A CETP polymorphism improves the diagnostic power of clinical examination in patients with cardiovascular disease

A case-control study

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Summary

Aims: Atherosclerosis is common and myocardial infarction, stroke and peripheral arterial occlusive disease are its devastating complications. Accurate risk prediction is urgently needed. We applied molecular tests to improve early clinical identification of patients threatened by a future course of complicated active atherosclerosis.

Methods and results: Participants were men and women seeking care in a department of general internal medicine at an academic teaching hospital in Basel, Switzerland, between September 2003 and March 2005. A maximum number of 57 patients with a medical history of proven cardiovascular events and 57 age- and gender-matched patients without cardiovascular events were selected from this cohort of 269 individuals. One of nine common single nucleotide polymorphisms (SNPs) reportedly linked to cardiovascular disease was significantly associated with cardiovascular events (p = 0.02). For CETPrs708272, the allele number per patient predisposing to cardiovascular events improved the discriminating power of clinical phenotyping for active versus inactive atherosclerosis. The area under the curve of receiver operating characteristic (ROC) for clinical examination alone was 0.627 (95% CI 0.525–0.730, p = 0.02) and increased to 0.672 (95% CI 0.571–0.772, p = 0.002) when the polymorphism was included in the assessment.

Conclusions: Information about common SNPs with high impact on the individual cardiovascular risk, such as CETPrs708272, may help to predict an active, symptomatic course atherosclerosis.

Key words: atherosclerosis; single nucleotide polymorphism; cholesterol ester transfer protein; physical examination

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Introduction

Molecular medicine can substantially improve the accuracy of clinical and laboratory procedures [1]. Precise and early identification of patients threatened by potentially devastating diseases, such as cancer or atherosclerosis, are increasingly appreciated in many modern diagnostic approaches and form the basis of personalised medicine [2]. Examples are molecular characterisation of fine needle aspirates from thyroid tumours [3] or early recognition of a predisposition to diabetes [4, 5] or obesity [6].

Germline genotypes are generally stable for a lifetime and represent an ideal substrate for early detection of individuals at risk. A large number of single nucleotide polymorphisms (SNPs) in candidate genes, i.e., genes coding for molecules involved in the pathogenesis of atherosclerosis, were reported to be associated with cardiovascular disease [7]. With the exception of uncommon single gene mutations with high penetrance (e.g., low density lipoprotein receptor or LDLR ligand mutations) causing rare monogenic forms of inherited atherosclerosis [8, 9], none of the more common genetic variants is used in daily clinical practice for cardiovascular risk estimation and patient management [10, 11].

In the present study we hypothesised that molecular tests would improve a recently developed method to quantitatively assess the clinical appearance or phenotype of patients with active,
CETP SNP in atherosclerosis

SNPs analysed: gene loci, polymorphisms, Table 1

carrier and allele frequency.

Remarkably accurate in discriminating between
disease activity score (cDAS), a biomarker that was
defined by the current or previous history of cardiovascular events in different organs.

between patients with and without symptomatic atherosclerosis [12].

We evaluated the diagnostic potential of nine common SNPs in seven candidate genes for
atherosclerosis involved in pathogenic key mechanisms such as lipid retention [14] and inflammation [15] (table 1). We determined these SNPs in
two groups of 57 age- and gender-matched inpatients with or without a lifetime history of cardiovascular events. We first tested the hypothesis that
alleles reportedly associated with cardiovascular disease were preferentially present in the group of patients with active atherosclerosis, i.e., with
cardiovascular events in their medical history. Second, we were interested in investigating whether the allele number per patient would improve the
diagnostic strength of clinical phenotyping, i.e., examination by simple, affordable and available bedside procedures.

