The risk of tendon xanthomas in familial hypercholesterolaemia is influenced by variation in genes of the reverse cholesterol transport pathway and the low-density lipoprotein oxidation pathway

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Introduction

Tendon xanthomas are cholesterol deposits in tendons. Their presence is a clinical sign of familial hypercholesterolaemia (FH, OMIM # 143890), an inherited disorder characterized by high low-density lipoprotein (LDL) cholesterol levels and premature cardiovascular disease (CVD). Recently, we demonstrated that the presence of xanthomas associates with a three-fold higher risk of CVD in patients with FH.¹ This finding suggests that xanthomas and atherosclerosis share pathophysiological mechanisms.

Reverse cholesterol transport (RCT) and LDL oxidation are two important pathophysiological pathways of atherosclerosis. The RCT pathway promotes cholesterol efflux from the arterial wall macrophages to the liver.² The ATP-binding cassette transporter A1 (ABCA1) enhances the cholesterol flux from macrophages to apolipoprotein AI (ApoAI) on high-density lipoprotein (HDL) particles. After transfer of cholesterol esters from these HDL particles to LDL particles by cholesteryl-ester transfer protein (CETP), the cholesterol is cleared together with its LDL particle by the LDL receptor (LDLR) on the liver. Variation in the genes

Aims

The presence of tendon xanthomas is a marker of high risk of cardiovascular disease (CVD) among patients with familial hypercholesterolaemia (FH). Therefore, xanthomas and atherosclerosis may result from the same pathophysiological mechanisms. Reverse cholesterol transport (RCT) and low-density lipoprotein (LDL) oxidation are pathophysiological pathways of atherosclerosis, and it is well established that genetic variation in these pathways influences CVD risk. We therefore determined whether genetic variation in these pathways is also associated with the occurrence of tendon xanthomas in FH patients.

Methods and results

Four genetic variants in each pathway were genotyped in 1208 FH patients. We constructed a gene-load score for both pathways. The odds of xanthomas increased with the number of the risk alleles in the RCT pathway (OR 1.21, 95% CI 1.08–1.36, \( P_{\text{trend}} = 0.0014 \)). Similarly, higher numbers of risk alleles in the LDL oxidation pathway were associated with the presence of xanthomas (OR 1.24, 95% CI 1.08–1.41, \( P_{\text{trend}} = 0.0015 \)).

Conclusion

The presence of tendon xanthomas in FH patients is associated with genetic variation in the RCT and LDL oxidation pathways. These results support the hypothesis that xanthomas and atherosclerosis share pathophysiological mechanisms.

Keywords

Xanthomas  •  Familial hypercholesterolaemia  •  Atherosclerosis  •  Reverse cholesterol transport  •  LDL oxidation

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of these proteins in this candidate pathway has been associated with CVD risk factors and the risk of CVD.\textsuperscript{2–6}

Similarly, genes in the LDL oxidation pathway also significantly influence atherosclerosis. Apolipoprotein AIV (ApoAIV), paroxonase 1 (PON1), nitric oxide synthase 3 (NOS3), and cystathione \(\beta\)-synthase (CBS) are antioxidants, which directly or indirectly neutralize free radicals and therefore diminish and prevent LDL oxidation, an important step in the formation of atherosclerotic plaques.\textsuperscript{7–9}

Because the effect of a single genetic variant is usually small and can be compensated by variants in other genes in the same pathway, pathway-based analyses are more informative.\textsuperscript{10} A number of studies suggest that the RCT pathway and the LDL oxidation pathway influence each other.\textsuperscript{11–13} We therefore determined the combined effect of genetic variation in each of these two pathways on the risk of tendon xanthomas and assessed interaction between these pathways.

**Methods**

**Study population**

Heterozygous FH patients were recruited from 27 lipid clinics in the Netherlands between 1989 and 2002. Detailed information on the study design and population has been described previously.\textsuperscript{14,15} Briefly, when FH is suspected in a patient, a blood sample is routinely submitted to a central laboratory for LDLR mutation analysis. Out of a random selection of 2400 unrelated patients with clinical FH, we included 1208 patients with genetically confirmed heterozygous FH and with information on the presence or absence of xanthomas. The majority was of Caucasian descent (99%). The Ethics Committee of each participating hospital approved the protocol, and informed consent was required to be included in the study.

The level of total cholesterol, HDL cholesterol, and triglycerides was measured using standard methods in patients withdrawn from lipid-lowering medication at least 6 weeks before blood sampling. The LDL cholesterol levels were calculated using the Friedewald formula.\textsuperscript{16} Xanthoma definition

The presence of tendon xanthomas was defined as (i) the presence of one or more unequivocal tendon xanthomas, (ii) the presence of a clear thickening of both Achilles tendons determined by observation and palpation during physical examination,\textsuperscript{17} (iii) a history of surgical removal of one or more xanthomas. As the presence of xanthomas was doubted in 68 patients, these patients were excluded from the study. The characteristics of these patients did not differ from those in the population examined (data not shown).

