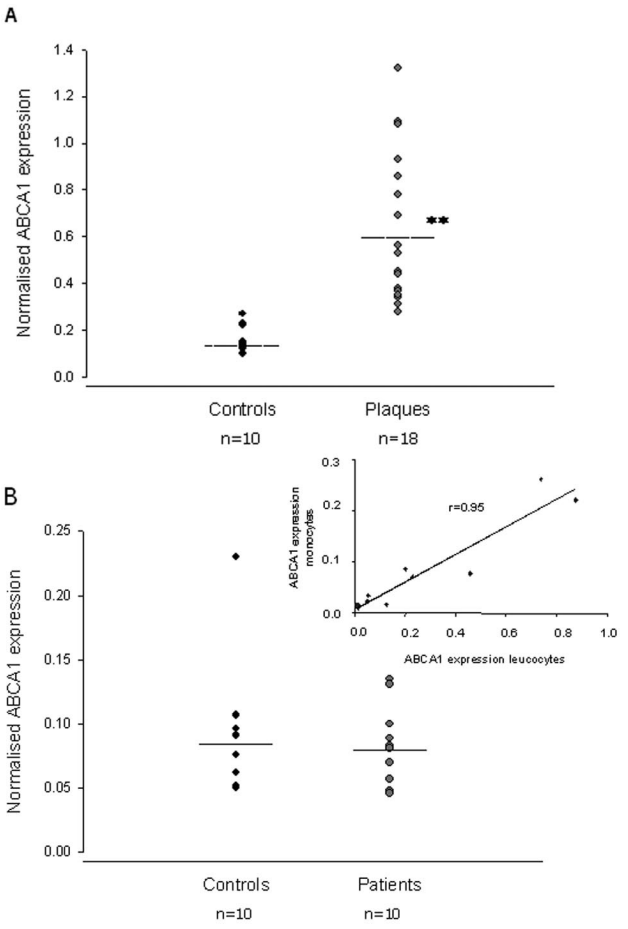

Figure 1. ABCA1 mRNA expression in plaques, control arteries, leukocytes, and monocytes. A, Quantitative, real-time polymerase chain reaction was performed on total RNA extracted from plaques (n=18) and control arteries (n=10). ABCA1 mRNA expression was normalized to β-actin as housekeeping gene. Values represent the average of 2 different measurements. Bars indicate mean values of each group; **denotes $P<0.0001$. B, Quantitative, real-time polymerase chain reaction was performed on total RNA extracted from leukocytes of patients (n=10) and control subjects (n=10). Inset: Correlation of ABCA1 mRNA expression between leukocytes and monocytes from whole blood (n=11) using magnetic beads technique. ABCA1 mRNA expression in leukocytes and monocytes was quantified with real-time polymerase chain reaction and normalised to GAPDH.

Results

ABCA1 Gene Expression

Real-time quantitative polymerase chain reaction was used to determine ABCA1 mRNA levels in plaques and control arteries. ABCA1 mRNA was significantly increased in plaques as compared with control arteries (Figure 1A, $P<0.0001$). To evaluate whether the upregulation of ABCA1

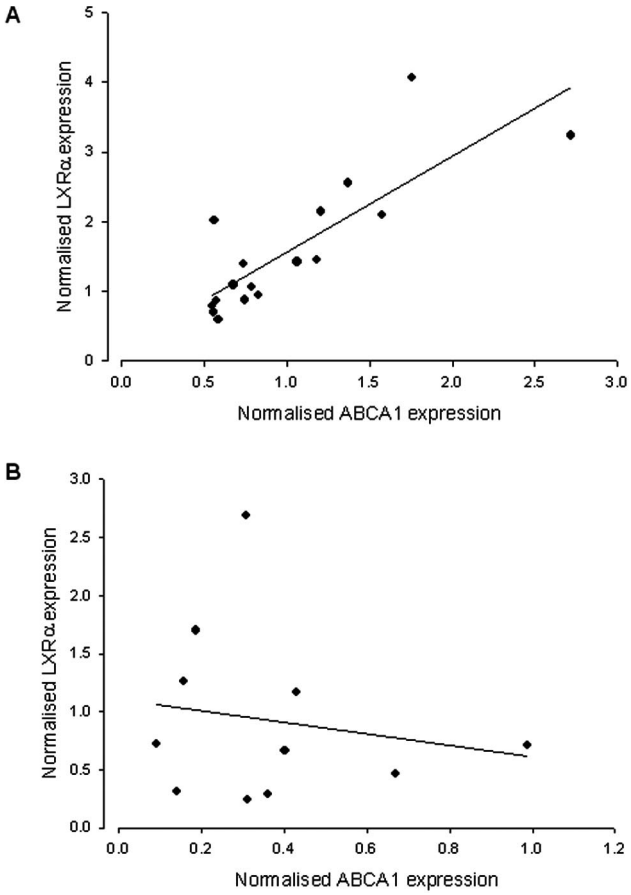

Figure 2. Association between mRNA levels of ABCA1 and LXRα in plaques and control tissue. ABCA1 and LXRα mRNA expression in plaques (A) and control arteries (B) was quantified with real-time polymerase chain reaction and normalized to β-actin.

was restricted to the diseased artery or reflected in the systemic circulation, we compared ABCA1 expression in leukocytes from patients undergoing CEA and age- and sex-matched controls. In previous experiments ABCA1 expression in leukocytes was comparable to monocytes (Figure 1B inset, $r=0.95$) and therefore seemed to reflect circulating monocyte ABCA1 expression. No difference in ABCA1 expression levels was found ($P=0.6741$; Figure 1B), indicating a localized upregulation of ABCA1 mRNA levels in the plaque tissue. To gain insight into potential underlying mechanisms, mRNA expression of LXRα was measured. The increase in ABCA1 mRNA detected in plaques was paralleled by a significant >2-fold increase in average LXRα mRNA levels ($P=0.0287$). Interestingly, in plaque tissue, a significant correlation between ABCA1 and LXRα mRNA expression levels was found (Figure 2A; $r=0.85$, $P<0.0001$), whereas in control arteries no association was detected (Figure 2B; $r=0.24$, $P=0.5070$).

ABCA1 Protein Expression

To assess protein expression, immunoblot analysis was performed. Total membrane fractions of plaques and control arteries were tested with antibodies against ABCA1 (Figure 3A; upper bands) and Na^+/K^+-ATPase (Figure 3A, lower bands), a plasma membrane protein used to ascertain equal sample loading. In contrast to mRNA levels, ABCA1 protein expression was significantly lower in plaques than in control arteries. Semiquantitative analysis using the OD of the bands confirmed the marked difference between controls and plaques ($P<0.0001$; Figure 3B) and remained highly significant after normalization of ABCA1 expression to Na^+/K^+-ATPase ($P=0.0004$; Figure 3C). No difference with regard to the Na^+/K^+-ATPase loading control was found ($P=0.8316$; Figure 3B).

Discussion

Primary and secondary prevention of stroke rely on the identification and treatment of modifiable risk factors such as hyperlipidemia, hypertension, hyperglycemia, and smoking. All of these conditions give rise to cerebral atherosclerosis, the commonest cause of stroke. Increasing interest and research in the field of the ABC transporters has raised the possibility of further new pharmacological interventions in atherosclerosis. The importance of ABCA1 in HDL formation has become apparent with the identification of mutations responsible for Tangier disease and familial HDL deficiency.[1,2] Although these investigations highlighted the importance of ABCA1 in reverse cholesterol transport and the development of early atherosclerosis, the potential role of ABCA1 in localized atherosclerosis in the absence of familial gene mutations has not been addressed.

We found both ABCA1 mRNA and protein were expressed in inferior mesenteric arteries with early asymptomatic atherosclerosis. Unsurprisingly, the histology of these age-matched control arteries showed early atheromatous changes (American Heart Association classification of atherosclerosis types I to III). These lesions ranging from foam cells to extracellular lipid pools in the intima do not result in clinically relevant sequelae, and may not progress to atheroma formation. The only previous report of ABCA1 localization in human atherosclerosis using in situ hybridization demonstrated that ABCA1 mRNA was predominantly localized to macrophages[14] in atherosclerotic lesions. However, it was not detected in normal arterial tissue, possibly because of the lower detection sensitivity of the in situ hybridization technique as compared with the quantitative real-time polymerase chain reaction (PCR) method that was used in the current study. ABCA1 protein has been detected in human endothelial and smooth muscle cell cocultures,[17] but there are no reports to date of ABCA1 protein detection in human arterial tissue in vivo. This study demonstrates the presence of ABCA1 protein in minimally atherosclerotic arteries.

The CEA specimens demonstrated significantly higher levels of ABCA1 mRNA compared with control arterial tissues. One potential source of confound might be related to the difference in drug usage between patient and control groups illustrated in Table 1. Therefore, linear regression modelling was used to assess ABCA1 expression in control and patient groups adjusted for age, sex, aspirin and statin usage. This analysis revealed that differences in ABCA1 expression between control and patient arteries remained significant once adjusted for drug usage (coef=0.646, $P=0.001$). This increase was not mirrored by an upregulation

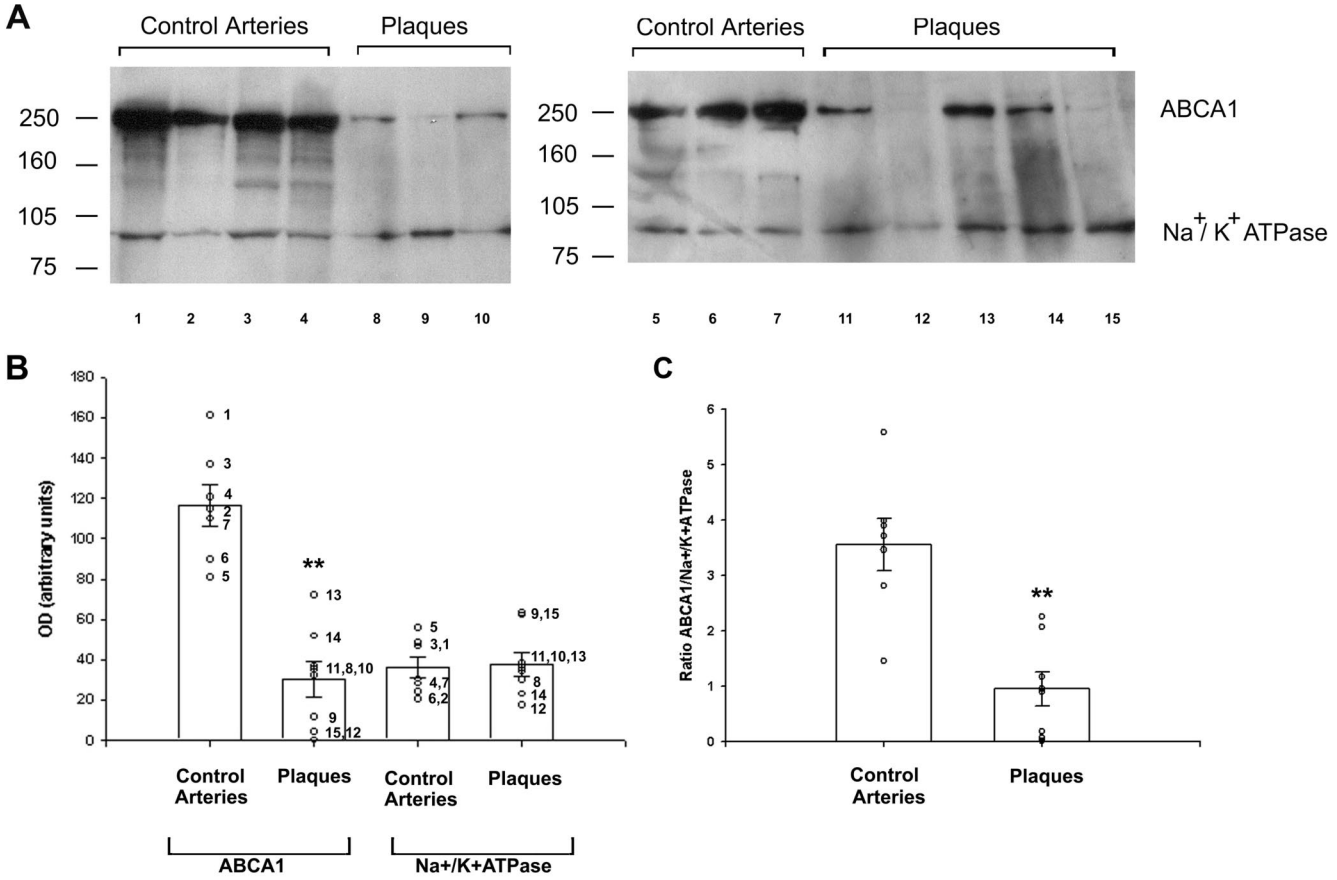

Figure 3. Western blot analysis of ABCA1 in plaques and control arteries. A, Representative immunoblots of ABCA1 protein expression are shown. Membrane proteins from plaques and control arteries were separated by reducing SDS-PAGE and immunoblotted with rabbit anti-ABCA1 and mouse anti-Na^+/K^+-ATPase antibodies. The positions of marker proteins (kDa) are indicated. Samples have been labelled with numbers 1 through 7 for controls and 8 through 15 for plaques. B, Quantification of protein expression was performed by assessing the OD of ABCA1 and Na^+/K^+-ATPase bands. Average values±SEM are shown. Individual values are also shown as open symbols with adjacent numbers representing the respective samples in panel A. **denotes $P<0.0001$. C, Ratio between ABCA1 and Na^+/K^+-ATPase protein expression according to results as presented in Figure 3B. **denotes $P=0.0004$.

of ABCA1 mRNA expression in peripheral leukocytes. This implies the observed upregulation in mRNA occurs predominantly at localized sites of disease.

The increase in *ABCA1* gene expression in diseased tissue is surprising given the fact that animal studies of *ABCA1* inactivation, as well as human *ABCA1* gene mutations, suggest loss of function is the key to lipid deposition and its sequelae. However, mRNA levels do not necessarily accurately reflect protein expression and, particularly for ABCA1, relative mRNA distribution in tissue shows significant discordance with protein expression patterns suggesting that post-transcriptional regulation may be important.[18] The relationship between ABCA1 mRNA and protein expression was assessed by analyzing the same specimen for both parameters. In control arteries, *ABCA1* gene expression was reflected by the presence of protein. Intriguingly, however, markedly lower levels of ABCA1 protein were present in advanced atherosclerotic lesions. Thus, despite increased transcription, a reduction in protein expression was observed. However, no positive or negative correlation was observed between the mRNA and protein levels in the control arteries and the plaques. This could be attributed to the low n-numbers, the semiquantitative nature of the protein data and various other influences on the intracellular sterol levels that regulate gene expression.

There is evidence that the composition and microenvironment of the atherosclerotic plaque could be associated with ABCA1 protein degradation. In advanced atherosclerotic lesions, macrophages tend to accumulate large amounts of free cholesterol.[19] Increased intracellular free cholesterol has been shown to accelerate the degradation of ABCA1 in macrophages.[20] Long chain fatty acids present in the plaque[21] can promote macrophage ABCA1 protein degradation.[22] Furthermore, it has recently been demonstrated that ABCA1 contains a PEST sequence that appears to enhance protein degradation.[23]

We have shown that in atherosclerotic tissues, both ABCA1 and LXRα mRNA levels were upregulated, and a clear correlation between mRNA levels of both genes was found. Nuclear receptors act as cholesterol sensors that respond to elevated sterol concentrations.[24] It has been previously shown in vitro that *ABCA1* transcription is stimulated by LXRα and the retinoid X receptor,[25] and the induction of *ABCA1* expression reflected that of *LXRα*.[26] Such a parallel increase in LXRα and ABCA1 mRNA was also found in our study. The observed upregulation of both LXRα and ABCA1 mRNA could be attributed to the

oxysterol-rich environment inside the plaque potentially amplified by low ABCA1 protein levels. Increased degradation of ABCA1 protein could hypothetically diminish cellular cholesterol efflux, resulting in increased free cholesterol, enhanced intracellular oxysterol loading, and stimulation of regulatory pathways involving LXRα.[6]

In conclusion, this study has shown that both the *ABCA1* gene and protein are expressed in mildly atherosclerotic arterial tissue in vivo. Advanced carotid atherosclerotic lesions are characterized by reduced ABCA1 protein levels despite significant upregulation of both ABCA1 and LXRα mRNA. This finding has potentially important clinical consequences. The observation that upregulation of ABCA1 mRNA fails to translate into ABCA1 protein implies that pharmacological targeting of the ABCA1 and LXRα pathways may not achieve the anticipated atheroprotective effect in advanced atherosclerotic lesions.

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mRNA Expression of Genes Involved in Lipid Efflux and Matrix Degradation in Occlusive and Ectatic Atherosclerotic Disease

Soni Soumian [1, 2]; Richard Gibbs [2]; Alun Davies [2]; Christiane Albrecht [1, 3]

[1] **MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College Hammersmith Hospital, Du Cane Road, London W12 0NN**

[2] **Department of Vascular Surgery, Charing Cross Hospital, London W6 8RF.**

[3] **Current affiliation - Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, Freising, Germany**

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Correspondence to: Soni Soumian, Department of Vascular Surgery, Imperial College, Charing Cross Hospital, Fulham Palace Road, London W6 8RF.

(**e-mail**: sonis@doctors.org.uk **Tel**:++44-20 8846 7320 **Fax**: ++44-208 846 7362)

Abstract

Aims

Atherosclerotic plaque behaviour is influenced by intraplaque inflammation, matrix turnover and the volume of the lipid core. Peroxisome-proliferator activated receptor γ (PPARγ) modulates atherosclerosis by its anti-inflammatory and anti-protease activity. PPARγ promotes lipid efflux through the liver-X-receptorα (LXRα) and the ATP binding cassette transporterA1 (ABCA1). Matrix metalloproteinase-9 (MMP-9) and cycloxygenase-2 (COX-2) are implicated in plaque instability. We assessed the expression of these genes in occlusive and ectatic atherosclerotic disease to determine the relationship between genes involved in lipid efflux and matrix degradation.

Methods

Carotid endarterectomy specimens from 16 patients (mean age-72 years) and aneurysm tissue from 16 patients (mean age-75 years) undergoing abdominal aortic aneurysm repair were used. Inferior mesenteric arteries from colectomy specimens of 12 patients (mean age-70 years) served as control. Total RNA was extracted from pulverised tissue and reverse transcribed into cDNA. Quantitative real-time PCR was performed using fluorescently labelled probes for ABCA1, LXR-α, PPAR-γ, COX-2 and MMP-9.

Results

PPARγ expression was significantly lower in both occlusive and ectatic atheroscelrotic disease ($p<0.001$) while LXRα and ABCA1 were significantly elevated ($p<0.01$). MMP-9 expression was significantly increased in diseased tissues ($p<0.0001$) though levels were markedly higher in occlusive disease ($p<0.01$). The increased mRNA levels of ABCA1 and MMP-9 were significantly correlated in

diseased tissues (p<0.01, r=0.71 and r=0.78). COX-2 expression was increased in ectatic but low in occlusive disease (p<0.01).

Conclusion

This observational study suggests a role for therapeutic upregulation of PPARγ, which could potentially upregulate lipid efflux through ABCA1 and inhibit matrix degradation through inhibition of MMP-9.