Table 1

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>SNP (rs number)</th>
<th>Risk allele</th>
<th>Amino acid change (HVGS name)</th>
<th>Carrier frequency (repository data)</th>
<th>Allele frequency (repository data)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>rs2230806G&gt;A</td>
<td>G(28)</td>
<td>NP_005493.2: p.R219K</td>
<td>0.965 (0.987*)</td>
<td>0.750 (0.842*)</td>
</tr>
<tr>
<td>ABCA1</td>
<td>rs2066715G&gt;A</td>
<td>A(29)</td>
<td>NP_005493.2: p.V825I</td>
<td>0.149 (n.a.)</td>
<td>0.075 (n.a.)</td>
</tr>
<tr>
<td>CETP</td>
<td>rs708272G&gt;T</td>
<td>C(21, 27)</td>
<td>non-coding</td>
<td>0.825 (0.870**)</td>
<td>0.592 (0.522**)</td>
</tr>
<tr>
<td>CYBA</td>
<td>rs4673T&gt;C</td>
<td>C(10)</td>
<td>NP_000902.2: p.Y72H</td>
<td>0.895 (0.867*) (0.875**)</td>
<td>0.640 (0.658*) (0.667**)</td>
</tr>
<tr>
<td>CX37</td>
<td>rs1764391G&gt;T</td>
<td>C(31)</td>
<td>NP_002051.2: p.P319S</td>
<td>0.904 (0.879*)</td>
<td>0.702 (0.681*)</td>
</tr>
<tr>
<td>MTHFR</td>
<td>rs1801133G&gt;T</td>
<td>T(32, 33)</td>
<td>NP_005948.3: p.A222V</td>
<td>0.596 (0.417*) (0.542**)</td>
<td>0.355 (0.242*) (0.292**)</td>
</tr>
<tr>
<td>SELE</td>
<td>rs1805193G&gt;T</td>
<td>T(34)</td>
<td>non-coding</td>
<td>0.184 (0.167*) (0.208**)</td>
<td>0.101 (0.092*) (0.104**)</td>
</tr>
<tr>
<td>TLR4</td>
<td>rs4986790A&gt;G</td>
<td>A(36)</td>
<td>NP_612564.1: p.D299G</td>
<td>1.00 (0.937*) (0.917**)</td>
<td>0.925 (0.967*) (0.958**)</td>
</tr>
</tbody>
</table>

1) Publications reporting increased cardiovascular risk with this allele. 2) TaqIB polymorphism. 3) CSHL-HAPMAP; HapMap-CEU.

Methods

Patients

Between September 2003 and March 2005, 269 patients treated for any reason at the department of general internal medicine gave written informed consent to par-
ticipate and were included prospectively in a cohort study designed to validate the diagnostic potential of various biomarkers (www.clinicaltrials.gov: NCT00863967). The protocol was approved by the ethical review board (Ethik-
kommission beider Basel, Basel, Switzerland). We defined active, symptomatic atherosclerosis by the current or previous history of cardiovascular events in different organs.

Clinical history and its verification by hospital records was the only classifying criterion for the assignment of individ-
ual patients to the group with active disease. According to this definition, cardiovascular events were a) for coronary heart disease: myocardial infarction, angina pectoris with concomitant signs of myocardial ischaemia, history of coronary bypass surgery or other revascularisation procedures, b) for cerebrovascular disease: ischaemic stroke, history of carotid surgery, c) for peripheral arterial occlusive disease: ankle brachial index 0.9 [16] and symptoms of inter-
mittent claudication, significant stenosis of arteries and symptoms of intermittent claudication, history of periph-
eral bypass surgery or other revascularisation procedures, d) for aorticatherosclerosis: symptomatic aortic aneurysm, diameter of infrarenal aorta >3 cm [17], aortic surgery for atherosclerosis and e) for arteriovenous of the kidney: renal artery stenosis, impaired renal function [18] with normal urinalysis, history of renal artery revascularisation pro-
cedures. A hundred of the 269 patients were affected by any of these manifestations of the disease, i.e., they had suf-
fered from proven cardiovascular events in the past and

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Table 2
Characteristics of the two patient groups.

