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CD36 mRNA EXPRESSION IS INCREASED IN CD14$^+$ MONOCYTES OF PATIENTS WITH CORONARY HEART DISEASE

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SUMMARY

1. Blood-derived monocytes/macrophages within the intima of the arterial wall are the main source of inflammatory cytokines and factors contributing to lesion growth, plaque instability and thrombotic events. In the present study, we assessed the hypothesis that mRNA expression levels of candidate genes of atherosclerosis in circulating CD14$^+$ blood monocytes are associated with coronary heart disease (CHD).

2. We investigated mRNA expression levels using reverse transcription–polymerase chain reaction of genes involved in cholesterol uptake (macrophage scavenger receptor (*MSR1*), scavenger receptor class B member 1 (*SRB1*), lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (*LOX1*), *CD36*, LDL receptor (*LDLR*)), reverse cholesterol transport (apolipoprotein E (*ApoE*), ATP-binding cassette sub-family A member 1 (*ABCA1*)) and inflammation (tumour necrosis factor-α (*TNF-α*), macrophage inflammatory protein-1α (*MIP-1α*), interleukin-6 (*IL-6*), *tissue factor*) in CD14$^+$ monocytes from 119 consecutively recruited patients and found that median *CD36* mRNA expression levels were significantly increased in patients with CHD compared with controls (111×10^3 *vs* 96×10^3 copies/10^6 copies *β-actin*, respectively; $n = 79$ and 40, respectively; $P < 0.05$), despite a high interindividual variability in gene expression.

3. A common T → C polymorphism (rs2151916) located only 14 bp upstream of the upstream transcriptional start site did not influence CD36 expression.

4. Expression levels of the other candidate genes investigated in the present study did not show any statistically significant differences between patients with CHD and controls.

5. We conclude that CD36 mRNA expression is significantly increased in patients with CHD and may serve as an indicator of CHD burden.

Key words: CD36, coronary heart disease, gene expression, monocytes, mRNA.

INTRODUCTION

Atherosclerotic coronary heart disease (CHD) is the major cause of morbidity and mortality in Westernized societies.[1] Epidemiological studies have revealed important genetic and environmental risk factors associated with CHD.[2] It is estimated that the major risk factors (cigarette smoking, hypertension, hypercholesterolaemia and diabetes mellitus) account for approximately 50% of the variability in CHD risk in high-risk populations.[3,4] The remaining risk is likely to be composed of yet to be identified minor risk factors or genetic influences.[2,5,6] The formation of atherosclerotic lesions is triggered by the cellular and immunological response of the vessel wall to metabolic injury.[7] In the early stages of atherogenesis, blood monocytes migrate into the intima of the arterial wall.[1,7] They transform into macrophages and become foam cells by accumulating cholesterol through scavenger receptor (SR)-dependent pathways. Monocyte-derived macrophages within the intima of the arterial wall are the main source of inflammatory cytokines, procoagulatory proteins and matrix-degrading metalloproteinases, thereby contributing to lesion growth, plaque instability and thrombotic events.[8] Previous work has focused on the identification of plasma components as indicators of cardiovascular risk, such as plasma lipoproteins, or markers of inflammation.[9] The aim of the present study was to assess the hypothesis that mRNA expression levels of candidate genes of atherosclerosis in circulating blood monocytes are associated with CHD. Thus, we investigated mRNA expression levels of genes involved in cholesterol uptake (macrophage scavenger receptor (*MSR1*), scavenger receptor class B member 1 (*SRB1*), lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (*LOX1*), *CD36*, LDL receptor (*LDLR*)), reverse cholesterol transport (apolipoprotein E (*ApoE*), ATP-binding cassette sub-family A member 1 (*ABCA1*)) and inflammation (tumour necrosis factor-α (*TNF-α*), macrophage inflammatory protein-1α (*MIP-1α*), interluekin-6 (*IL-6*), *tissue factor*) in monocytes isolated from patients with and without CHD.