**Selection and genotyping of the single nucleotide polymorphisms**

On the basis of a panel of CVD candidate single nucleotide polymorphisms (SNPs)\textsuperscript{18} and existing data derived from literature, we selected four genes of the RCT pathway and four genes of the LDL oxidation pathway. The selection of the variants in these genes was based on functionality and/or associations with CVD in earlier studies and HapMap tagging. In the RCT pathway, we chose rs1800977 of the ABCA1 gene,\textsuperscript{2,19} rs670 of the ApoA1 gene,\textsuperscript{2,20,21} rs708272 of the CETP gene,\textsuperscript{22–24} and rs5742911 of the LDLR gene.\textsuperscript{2,25,26} We selected rs675 of the ApoA4 gene,\textsuperscript{9,27} rs854560 of the PON1 gene,\textsuperscript{28–31} rs3918226 of the NOS3 gene,\textsuperscript{8} and rs5742905 of the CBS gene\textsuperscript{32–34} of the LDL oxidation pathway.

The risk allele for each polymorphism was chosen on the basis of the previous studies examining associations with CVD or intermediate traits: the C allele of rs1800977 of the ABCA1 gene,\textsuperscript{19} the A allele of rs670 of the ApoA1 gene,\textsuperscript{21} the T allele of rs708272 of the CETP gene,\textsuperscript{23,25} the A allele of rs854560 of the PON1 gene,\textsuperscript{28} the C allele of rs3918226 of the NOS3 gene,\textsuperscript{37} and the T allele of rs5742905 of the CBS gene.\textsuperscript{34} Owing to discrepancies in literature and to lack of studies with a population similar to ours, we chose the risk allele for the following polymorphisms based on our data: the G allele of the LDLR gene and the T allele of rs675 of the ApoA4 gene.

DNA was extracted from peripheral blood samples using standard methods.\textsuperscript{18} To genotype the ApoA1 SNP rs670, we used TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA, USA). The forward and reverse primer sequences were 5'-CAGGACCAGTGACGCAA-3' and 5'-GGCTGGAGGCTGA TAAGC-3', respectively. The probes used were VIC-CCAGGCC TGGCCCT and FAM-CCAGGCCGTGCCCT. The polymorphisms of the other genes were performed previously by Roche Molecular Systems, CA, USA. The genotypes were generated using PCR and immobilized probe assay, as described previously.\textsuperscript{18} Results were scored blinded to the presence of xanthomas.

**Statistical analysis**

All statistical analyses were performed with SPSS 14.0 for Windows. The \(\chi^2\) test and \(t\)-test were used to compare characteristics between patients with xanthomas and those without. Hardy–Weinberg equilibriums (HWE) of the polymorphisms were tested using a \(\chi^2\) test.

We used logistic regression models to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for each individual polymorphism, adjusted for age and sex (Model 1) and additionally adjusted for body mass index (BMI) and untreated LDL cholesterol levels (Model 2).

Haplotype analysis was not possible because of low linkage disequilibrium (LD) between the different polymorphisms, as one would expect of polymorphisms located at different chromosomes or located more than 35 000 000 bp apart from each other. Low LD is a prerequisite for gene-load score analysis.

To calculate the gene-load score, the absence of risk alleles was coded 0, the presence of one risk allele was coded 1, and the presence of two risk alleles was coded 2. To assess the combined effects of the four polymorphisms of the RCT pathway genes, a variable was created which included these four polymorphisms. This resulted in a score ranging from 0 to 8, representing the total number of risk alleles. Similarly, we assessed the combined effects of the risk alleles of the four LDL oxidation pathway genes. Because very few individuals carried no or little risk alleles for the RCT pathway, we combined 0 and 1 risk alleles into one group in our logistic regression analyses (adjusted for age and sex). For LDL oxidation pathway gene-load score, we combined 1 to 3 risk alleles into one group.

To assess if there was evidence for multiplicative interaction between the pathways, we performed a logistic regression analysis including the grouped RCT pathway, the grouped LDL oxidation, and an interaction term, adjusted for age and sex.

**Results**

Table 1 shows the general characteristics of our study population. The FH patients with tendon xanthomas were older, had...
higher BMI, higher total cholesterol and LDL cholesterol levels than those without xanthomas (P < 0.01). The frequency of CVD was higher among patients with xanthomas (P < 0.01).

All polymorphisms were in HWE, with the exception of the CBS rs5742905 polymorphism, which was not in HWE due to absence of the TT genotype (P = 0.00053).