<table>
<thead>
<tr>
<th>Cardiovascular risk factors</th>
<th>No cardiovascular events (n = 57 controls)</th>
<th>Cardiovascular events (n = 57 cases)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n (%)</td>
<td>31 (54)</td>
<td>31 (54)</td>
<td>1.0</td>
</tr>
<tr>
<td>Age, y</td>
<td>69 ± 11</td>
<td>68 ± 10</td>
<td>0.64</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>11 (19)</td>
<td>16 (28)</td>
<td>0.27</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.5 ± 4.2</td>
<td>26.7 ± 4.7</td>
<td>0.69</td>
</tr>
<tr>
<td>Hypercholesterolaemia, n (%)</td>
<td>33 (58)</td>
<td>31 (54)</td>
<td>0.70</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>36 (63)</td>
<td>32 (56)</td>
<td>0.44</td>
</tr>
<tr>
<td>Positive family history, n (%)</td>
<td>31 (54)</td>
<td>38 (67)</td>
<td>0.18</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>38 (67)</td>
<td>35 (61)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

History of cardiovascular disease

| Coronary heart disease, n (%)               | 0 (0)                                     | 37 (65)                             | <0.001|
| Cerebrovascular disease, n (%)             | 0 (0)                                     | 15 (26)                             | <0.001|
| Arterial occlusive disease, n (%)          | 0 (0)                                     | 16 (28)                             | <0.001|
| cDAS                                      | 1.14 ± 0.37                               | 1.12 ± 0.43                         | 0.02  |
| cDAS<sub>CETP</sub>                       | 2.17 ± 0.80                               | 2.65 ± 0.79                         | 0.002 |
| Statin treatment, n (%)                    | 15 (26)                                   | 38 (67)                             | <0.001|
| HDL plasma levels, mmol/L                  | 1.1 ± 0.4                                 | 1.0 ± 0.4                           | 0.34  |

1) Clinical disease activity score (cDAS) is a variable calculated per patient with a range between 0 for no, and 3 for maximal activity of atherosclerosis (fig. 1A).

2) cDAS that is endorsed with the number of proatherosclerotic CETP alleles per patient is labeled cDAS<sub>CETP</sub> (fig. 1B).

Figure 1
The novel method for clinical phenotyping and the calculation of the clinical disease activity score (cDAS).

A. The 25 variables that differed significantly between patients with and without symptomatic atherosclerosis (12) are shown as labelled colour-coded columns. Each column visualises the quartile distribution of the symptomatic patients’ variables. Green, yellow, orange and red color represent the 1st, 2nd, 3rd and 4th quartile range and are quantitatively scored with 0, 1, 2 and 3 scoring points respectively. Each individual patient’s variable data are weighed according to the quartile colour or score point. An example is given with the white circles indicating data from a single patient. This patient has a body mass index of 28 and is therefore given an orange label for this variable, or 2 scoring points contributing to the clinical disease activity score. The clinical disease activity score (cDAS) is calculated as the average variable score determined by appropriate quartile allocation. It is a continuous numerical variable with a maximal range of 0–3. B. cDAS resulting from the clinical phenotype can be endorsed by genotyping. The number of CETP rs708272C/TaqIB1 alleles per patient is added to the individual cDAS and results in cDAS<sub>CETP</sub> (maximal range: 0–5).
110 individuals did not. For the 59 remaining patients, active atherosclerosis was neither definitely confirmed nor ruled out. The patient group with symptomatic atherosclerosis was significantly older and included more male participants [12]. To examine the diagnostic power of proatherosclerotic single nucleotide polymorphisms we selected a maximal total of 52 age-matched women and 62 age-matched men from the two patient groups for further analysis (table 2). Within this selected patient cohort, 65% of the 57 patients with symptomatic atherosclerosis had coronary heart disease, 26% suffered from stroke and 28% had a history of peripheral arterial occlusive disease. These prevalence numbers are similar to previously reported data from private practice out-patient registries [19, 20].