Introduction

The clinical progression of the atherosclerotic plaque is governed by the key processes of inflammation, lipid deposition and matrix degradation. Increased uptake of modified low density lipoprotein (LDL) by monocytes/macrophages results in foam cell formation, the hallmark of an atheromatous plaque [1]. A large lipid core, formed from the extracellular accumulation of these foam cells [2], is recognized as a risk factor for plaque rupture [3]. Increased activity of both pro-inflammatory cytokines and the matrix degrading matrix metalloproteinases, particularly in the region of the fibrous cap, can render the atherosclerotic plaque potentially unstable [4]. Ubiquitously expressed NF-k B transcription pathways [5], involved in the activation of cytokines such as cycloxygenase 2 (COX-2) [6;7], have been implicated in plaque instability [8]. COX-2, mainly expressed in atherosclerotic lesions [9], is involved in the release of matrix metalloproteinase 9 (MMP-9) [10], a matrix degrading protease, which has been reported to play a major role in atheromatous plaque rupture [11]. A strategy that could combine both promotion of cholesterol efflux, as well as inhibition of inflammation and matrix degradation, could be of great benefit in treating or modifying plaque behaviour.

One of the most exciting discoveries in recent years has been the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor, which is involved in the regulation of both the inflammatory response and lipid homeostasis in the macrophage [12]. Nuclear receptors like PPARγ and the liver-X-receptor α (LXRα) are known to modulate atherogenesis at various stages from cell recruitment, lipid accumulation and local inflammatory response [13-16] . PPARγ is known to suppress monocyte chemoattractant protein-1 expression [17], inhibit adhesion molecules [18;19] and facilitate cholesterol efflux [20;21]. It promotes cholesterol efflux via ATP-binding cassette transporter A1 (ABCA1) (through the upregulation of LXRα)

dependent [20] and distinctly ABCA1 independent pathways [21]. ABCA1, a membrane protein involved in cholesterol efflux is highly expressed in macrophages [22] and plays a major role in high-density lipoprotein (HDL) metabolism [23]. It is also implicated in promoting macrophage engulfment of apoptotic cells [24]. Patients with ABCA1 deficiency states like Tangier disease [25] have virtually absent HDL and are predisposed to premature atherosclerosis [26].

PPARγ and LXRα are reported to suppress the synthesis of COX-2 and MMP-9 through its inhibitory effect on NF-κB pathways [13;14;27]. It is interesting to note that PPAR agonists can effectively increase ABCA1 expression in addition to its suppression of pro-inflammatory pathways including COX-2 and MMP-9 [16;28]. All of these functions are pivotal in stabilising a potentially unstable atherosclerotic plaque. PPARγ has the potential to exert pleiotropic effects on the plaque by upregulation of lipid efflux and down regulation of genes governing inflammation. We aimed to evaluate the mRNA expression of the genes involved in this lipid efflux pathway and to assess its relationship with COX-2 and MMP9 in occlusive and ectatic atherosclerotic disease.

Methods

Subjects and specimens

Sixteen carotid plaques were collected from subjects with internal carotid artery stenoses of >70% undergoing carotid endartercetomy (CEA). Fourteen patients were diagnosed with symptomatic carotid disease with a history of transient ischaemic attacks, strokes or amaurosis fugax, whilst two were asymptomatic. Aneurysm wall specimens were collected from sixteen patients undergoing elective abdominal aortic aneurysm repair. Twelve macroscopically normal inferior mesenteric arteries (IMA) dissected from colectomy specimens of subjects having

elective operation served as controls. These patients were phenotypically free of symptomatic atherosclerotic disease by history, examination and a normal ECG tracing. The study had ethical approval from the Riverside Research Committee and informed consent was obtained from subjects. Demographic details of patient and control groups are listed in Table 1

Table 1

Characteristics	CEA patients (n = 16)	Aneurysm patients (n = 16)	Controls (n = 12)
Age (mean)	72	75	70
Sex:			
Males	8	14	6
Females	8	2	6
Smoking	1	6	2
Diabetes	4	2	1
Hypertension	12	13	5
Symptomatic peripheral vascular disease	1	4	0
Drugs:			
Aspirin	12	10	2
Clopidogrel	2	1	0
Warfarin	2	1	0
Statins	9	5	2

Gene expression studies

RNA isolation and cDNA preparation

Plaques, aneurysm specimens and control arteries were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C. Total RNA was extracted from approximately

30mg of pulverised frozen tissue with the RNeasy Mini or Lipid Tissue Mini kit (Qiagen) according to the manufacturer's instructions.

For cDNA synthesis, total RNA (100 ng) was transcribed with a first strand cDNA synthesis kit for RT-PCR (Roche, UK), according to the supplier's instructions.

Real-time quantitative RT-PCR (Taqman®)

Primers and probe for Taqman® analysis of ABCA1 mRNA were designed to span two adjacent exons with PrimerExpress software (PE Applied Biosystems, UK). Primers and probes for all genes investigated are listed in Table 2A &B.

Table 2A

Genes	Probe (5'- 3')
ABCA1	AACTTTAACAAATCCATTGTGGCTCGCCTGT
***LXR*α**	CACTTCTAGGAGGCAGCCAC
PPARγ	ACTTCAAGAGTACCAAAGTGCAATCAAAGTGGAG
COX-2	ATGATTGCCCGACTCCCTTGGGTGT
MMP-9	CCCAGCGAGAGACTCTACACCCGG

Table 2B

Genes	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
ABCA1	GGGAGGCTCCCGGAGTT	GTATAAAAGAAGCCTCCGAGCATC
***LXR*α**	CAAGTGTTTGCACTGCGTCT	CAGGAATGTTTGCCCTTCTC
PPARγ	CCAGTGGTTGCAGATTACAAGTCTG	TTGTAGAGCTGAGTCTTCTCAGAATAATAAG
COX-2	GCCCTTCCTCCTGTGCC	AATCAGGAAGCTGCTTTTTACCTTT
MMP-9	GACGACCGGTTTGGCTTCT	AGGGTTTCCCATCAGCATTG

Single tube Taqman analysis was performed on an ABI Prism 7700 sequence detection system with 300nM of forward and reverse primers in the presence of 200nM 5'FAM-3'TAMRA-tagged probe for *ABCA1, COX-2, MMP-9* and *PPARγ* and 900nM of forward and reverse primers in the presence of 300nM 5'FAM-3'TAMRA-tagged probe for *LXR*α. The internal standard was β-actin mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were carried out in duplicate and contained 5µl of undiluted cDNA in a total volume of 25µl.

Quantitation

The amount of mRNA in cells was calculated according to the relative standard curve method described in the PE User bulletin no2. Target quantity was calculated from the standard curve and normalised to β-actin.

Statistical Methods

For statistical evaluations the SAS 8.1 program package [29] was used. For statistical analysis of mRNA expression, the delta ct values (ct target gene – ct β -actin) were used. Samples were tested for normality by using the UNIVARIATE procedure and the Shapiro-Wilk W test. The null hypothesis was not rejected in any sample. We used analysis of variance to test differences among the control group, plaques and arteries; pairwise significant differences were established using Bonferroni-corrected *t*-tests. Sex differences between the patient and control groups were assessed using the χ^2 test (FREQ procedure). We tested the significance of the Pearsons correlation coefficient in order to evaluate the relationships between normalized ABCA1 and normalized MMP-9. A p-value < 0.05 was considered significant.

Results

Expression of genes implicated in lipid efflux (ABCA1, PPARγ, LXRα)

Real-time quantitative PCR was used to determine PPARγ mRNA levels in plaques and control arteries. PPARγ expression levels were significantly lower in occlusive and ectatic atherosclerotic tissues , compared to arterial control tissue (Figure1A, p<0.001); *LXRα* and *ABCA1 mRNA* levels were significantly higher in both tissues (Figure1B & 1C, $p<0.01$). *LXRα* and *ABCA1* mRNA levels were markedly increased in occlusive as compared to ectatic disease ($p<0.01$ and $p<0.05$, respectively).

Expression of genes implicated in inflammation and matrix degradation (MMP-9, COX-2)

MMP-9 levels were significantly higher in ectatic and stenotic arteries (Figure 2A, $p<0.01$). Highest levels were found in occlusive disease. *COX-2* levels were significantly higher in aneurysm tissues (Figure2B, $p<0.01$) while they were markedly reduced in atherosclerotic plaques as compared to control tissues ($p<0.01$).

Association between ABCA1 and MMP-9

ABCA1 and *MMP-9* mRNA expression levels were associated in both occlusive and ectatic atherosclerotic tissues (Figure 3A & 3B, $r=0.71$ & 0.78 for plaques and aneurysms, respectively, $p<0.01$).

Discussion

PPARγ has attained remarkable interest in terms of atherosclerosis because of it's potential beneficial effects. It is expressed by all major cells of the vasculature, including endothelial cells, vascular smooth muscle cells and monocytes/macrophages [30]. This receptor can be therapeutically targeted using the thiazolidinediones (TZD), a group of drugs such as pioglitazone, rosiglitazone and troglitazone, agents that have been recently used in the treatment of type 2 diabetes and proved to be very effective in reducing insulin resistance [31-34].

It has been demonstrated that PPARγ activation inhibits angiogenesis which plays an important role in plaque progression and aneurysm formation [35] and stimulates nitric oxide release from endothelial cells [36] which is critical for maintenance of normal vascular physiology. Furthermore drugs like thioglitazones have been found to inhibit intimal hyperplasia [37] and promote lipid efflux in addition to reducing inflammation [38]. The clinical relevance of this has been demonstrated by the fact that less neointima formation was seen after coronary artery stent placement in type 2 diabetic patients when they were treated with troglitazone [39]. In vivo studies using LDL receptor knockout mice have demonstrated that PPARγ agonists reduced the development of atherosclerotic lesion formation [15;40].

In the present study we found a reduced expression of PPARγ in human atherosclerotic tissues, both occlusive and ectatic, when compared to normal arterial controls while ABCA1 and LXRα mRNA were significantly upregulated in both types of diseases. We have previously reported that ABCA1 protein was low in carotid atherosclerotic plaques despite increased mRNA expression [41]. We hypothesised that reduced ABCA1 protein leads to an oxysterol rich plaque microenvironment, which in turn stimulates LXRα with consequent upregulation of the ABCA1 gene.

This could explain the high mRNA levels of LXRα and ABCA1 in these tissues. The variable levels of ABCA1 and LXRα upregulation in both occlusive and aneurysmal disease could be attributed to the difference in the availability of ligands activating these genes. The reduced expression of PPARγ in these tissues could potentially be due to the increased amount of cytokines in the plaque microenvironment [42].

MMP-9, implicated in degradation of the plaque fibrous cap, was found to be significantly elevated in both types of diseased tissues, more so in occlusive disease [11;43]. Although its role on aneurysm formation is not quite clear, MMP-9 has been extensively studied in the context of plaque pathophysiology [43;44]. Surprisingly COX -2 was decreased in atheromatous plaques in this study whereas the levels were significantly higher in aneurysms. Oxidised LDL has been reported to inhibit COX-2 in human macrophages in *in vitro* work [45] suggesting that the impact of macrophage COX-2 may be attenuated in advanced atherosclerotic lesions [46]. Moreover the levels of oxidised LDL may reach higher concentrations in the plaque than in the aneurysm wall. This also seems to reflect the recent findings that therapeutic COX-2 inhibition may not be beneficial in stabilising the plaque considering the fact that majority of these plaques were symptomatic [47;48].

ABCA1 mRNA levels were found to correlate with MMP-9 levels in both aneurysmal and occlusive specimens in our study. Low protein levels of ABCA1 in atherosclerotic plaques [41] could account for the increased ABCA1 mRNA levels in these tissues. Proteases like calpain have been reported to be involved in ABCA1 protein degradation [49]. MMP's are also known to degrade non-extracellular matrix proteins in addition to matrix proteins [50] and were also found to be correlated to calpain in *in vitro* work [51;52]. This raises the possibility that common protein degradation pathways involving MMP-9 and calpain may be involved in ABCA1 protein degradation thus promoting matrix degradation whilst at the same time reducing lipid

efflux. In this study, a significant proportion of subjects in the CEA and aneurysm groups had co-morbid conditions and were on aspirin and statins. Conditions like diabetes mellitus can potentially decrease ABCA1 expression [53;54] however the effect of hypertension on the expression of these genes has not been reported. The evidence regarding the influence of statins on ABCA1expression is conflicting [55;56] although its positive effect on PPAR-γ expression and suppression of MMP-9 and COX-2 has been reported by various *in vitro* studies [57;58] .

PPARγ is known to suppress the synthesis of both COX-2 and MMP-9 [59-61]. Considering the low levels of PPARγ in these specimens, it is tempting to speculate that PPARγ upregulation through pharmacological means using TZD's or synthetic ligands could potentially be beneficial in increasing lipid efflux through LXRα and ABCA1 and reducing inflammation through the inhibition of COX-2 and MMP-9 thus stabilising the atherosclerotic plaque.

In conclusion our observational study revealed low expression of PPARγ in ectatic and occlusive disease and underlined the potential link between genes involved in lipid efflux and matrix degradation. The interesting finding that reduced PPARγ expression is observed in atherosclerotic tissues raises the possibility that upregulation of this pathway may be beneficial in the context of treating atherosclerosis.

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Competing interest statement and copyright

Figure legends

Figure 1. mRNA expression of PPARγ, LXRα and ABCA1 in aneurysms, plaques and control arteries. Quantitative, real-time PCR was performed on total RNA extracted from aneurysms (n=16), plaques (n=16) and control arteries (n=12). mRNA expression of PPARγ, LXRα and ABCA1 was normalised to β-actin as housekeeping gene. Bars indicate mean values of each group; a, b, c: means without common letter are significantly different ($p < 0.05$).

(A) PPARγ expression in aneurysms, plaques and controls

(B) LXRα expression in aneurysms, plaques and controls

(C) ABCA1 expression in aneurysms, plaques and controls

Figure 2. mRNA expression of COX-2 and MMP-9 in aneurysms, plaques and control arteries. Methodology and annotation see Figure 1. mRNA expression of COX-2 and MMP-9 was normalised to β-actin.

(A) MMP-9 expression in aneurysms, plaques and controls

(B) COX-2 expression in aneurysms, plaques and controls

Figure 3. Association between mRNA levels of ABCA1 and MMP-9 in plaques and aneurysm tissue.

ABCA1 and MMP-9 mRNA expression in plaques (A) and aneurysm tissues (B) was quantified with real-time PCR and normalised to β-actin.

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Figure 1

A

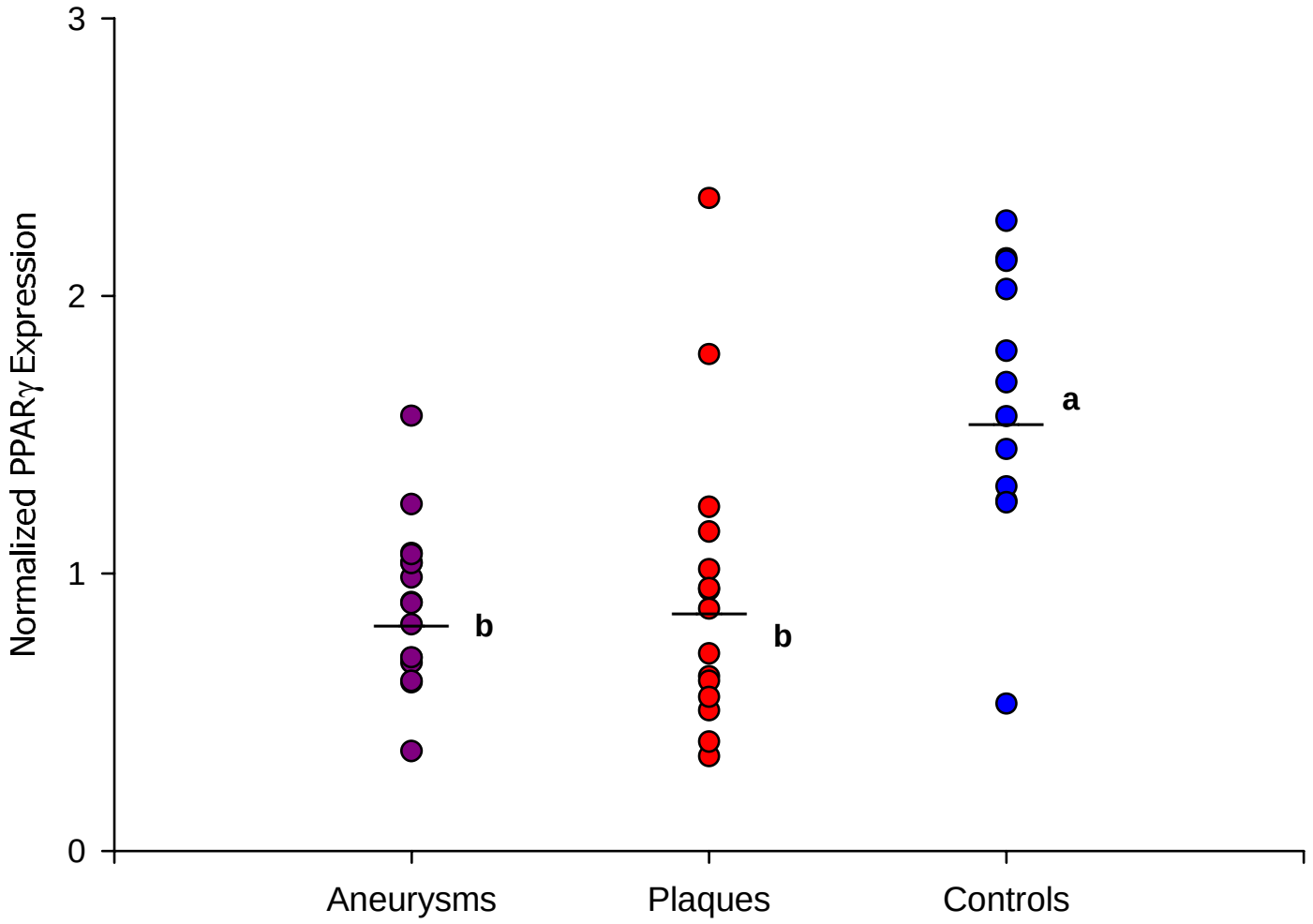

Normalized PPARγ Expression
3
2
1
0
b
b
a
Aneurysms
Plaques
Controls

Figure 1

B

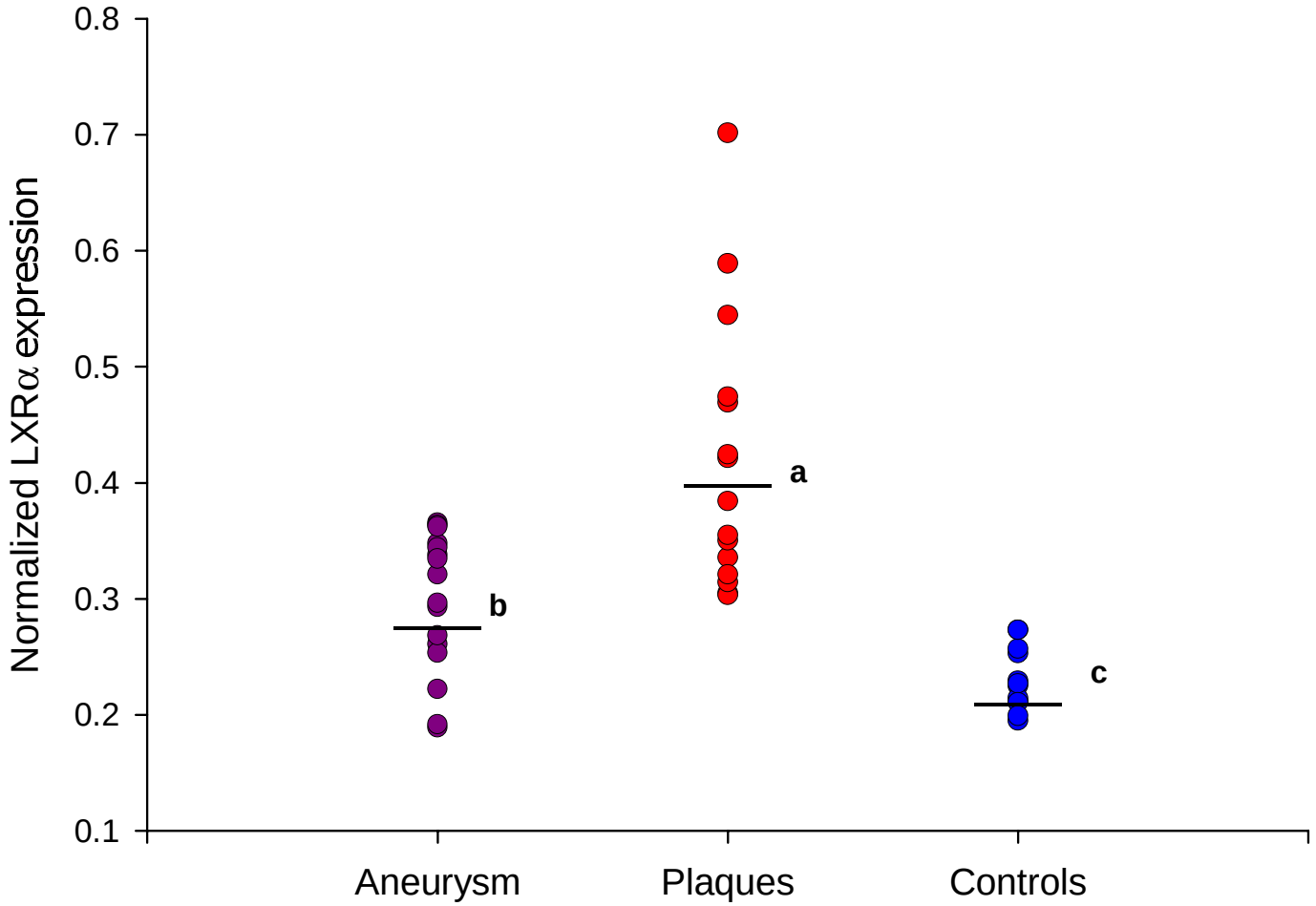

Normalized LXRα expression
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
a
b
c
Aneurysm
Plaques
Controls