The odds of xanthomas per individual SNP in each pathway is shown in Table 2. In the RCT pathway, the T allele of the CETP rs708272 polymorphism was significantly associated with the presence of xanthomas (OR 1.25, 95% CI 1.04–1.50, P = 0.02). More carriers of a G allele of the LDLR rs5742911 polymorphism had xanthomas than non-carriers (additive model: OR 1.28, 95% CI 1.04–1.58, P = 0.02, Table 2).

Table 2 shows that, in the LDL oxidation pathway, carriers of a T allele of the ApoA4 polymorphism had a higher odds of xanthomas than those carrying A alleles (OR 1.31, 95% CI 1.05–1.64, P = 0.02). The other polymorphisms were not significantly associated with xanthomas.

Figure 1 shows the gene-load scores of the RCT and the LDL oxidation pathway. The odds of xanthomas increased with the number of the risk alleles in the RCT pathway (OR 1.21, 95% CI 1.08–1.36, Pr = 0.00144). Each increase in the number of risk alleles in the LDL oxidation pathway was accompanied by an increase in the frequency of xanthomas (OR 1.24, 95% CI 1.08–1.41, Pr = 0.0015). Although these associations were significant, the areas under the ROC curves (AUCs) found were relatively small: for the RCT pathway 0.56 (95% CI 0.52–0.60) and for the LDL oxidation pathway 0.55 (95% CI 0.52–0.59).

We did not find evidence for multiplicative interaction between the RCT and the LDL oxidation pathway (OR interaction 0.98, 1.24, 95% CI 1.08–1.41, Pr = 0.02). The other polymorphisms were not significantly associated with xanthomas.

Table 1 General characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Xanthomas</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>567</td>
<td>641</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.0 ± 12.1</td>
<td>40.3 ± 12.7</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>45.9</td>
<td>47.0</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>69.9</td>
<td>67.4</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.9 ± 3.5</td>
<td>24.2 ± 3.2</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>131.9 ± 17.7</td>
<td>131.5 ± 18.0</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>80.3 ± 10.8</td>
<td>79.5 ± 10.3</td>
</tr>
</tbody>
</table>

Continuous variables: mean ± standard deviation.

Table 2 The odds of xanthomas and variants in genes of two pathophysiological pathways

<table>
<thead>
<tr>
<th>Gene (rs number)</th>
<th>Allele</th>
<th>Frequency</th>
<th>Model 1^a</th>
<th>Model 2^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P-value</td>
</tr>
<tr>
<td>The reverse cholesterol transport pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA1 (rs1800977)</td>
<td>T</td>
<td>0.34</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.66</td>
<td>1.11</td>
<td>0.92–1.34</td>
</tr>
<tr>
<td>ApoA1 (rs670)</td>
<td>G</td>
<td>0.85</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.15</td>
<td>1.10</td>
<td>0.86–1.40</td>
</tr>
<tr>
<td>CETP (rs708272)</td>
<td>C</td>
<td>0.57</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.43</td>
<td>1.25</td>
<td>1.04–1.50</td>
</tr>
<tr>
<td>LDLR (rs5742911)</td>
<td>A</td>
<td>0.76</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.24</td>
<td>1.28</td>
<td>1.04–1.58</td>
</tr>
<tr>
<td>The LDL oxidation pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA4 (rs675)</td>
<td>A</td>
<td>0.19</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.81</td>
<td>1.31</td>
<td>1.05–1.64</td>
</tr>
<tr>
<td>PON1 (rs854560)</td>
<td>T</td>
<td>0.37</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.63</td>
<td>1.10</td>
<td>0.92–1.32</td>
</tr>
<tr>
<td>NOS3 (rs3918226)</td>
<td>T</td>
<td>0.07</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.93</td>
<td>1.36</td>
<td>0.95–1.93</td>
</tr>
<tr>
<td>CBS (rs5742905)</td>
<td>C</td>
<td>0.90</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.10</td>
<td>1.34</td>
<td>0.98–1.84</td>
</tr>
</tbody>
</table>

^aLogistic Model 1: adjusted for age and sex.

^bLogistic Model 2: adjusted for age, sex, LDL cholesterol and BMI.
Discussion

In this study, we have demonstrated that variants in genes of the RCT pathway and the LDL oxidation pathway are associated with the presence of tendon xanthomas in FH patients. The odds of xanthomas increased with increasing numbers of risk alleles in the genes of both pathways.