DNA isolation and genotyping

Whole blood was drawn into 1/10 volume adenosine-citrate-dextrose solution (RDB8652, Baxter, Maurepas, France). Peripheral blood mononuclear cells were immediately separated from anticoagulated blood by ficoll density-gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Genomic DNA was isolated from 2x10^9 cells by MagNA Pure LC DNA Isolation Kit I (3003990, Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions and stored at 4°C. The relevant nucleotide fragment was amplified by PCR at 55°C annealing temperature with 40 amplification cycles using Go Taq Flexi DNA polymerase (MB305, Promega AG, Dübendorf, Switzerland) according to standard protocols. The 9 SNPs (table 1) were examined by RFLP (figure 2). Details for primer sequences and restriction enzymes (all from New England Biolabs Inc, Hitchin, UK) are shown in figure 3.

Clinical phenotyping and clinical disease activity score (cDAS)

Clinical phenotyping is a novel approach to the description of patients with common chronic disorders such as atherosclerosis, and the method has been described in detail before [12]. Basically, the procedure relies on a clinical dataset consisting of routine bedside examination (e.g., personal history, systolic blood pressure, waist circumference etc) assessed in a contemporary cohort of individuals requiring medical attention in a certain healthcare environment (e.g., a hospital, private practice, etc.). From this comprehensive dataset variables are selected that are significantly different between patients with and without symptomatic atherosclerosis (according to the definition mentioned above). For symptomatic atherosclerosis, we found 25 variables to be different from the control dataset (fig. 1A). The frequency distribution of each of these variables can be used to describe quartile ranges (fig. 1A) which indicate in an apparently how much the observed parameter deviates from those of the patient population free of cardiovascular disease. When an individual patient’s measured value is in the first quartile, this deviation is mild (indicated by the green color or 0 scoring points). When it is in the second quartile (yellow color =1 scoring point), the observed deviation stands out more prominently, and in the third and fourth quartile (orange or red color, = 2 or 3 scoring points, respectively), differences become more marked. A patient’s individual scoring point assigned to the quartile distributions can then be used to determine his clinical disease activity score (cDAS). The comprehensive cDAS is calculated as the average of all scoring points determined from the appropriate quartile allocation of each individual patient’s value for all 25 variables (fig. 1A). For example, if a patient’s body mass index is 28 kg/m², he scores 2 points for this variable, for a waist circumference of 103 cm, another 2 scoring points are added, and so forth.
Statistics

cDAS is normally distributed in symptomatic but not in asymptomatic patients. The means of numerical variables in the two patient groups were therefore compared using the Mann Whitney U test and the frequency distribution of categorical variables with the χ² test. To analyse the diagnostic power of cDAS and cDAS CETP which are both continuous variables, we used receiver operating characteristic (ROC) curves. This plots sensitivity and specificity as a function of a changing discrimination threshold. Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago IL, USA) and STAT version 11 (Stata Corporation, College Station TX, USA).

Results

Conventional cardiovascular risk factors were distributed similarly in the two age- and gender-matched patient groups (table 2). The calculated clinical disease activity score (cDAS) was significantly higher in patients with symptomatic atherosclerosis (1.32 ± 0.43 versus 1.14 ± 0.37 in controls, p = 0.02).

The wild type allele of CETPrs708272 is reportedly associated with cardiovascular disease [21]. In this study we found this allele (rs708272C or TaqIB1), as expected, significantly more often in the patient group with symptomatic atherosclerosis (table 3). The allele frequency of proatherosclerotic CETPrs708272C/TaqIB1 was 52% in asymptomatic patients and 66% in patients with symptomatic atherosclerosis (p = 0.02). 35% of the homozygous carriers of the atheroprotective CETPrs708272T/TaqIB2 allele had symptomatic atherosclerosis, 45% of heterozygous carriers had suffered from cardiovascular events in the past, and 63% of the homozygous carriers of proatherosclerotic CETPrs708272C/TaqIB1 had active disease.

