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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 monogenetic 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,

symptomatic atherosclerosis [12]. Comprehensive, quantitative phenotyping in the field of cardiovascular diseases was first discovered and developed by physiologists and formed the basis for sophisticated phenotype-genotype correlations in an animal model of hypertension [13]. We adapted this approach to the field of clinical medicine and applied the rules of differential display to analyse data obtained during comprehensive bedside examination of patients. This method of clinical disease phenotyping revealed 25 numerical variables that were significantly different between patients with and without symptomatic atherosclerosis and contributed to the empirical clinical profile of atherosclerosis. These quantitative variables were transformed mathematically into score points and the average score per patient was named clinical disease activity score (cDAS), a biomarker that was remarkably accurate in discriminating 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

SNPs analysed: gene loci, polymorphisms, carrier and allele frequency.

Gene symbol	SNP (rs number)	Risk allele[1)]	Amino acid change (HVGS name)	Carrier frequency (repository data)	Allele frequency (repository data)
ABCA1	rs2230806G>A	G(28)	NP_005493.2: p.R219K	0.965 (0.987*)	0.750 (0.842*)
ABCA1	rs2066715G>A	A(29)	NP_005493.2: p.V825I	0.149 (n.a.)	0.075 (n.a.)
CETP	rs708272C>T[2)]	C(21, 27)	non-coding	0.825 (0.870**)	0.592 (0.522**)
CYBA	rs4673T>C	C(30)	NP_000092.2: p.Y72H	0.895 (0.867*) (0.875**)	0.640 (0.658*) (0.667**)
CX37	rs1764391C>T	C(31)	NP_002051.2: p.P319S	0.904 (0.879*)	0.702 (0.681*)
MTHFR	rs1801133C>T	T(32, 33)	NP_005948.3: p.A222V	0.596 (0.417*) (0.542**)	0.355 (0.242*) (0.292**)
SELE	rs1805193G>T	T(34)	non-coding	0.184 (0.167*) (0.208**)	0.101 (0.092*) (0.104**)
SELE	rs5361A>C	C(35)	NP_000441.2: p.S149R	0.193 (0.167*)	0.105 (0.092*)
TLR4	rs4986790A>G	A(36)	NP_612564.1: p.D299G	1.00 (0.933*) (0.917**)	0.925 (0.967*) (0.958**)

[1)] Publications reporting increased cardiovascular risk with this allele. [2)] TaqIB polymorphism. * CSHL-HAPMAP; HapMap-CEU. ** PERLEGEN; AFD_EUR_PANEL. n.a. no data from a population of European descent available.

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 participate 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 (Ethikkommission 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 individual 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 intermittent claudication, significant stenosis of arteries and symptoms of intermittent claudication, history of peripheral bypass surgery or other revascularisation procedures, d) for *aortic atherosclerosis*: symptomatic aortic aneurysm, diameter of infrarenal aorta >3 cm [17], aortic surgery for atherosclerosis and e) for *atherosclerosis of the kidney*: renal artery stenosis, impaired renal function [18] with normal urinalysis, history of renal artery revascularisation procedures. A hundred of the 269 patients were affected by any of these manifestations of the disease, i.e., they had suffered from proven cardiovascular events in the past and

Table 2

Characteristics of the two patient groups.

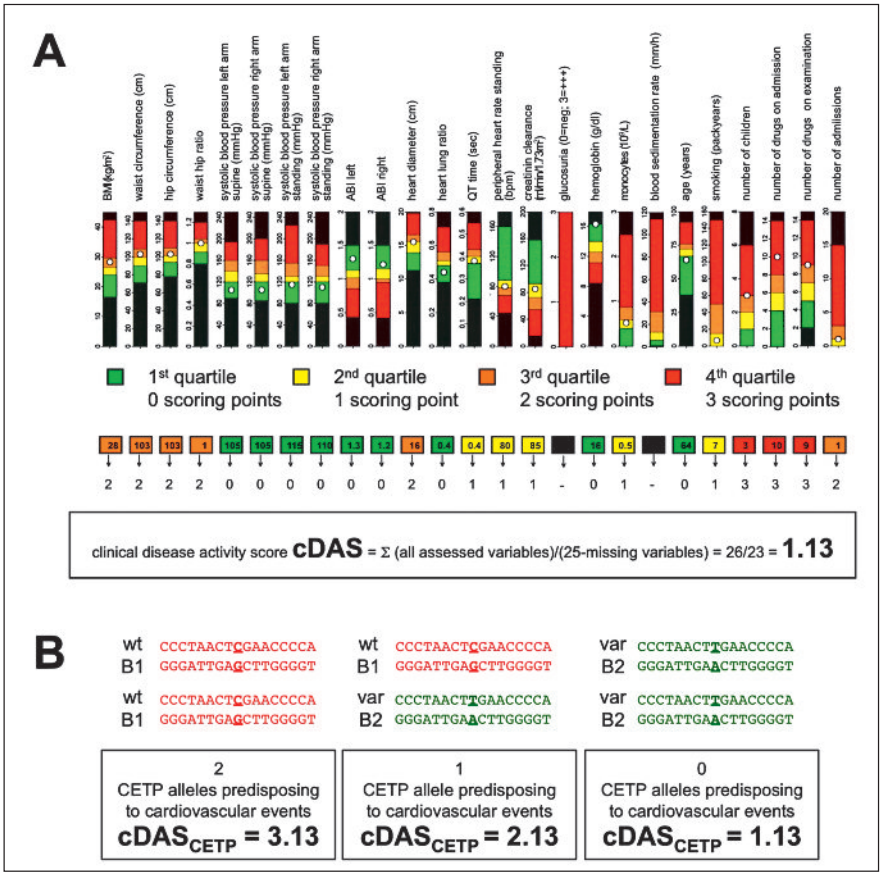
	No cardiovascular events (n = 57 controls)	Cardiovascular events (n = 57 cases)	P
Cardiovascular risk factors			
Male sex, n (%)	31 (54)	31 (54)	1.0
Age, y	69 ± 11	68 ± 10	0.64
Diabetes mellitus, n (%)	11 (19)	16 (28)	0.27
Body mass index, kg/m^2	26.5 ± 4.2	26.7 ± 4.7	0.69
Hypercholesterolaemia, n (%)	33 (58)	31 (54)	0.70
Hypertension, n (%)	36 (63)	32 (56)	0.44
Positive family history, n (%)	31 (54)	38 (67)	0.18
Smoking, n (%)	38 (67)	35 (61)	0.56
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)]	1.14 ± 0.37	1.32 ± 0.43	0.02
cDAS$_{CETP}$ [2)]	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$_{CETP}$ (fig. 1B).