Figure 1

C

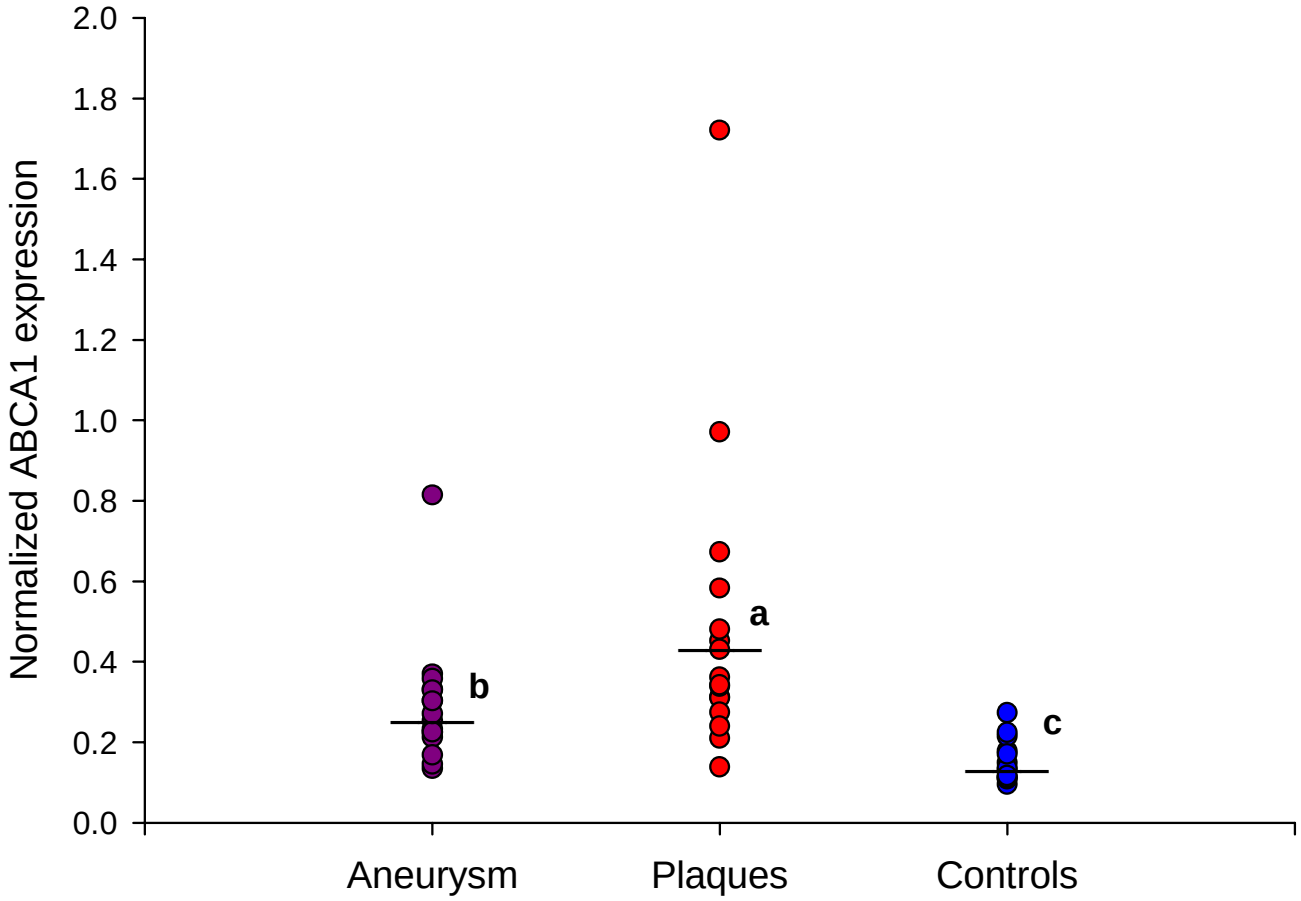

Normalized ABCA1 expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
a
c
Aneurysm
Plaques
Controls

Figure 2

A

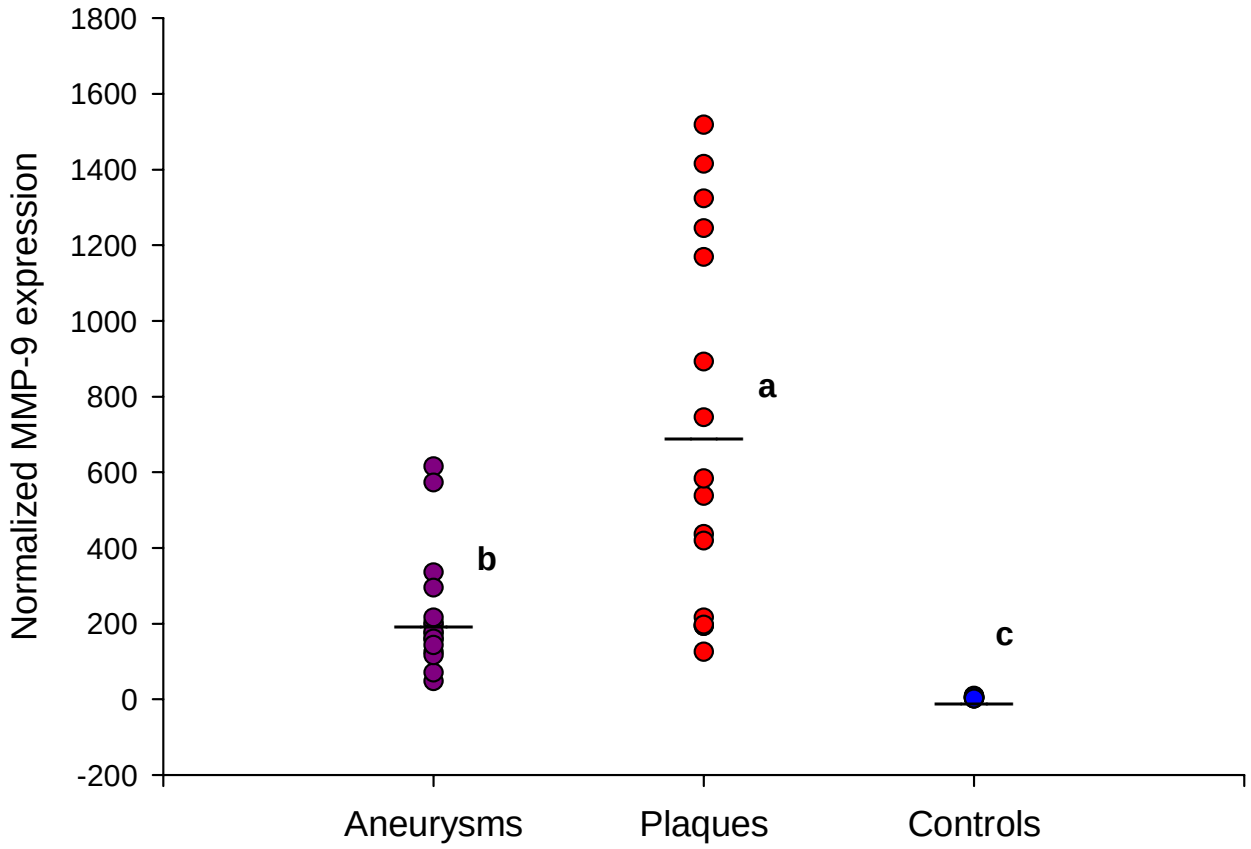

Normalized MMP-9 expression
1800
1600
1400
1200
1000
800
600
400
200
0
-200
a
b
c
Aneurysms
Plaques
Controls

Figure 2

B

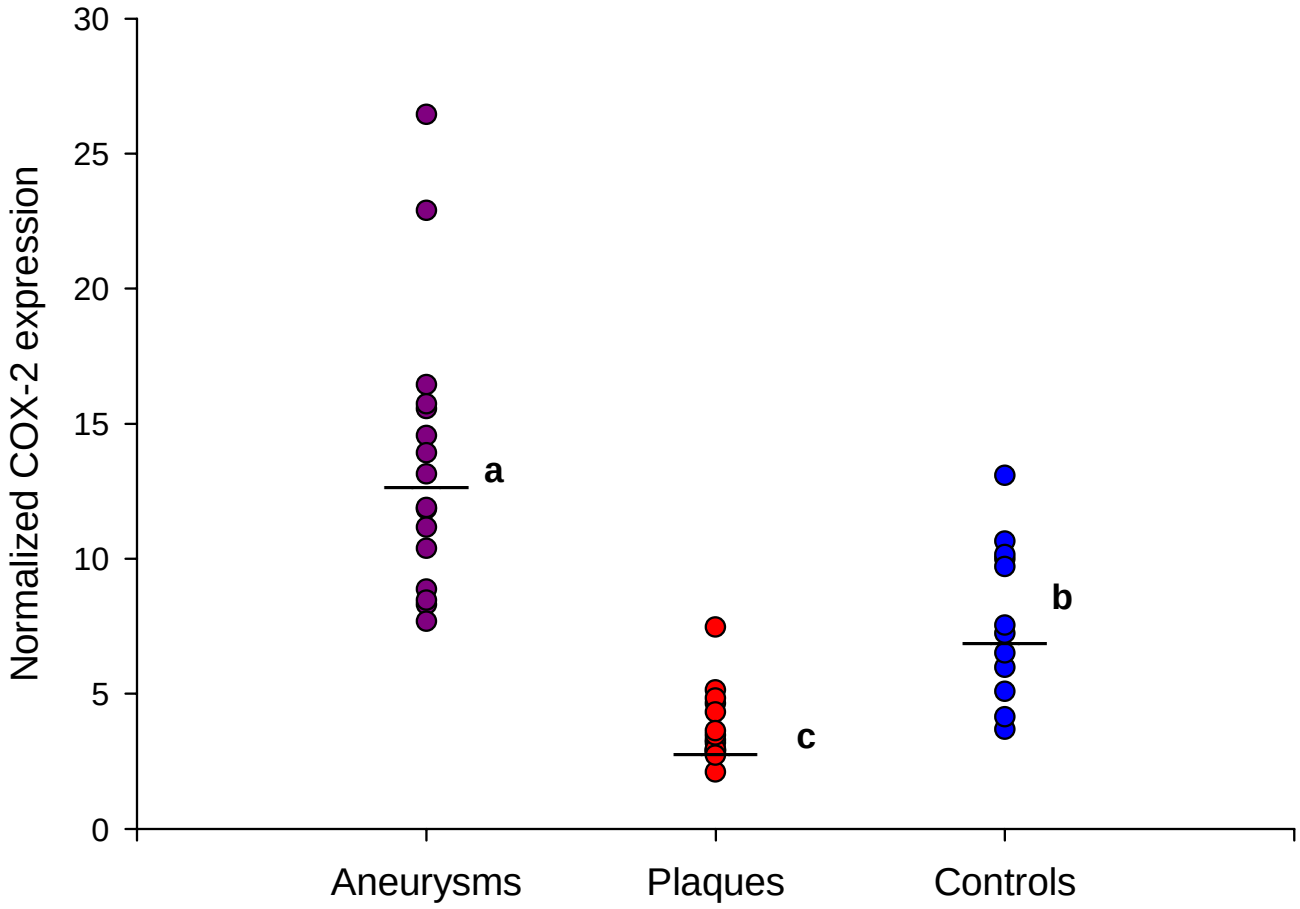

Normalized COX-2 expression
30
25
20
15
10
5
0
a
b
c
Aneurysms
Plaques
Controls

Figure 3

A

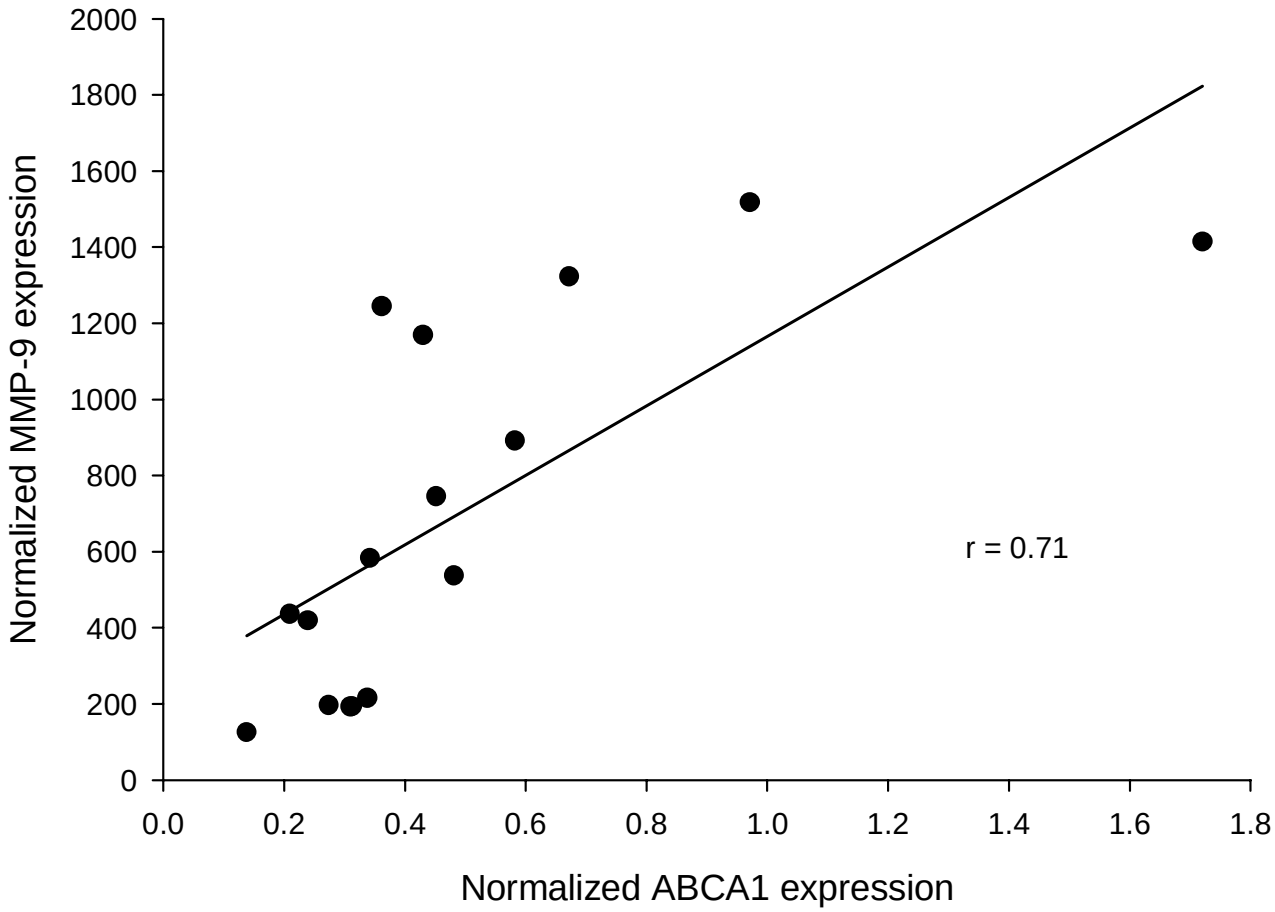
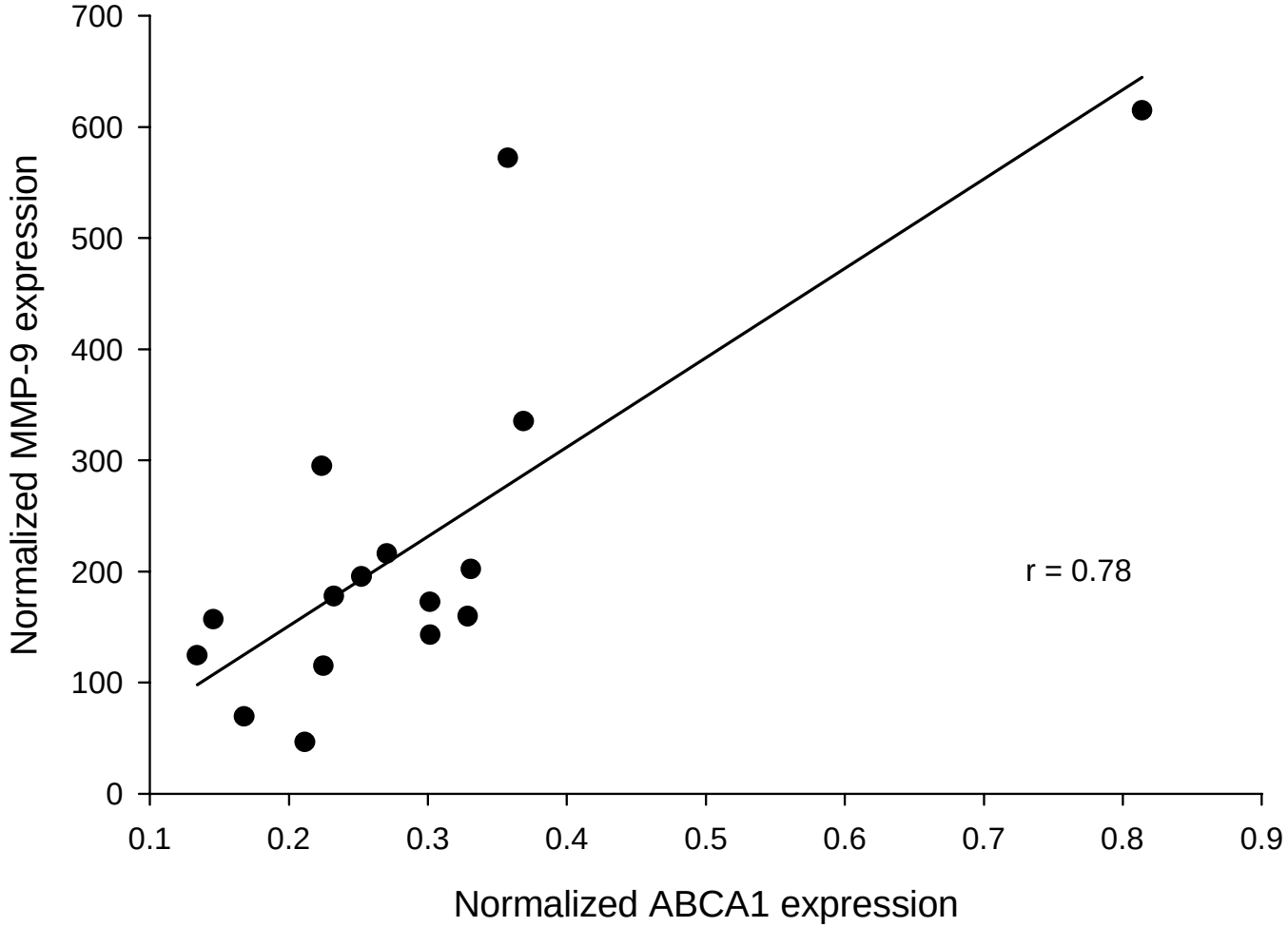