All patients, particularly the symptomatic group, were treated for various cardiovascular risk factors according to standard practice. 26% of the patients without a history of cardiovascular events and 67% of the symptomatic patients were on statins. Since statin use affects plasma lipoproteins [22], we measured HDL levels in the 61 patients without statins and determined the effect of CETPrs708272 polymorphism: patients homozygous for the atheroprotective CETPrs708272T/TaqIB2 allele (n = 12) had 1.16 ± 0.5 mmol/L HDL, patients with one CETPrs708272C/TaqIB1 allele (n = 27) had 1.08 ± 0.4 mmol/L HDL, and patients with two CETPrs708272C/TaqIB1 alleles (n = 22) had 1.01 ± 0.4 mmol/L HDL. This confirms reports on the effect of CETPrs708272 polymorphisms on HDL [21].

Figure 4
Receiver operating characteristic (ROC) curve for cDAS and cDAS CETP. The area under the curve (AUC) of the ROC curve is 0.627 (95% CI 0.525–0.730) for cDAS and 0.672 (95% CI 0.571–0.772) for cDAS CETP. The two ROC curves are not significantly different (p = 0.43).

Table 3
Distribution of the risk alleles predisposing to cardiovascular events (bold) in the two patient groups.

| Gene symbol (SNP) | No cardiovascular events (n = 57 controls) | Cardiovascular events (n = 57 cases) | OR (95%CI) | P
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC1A(rs2230806G&gt;A)</td>
<td>88/114</td>
<td>83/114</td>
<td>0.79 (0.43–1.44)</td>
<td>0.444</td>
</tr>
<tr>
<td>ABC1A(rs2066715G&gt;A)</td>
<td>10/114</td>
<td>7/114</td>
<td>0.68 (0.25–1.85)</td>
<td>0.449</td>
</tr>
<tr>
<td>CETP (rs708272G&gt;T)</td>
<td>59/114</td>
<td>76/114</td>
<td>1.86 (1.09–3.18)</td>
<td>0.02**</td>
</tr>
<tr>
<td>CYBA (rs4673T&gt;C)</td>
<td>72/114</td>
<td>70/114</td>
<td>0.93 (0.54–1.58)</td>
<td>0.941</td>
</tr>
<tr>
<td>CX37 (rs1764919G&gt;T)</td>
<td>77/114</td>
<td>83/114</td>
<td>1.69 (0.39–7.28)</td>
<td>0.385</td>
</tr>
<tr>
<td>MTHFR (rs1801133C&gt;T)</td>
<td>42/114</td>
<td>39/114</td>
<td>0.80 (0.46–1.39)</td>
<td>0.678</td>
</tr>
<tr>
<td>SELE (rs1805193G&gt;T)</td>
<td>14/114</td>
<td>9/114</td>
<td>0.61 (0.25–1.48)</td>
<td>0.272</td>
</tr>
<tr>
<td>SELE (rs5361A&gt;C)</td>
<td>15/114</td>
<td>9/114</td>
<td>0.60 (0.25–1.49)</td>
<td>0.271</td>
</tr>
<tr>
<td>TLR4 (rs4986790G&gt;A)</td>
<td>105/114</td>
<td>106/114</td>
<td>1.13 (0.42–3.05)</td>
<td>0.800</td>
</tr>
</tbody>
</table>

** Significant difference between the two patient groups. χ² test.
none of the five alleles was this finding statistically significant.