Figure 1

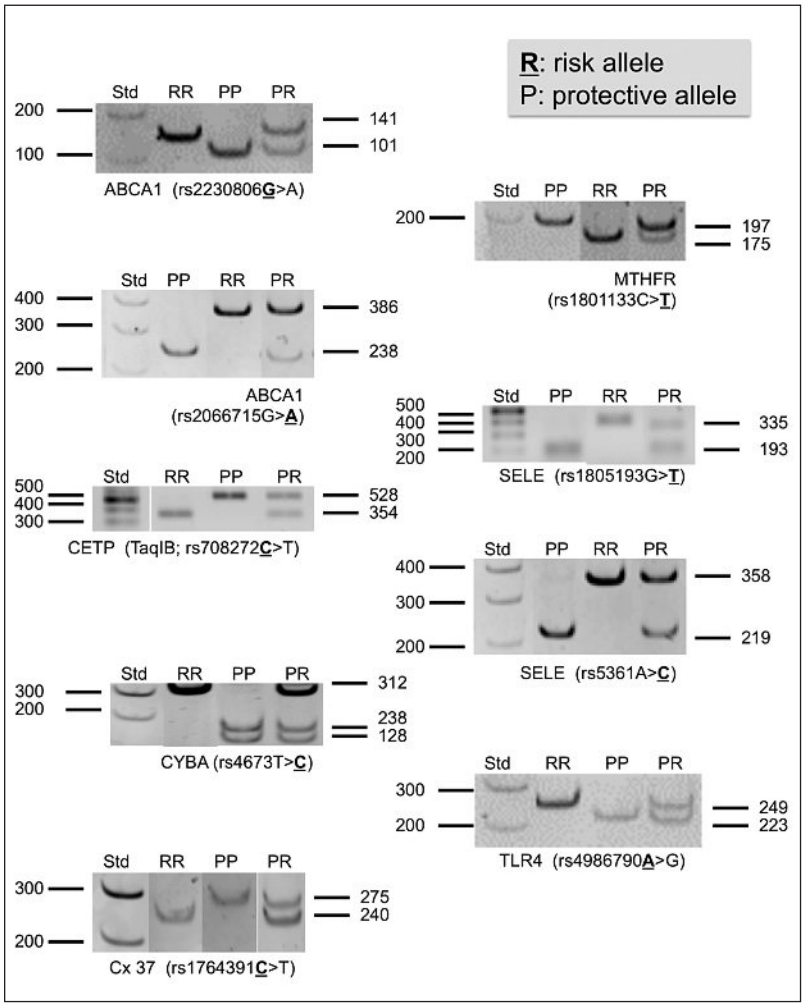
The novel method for clinical phenotyping and the calculation of the clinical disease activity score (cDAS).
A. The 25 variables that differed significantly between patients with and without symptomatic atherosclerosis (12) are shown as labelled colour-coded columns. Each column visualises the quartile 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 CETPrs708272C/TaqIB1 alleles per patient is added to the individual cDAS and results in cDAS$_{CETP}$ (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].

Figure 2

Restriction fragment length polymorphisms used to identify the nine SNPs. Restriction enzyme digests of PCR products were separated by gel electrophoresis and stained with ethidium bromide. By applying this method, an unambiguous result was obtained for all participants in this study.



