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

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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-

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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-

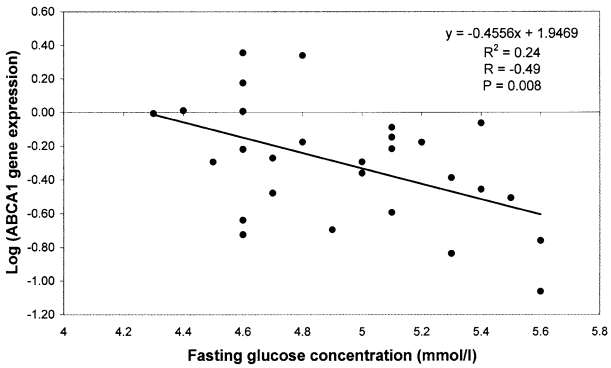

Fig 1. *ABCA1* gene expression ($\log_e$-transformed) *v* fasting glucose concentration (N = 28).

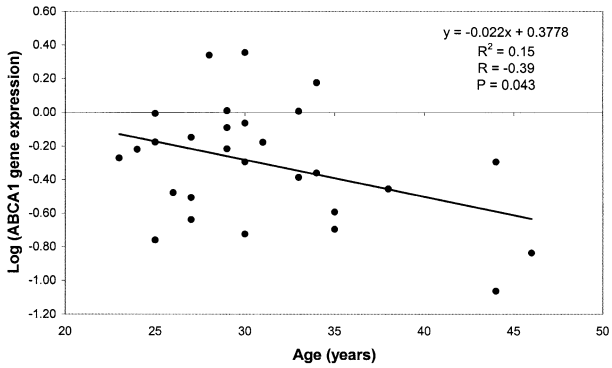

Fig 2. *ABCA1* gene expression ($\log_e$-transformed) *v* age (N = 28).

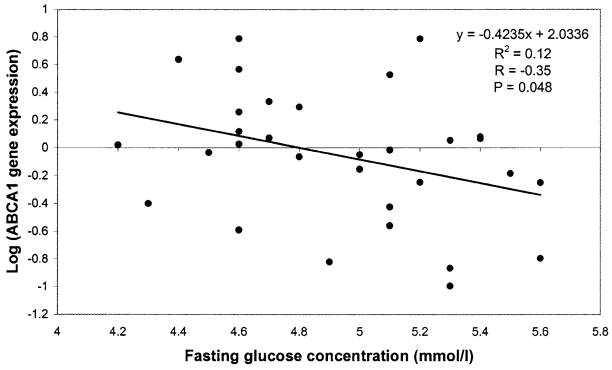

Fig 3. *ABCA1* gene expression (log_e-transformed) measured using β-actin as the internal standard *v* fasting glucose concentration (N = 32).

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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