In this patient cohort CETPrs708272C/TaqIB1, i.e. the proatherosclerotic allele, enhances the odds ratio of symptomatic atherosclerosis to 1.86 (95% CI 1.09–3.18). Although a modest effect that is contributed by CETPrs708272C/TaqIB1 itself, it improves the discriminating power when combined with the clinical disease activity score cDAS. To integrate molecular and clinical information, the number of CETPrs708272C/TaqIB1 alleles were simply added to the patient’s cDAS (fig. 1B). Thus, if a patient has a cDAS of 1.13 and 2 CETPrs708272C/TaqIB1 alleles, his combined score (cDAS_{CETP}) is 3.13. A patient’s cDAS_{CETP} is 2.13 if he has 1 CETPrs708272C/TaqIB1 allele and the score remains 1.13 if he has only atheroprotective CETPrs708272T/TaqIB2 alleles. cDAS_{CETP} resulting from combined phenotypical and genotypical data had a better discriminating power than cDAS alone (table 2). The area under the curve of the receiver operating characteristic (ROC) was 0.627 (95% CI 0.525–0.730) for cDAS and 0.672 (95% CI 0.571–0.772) for cDAS_{CETP} (p = 0.43; fig. 4). On average, cDAS_{CETP} was 2.65 ± 0.79 for patients with symptomatic atherosclerosis and 2.17 ± 0.80 for patients without cardiovascular events (p = 0.002). No other SNP analysed in this study changed the diagnostic accuracy of cDAS.

Discussion

In this study we evaluated common, reportedly proatherosclerotic SNP alleles of candidate genes as a diagnostic instrument by themselves, but, more importantly, as a tool to enhance the potential of a recently developed clinical method of assessing cardiovascular disease. Among the nine polymorphisms analysed, only CETPrs708272C/TaqIB was able to discriminate between symptomatic and asymptomatic patients. There was a clear allele dose effect for CETPrs708272C/TaqIB1 and symptomatic atherosclerosis, and the number of proatherosclerotic CETPrs708272C/TaqIB1 alleles per patient improved the diagnostic accuracy of clinical phenotyping by the method of clinical disease activity scoring [12].

Cholesterol ester transfer protein is involved in shuttling cholesterol from high to low density lipoproteins [23] and triglycerides from very low density lipoproteins to LDL and HDL [24]. Several genetic CETP variants with reduced functional activity resulting in higher HDL levels were described but the impact of CETP on arterial health remained ambiguous. In this context, a recent phase III trial with a potent CETP inhibitor revealed an increase in death and cardiovascular events in high-risk patients who received torcetrapib [25]. This now being largely explained by off-target hypertensive effects of the drug [26], an increasing and consistent amount of epidemiological evidence supports a robust association of CETPrs708272 with cardiovascular outcome [21, 27]. Our findings are in line with these data.

The present single centre case-control study is limited by the small sample size and lacks prospective outcome data. However, despite these limitations it may contribute two important, novel aspects to the field of personalised medicine. First, the patients included in this cohort were representative of a wide range of unselected ill individuals who seek medical attention in a Swiss hospital’s department of general internal medicine. A molecular diagnostic test is particularly valuable in this setting if the allele frequency is high and the pathogenic impact of the polymorphism is reasonably strong, as suggested by our results for CETPrs708272. Second, we show here that the discriminating power of genotyping is improved by careful, standardised and clearly structured clinical examination. To date not many genetic tests for complex polygenic disorders have been evaluated by such an approach. Taking into account the impact that CETPrs708272C/TaqIB1 alleles may have on the incidence of cardiovascular events in the individual patient, and considering the high allele and carrier frequency of about 59% and 82% respectively, CETPrs708272C may become an essential part of algorithms developed to predict an active, symptomatic course of atherosclerosis. Genetic testing has several advantages over plasma biomarker assessment: a) DNA tests can be performed under lipid lowering therapy which may influence lipoprotein levels, b) testing has to be performed once in a lifetime and not repetitively and c) SNPs affecting the functional activity of an enzyme or protein that is not accessible in the plasma compartment may reflect functional state more accurately than plasma levels of substrates.

If confirmed by prospective outcome data in a larger multicentre trial involving asymptomatic patients at baseline, our approach may evolve into a better way of allocating people at risk to a more intensive treatment regime of cardiovascular risk factors.

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References