Normalized MMP-9 expression
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Normalized ABCA1 expression
r = 0.71

Figure 3

B


700
600
500
400
300
200
100
0
Normalized MMP-9 expression
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Normalized ABCA1 expression
r = 0.78

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Review article

ABCA1 and atherosclerosis

S Soumian[a], C Albrecht[b], AH Davies[a] and RGJ Gibbs[a]

Abstract: ATP binding cassette transporter A1 (ABCA1) mediates the cellular efflux of phospholipids and cholesterol to lipid-poor apolipoprotein A1 (apoA1) and plays a significant role in high density lipoprotein (HDL) metabolism. ABCA1's role in the causation of Tangier disease, characterized by absent HDL and premature atherosclerosis, has implicated this transporter and its regulators liver-X-receptorα (LXRα) and peroxisome proliferator activated receptorγ (PPARγ) as new candidates potentially influencing the progression of atherosclerosis. In addition to lipid regulation, these genes are involved in apoptosis and inflammation, processes thought to be central to atherosclerotic plaque progression. A Medline-based review of the literature was carried out. Tangier disease and human heterozygotes with ABCA1 mutations provide good evidence that ABCA1 is a major candidate influencing atherosclerosis. Animal and in vitro experiments suggest that ABCA1 not only mediates cholesterol and phospholipid efflux, but is also involved in the regulation of apoptosis and inflammation. The complex and beneficial interactions between apoA1 and ABCA1 seem to be pivotal for cholesterol efflux. The expression of the ABCA1 is tightly regulated. Furthermore the plaque microenvironment could potentially promote ABCA1 protein degradation thus compromising cholesterol efflux. PPAR-LXR-ABCA1 interactions are integral to cholesterol homeostasis and these nuclear receptors have proven anti-inflammatory and anti-matrix metalloproteinase activity. Therapeutic manipulation of the ABCA1 transporter is feasible using PPAR and LXR agonists. PPAR agonists like glitazones and ABCA1 protein stabilization could potentially modify the clinical progression of atherosclerotic lesions.

Key words: apolipoprotein A1; atherosclerosis; ATP binding cassette transporter 1; HDL cholesterol; Tangier disease

Introduction

Atherosclerosis, a multifactorial disease of great complexity, starts in fetal life, progresses slowly through childhood and adolescence and accelerates in adult life. The characteristic component of the atherosclerotic plaque is the macrophage derived foam cell.[1] This cholesterol laden macrophage is thought to result from the uptake of oxidatively modified low density lipoprotein (LDL).[2] However, intracellular free cholesterol (FC) can be toxic to the cell[3] and therefore an efficient cholesterol efflux mechanism in the macrophage is mandatory to prevent cholesterol accumulation. High density lipoprotein (HDL) and its apolipoproteins, especially apolipoprotein A1 (apoA1), are responsible for the transfer of cholesterol from peripheral cells to the liver for biliary excretion, the process of reverse cholesterol transport.[4] ATP binding cassette transporter A1 (ABCA1), a membrane transporter abundant in macrophages, mediates this cholesterol and phospholipid (PL) efflux to lipid-poor apoA1, the precursor of HDL, and plays a major role in cholesterol homeostasis and reverse cholesterol transport.[5]

The various functions of ABCA1 became apparent after the discovery in 1999 that mutations in the ABCA1 gene caused Tangier disease (TD), an autosomal recessive hereditary disorder characterized by severe HDL deficiency, sterol deposition in macrophages and premature atherosclerosis.[6] TD patients with homozygote mutations in the ABCA1

[a]Department of Vascular Surgery, Faculty of Medicine, Imperial College, Charing Cross Hospital, London, UK; [b]MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Hospital, London, UK

Address for correspondence: Soni Soumian, Department of Vascular Surgery, Imperial College, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, UK. Tel: + 44 20 8846 7320; Fax: + 44 208 846 7362; E-mail: s.soumian@imperial.ac.uk

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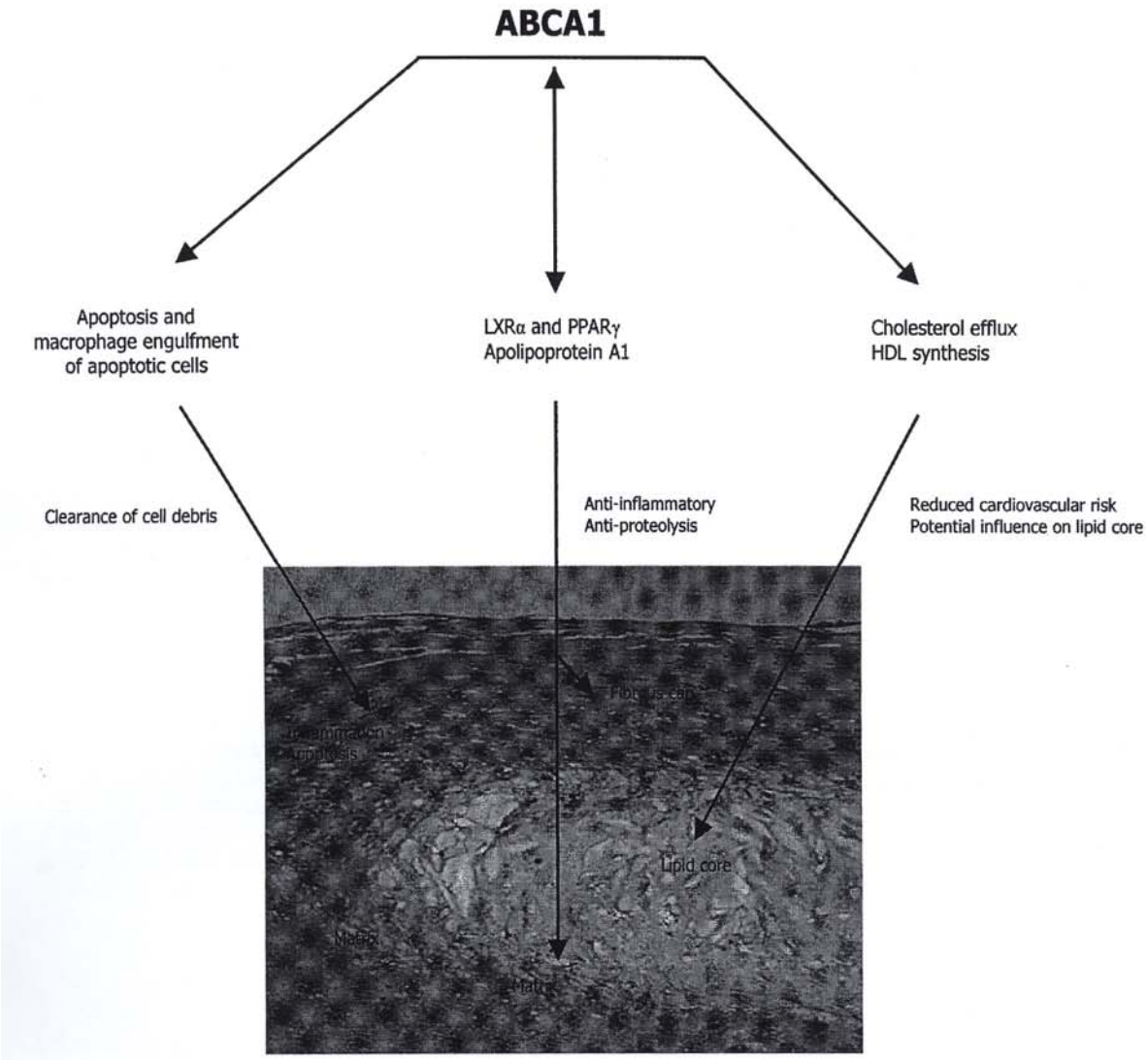

Figure 1 The influence of ABCA1 on the atherosclerotic plaque.

gene have virtually absent HDL and apoA1, decreased low density lipoprotein (LDL) level (40% normal) and hypertriglyceridemia.[7] The association between ABCA1 and HDL is ascertained by the fact that reduction in ABCA1 activity is associated with a significant decrease in plasma HDL levels.[8] Apart from its role in lipid metabolism, ABCA1 has also been implicated in promoting engulfment of apoptotic cells,[9] LDL oxidation[10] and the release of inflammatory mediators.[11,12] ABCA1 also binds to other lipoproteins including apolipoprotein E thus playing a role in its anti-atherogenic effect.[13]

Atherosclerotic plaque composition is the major determinant of plaque disruption and the ensuing complications. The aetio-pathogenesis of an atheromatous plaque is influenced by lipid metabolism, disordered cell turnover and extracellular matrix turnover within its structure. ABCA1 plays a role in all of these events, thus potentially implicating this transporter in the initiation, progression and pathogenesis of atherosclerotic vascular disease (Figure 1). In this review we will focus on the potential role of ABCA1 in atherosclerotic vascular disease.

Structure and distribution

ABCA1 is a member of the superfamily of ABC transporters that utilize ATP as a source of energy to transport various molecules across membranes.[14] The ABCA1 gene consists of 50 exons spanning 149 kb and encodes a protein that contains 2261 amino acids.[15] Immunofluorescence studies suggest that ABCA1 localises in the plasma membrane[16] although

shuttling of ABCA1 between intracellular endocytic components and the plasma membrane has been reported.[17] The structure of ABCA1 is characterized by the presence of two transmembrane domains with six helices each and a nucleotide binding domain containing two conserved peptide motifs (Walker A and B) that are characteristic for the superfamily of ABC transporters. It also has two large extracellular loops joined by a disulfide linkage which are thought to be important for the binding of apoA1.[18]

ABCA1 has been shown to be highly expressed in macrophages.[19,20] Though a near ubiquitous tissue expression has been proposed, Wellington et al[21] have shown a significant discordance between relative mRNA and protein expression patterns of ABCA1 in murine tissues.

Mechanism of cholesterol efflux

Cholesterol efflux occurs through different mechanisms, ABCA1 being one of them. The efflux of FC promoted by ABCA1 is considered to be unidirectional and by active transport to apoA1. It is suggested that the golgi apparatus processes excess intracellular FC into vesicles that are translocated to plasma membrane ABCA1 for exocytosis.[22] Recent literature suggests that ABCA1 mediates concurrent transport of FC and PL to apolipoproteins[23] though the availability of different lipids in the vicinity of ABCA1 may result in modification of the ratio of cholesterol/phospholipid undergoing efflux.[24] It also has been recently shown in transgenic mice that in vivo modification of the PL/apoA1 ratio by various lipases could play a major role in directing FC efflux through either the ABCA1 or the scavenger receptor B1 (SR-B1) pathway.[25]

ApoA1 and ABCA1

It is well established that free apolipoproteins play a role in mediating removal of cellular cholesterol.[26] ApoA1 is the most abundant apolipoprotein in HDL and is known to play a role in cholesterol efflux through ABCA1 dependent[27] and independent mechanisms.[28]

To date no clear consensus exists regarding the process by which apoA1 removes cholesterol and PL from the plasma membrane. There are studies supporting the theory of membrane solubilization wherein apoA1 and ABCA1 simultaneously remove both PL and cholesterol through a single step process.[29] Another proposed mechanism includes a two step process in which ABCA1 lipidates apoA1 with PL first to form phospholipid rich nascent HDL particles which then remove cholesterol from cells by diffusion.[30,31] The flopping of phosphatidylserine (PS) from the inner to the outer leaflet which is promoted by ABCA1 was also thought to play a role in apoA1 mediated lipid efflux[32] although a recent study suggests that the small increase in PS translocation caused by ABCA1 might be insufficient for apoA1 binding and lipid efflux.[33]

There is increasing evidence that apoA1 directly interacts with ABCA1.[27,34] Further proof of this are studies which showed that mutations in the extracellular loops of ABCA1 impaired both cross-linking with apoA1 and lipid efflux.[18] Moreover, truncation mutations of apoA1 lacking helix 10 impaired cholesterol transport through the ABCA1 pathway.[35] For efficient apoA1 mediated transport, a functional ABCA1 protein and specific binding at the plasma membrane[32] is crucial as mutated ABCA1 seen in diseases like TD[6] and familial HDL deficiency,[36] can result in increased catabolism of apoA1 thus affecting HDL production.[37]

Animal studies have revealed that apoA1 itself, independent of HDL cholesterol, has properties that protect against atherosclerosis.[38] ApoA1 has documented roles in inhibiting LDL oxidation,[10,39,40] and also has an anti-inflammatory effect both by inhibiting secretion of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α)[41] and via the removal of reactive oxygen species. It has been suggested that ABCA1 may be linked to these functions via apoA1.[10]

The apoA1 concentration in the extracellular fluid is dependent on synthesis, catabolism, dissociation and its reassociation with the lipoproteins in the plasma compartment represented by the HDL component.[42] This in turn could potentially influence lipid efflux in the plaque environment.

ABCA1 and the atherosclerotic plaque

The atherosclerotic plaque is a dynamic structure composed of lipids, cells and extracellular matrix. Plaques with high levels of collagen and smooth muscle cells providing a thick fibrous cap are characteristic of an advanced stable plaque, while unstable plaques have a thin cap, dense inflammatory infiltrate and a large lipid core.[43] Unstable plaques are prone to rupture with subsequent in situ thrombosis and embolism,[44] leading to significant clinical consequences. The behaviour of these plaques is highly unpredictable and the precise mechanisms causing the transition to instability are incompletely understood. The development and subsequent progression of plaques are determined by changes in its three major constituents, and ABCA1 could potentially influence all of the processes governing plaque architecture, namely:

(1) lipid accumulation and metabolism;
(2) alterations in cell turnover involving apoptosis and inflammation;
(3) altered extracellular matrix turnover.

ABCA1 and lipid metabolism

A large lipid core in the atheromatous plaque is one of the major indicators of potential instability. Inefficient macrophage function to clear lipids and cell debris could lead to an increase in the volume of lipid within the central core of the plaque. In the Tromso study[45,46] it was reported that lipid rich echolucent carotid plaques are associated with low levels of HDL cholesterol and increased risk of ischemic cerebrovascular events independent of the degree of stenosis and cardiovascular risk factors. Furthermore low HDL levels were shown to be associated with increased carotid intima-media thickness independent of other risk factors.[47]

Cholesterol from peripheral cells is returned to the liver by ABCA1 dependent and independent mechanisms. ApoA1 that is secreted or regenerated from HDL by the liver is lipidated by ABCA1 in peripheral cells to form pre-β HDL (nascent HDL) which then collects cholesterol from various cells. Although a basal level of phospholipidation of apoA1 may occur through an ABCA1 independent pathway,[48] the major part of it is done by ABCA1. It is also thought that the cholesterol processed from LDL receptor uptake in the liver is transferred to HDL by hepatic ABCA1 to be secreted into bile. In addition ABCA1 might also be involved in the removal of FC through apoA1 independent mechanisms via the formation of large membrane FC rich vesicles.[49]

Animal studies have provided further proof of ABCA1 function. Classical examples are ABCA1 knock out[42] and transgenic mice[50–53] as well as the Wisconsin Hypoalpha Mutant Chicken (WHAM)[54,55] which is the naturally occurring animal model of TD. In the context of human studies on ABCA1, a 2–3-fold increase in cardiovascular disease in TD patients and obligate ABCA1 heterozygotes as compared to age and sex matched controls from the Framingham study[56] was found. A recent study revealed that heterozygotes with ABCA1 mutations had lower amounts of cholesterol efflux, lower HDL concentrations and greater carotid intima-media thickness than the control group.[57] This study also indicated that the upper limit of normal intima-media thickness (0.80 mm) is reached in ABCA1 heterozygotes at the age of 55 as compared to 80 years in the control group.

In this context, a potentially variable role of ABCA1 in hepatocytes and macrophages towards its contribution to HDL formation has been suggested. Studies in mice have highlighted the importance of both hepatic and macrophage ABCA1. Mice specifically overexpressing ABCA1[58] in hepatocytes and macrophages showed significant increases in HDL levels. However, selective inactivation studies of ABCA1 in macrophages showed clear evidence of increased atherosclerosis in hyperlipidemic mice[58] but had very minimal impact on HDL formation.[59] Similarly mice selectively deficient in leukocyte ABCA1 developed large advanced atherosclerotic lesions without any change in plasma HDL levels.[60] This implies that hepatic ABCA1 may be responsible for the main contribution of ABCA1 to plasma HDL. Recent studies by Sahoo et al[61] seem to support this finding. Taken together, these findings suggest that hepatic ABCA1 exerts a generalized anti-atherogenic effect via its contribution to HDL formation while macrophage ABCA1 provides a peripheral anti-atherogenic effect on the vasculature.

However, interesting questions did arise by studying TD and ABCA1 deficient mice. First, the accumulation of cholesteryl esters in these conditions is localized primarily to macrophages and certain tissues.[7] The reason for this observation is still under investigation, but it has been suggested that lipids accumulate predominantly in tissues with high cell turnover and a large population of macrophages. Secondly, TD patients have only a 4-fold increase in atherosclerotic disease compared to the general population, despite the fact that more severe atherosclerosis would be expected as a consequence of the complete absence of HDL.

It has been proposed[7] that the source of cholesterol may differ between different macrophage subtypes with arterial macrophages primarily obtaining cholesterol from lipoproteins while other peripheral macrophages accumulate cholesterol from phagocytosed cell membranes. Thus despite ABCA1 deficiency and low HDL levels in TD patients, the expected aggressive atherosclerosis fails to develop in the background of a less atherogenic lipid profile (less than 40% LDL) which provides a potentially diminished cholesterol pool for arterial macrophages. Hence it may be speculated that in the presence of normal or increased levels of LDL, ABCA1 deficiency could potentially enhance atherosclerosis.