METHODS

Study population

A total of 119 patients undergoing cardiac catheterization at the University Hospital Munich, Grosshadern (Department of Medicine I) were included in the study. All patients gave informed consent prior to enrolment. Arterial blood was drawn during cardiac catheterization using the standard Judkins technique. The study protocol was approved by the Ethics Committee of the Faculty of Medicine. Patient characteristics are listed in Table 1.

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Table 1 Demographic, clinical and angiographic characteristics fo patients with and without coronary heart disease

	No CHD ($n = 40$)	CHD ($n = 79$)	*P* value
No. men	19 (48%)	65 (82%)	< 0.001
Age (years)	57 ± 12	64 ± 10	< 0.05
BMI (kg/m^2)	27.6 ± 6.6	25.9 ± 3.8	NS
LVEF (%)	64 ± 18	66 ± 17	NS
No. patients with:			
Hypertension	13 (33%)	41 (52%)	< 0.05
Current smoker	4 (10%)	12 (15%)	NS
CHD family history	13 (33%)	26 (33%)	NS
Diabetes mellitus	2 (5%)	12 (15%)	< 0.05
No. patients using:			
Beta-blockers	17 (43%)	46 (58%)	NS
Nitrates	9 (23%)	34 (43%)	< 0.05
ACEI	11 (28%)	27 (34%)	NS
Calcium antagonists	3 (8%)	7 (9%)	NS
Aspirin	15 (38%)	62 (79%)	< 0.001
Vitamin supplements	4 (10%)	2 (3%)	NS
Statins	6 (15%)	31 (39%)	< 0.01

Values are mean±SD or the number of patients, with the percentage given in parentheses.

BMI, body mass index; LVEF, left ventricular ejection fraction; CHD, coronary heart disease; ACEI, Angiotensin-converting enzyme inhibitors.

Plasma analyses and leucocyte count

All plasma analyses and the leucocyte count were performed on automated analysers according to *Richtlinie der Bundesärztekammer zur Qualitätssicherung quantitativer laboratoriumsmedizinischer Untersuchungen* (http://www.bundesaerztekammer.de/page.asp?his=1.120.121.1047.1053&all=true).

Isolation of CD14$^+$ monocytes

Mononuclear cells were isolated from 16 mL whole blood (Vacutainer CPT; Becton Dickinson, Heidelberg, Germany) by Ficoll density gradient centrifugation (20 min, 1500 *g*, 4°C). Cells were pooled, washed with phosphate-buffered saline (PBS; containing 2 mmol/L EDTA and 0.5% bovine serum albumin (BSA)) and monocytes were separated by magnetic cell sorting on a MACS LS+ separation column after incubation with 80 μL CD14-MicroBeads (15 min, 6°C; MidiMACS separation unit; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of monocytes was 95% according to morphological and cytochemical criteria.

Quantitative reverse transcription–polymerase chain reaction

Monocyte RNA was isolated with the monophasic phenol–guanidine isothiocyanate TRIzol reagent (Invitrogen, Karlsruhe, Germany). The cDNA was synthesised from 2 μg RNA by reverse transcription (RT) with Super-Script II RNase H$^-$ reverse transcriptase (Invitrogen) and random hexamer primers. Gene expression was determined by quantitative fluorogenic RT-polymerase chain reaction (PCR; ABI PRISM 7700 SDS; PE Applied Biosystems, Foster City, CA, USA) using specific primers and probes. Primers and probes were selected to span two exons in order to prevent amplification of genomic DNA (Table 2). The PCR was prepared in a final volume of 50 μL reaction mixture containing 10 μL cDNA (diluted 1 : 10), 5 mmol/L $MgCl_2$, 5 μL 10× AmpliTaq buffer A, 200 μmol/L each dNTP, 0.5 U AmpErase, 1.25 U AmpliTaq Gold (Applied Biosytems), 200 nmol/L 6-carboxyfluorescein (FAM)/carboxytetramethyl rhodamine (TAMRA)-labelled TaqMan probe and 900 nmol/L of each oligonucleotide primer. The cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 two-step cycles of 95°C for 15 s and 60°C for 1 min. Standardization was performed using serial dilutions of linearized plasmid cDNA ranging from 10 to 10^7 copies. Data were analysed with ABI PRISM sequence detection software. The intra- and interassay coefficients of variation were 5–15% and 7–27%, respectively. In the present study, all mRNA gene expression results are given as the copy number of a particular candidate gene normalized to 10^6 copies of *β-actin*.