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 $2x10^6$ cells by MagNA Pure LC DNA Isolation Kit I (3003990, Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions and stored at 4°. The relevant nucleotide fragment was amplified by PCR at 55° annealing temperature with 40 amplification cycles using Go Taq Flexi DNA polymerase (M8305, 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 apparent way 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 colour 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 colour, = 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/m^2, he scores 2 points for this variable, for a waist circumference of 103 cm, another 2 scoring points are added, and so forth.

Figure 3

Technical data (primer sequence, restriction enzymes used, fragment length of PCR product and RE digests) for SNP identification by PCR and RFLP.

Gene symbol	SNP (rs number)	upper primer (5')	lower primer (3')	full length product (bp)	RE	wt fragments (bp)	var fragments (bp)
ABCA1	rs2230806G>A	GCAAGGCTACCAGTTACATT	GAACGAAGTACTCGCTCTGC	141	EcoN I	141	101/40
ABCA1	rs2066715G>A	GAACGAAGTACTCGCTCTGC	CCCATGCACTGCAGAGATTC	386	Bsa I	238/148	386
CETP	rs708272C>T	CACTAGCCCAGAGAGAGGAGTGCC	CTGAGCCCAGCCGCACACTAAC	528	Taq I	354/174	528
CYBA	rs4673C>T	TGCTTGTGGGTAAACCAAGGCCGGTG	AACACTGAGGTAAGTGGGGGTGGCTCCTGT	312	Rsa I	312	184/128
Cx37	rs1764391C>T	CTGGACCCACCCCCTCAGAATGGCCAAAGA	AGGAAGCCGTAGTGCCTGGTGG	275	Drd I	240/35	275
MTHFR	rs1801133C>T	TGAAGGAGAAGGTGTCTGCGGGA	GGACGGTGCGGTGAGAGTG	197	Hinf I	197	175/22
SELE	rs1805193G>T	TTGCCCAAAATCTTAGGATG	AAGCCCAGGGAAGAACACAT	335	Hph I	193/142	335
SELE	rs5361A>C	ATGGCACTCTGTAGGACTGCT	GTCTCAGCTCACGATCACCAT	358	Pst I	219/139	358
TLR4	rs4986790A>G	GATTAGCATACTTAGACTACTACCTCCATG	GATCAACTTCTGAAAAAGCATTCCCAC	249	Nco I	249	223/26

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 χ^2 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].

Proatherosclerotic polymorphisms of connexin 37 (CX37) and toll receptor 4 (TLR4) were more common in the symptomatic patients, but these differences did not reach statistical significance. For six of the nine polymorphisms (ABCA1101051G>A, ABCA12868G>A, CYBA 4673T>C, MTHFR677C>T, SELE98G>T and SELE561A>C), the reportedly proatherosclerotic allele was found less frequently in the patient group with symptomatic disease. However, for

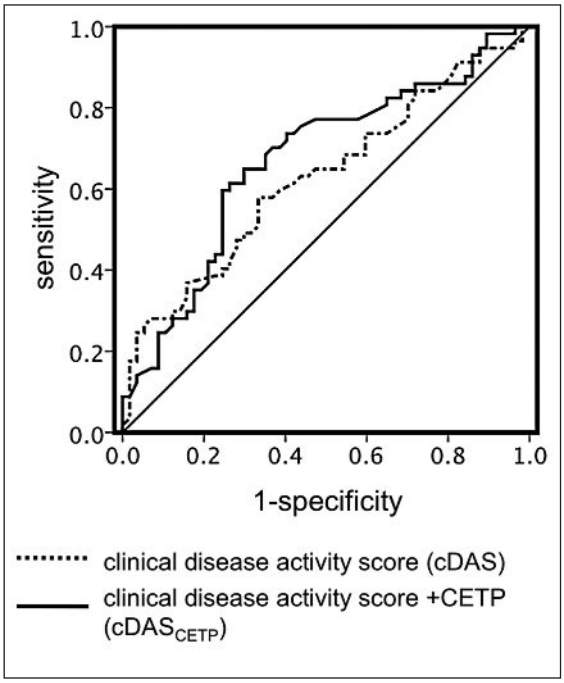


Figure 4

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‡
ABCA1(rs2230806**G**>A)	88/114	83/114	0.79 (0.43–1.44)	0.444
ABCA1(rs2066715G>**A**)	10/114	7/114	0.68 (0.25–1.85)	0.449
CETP (rs708272**C**>T)	59/114	76/114	1.86 (1.09–3.18)	0.022**
CYBA (rs4673T>**C**)	72/114	70/114	0.93 (0.54–1.58)	0.941
CX37 (rs1764391**C**>T)	77/114	83/114	1.69 (0.39–7.28)	0.385
MTHFR (rs1801133C>**T**)	42/114	39/114	0.80 (0.46–1.39)	0.678
SELE (rs1805193G>**T**)	14/114	9/114	0.61 (0.25–1.48)	0.272
SELE (rs5361A>**C**)	15/114	9/114	0.60 (0.25–1.49)	0.271
TLR4 (rs4986790**A**>G)	105/114	106/114	1.13 (0.42–3.05)	0.800

** Significant difference between the two patient groups. ‡ χ^2 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 CETPrs708272/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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