ABCA1 and alterations in cell turnover

Apoptosis

Apoptosis is known to play a major role in regulating cell turnover in the plaque. Apoptosis (Greek: leaf-fall), often used synonymously with programmed cell death, is a physiologically highly selective method to eliminate old or injured cells before the leakage of intracellular contents can induce an immune response. The initial phase of apoptosis has to be followed by an efficient phagocytic phase to render the process immunologically silent. Therefore recognition of an apoptotic cell by the phagocyte is crucial. After stimulation, PS, an anionic phospholipid which resides in the inner leaflet, is translocated to the outer leaflet, a process which is sufficient to guarantee recognition by the phagocyte.[62] ABCA1 was found to be involved in PS translocation and in promoting engulfment of apoptotic cells.[9] This study revealed that phagocytosis

is impaired in ABCA1 deficient macrophages and that forced expression of ABCA1 can confer engulfment ability to non-phagocytic cells. ABCA1 null animals were found to manifest reduced externalization of PS and similar findings were obtained in other studies.[33] The externalization of PS is paramount in the atherosclerotic plaque due to its known tendency to promote procoagulant potential[63,64] and the coagulation cascade. ABCA1 was also shown to interact with the Fas associated death domain (FADD) protein,[65] a process which may play a role in regulating apoptosis.

Inflammation

Cellular cholesterol levels determine the levels of intracellular oxygenated sterols which in turn regulates LDL oxidation, activation of IL-1[66] and promotion of apoptotic pathways.[67] ABCA1 which is proven to selectively remove cytotoxic FC from the cell[68] could potentially control intracellular oxysterol levels. Macrophages and activated T lymphocytes in the plaque[69] release proinflammatory cytokines, mainly interferon-gamma (IFN-γ) from T lymphocytes), TNF and IL-1 (both from macrophages). These cytokines have been reported to decrease the mRNA and protein levels of ABCA1.[16,70–73] Moreover, lipopolysaccharide found in infectious agents like *Chlamydia* was also reported to downregulate ABCA1 expression.[70,74] It has been demonstrated that IFN-γ can cause a 3-fold downregulation of ABCA1 and simultaneously an increase in acyl- CoA: cholesterol acyltransferase (ACAT) activity[71,73] thus promoting atherosclerosis. IL-1β, responsible for the activation of MMPs and other interleukins have also been reported to inhibit ABCA1 function thus promoting lipid accumulation.[72] It has been recently reported that myeloperoxidase present in atheromas[75] promotes atherogenesis by impairing ABCA1 function.[76] There is evidence that ABCA1 is involved in the secretion of IL-1β and macrophage inhibitory factor (MIF) from macrophages and monocytes respectively.[11,12,77] However, the role of ABCA1 in the transport of these proinflammatory cytokines needs further evaluation.

Hence it could be speculated that a vicious cycle might ensue where the initial cytokine induction could cause ABCA1 deficiency, which in turn results in inadequate clearance of apoptotic and necrotic cells that further aggravates inflammatory mediators leading to plaque instability. All of the previously mentioned findings suggest that therapeutic upregulation of ABCA1 expression could be a promising approach in the context of treatment of atherosclerosis.

ABCA1 and extracellular matrix turnover

The integrity of the extracellular matrix (ECM) which influences the strength of the fibrous cap in the plaque[78] is determined by the balance between matrix metalloproteinases (MMP) and tissue inhibitors of MMPs.[79] There is general agreement that increased levels of intra-plaque MMPs could lead to plaque rupture[80] and clinically significant plaque instability has been shown to be associated with increased levels of MMP-9.[81] The MMPs which are secreted by inflammatory cells promote VSMC migration and ECM degradation.[82] The proinflammatory cytokine IL-1β is reported to upregulate MMP-9 production.[83,84] The interaction of ABCA1 with apoA1 may reduce MMP-9 production as apoA1 is documented to suppress cytokine IL-1β production.[41] Furthermore the nuclear receptors liver-X-receptor (LXR) and peroxisome proliferator-activated receptor (PPAR), which are the key regulators of ABCA1 function, have documented anti-inflammatory and anti-MMP activity.[85–89]

Regulation of ABCA1

The pivotal role played by ABCA1 in cholesterol homeostasis mandates a tight regulatory pathway. In addition to increased lipid efflux, a consequence of ABCA1 overexpression could also be the potential alteration of membrane structure with subsequent detrimental effects.[16] Evidence for the tight regulation of ABCA1 is the short half life of the ABCA1 protein[90,91] and its rapid turnover in macrophage cell lines.[92] It is regulated at transcriptional and post-transcriptional levels (Figure 2).

Transcriptional regulation

Sterols

The large intracellular sterol concentration in foam cells plays a major role in ABCA1 regulation. Acetylated LDL upregulates ABCA1 mRNA and protein which can be reversed by incubation of these macrophages in HDL.[19] It was also reported that ABCA1 mRNA and protein were upregulated in a time and dose dependent fashion by native LDL.[93]

Nuclear receptors

Nuclear receptors are ligand activated transcription factors that regulate the expression of their target genes. The tissue specific expression and ligand availability tightly controls its activity.[94] LXR and PPAR, belonging to a subgroup called adopted orphan nuclear receptor group, are the major players in ABCA1 regulation and will therefore be discussed below.

LXRs act as cholesterol sensors that respond to elevated sterol concentrations and activate a cadre of genes that govern transport, catabolism and elimination of cholesterol.[95] LXRα expressed in the macrophage is important in terms of plaque physiology as it seems to be capable of activating and suppressing the ABCA1 gene based on ligand availability.[96] The ligands that activate these receptors in the macrophage are mainly oxysterols like 27-hydroxycholesterol.[97]

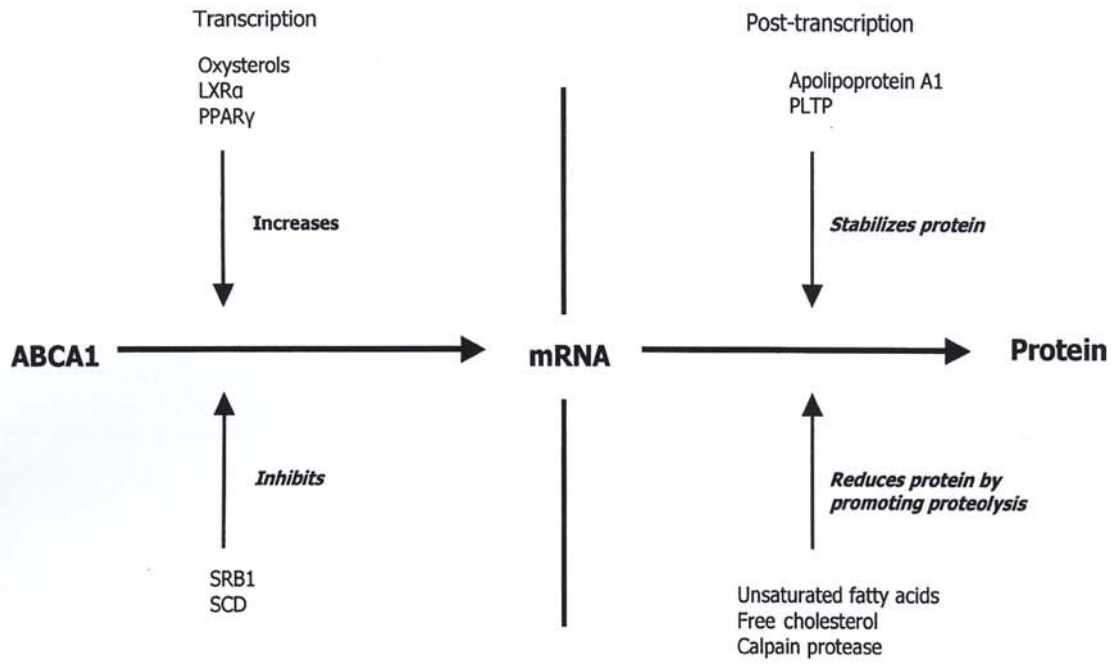

Figure 2 Regulation of ABCA1 at transcriptional and post-transcriptional levels.

LXRα influences ABCA1 expression transcriptionally and post-transcriptionally (Figure 3). Studies have reported that the oxysterol induction of ABCA1 is partly through the LXR pathway.[98,99] Recent studies have highlighted the anti-atherogenic activity of LXR.[100,101] In human macrophages exclusively, it has been observed that the LXRα gene itself is a target for the LXR signaling pathway, an effective autoregulatory mechanism to amplify the ABCA1 lipid efflux pathway.[102,103]

PPARs have been implicated in the regulation of both inflammation and lipid homeostasis in the macrophage and polyunsaturated fatty acids serve as their ligands.[94] PPARγ enhances cholesterol efflux by inducing the transcription of LXRα and thus ABCA1.[104,105] The observation that PPAR agonists and LXR ligands are ineffective in macrophages of TD patients[99,105] indicates that functional ABCA1 is required for this pathway of cholesterol efflux. With regard to the role of these nuclear receptors in the context of plaque pathophysiology, LXRα and PPARγ have been documented to have anti-inflammatory[86–88] and anti-MMP activity.[85,89] The PPARγ–LXRα –ABCA1 cascade does represent a powerful means of cholesterol efflux from the macrophage and therefore could play a significant role in influencing the progression of atherosclerotic plaques.

Post-transcriptional regulation

Studies in mice have revealed a significant discordance between ABCA1 protein and mRNA levels, suggesting that post-transcriptional regulation plays a major role[21] in ABCA1 protein expression. Unsaturated fatty acids have been found to promote ABCA1 protein degradation directly[92,106] and indirectly.[107,108] This may be significant in disorders like type 2 diabetes and insulin resistance, conditions with increased levels of fatty acids where accelerated atherosclerosis is observed. Cytotoxic levels of intracellular FC[109] were also found to promote ABCA1 protein degradation through the proteolysis pathway. ABCA1 phosphorylation, which is reported to be influenced by apoA1[110] and protein kinase C,[111] may also have a major effect on protein stability. The PEST sequence identified in ABCA1 by Wang et al[112] appears significant in protein degradation as deletion of this motif resulted in a 4–5-fold increase in ABCA1 protein, increased ABCA1 mediated efflux and enhanced apoA1 binding.

In vitro experiments with peritoneal macrophages, transfected cells and mouse primary hepatocytes have shown that apoA1 binding increased ABCA1 protein without affecting mRNA levels.[112] Interestingly this apoA1 mediated stabilization of ABCA1 protein is achieved by inhibition of PEST sequence mediated degradation by proteases.[90,112] There is also evidence that PLTP interacts with ABCA1 for its function in cholesterol efflux and also stabilizes ABCA1 protein.[113]

We have reported that, in human carotid atherosclerotic plaques, ABCA1 protein is significantly reduced despite increased mRNA.[114] The observed upregulation in this study of both LXRα and ABCA1

mRNA in atherosclerotic tissues could be attributed to the oxysterol-rich environment inside the plaque potentially amplified by low ABCA1 protein levels. It is possible that the degradation of ABCA1 protein in the plaque microenvironment might be the key factor influencing cholesterol homeostasis at the macrophage level. The resulting localized deficiency in ABCA1 function could lead to decreased lipid efflux, accumulation of oxysterols and acceleration of the atherosclerotic process.

Therapeutic modulation

The nuclear receptors PPARγ and LXRα are promising targets for pharmacological manipulation of the ABCA1 pathway. In vitro studies have shown that PPAR agonists such as the glitazones used in type 2 diabetes induce cholesterol efflux from macrophages through the activation of ABCA1.[105,115] It is interesting to note that PPAR agonists can effectively override the cytokine IL-1β induced suppression of ABCA1 and promote cholesterol efflux.[72] This effect in itself may be valuable in terms of plaque pathophysiology. ACAT inhibitors which increase intracellular FC levels have also been reported to upregulate ABCA1 both at the mRNA and protein level[116] but are unlikely to be considered for general use.

Studies on rats showed that clofibrate, widely used as a hypolipidemic agent, increases ABCA1 mRNA through activation of LXRα.[117] Recently verapamil was shown to enhance both ABCA1 mRNA and protein expression through LXR independent mechanisms.[118] Ando et al[119] demonstrated that in vivo pravastatin increased LXRα mRNA levels. Statins increase HDL levels in addition to the significant reductions in total cholesterol and triglycerides.[120,121] In fact it is suggested that the increase in HDL cholesterol produced by statins could partly also be through the upregulation of ABCA1 and apoA1.[122,123] PPAR and LXR agonism may be beneficial in terms of its generalized effect on HDL and inflammation but its effect on the localized atheromatous plaque has not been studied in detail.

The benefits of transcriptional upregulation may be potentially annulled by the in vivo plaque microenvironment that promotes ABCA1 protein degradation.[114] In addition to ABCA1 transcriptional upregulation, ABCA1 protein stabilization may be crucial to promote function of cholesterol efflux.

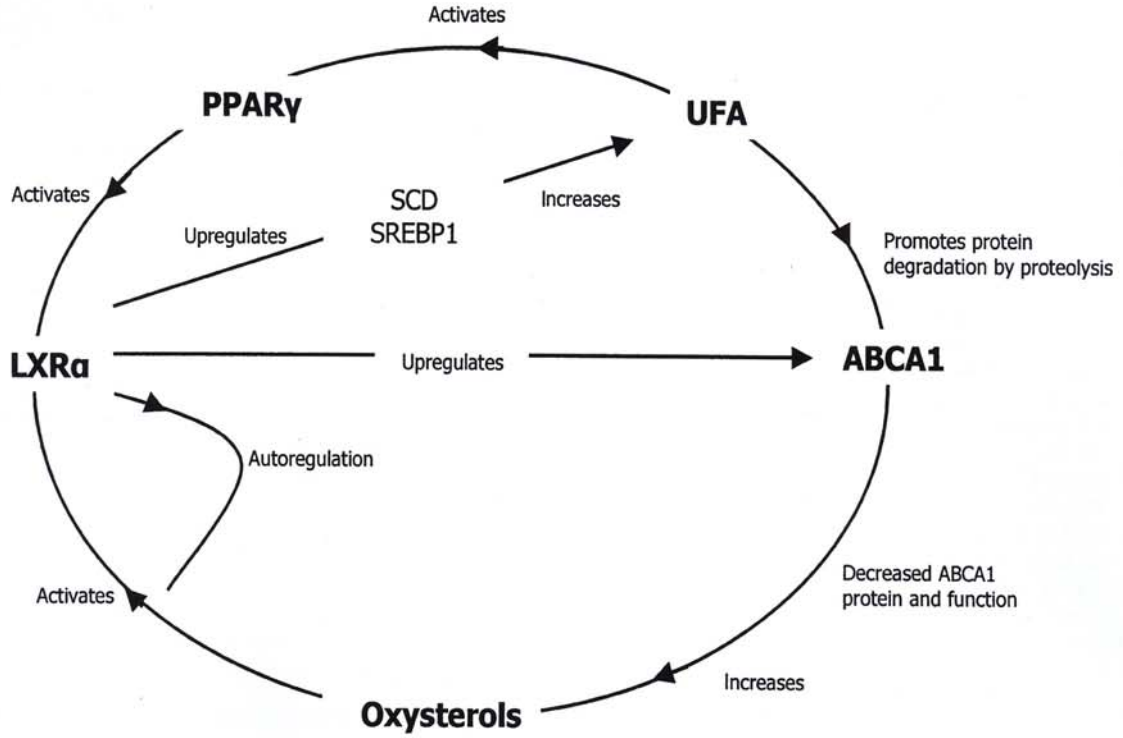

Figure 3 Influence of nuclear receptors LXRα and PPARγ on ABCA1 expression. LXRα has been shown to activate the sterol regulatory element binding protein-1 (SREBP-1)[124] and stearoyl-CoA desaturases-1 and 2 (SCD)[108] which results in increased unsaturated fatty acid synthesis. These unsaturated fatty acids serve as ligands for PPAR activation[94] but have been implicated in causing ABCA1 protein degradation.[92,106] Therefore the enhanced ABCA1 transcription induced by LXR ligands may be counteracted by increased ABCA1 protein degradation.

Synthetic peptides based on the structure of apoA1 have been found to be effective in stabilizing ABCA1 protein.[125,126] Calpain protease inhibitors and specific LXR agonists[127] are further venues for exploration in the quest to promote cholesterol efflux therapeutically. Therefore it would be interesting to assess the effect of combined transcriptional upregulation with protein stabilization of ABCA1 in the context of treatment of atherosclerosis.

Conclusion

The critical role of ABCA1 at the macrophage and hepatic level regarding lipid efflux and HDL formation could have a major influence on the biogenesis and progression of atheromatous plaques. Further studies need to be focused on apoA1 and ABCA1 interactions that seem to regulate each other and have a potent role in protection against atherosclerosis. The PPAR–LXR–ABCA1 cascade has a substantial role in cholesterol homeostasis and inflammation and therefore has a promising potential for therapeutic manipulation. The efficacy of combined therapy with ABCA1 transcriptional upregulation and protein stabilization needs to be evaluated. Further work needs to be undertaken to unravel other functions of this transporter and its pathways so that therapeutic strategies could be devised to prevent atherosclerotic disease and its complications.

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Leukocyte *ABCA1* Gene Expression Is Associated With Fasting Glucose Concentration in Normoglycemic Men

C. Albrecht, I. Simon-Vermot, J.I. Elliott, C.F. Higgins, D.G. Johnston, and J. Valabhji

Adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) mediates the efflux of cholesterol to apolipoprotein A1, a process necessary for high-density lipoprotein (HDL) formation and reverse cholesterol transport. In patients with Tangier disease, mutations in *ABCA1* result in low circulating HDL-cholesterol and predisposition to coronary heart disease (CHD). *ABCA1* gene expression is decreased in diabetic mice. In humans, glycated hemoglobin (HbA_{1c}) predicted future CHD events, even within the normal range. We hypothesised that leukocyte *ABCA1* gene expression would be inversely associated with indices of glycemia in normoglycemic men. Fasting blood samples were taken from 32 healthy, nonsmoking, normoglycemic men (age 23 to 46 years). *ABCA1*, peroxisome proliferator–activated receptor gamma (*PPARγ*), and liver X receptor alpha (*LXRα*) gene expressions in circulating leukocytes were measured using TaqMan technology. Significant inverse associations between *ABCA1* gene expression and both fasting glucose concentration ($r = -0.49$, $P = .008$) and age ($r = -0.39$, $P = .043$) were found. There was no association with HbA_{1c} ($r = -0.23$, $P = .238$) or HDL-cholesterol concentration ($r = 0.02$, $P = .904$). In a multiple regression model, fasting glucose remained a significant independent predictor ($P = .037$), whereas age did not ($P = .226$). Mechanisms underlying the association were explored; there were no significant associations between fasting glucose concentration and leukocyte *PPARγ* gene expression, or between fasting glucose concentration and leukocyte *LXRα* gene expression. This is the first demonstration of an association between *ABCA1* gene expression and fasting glucose concentration in vivo.