In addition to the higher risk of CVD in FH patients with xanthomas compared with that in those without xanthomas, there are other findings suggesting that xanthomas and atherosclerosis are related and share pathophysiological pathways. The composition of xanthomas and atherosclerotic plaques show similarities as both lesions consist of connective tissue matrix containing macrophages transformed into foam cells. In addition, both xanthomas and plaques can be prevented or reduced by the use of cholesterol-lowering drugs. Recently, we have shown that variation in the 5-lipoxygenase activation protein gene, which is involved in inflammation, contributed to the risk of xanthomas in FH. The present study is the first study to extend the analysis of xanthomas to genetic variation in the RCT and LDL oxidation pathways. Previously, genetic variation in these pathways has been related to atherosclerosis or one of its risk factors. These findings strongly suggest that xanthomas and atherosclerosis share pathogenic mechanisms.

We assigned the same score to each risk allele of each polymorphism. As an alternative method, we also made a weighted gene score for each patient, in which the absence of risk alleles was coded 0 and the presence of one or two risk alleles were coded as 1 LN(OR) and 2 LN(OR), respectively. The resulting continuous gene score was divided into five equal groups of patients. The results of this alternative approach were similar to the unweighted approach (data not shown).

In gene-load score analyses, additive risk-allele effects are used as markers of the susceptibility of particular pathway to deteriorate and contribute to signs and symptoms. The dosage effect observed in both pathways supports involvement of these pathways in the development of xanthomas. The effect of both the RCT pathway and the LDL oxidation pathway clearly showed that the odds of xanthomas increased according to the number of risk alleles. Although the previous literature has suggested an interaction between the studied pathways, we did not find a multiplicative interaction between the pathways. In line with this, combining the gene scores of both pathways showed a trend similar to that of the individual pathways.

The AUC values demonstrate that the gene-load scores of these pathways contributed modestly to xanthomas, and other (unknown) pathogenetic factors need to be elucidated to understand the pathogenesis of xanthomas more completely. On the other hand, we may have underestimated the contribution of the two pathways by studying only a few genetic variants of each pathway.

HDL plays an important role in the RCT pathway; however, HDL cholesterol levels itself were not associated with the presence of xanthomas (Table 1). Perhaps the functionality of HDL particles is of more importance than the quantity. Previously a probucol study indeed demonstrated that the reduced HDL particle size was linked to xanthoma regression. Unfortunately, we had no data about the subdivision of the particles.
In our study, the CBS rs5742905 polymorphism was out of HWE. Because homozygosity of the CBS rs5742905 T allele leads to CBS deficiency that causes hyperhomocystinemia (OMIM #236200), the absence of TT carriers in our population can be explained by the preferential referral of CBS-deficient patients to other outpatient clinics. The results of the analyses, including and excluding the CBS variant, were similar (data not shown).

To maintain sufficient patients per number of risk alleles, we could not include all genes involved in both pathways. For example, both lecithin-cholesterol acyltransferase and scavenger-receptor B1 (SR-B1) are known to be involved in the RCT pathway too. Lecithin-cholesterol acyltransferase converts cholesterol in HDL particles to cholesterol esters, and SR-B1 binds HDL particles and facilitates removal of remaining cholesterol esters from these particles. However because of discrepancies in literature about their importance in the RCT pathway and because of differences in outcome per sex and age of polymorphism analysis, we did not genotype a variant in these genes. Variation in another important candidate gene, the E2–E3–E4 variation in the apolipoprotein E (ApoE) gene, was previously found not to be associated with xanthomas. In addition, we decided not to include any polymorphisms of genes involved in the more down-stream bile acid synthesis and excretion.

It is well known that the natural history of atherosclerosis differs between the two sexes. Similar to CVD, the male gender is also associated with the presence of xanthomas. In the present study, the percentage of males was not significantly different between the FH patients with and without tendon xanthomas. Stratified analyses of the individual polymorphisms demonstrated that all variants with the exception of one increase the odds of xanthomas more in males than in females (data not shown). However, because all ORs were in the same direction in both sexes, and the CIs of these ORs clearly overlapped, we decided to perform a combined analysis corrected for sex (and age) preserving optimal power for the current analyses.

The presence of xanthomas can be determined by physical examination of the tendons without any discomfort for the patient. However, there are no data on inter-observer variability, which could be a problem in the detection of small xanthomas. Xanthomas can be identified in patients with ultrasonography. Unfortunately, we had no ultrasonography of the tendons without any discomfort for the patients included in our study. Therefore, it could be argued that we did not study the presence of xanthomas, but instead analysed the occurrence of severe xanthomas. Nonetheless, this diagnosis is relevant for daily clinical practice.

In conclusion, we observed that the presence of tendon xanthomas in FH patients is influenced by genetic variation in the RCT and LDL oxidation pathways. Our results support the hypothesis that xanthomas and atherosclerosis share pathophysiological pathways. The presence of xanthomas may therefore indicate FH patients with a more severe CVD risk.

**Funding**

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