A LOW CONCENTRATION of high-density lipoprotein (HDL)-cholesterol is a powerful and independent predictor of coronary heart disease (CHD),[1,2] and this is thought, though not proven, to relate to the role of HDL in reverse cholesterol transport. Recent studies have demonstrated that adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) is highly regulated in macrophages and directly or indirectly mediates the efflux of cholesterol and phospholipids to apolipoprotein A1, a process necessary for HDL formation, and the first step in reverse cholesterol transport.[3-5] Whether ABCA1 is itself the cholesterol transporter or whether it regulates the transport process is not yet known. The identification of mutations in *ABCA1* in patients with Tangier disease and familial HDL deficiency[6-8] demonstrated that inadequate transport of phospholipid and cholesterol to the extracellular space results in the hypercatabolism of lipid-poor nascent HDL particles, low circulating HDL-cholesterol, and the predisposition to CHD. More common variants in the gene encoding ABCA1, such as the R219K variant, which has a carrier frequency of 46% in Europeans, are also associated with altered lipid levels and a modified risk of CHD.[9]

The association between ABCA1 and CHD is only partly explained by HDL-cholesterol concentrations. Indeed, in apparently normal human populations, an association between ABCA1 and HDL-cholesterol has not been clearly demonstrated. Common variants in the gene encoding ABCA1 are not associated with changes in HDL-cholesterol, despite modifying CHD risk.[10] ABCA1 regulatory variants influence CHD-independent of effects on HDL-cholesterol.[11] In low-density lipoprotein (LDL)-receptor knockout mice, leukocyte ABCA1 plays a critical role in the protection against atherosclerosis, but disruption of leukocyte ABCA1 function does not affect plasma HDL-cholesterol concentrations despite increasing the atherosclerotic risk.[12] Experiments involving bone marrow transplantation in ABCA1-deficient and wild-type mice suggest that macrophage expression of ABCA1 makes minimal contribution to plasma HDL concentrations.[13]

Diabetes is associated with increased CHD risk.[14,15] The risk is in excess of that predicted by lipid parameters, blood pressure, smoking status, and age. Whether hyperglycemia itself mediates the excess risk is unproven. *ABCA1* gene expression is decreased in the liver and peritoneal macrophages of diabetic compared to control mice.[16] Reduced ABCA1 function could therefore contribute to the excess CHD risk in human diabetes, and this could be independent of HDL-cholesterol. The potential importance of glucose itself is suggested by a recent prospective population-based study in men, the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition. This study has demonstrated that glycated hemoglobin (HbA_{1C}), a measure of average circulating glucose over 6 to 8 weeks, predicted future CHD events even with HbA_{1C} levels in the normal range.[17]

The direct effect of glucose on *ABCA1* gene expression has been examined in vitro, although results have been conflicting. Cultured HepG2 cells were grown in medium with 5.5, 12.5, or 25 mmol/L glucose. Increasing glucose concentration in the medium was associated with increasing ABCA1 mRNA levels.[18] However, incubation of cultured HepG2 cells with glucose has also been shown to have no effect on ABCA1 mRNA.[16]

We have examined expression of the *ABCA1* gene in human leukocytes obtained from healthy, nondiabetic men and inves-

From the Medical Research Council Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Hospital; and the Department of Endocrinology and Metabolic Medicine, Imperial College School of Medicine, St Mary's Hospital, London, UK.

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Address reprint requests to Jonathan Valabhji, MD, Department Endocrinology and Metabolic Medicine, Imperial College School of Medicine, St Mary's Hospital, Praed Street, London W2 1NY, UK.

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tigated its relationship to circulating glucose concentrations and to lipid, lipoprotein, and apolipoprotein parameters, including HDL-cholesterol. We hypothesized that leukocyte *ABCA1* gene expression would be associated with indices of glycaemia.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate lipid and glucose metabolism and cellular differentiation. PPARγ activators induce *ABCA1* gene expression.[19] Liver X receptors (LXR) also regulate *ABCA1* expression by binding to response elements in the *ABCA1* gene.[20] In order to investigate potential underlying mechanisms for an association between glucose and ABCA1, we also measured leukocyte mRNA expression of PPARγ and LXRα.

MATERIALS AND METHODS

Subjects

Thirty-two healthy male subjects between 20 and 50 years of age were recruited from hospital staff. All subjects were nonsmokers, and were not known to have impaired glucose tolerance, diabetes, hyperlipidemia, or impaired renal or thyroid function. None were known to be hypertensive or to have any significant medical disorders. Subjects were assessed clinically by the same investigator, following an 8-hour overnight fast. Blood pressure was measured once using an Omron 711 automatic sphigmomanometer (Omron Healthcare, Henfield, UK) after the subject was rested sitting for 10 minutes. Venous blood samples were obtained in the overnight fasting state. Ethical committee approval was obtained from the Local Research Ethics Committee, St Mary's NHS Trust, and informed consent was obtained from all participating subjects.

Laboratory Measurements

Creatinine, HbA_{1c}, and fasting glucose concentrations were measured using standard laboratory techniques. Cholesterol, triglyceride, and HDL-cholesterol concentrations were measured using the appropriate Olympus System Reagents (6116 and 6216, 6133, and OSR 6156, respectively; Olympus Diagnostic Systems, Southall, UK), on an Olympus AU640 analyzer system. Apolipoprotein concentrations were measured using rate immunonephelometry on an Array 360 Protein System (Beckman Coulter UK, High Wycombe, UK).

ABCA1 Gene Expression

cDNA preparation. Leukocytes were isolated from 10 mL whole blood according to the Qiagen RNeasy Midi manual (Qiagen, Crawley, UK). Briefly, erythrocytes were selectively lysed in erythrocyte lysis buffer (EL; Qiagen). Leukocytes were pelleted by centrifugation at $400 \times g$ for 10 minutes at 4°C, washed twice with EL, and resuspended in 4 ml RLT buffer (Qiagen) containing β-mercaptoethanol. Samples were homogenized for 1 minute using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) and total RNA was extracted using RNeasy midi columns (Qiagen). Contaminating DNA was removed from RNA by incubation with DNAse I (Promega, Southampton, UK). For cDNA synthesis, RNA (1 μg) was transcribed with a first strand cDNA synthesis kit for reverse-transcription polymerase chain reaction (RT-PCR; Roche, Hertfordshire, UK), according to the supplier's instructions. The RT reaction consisted of 0.08 U random hexamer primers, 20U Avium myeloblastosis virus (AMV) RT, 5 mmol/L $MgCl_2$, 1 mmol/L deoxynucleotide mix, 40 U RNAse inhibitor, and 1x reaction buffer (Roche). For Taqman analysis, the cDNA was diluted 5-fold with nuclease-free water.

Real-time quantitative RT-PCR (TaqMan). Primers and probe for TaqMan analysis of ABCA1 mRNA were designed to span 2 adjacent exons with PrimerExpress software (PE Applied Biosystems, Warrington, UK) and the reaction optimized according to PE User bulletin no. 2 (PE Applied Biosystems, *www.pebio.com*). The forward primer was GGGAGGCTCCCGGAGTT in exon 3, the reverse primer was GTATAAAAGAAGCCTCCGAGCATC in exon 4, and the FAM-labeled probe, spanning exons 3 and 4, was AACTTTAACAAATC-CATTGTGGCTCGCCTGT. Primers and FAM-labeled fluorescent probes for LXR alpha (5′-3′ CAAGTGTTTGCACTGCGTCT, 5′-3′ CAGGAATGTTTGCCCTTCTC, probe 5′-3′ CACTTCTAGGAG-GCAGCCAC) and PPARγ (5′-3′ CCAGTGGTTGCAGATTA-CAAGTCTG, 5′-3′ TTGTAGAGCTGAGTCTTCTCAGAATAATAAG, probe 5′-3′ ACTTCAAGAGTACCAAAGTGCAATCAAAGTGGAG) were kindly provided by Drs Patel and Smith (Glaxo Smith Kline, Uxbridge, UK). Single-tube TaqMan analysis was performed on an ABI prism 7700 sequence detection system with 300 nmol/L of forward and reverse primers in the presence of 200 nmol/L 5′FAM-3′TAMRA-tagged probe for ABCA1. TaqMan measurements for LXRα and PPARγ were performed with 900 nmol/L of forward and reverse primers and 300 nmol/L 5′FAM-3′TAMRA-tagged probe. The internal standard was glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were performed in triplicate and contained 5 μL of diluted cDNA in a total volume of 25 μL.

Quantitation. The amount of ABCA1, LXRα, and PPARγ mRNA in cells was calculated according to the relative standard curve method described in the PE User bulletin no. 2. Briefly, standard curves were determined for all target genes (*ABCA1*, *LXRα*, *PPARγ*) and G3PDH using human liver RNA samples. Target quantity was calculated from the standard curve, normalized to G3PDH (as is conventional with measurements of this kind) and expressed relative to a calibrator, which in our experimental design was defined as the mean of all ABCA1 measurements after normalization. Thus, all quantities are unitless and expressed as n-fold differences relative to the calibrator.

Validation. Following extraction of mRNA, subject samples were batched. RT reactions were performed and *ABCA1* gene expression was then determined for all the samples in one assay. Both RT reactions and TaqMan measurements of *ABCA1* gene expression were repeated for all samples in a second assay measured on a different day. It was therefore possible to calculate the interassay coefficient of variation for measurement of *ABCA1* gene expression. For subsequent analyses, the means of these 2 values for *ABCA1* gene expression were used. Four subjects had very high measured levels of *ABCA1* gene expression after normalization with G3PDH due to a very low signal for G3PDH in the TaqMan determinations (between 16- and 23-fold lower compared to the mean G3PDH value for the other subjects). It was important to determine whether these 4 values were true values for *ABCA1* gene expression, or whether they represented either different levels of expression of the internal standard, G3PDH, or interference with the G3PDH fluorescence-based assay. A third TaqMan assay was therefore performed using a probe and oligos for β-actin (PE Applied Biosystems) as internal standard. When β-actin was used as the internal standard for the TaqMan determinations and analysis, the levels of *ABCA1* gene expression for these 4 subjects fell within the range of values for the other subjects. It was therefore assumed that the high measured levels of *ABCA1* gene expression in these 4 subjects resulted from different levels of expression of the internal standard G3PDH or an interference with the fluorescence signal. These 4 subjects were therefore excluded from the main analyses. The interassay coefficient of variation for measurement of *ABCA1* gene expression was 24%. Similarily, *LXRα* and *PPARγ* expression levels in leukocytes were determined both with GAPDH and β-actin as housekeeping genes and analyzed as mentioned above for ABCA1. The interassay coefficient of variation for *LXRα* and *PPARγ* gene expression were 29% and 23%, respectively.

Table 1. Subject Characteristics (N = 32)

Parameter	Mean (or median)	Standard Deviation (or interquartile range)	Range
Age (yr)	30 (median)	27-34 (interquartile range)	23-46
Body mass index (kg/m^2)	25.6	3.1	20.3-33.3
Systolic blood pressure (mm Hg)	121	10	90-143
Diastolic blood pressure (mm Hg)	82	8	60-97
Fasting glucose (mmol/l)	4.9	0.4	4.2-5.6
HbA_{1c} (%)	4.8	0.4	4.1-5.7
Creatinine (μmol/L)	105	8	93-128
Total cholesterol (mmol/L)	4.94	0.94	2.86-7.54
Triglyceride (mmol/l)	0.89 (median)	0.69-1.53 (interquartile range)	0.41-4.27
HDL-cholesterol (mmol/L)	1.32	0.29	0.88-2.02
Apolipoprotein B100 (mg/dL)	99	28	47-176
Apolipoprotein A1 (mg/dL)	129	15	104-156

Statistical Analysis

Continuous variables with normal distributions are expressed as means with standard deviations. Continuous variables with skewed distributions are expressed as medians with interquartile ranges. Linear regression was used to assess the relationship between continuous variables. Residual analyses were performed: a scatterplot of the residuals versus the fitted values established homoscedasticity; the residuals were tested for normality using the Shapiro-Wilk W test. If the residuals did not have constant variance or were not normally distributed, then a variable was $\log_e$-transformed prior to regression. A 2-tailed P value less than .05 was considered significant. The Arcus Quickstat Biomedical package was used for the analyses (Longman Software Publishing, Cambridge, UK).

RESULTS

Thirty-two subjects were recruited. Four subjects were excluded (as described in above), so that 28 subjects were included in the main analyses. Table 1 shows subject characteristics, including blood pressure, lipid, and glucose parameters. The characteristics of the 4 excluded subjects were not significantly different (data not shown).

There was a strong inverse association between *ABCA1* gene expression ($\log_e$-transformed) and fasting glucose concentration ($r = -0.49$, $P = .008$; Fig 1). There was no significant association with HbA_{1c} ($r = -0.23$, $P = .238$). There was a significant inverse association between *ABCA1* gene expression and age ($r = -0.39$, $P = .043$; Fig 2). There were no significant associations between *ABCA1* gene expression and HDL-cholesterol concentration ($r = 0.02$, $P = .904$), apolipoprotein A1 concentration ($r = -0.17$, $P = .400$), total cholesterol concentration ($r = -0.11$, $P = .587$), triglyceride concentration ($\log_e$-transformed) ($r = 0.06$, $P = .773$), apolipoprotein B100 concentration ($r = -0.09$, $P = .646$), systolic blood pressure ($r = 0.10$, $P = .624$), or diastolic blood pressure ($r = -0.07$, $P = .720$).

There was an association between fasting glucose concentration and age ($r = 0.39$, $P = .040$). In a multiple linear regression model with *ABCA1* gene expression ($\log_e$-transformed) as the dependent variable and age and fasting glucose as predictors, fasting glucose remained a significant independent predictor ($P = .037$), whereas age did not ($P = .226$).

There were no significant associations between fasting glucose concentration and *PPARγ* gene expression ($r = 0.32$, $P = .113$) or between fasting glucose concentration and *LXRα* gene expression ($\log_e$-transformed) ($r = 0.12$, $P = .546$). There were no significant associations between *ABCA1* gene expression ($\log_e$-transformed) and *PPARγ* gene expression ($r = 0.148$, $P = .471$) or between *ABCA1* gene expression ($\log_e$-transformed) and *LXRα* gene expression ($r = -0.34$, $P = .082$).

To confirm the validity of the association between *ABCA1* gene expression and fasting glucose concentration, the association between *ABCA1* gene expression measured relative to beta actin as the internal standard and fasting glucose concen-

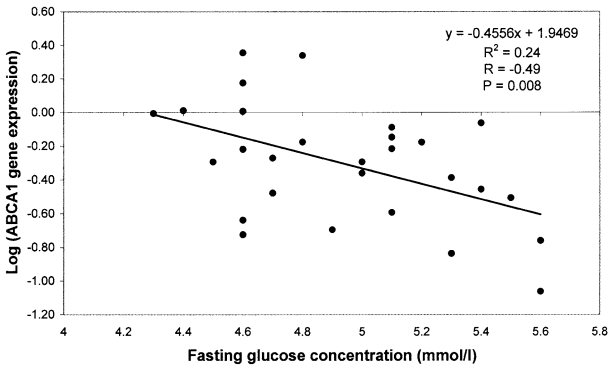

Fig 1. *ABCA1* gene expression ($\log_e$-transformed) *v* fasting glucose concentration (N = 28).

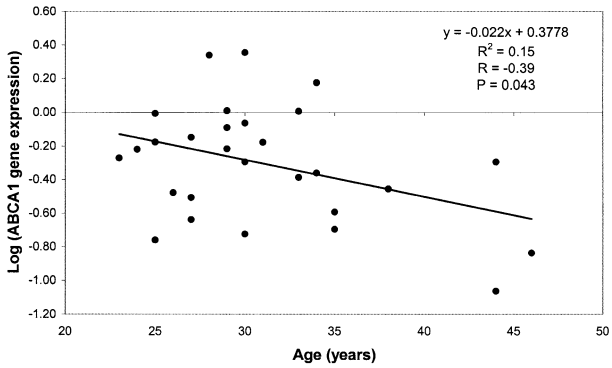

Fig 2. *ABCA1* gene expression ($\log_e$-transformed) *v* age (N = 28).

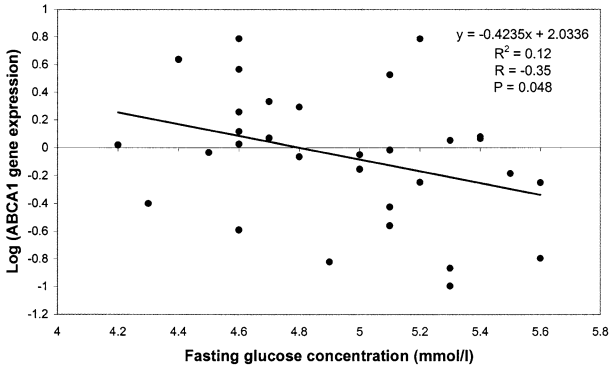

Fig 3. *ABCA1* gene expression (log_e-transformed) measured using β-actin as the internal standard *v* fasting glucose concentration (N = 32).

tration was assessed in all 32 subjects. The association remained significant ($r = -0.35$, $P = .048$; Fig 3).

DISCUSSION

This is the first demonstration of an association between *ABCA1* gene expression and fasting glucose concentration in vivo. The fact that the significant association was with fasting glucose concentration, and not HbA_{1c}, may reflect the fact that the time-scale for changes in *ABCA1* gene expression more closely resembles that for glucose (minutes) than that for HbA_{1c} (weeks).

Results from the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition demonstrated that an index of glycemia (HbA_{1c}), even within the normal range, predicted future CHD events.[17] While an association between *ABCA1* gene expression and fasting glucose concentration could link indices of glycemia and CHD events, no marker for CHD was assessed in the current study.

It has been demonstrated that unsaturated fatty acids decrease ABCA1 activity in cultured macrophages and, since type 2 diabetes is associated with increased circulating nonesterified fatty acids, it has been suggested that this could explain the excess CHD risk in this condition.[21] However, in the same study, the fatty acid effect was through increased ABCA1 protein degradation, and levels of ABCA1 mRNA were not affected.[21] Others, however, have been able to demonstrate that unsaturated fatty acids downregulate ABCA1 mRNA.[16] Unfortunately, fatty acids were not measured in the current study.

Hyperglycemia is common to both type 1 and type 2 diabetes. Type 1 diabetes is associated with normal or even increased HDL-cholesterol concentrations,[22] whereas type 2 diabetes is associated with decreased HDL concentrations.[23] The results suggest that, if low *ABCA1* gene expression is involved in the higher CHD risk of both type 1 and type 2 diabetes, this could be through an association with glucose concentration, rather than HDL-cholesterol.

Despite the prespecified young age of the cohort of subjects in the present study, there was a significant univariate association between *ABCA1* gene expression and age. This is the first study to demonstrate such a relationship. It has been observed that in individuals heterozygous for *ABCA1* mutations, HDL-cholesterol is inversely associated with age.[24] This was assumed to be mediated by decreased ABCA1 activity, although no ABCA1 data were reported.[24]

The association between age and CHD is so strong that any important pathophysiological step in the disease process would also have to show a strong association with age. Age is a surrogate marker for physiological or pathological characteristics that change with time. An important challenge is to identify which physiological or pathological characteristics mediate the association between age and CHD. It is possible that decreased *ABCA1* gene expression is one such characteristic and may result from increasing glucose concentrations (even within the normal range) with ageing. This is suggested by the observation in the current study that age is no longer a significant independent predictor of *ABCA1* gene expression when fasting glucose concentration is considered.

Although ABCA1 activity appears to play an important role in reverse cholesterol transport, it is one of many factors that contribute to the amount of cholesterol carried on HDL particles. Other important factors include lecithin:cholesterol acyltransferase and cholesterol ester transfer protein activities, lipoprotein and hepatic lipase activities, triglyceride concentrations, and activity of the hepatic scavenger receptor B-1, as well as concentrations of the apolipoproteins A1 and A2. In a murine model, discordance was seen between relative ABCA1 mRNA and protein expression in different tissues, suggesting the possibility of post-transcriptional regulation of *ABCA1* expression.[25] In turn ABCA1 protein levels may not be an accurate determinant of ABCA1 activity, if activity is regulated. These factors may all contribute to the lack of a demonstrable association between *ABCA1* gene expression and HDL-cholesterol concentration.

PPARγ activators induce *ABCA1* gene expression and also increase apolipoprotein AI–induced cholesterol efflux from macrophages.[19] PPARγ activation in vivo, using thiazolidinedione agents, results in reduced glucose concentrations.[26] However, there was no association between glucose and levels of *PPAR*γ gene expression. There was also no association between glucose and levels of *LXR*α gene expression. While this may suggest that the association between glucose and *ABCA1* expression is mediated via other regulatory pathways, it should be noted that we were also unable to demonstrate associations between *ABCA1* expression and expressions of *PPAR*γ and *LXR*α, despite their acknowledged roles in the regulation of *ABCA1* expression. Given the small numbers in the study, the lack of associations between glucose and *PPAR*γ expression and *LXR* expression may therefore represent type 2 errors.

In summary, we have demonstrated an inverse association between *ABCA1* gene expression and fasting glucose concentration in vivo. The association could have implications regarding the higher CHD risk associated with hyperglycemic states.

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Placental ABCA1 expression is reduced in primary antiphospholipid syndrome compared to pre-eclampsia and controls.

Albrecht C*[1,5], Soumian S*[1], Tetlow N*[2], Patel P[2], Sullivan M[2], Lakasing L[3], Nicolaides K[4], Williamson C[2].

[1]MRC Clinical Sciences Centre and [2]Institute of Reproductive and Developmental Biology, Imperial College London, Du Cane Road, London W12 0NN. [3]Department of Obstetrics and Gynaecology, St Mary's Hospital, London W2 1PG. [4]Harris Birthright Centre for Fetal Medicine, Denmark Hill, London SE5 9RS. [5]Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, 85354 Freising, Germany

*these authors contributed equally to the work

Author for correspondence:
Catherine Williamson
Institute of Reproductive and Developmental Biology
Imperial College London
Du Cane Road
London W12 0NN

Tel. +44 20 7594 2197
Fax. +44 20 7594 2154
Email: catherine.williamson@imperial.ac.uk

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Introduction

Placental cholesterol metabolism and lipid transport are crucial to the survival and development of the fetus [1,2]. In addition to significant endogenous cholesterol production, [3,4] the fetus may obtain cholesterol from exogenous sources including the placenta and maternal circulation. Furthermore cholesterol is the substrate for human placental steroid hormone synthesis[5] which is central to maintenance of pregnancy through its promotion of Th2 cytokines [6,7]. The placenta is involved in the metabolism of lipoproteins [8,9] and recent studies have indicated that maternal cholesterol is a source for fetal cholesterol [10,11]. The ATP binding cassette transporter A1 (ABCA1), a membrane protein, mediates cholesterol and phospholipid efflux to lipid-poor apolipoprotein (apo) A1, the precursor of high density lipoprotein (HDL), and plays a major role in cholesterol metabolism [12]. ABCA1 deficiency results in Tangier disease and familial HDL deficiency which are characterised by premature atherosclerosis and lipid deposition in reticuloendothelial tissues[12-14].

ABCA1 is highly expressed in human tissues that are involved in cholesterol metabolism and steroidogenesis including the liver, adrenals and testis [15]. Apart from being implicated in cellular cholesterol homeostasis, ABCA1 is also known to play a role in apoptosis [16] and inflammation [17]. It has been reported that ABCA1 is involved in phosphatidylserine (PS) translocation which in turn influences the rate of apoptosis, and which also can promote the coagulation cascade [16,18, 59]. Apoptosis and PS translocation have critical roles in placental development [19,20] and its regulation is imperative for a successful pregnancy. In addition, the regulatory pathways of ABCA1 involving peroxisome proliferator-activated receptor-gamma (PPARγ) [21] have anti-inflammatory roles [22] and are linked to the nuclear factor-kappa B (NF-KB) pathways [23]. PPARγ is an orphan nuclear receptor with diverse biological functions [24] and is pivotal to trophoblast differentiation and maturation to establish maternal-fetal transport and also to fetal tissue development [25,26].

In murine placentas *ABCA1* mRNA expression has been localised to the lining of decidual maternal blood vessels and the labyrinthine trophoblast layer [27]. This localisation is consistent with ABCA1 playing a role in cholesterol transport. ABCA1 may also mediate apoptotic cell death in the placenta, as it does in the embryo[28]. Loss of functional ABCA1 in null mice results in severe placental malformation with structural

abnormalities, haemorrhage and cell debris in the spongiotrophoblast and labyrinthine trophoblast [27]. This is associated with intrauterine growth restriction and increased rates of neonatal death.

The localisation and role of ABCA1 in human placentas have not been established. In addition there have been no studies of ABCA1 expression in disorders associated with abnormal placentation, such as pre-eclampsia and antiphospholipid syndrome (APS). APS is characterised by recurrent miscarriage, late fetal loss, thrombocytopenia and thrombosis in conjunction with positive antiphospholipid antibodies or lupus anticoagulant. Histological studies of APS placentas demonstrate necrosis, thrombus formation, acute and chronic inflammation [29,30]. Pre-eclampsia affects approximately 3% of primigravidae is characterised by endothelial dysfunction and abnormal placentation with abnormal trophoblast invasion. Histological studies of placentas from affected pregnancies demonstrate parenchymal infarcts, decidual vessel atherosis and increased syncytial knot formation [31-33]. There are no specific placental histological changes that distinguish between placentas from pregnancies complicated by primary APS or pre-eclampsia, and there is some overlap in the clinical features and the aetiology of the fetal complications of both conditions. APS and pre-eclampsia [34,35] are both associated with placental apoptosis.

This study aimed to establish the localisation of ABCA1 in term placentas and to investigate whether ABCA1 mRNA and protein expression is altered in placentas from pregnancies complicated by pre-eclampsia or APS.

Materials and Methods

Subjects

Patients with pre-eclampsia were recruited prospectively at Kings College Hospital, London. Preeclampsia was defined according to the International Society for the Study of Hypertensive Disorders in Pregnancy criteria as ≥2 blood pressure recordings of ≥ 140/90 mm Hg ≥4 hours apart at >20 weeks' gestation in a previously normotensive patient. Proteinuria was defined as 0.3 g protein/24 h urine excretion. Intrauterine growth restriction was defined as birth weight <10th percentile for gestational age and sex. The study was approved by the local ethics committee. Patients with antiphospholipid syndrome were recruited prospectively from a specialist antenatal clinic at St Thomas' Hospital, London. The condition was diagnosed in women with the clinical features of the syndrome in addition to the following biochemical results: either anticardiolipin antibodies (immunoglobulin G >20 GPL [immunoglobulin G phospholipid] units or immunoglobulin M >6 MPL [immunoglobulin M phospholipid] units) or lupus anticoagulant (dilute Russell viper venom test ratio >1.1) on ≥ 2 separate occasions > 6 weeks apart. All patients were treated with 75mg aspirin daily. Three patients also received low molecular weight heparin (fragmin, 5000 units subcutaneously daily until 20 weeks of gestation, and then twice daily until delivery) and seven cases were treated with warfarin. The study was approved by the local ethics committee. Placentas from women with uncomplicated pregnancies were obtained prospectively from women delivering at Queen Charlotte's Hospital, London. The study was approved by the local ethics committee.

ABCA1 mRNA localisation

A riboprobe specific for human ABCA1 mRNA was designed according to Genebank accession number AF285167. Sequence analysis of the putative resulting amplicon was screened against other genes, especially other members of the ABC transporter family, to avoid non-specific binding. For cDNA synthesis total RNA (1μg) from human liver was reverse transcribed with a first strand cDNA synthesis kit for RT-PCR (Roche, UK). The RT reaction comprised 0.04U of oligo-p$(dT)_{15}$ primer, 5mM $MgCl_2$, 1mM of each dNTP, 40U RNAse inhibitor, 1X reaction buffer (Roche) and 20U AMV reverse transcriptase (Roche). PCR amplification of liver cDNA with ABCA1 specific primers (forward 5'-GAATTCTTCAACAGGGAAAACAG 3'; reverse 5'- GAGGAACCGAAGTAAGGAGTTGC

3') was performed using standard PCR conditions (25µl reaction volume containing 150ng of cDNA, 250nM of each primer, 200µM of each dNTP, 1X Thermopol buffer (New England Biolabs, UK) and 1U of *Vent* polymerase (New England Biolabs); amplification conditions: initial denaturation for 1 min at 97°C, 35 cycles of 1 min at 96°C, 1 min at 55°C and 1 min at 72°C, with a final extension at 72°C for 10 min). The amplification product was analysed on 2% low melting agarose gels, purified with a gel extraction kit (Qiagen) and sequenced on an ABI 3700 Prism automated sequencer. The amplicon (730 bp) was cloned into pCR-Blunt II-TOPO vector (Invitrogen, UK) according to the manufacturer's instructions and subloned into pGem 3 Zf (-) vector (Promega, UK) with specific restriction sites.

In situ hybridisation: 10µm sections of formalin-fixed, term control placenta and liver were dewaxed and hydrated through alcohol series and rinsed in PBS followed by 5 minutes in PBS/1mM EDTA. Placental sections were digested with proteinase K 1µg/ml in PBS/1mM EDTA for 30 minutes at 37°C. Sections were treated with PBS/ 0.2% glycine for 5 minutes. Internal alkaline phosphatase activity was blocked by immersing sections in 20% acetic acid in methanol at 4°C for 30 seconds. Sections were then rinsed twice in PBS for 5 minutes each. 20-50 µl of 1/100 diluted probe was applied to the air dried slides and hybridised at 70°C overnight. Sections were digested with RNAseA in 2XSSC (100 µg/ml) for 30 minutes at 37°C. Sections were washed from 2 X SSC to 1 X SSC, 2 times each for 10 minutes each. Sections were washed in TBS and AP1 for 5 minutes each. Non-specific binding was blocked by incubating with alkaline phosphatase (AP) 1/ 3% BSA for 30 minutes at room temperature. Sections were incubated with anti-digoxigenin-AP conjugate (1/500 diluted in AP1/BSA) for 1-2 hours at room temperature. Sections were rinsed in AP1 and AP2 and colour developed by using the Vector laboratories alkaline phosphatase substrate kit according to the manufacturer's protocol. The probe signal was visualised by microscopy.

Protein localisation and immunohistochemistry

10µm sections were cut from formalin-fixed, wax embedded control term placental tissues and dewaxed and hydrated through a series of washes from histoclear to 30% ethanol and finally in PBS/ 0.1% triton X 100. Antigen retrieval was performed by boiling the sections in citrate buffer for 10 minutes, followed by a PBS wash. Sections were incubated with normal goat serum for 1 hour (rabbit-Vectastain ABC kit, Vector Lab CA, USA). Sections were incubated with 1:200 diluted primary antibody (ab 7360 abcam,

Cambridge UK) overnight at room temperature. Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogenperoxidase for 30 minutes, followed by three PBS/ triton washes. Sections were incubated with secondary antibody using Vectastaining kit as per instructions for 1 hour. Colour was developed with 2.4μg/ μl 3.3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, UK) and 0.06% hydrogen peroxide. The signal was visualised by microscopy.

RNA preparation and quantitative *ABCA1* mRNA measurement

Synthesis of the first strand cDNA was performed using 1μg total RNA and 200 U MMLV-reverse transcriptase (Promega, USA). The reverse transcription (RT) reaction was carried out according to the manufacturer's instructions in 20μL reaction volume in a gradient cycler (Biometra, Göttingen, Germany), and achieved by successive incubations at 25ºC for 10 minutes and 42ºC for 50 minutes, finishing with enzyme inactivation at 90ºC for 2 minutes.

Quantitative reverse transcription PCR of *ABCA1* in human placenta samples was carried out using LightCycler DNA Master SYBR Green technology (Roche Diagnostics, Germany). A primer pair was designed covering two exon boundaries in order to avoid amplification of genomic DNA. A forward primer spanning exon junctions 2 and 3 (5'-CTATGAACATGAATGCCATT3') was combined with a reverse primer spanning exon boundaries 4 and 5 (5'-GCTTCAAGTTTGAGCTGGAT3') assessing a 278nt *ABCA1* cDNA fragment. *β-actin* primers (forward 5'- AACTCCATCATGAAGTGTGACG 3'; reverse 5'- GATCCACATCTGCTGGAAGG 3') amplifying a 214 bp fragment were used for normalisation. PCR reactions contained 3mM $MgCl_2$, 0.4 μM forward and reverse primer and 1 μl LightCycler DNA Master SYBR Green I (10x, Roche). Before amplification an initial denaturation step (60s/95°) was performed to activate FastStart DNA polymerase and to ensure complete denaturation of the cDNA. LightCycler PCR was performed with 40 cycles using following amplification conditions: denaturation 15s/95°C, annealing 10s/62°C, elongation 25s/72°C. To each amplification cycle a fourth segment with an elevated temperature fluorescence acquisition point was added to remove unspecific signals before SYBR Green I quantification (3 seconds at 80°C and 85°C for *ABCA1* and *β-actin*, respectively). Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification. Data were analyzed using the 2nd Derivate Maximum calculation described in the LightCycler Relative Quantification Software. All runs included a negative control consisting of PCR-grade

water, and each sample was measured in duplicate. *ABCA1* mRNA values were expressed relative to *β-actin*.

Protein preparation and western blotting

Snap-frozen tissues were pulverised under liquid nitrogen. Approximately 150-180 mg was resuspended in 600 µl ice cold lysis buffer (50 mM mannitol, 2 mM EDTA, 50 mM Tris HCl, pH 7.6) plus complete mini protease inhibitors (Roche) and homogenised on ice for 4 X 30 seconds with 30 second intervals using an Ultra-Turrax homogeniser. Nuclei were precipitated from the homogenate by a 10 minute 500 *xg* centrifugation step and the supernatants loaded onto 800 µl of a 300 mM mannitol 2 mM EDTA 50 mM Tris HCl, pH 7.6 cushion. The samples were centrifuged at 100000 *xg* for 55 minutes to pellet the cell membranes. Pellets were resuspended in lysis buffer, incubated with 25 U of benzonuclease (Sigma) for one hour on ice and ASB-14 added to a final concentration of 1%. 100 µg of protein was mixed 1:1 with Laemmli buffer and incubated at 37˚C for 5 minutes prior to loading on a 7% SDS-PAGE gel. Samples were transferred onto polyvinylidine difluoride membranes (Immobilion-P, Millipore). Blots were blocked in 5% milk powder in PBS for 1 hour and incubated overnight at 4˚C with a polyclonal antibody against ABCA1 (1:1500, Abcam) and a monoclonal antibody against Na^+/K^+-ATPase (1:4000, Research Diagnostics). The membranes were washed three times for 5 minutes with PBS and incubated with anti-rabbit and anti-mouse IgG HRP secondary antibodies (1:1000, DAKO) for one hour at room temperature. Signal was detected using chemiluminescence (Amersham ECL system).

Statistical analysis

For statistical evaluations the SAS 8.1 program package was used (SAS Version 8.e. SAS Inst., Inc., Cary, US). Labour and pre-labour control specimens were tested for significant differences by Student's t-test. As *ABCA1* mRNA expression was not significantly different ($p>0.1$), labour and pre-labour controls were combined in one control group for further analysis. *ABCA1* mRNA expression in APS placenta samples (n=8) was compared to controls (n=8; 4 labour, 4 pre-labour) using Student's t-test. Accordingly PEC placenta samples (n=14) were compared to correspondingly processed control specimens (n=11; 6 labour, 5 pre-labour).

Results

Subjects

Clinical features of the women with APS ad pre-eclampsia are summarised in Table 1 (still lacking).

ABCA1 mRNA and protein localisation

ABCA1 mRNA was localised to the syncytium of placental villi and endothelia of fetal blood vessels within the villi using the riboprobe (Figure 1a,b,c). Stromal cells of placental villi did not seem to express mRNA for *ABCA1*. No staining could be seen with the sense control riboprobe (Figure 1d). Positive staining for mRNA for ABCA1 was not homogeneous in syncytia or endothelia, but seemed to be present in all villi.

ABCA1 protein was investigated by immunohistochemistry, and could be localised to the syncytium and vascular endothelial cells of placental villi (Figure 2a). No staining could be seen in controls in which the primary antibody was omitted (Figure 2b). At higher magnification the most intense staining seemed to be in the microvillous surface of the syncytium, as well as throughout the endothelial cells of the placental vessels (Figure 2c).

ABCA1 gene expression

As the onset of labour has been shown to influence the placental mRNA expression of another ABC transporter, *MDR3* (Patel et al. 2003), we compared expression in pre-labour and labour placentas, but we did not identify any significant alterations in *ABCA1* mRNA expression with the onset of labour ($p>0.1$; Figure 3A and B, black circles). There was no difference in *ABCA1* mRNA expression between placentas from pregnancies complicated by pre-eclampsia (PEC) and 3rd trimester controls ($p>0.1$, Figure 3B). However, *ABCA1* mRNA expression was reduced in the placentas from women with APS when compared to controls ($p<0.001$, Figure 3A,).

ABCA1 protein expression

Western blot analysis was carried out on samples from each subgroup in order to determine whether the decreased mRNA expression of *ABCA1* is paralleled by decreased protein expression. Total membrane fractions of placenta (100 μg protein per lane) were tested with antibodies against ABCA1 and Na^+/K^+-ATPase, a plasma membrane protein used to ascertain equal sample loading. While there were no obvious

differences in ABCA1 protein expression between placental samples from women with PE and controls, there was reduced expression in placentas from women with APS.

Discussion

We have confirmed previous data that demonstrate that ABCA1 is expressed within the human placenta, and have localised *ABCA1* mRNA and protein to the syncytial membrane and endothelia in term placentas from uncomplicated pregnancies. The localisation of ABCA1 to the syncytia and vascular endothelia of human placenta is consistent with a role in cholesterol and phospholipid transport between the maternal and fetal circulations, and the high levels of protein in the microvillli of the syncytia imply that the main activity may be in the maternal to fetal direction. Although originally identified as a cholesterol transporter it has become clear that this protein is multifunctional, and that it also plays a role in PS translocation and apoptosis.

Any decrease in ABCA1 protein, or in functional activity could be predicted to have two different effects. Firstly, a decrease in cholesterol transport to the fetus would affect the formation of cell membranes, and also decrease the level of substrate available for fetal steroidogenesis (Sullivan, 2004 review). Though maternal HDL-C concentrations affect fetal mass and extra embryonic fetal sterol metabolism in the mouse [36], no such findings have been reported with human subjects. Elevated placental uptake of HDL [37] is probably due to the need for steroidogenesis which depends on LDL and HDL uptake [38]. Uptake of maternal lipoproteins by the placenta and the concentrations of fetal lipoproteins are key determinants of progesterone production [39]. After 6-8 weeks of pregnancy the placental trophoblast takes over as the main source of progesterone production [40] which in turn determines the survival of the fetus [41,42]. Investigations in humans [43] and baboons [44] have demonstrated the strong influence of maternal lipoproteins on placental production of progesterone { thus confirming the importance of transplacental lipid transport. Studies in ABCA1 null mice have clearly revealed the catastrophic effect ABCA1 deficiency has on the placenta, characterised by disrupted architecture, haemorrhage and ragged spongioblast inclusions {Christiansen-Weber, 2000 344 /id} ABCA1 deficiency could thus potentially affect human placental steroid hormone synthesis by its impact on lipid transport.

A decrease in ABCA1 protein may also influence the process of syncytialisation in human placenta as this is functionally similar to apoptosis[19] both involve condensation of nuclear chromatin, externalisation of annexin V, redistribution of PS, and the controlled loss of cell fragments. Decreased activity or loss of ABCA1, could decrease this process, thus leading to abnormal placentation. In this context a report of decreased

annexin V in placentas from patients with APS (Rand et al, 1997) is consistent with this perspective, although other work showing no change in such factors (Lakasing et al, 1999) suggest that interpretation of these findings needs to be cautious.

We found decreased levels of mRNA and protein for ABCA1 in placentas from patients with APS (Figures 3 and 4), but not in women with pre-eclampsia. These pathophysiology of APS and pre-eclampsia share a number of similarities, including the involvement of prothrombotic and inflammatory processes, increased apoptosis and decreased cell growth. Therefore the difference in ABCA1 expression indicates that the apparent similarities result from the convergence of different processes.

In APS, antiphosphlipid antibodies are specifically targeted to decidual tissue and can cause a rapid increase in decidual and systemic tumour necrosis factor (TNF) α levels [45] and this acts through the activation of the complement pathway which is a central mechanism in antiphospholipid antibody induced pregnancy loss and IUGR [46,47]. Moreover, the protective effects of TNF α deficiency and TNF blockade seem to support these findings [45]. The increased level of these cytokines may account for the downregulation of ABCA1 expression in APS placentas.

Th1 cytokines like TNF α have wide ranging effects on placental architecture, hormone synthesis and embryonic development [48]. Furthermore it is known to inhibit membrane transport proteins like ABCA1 and its regulatory pathways including PPAR-gamma [49,50]. The production of Th2 cytokines by the decidual T cells contributes to the maintenance of pregnancy while an exaggerated Th1 response seem harmful to the fetus [51,52]. Levels of Th1 cytokines like TNF-A, IL-2 and IFN-G have been found to be higher in patients who had recurrent miscarriages [53,54].

Although Th1/Th2 cytokine imbalances have a role in the aetiopathogenesis of pre-eclampsia and trophoblastic diseases, there is no clear understanding regarding the exact course of events [55,56]. The extent of the inflammatory process in the placenta could have an impact on the ABCA1 expression levels. It was recently reported that that TNFα levels, despite its elevation in the peripheral blood, are not elevated in the placenta of pre-eclamptic patients [55,57]. The degree of ABCA1 downregulation in the APS placentas may thus be a reflection of the extent of the inflammatory process in the placenta characterised by significantly higher number of inflammatory cells [58].

Figure legends

Figure 1: Localisation of *ABCA1* mRNA using *in situ* hybridisation. a-c: antisense riboprobe; d= sense riboprobeThe top figure demonstrates that *ABCA1* mRNA is localised in the syncytium and in endothelia.

Figure 2: Immunohistochemistry demonstrating ABCA1 expression in the syncytium and endothelia in a control placenta. (a) Low power with 1:200 antibody to ABCA1. x100 magnification; (b) negative control (no antibody used); (c) 1:200 antibody to ABCA1. x750 magnification.

Figure 3: Expression of *ABCA1* mRNA in placentas from (A) APS and (B) pre-eclampsia (PEC) patients . Controls: white circles indicate pre-labour, black circles labour samples.

Figure 4: Western blot analysis of (A) ABCA1 and (B) Na^+-K^+-ATPase in different conditions of pregnancy. Representative immunoblots of ABCA1 protein expression are shown. Membrane proteins from placenta were separated by reducing SDS-PAGE and immunoblotted with rabbit anti-ABCA1 and mouse anti-Na^+/K^+-ATPase antibodies. Samples have been labelled with numbers 1-3 for controls, 4-5 for Pre-eclampsia and 6-9 for APS.

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FIGURE 1

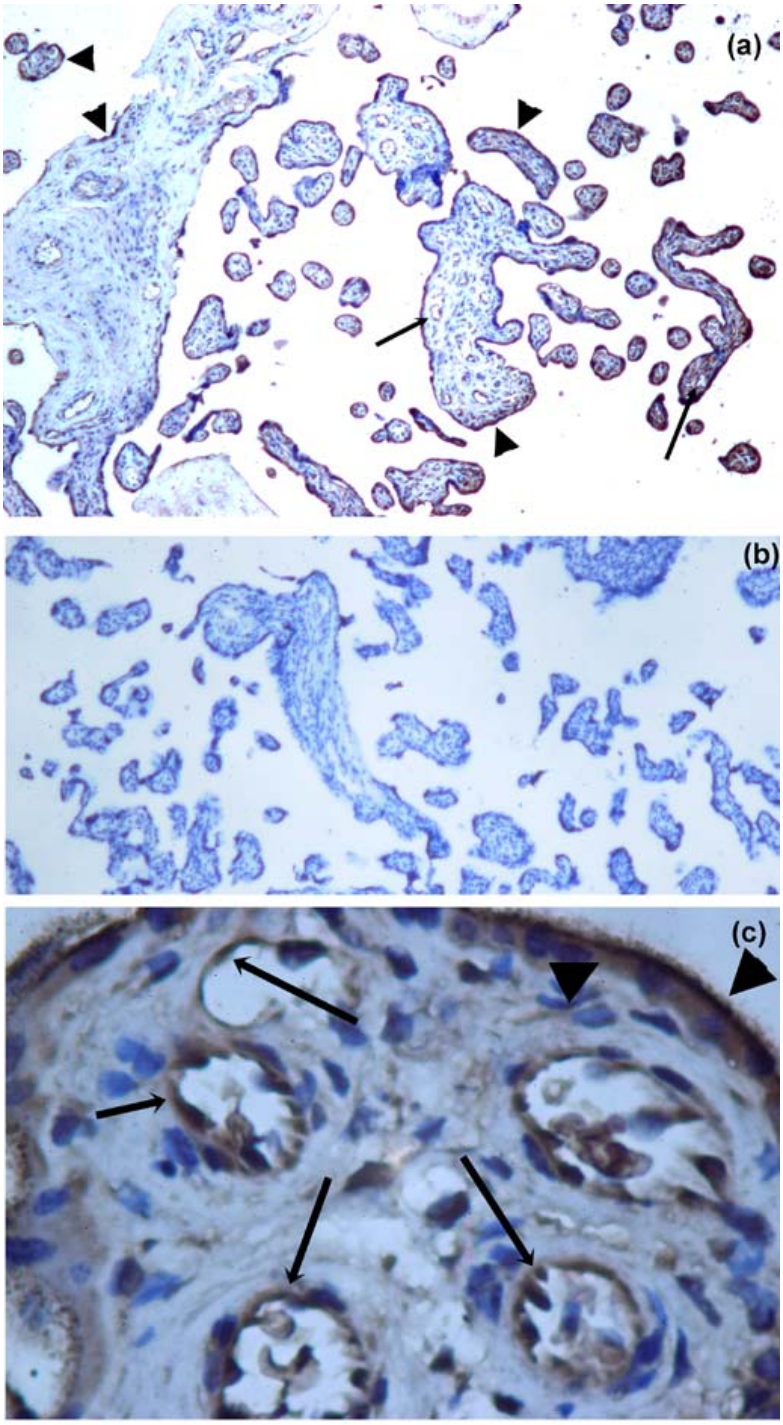


FIGURE 2

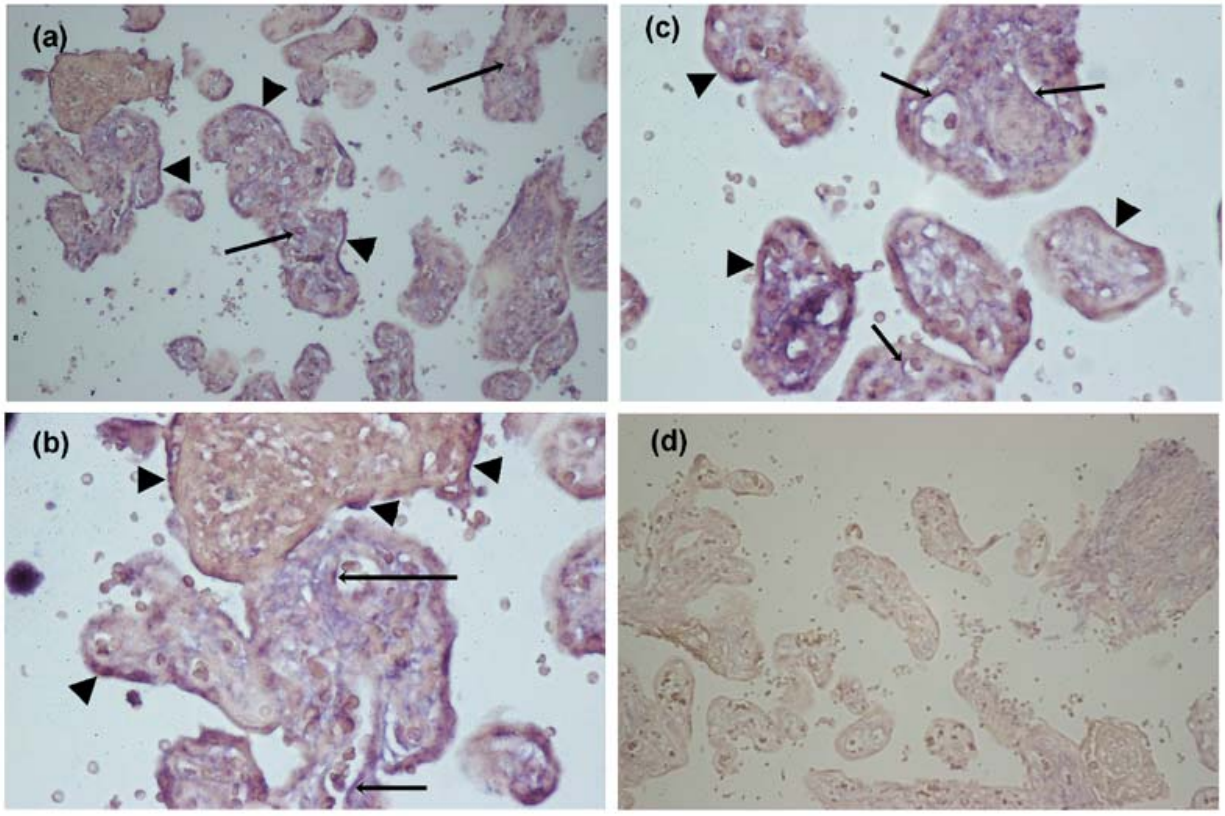
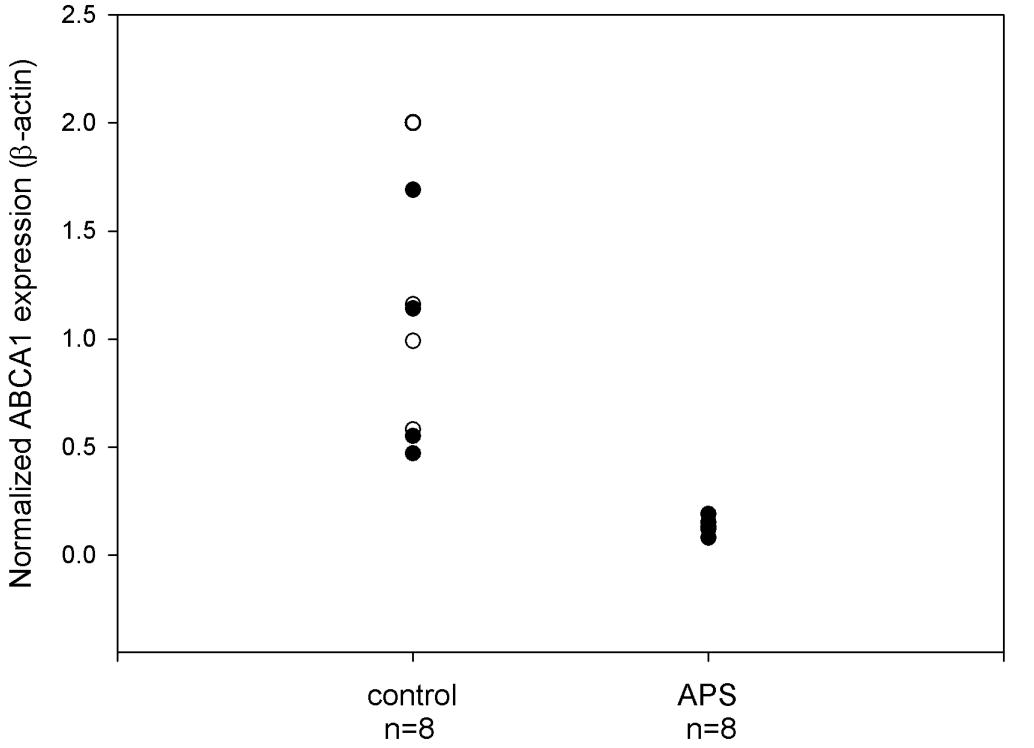


FIGURE 3


Normalized ABCA1 expression (β-actin)
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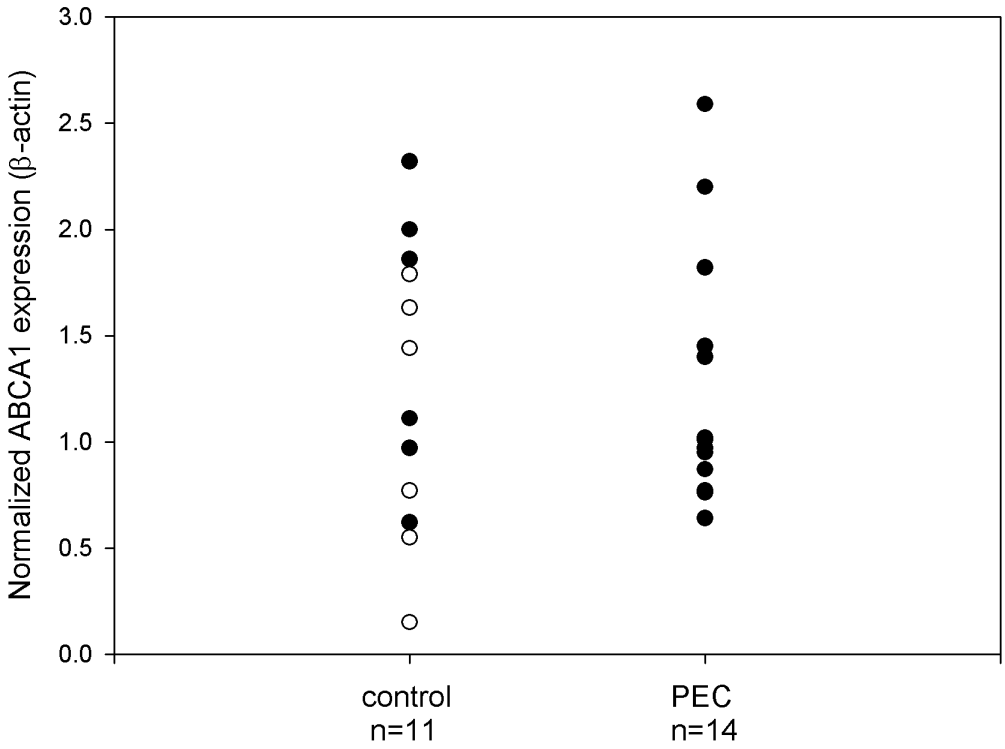
2.0
1.5
1.0
0.5
0.0
control
n=8
APS
n=8


Normalized ABCA1 expression (β-actin)
3.0
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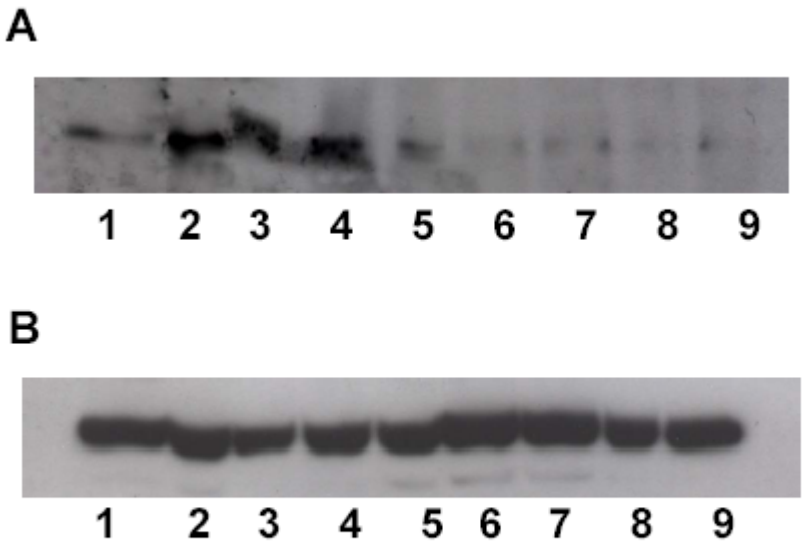
2.0
1.5
1.0
0.5
0.0
control
n=11
PEC
n=14

FIGURE 4



2 OTHER PUBLICATIONS IN THE CONTEXT OF ABC TRANSPORTER PROTEINS

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E. Viturro, C. Farke, H.H.D. Meyer, and **C. Albrecht**. Identification and sequence analysis of the sterol transporters ABCG5 and ABCG8 in the bovine mammary gland. In press J Dairy Sci. Sept 2005.

Manuscript submitted

C. Farke, E. Viturro, H.H.D. Meyer, and **C. Albrecht**. Identification, sequence analysis and tissue distribution of the bovine ATP-binding cassette transporter A1 - an analogue to the human cholesterol efflux regulatory protein. Manuscript submitted to Gene, Oct 2005.

C. Albrecht and E. Viturro. The A-subfamily of ABC transporters – genes, functions and implications. Invited review submitted to Eur J Physiol. Oct 2005

3 OTHER PUBLICATIONS (DIVERSE)

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Manuscript submitted

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B. Lutz, **C. Albrecht** and H.H.D. Meyer. Transgene Futtermittel – Einfluss auf Milch? Eine objektive Betrachtung. Deutsche Molkereizeitung 2005; 6:30-33.

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4 Abbreviations

ABC: ATP binding cassette
apo: apolipoprotein
APS: antiphospholipid syndrome
ATP: adenosine triphosphate
CEA: carotid endarterectomy
CHD: coronary heart disease
COX: cyclooxygenase
EBV: Epstein-Barr virus
EGFP: enhanced green fluorescent protein
ER: endoplasmic reticulum
GCA: giant cell arteritis
HDL: high density lipoprotein
IBD: inflammatory bowel disease
IFN: interferon
IL: interleukin
LDL: low density lipoprotein
LXR: liver-X-receptor
MMP: matrix metalloproteinase
PCR: polymerase chain reaction
PEST: sequence rich in proline (P), glutamate (E), serine (S) and threonine (T)
PLTP: phospholipid transfer protein
PPAR: peroxisome proliferator activated receptor
PS: phosphatidylserine
RA: rheumatoid arthritis
RT: reverse transcription
SCD: stearoyl-CoA desaturase
SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase
SLE: systemic lupus erythematosus
SRB1: scavenger receptor B1
SREBP: sterol regulatory element binding protein
SS: Scott syndrome
TD: Tangier disease
Th: T-helper cells
TNF: tumour necrosis factor
UFA: unsaturated fatty acid