Table 2 Sequences of TaqMan primers and probes

Gene	Sense primer (s), antisense primer (a) and fluorogenic probe (p)
MSRI	(s) 5′-TTT ACC TCC TCG TGT TTG CAG TT-3′
	(a) 5′-CAA TTC TTC GTT TCC CAC TTC AG-3′
	(p) 5′-TCA TCC CTC TCA TTG GAA TAG TGG CAG C-3′
CD36	(s) 5′-AAG TCA CTG CGA CAT GAT TAA TGG-3′
	(a) 5′-GAA CTG CAA TAC CTG GCT TTT CTC-3′
	(p) 5′-ACA GAT GCA GCC TCA TTT CCA CCT TTT GT-3′
LOX1	(s) 5′-GAG AGZ AGC AAA TTG TTC AGC TCC TT-3′
	(a) 5′-TGA GCC CGA GGA AAA TAG GTA AC-3′
	(p) 5′-CCG CAA GAC TGG ATC TGG CAT GGA-3′
ApoE	(s) 5′-GCT CAG CTC CCA GGT CAC-3′
	(a) 5′-GGC CTT CAA CTC CTT CAT GGT-3′
	(p) 5′-CGT CCA TCA GCG CCC TCA GTT CCT-3′
ABCA1	(s) 5′-GCC AGT GTA GCA GCG ACA AA-3′
	(a) 5′-CCA TAC CTA AAC TCA TTC ACC CAG A-3′
	(p) 5′-CTT TAA GCT TTT GGC TAT GAT CTG CAC ATA CGT CTT C-3′
SRBI	(s) 5′-GCC GTC GCT CAT CAA GCA-3′
	(a) 5′-GAT AGG GAT CTC CTT CCA CAT GTT G-3′
	(p) 5′-CCT TAA GAA CGT GCG CAT CGA CCC C-3′
LDLR	(s) 5′-TGC CAG TGT GAG GAA GGC TT-3′
	(a) 5′-TGC CGG TTG GTG AAG AAG AG-3′
	(p) 5′-AGC TGG ACC CCC ACA CGA AGG C-3′
MIP-1α	(s) 5′-TGA GAC GAG CAG CCA GTG CT-3′
	(a) 5′-GCA CAG ACC TGC CGG CTT-3′
	(p) 5′-AAG CCC GGT GTC ATC TTC CTA ACC AAG C-3′
TNF-α	(s) 5′-TCT TCT CGA ACC CCG AGT GA-3′
	(a) 5′-GGA GCT GCC CCT CAG CTT-3′
	(p) 5′-AGC CTG TAG CCC ATG TTG TAG CAA ACC CT-3′
IL-6	(s) 5′-GGT ACA TCC TCG ACG GCA TC-3′
	(a) 5′-CCA GTG CCT CTT TGC TGC TT-3′
	(p) 5′-CAG CCC TGA GAA AGG AGA CAT GTA ACA AGA CTA AC-3′
Tissue factor	(s) 5′-TGA TTC CCT CCC GAA CAG TTA A-3′
	(a) 5′-CCA CAG CTC CAA TGA TGT AGA ATA TT-3′
	(p) 5′-CGG AAG AGT ACA GAC AGC CCG GTA GAG TG-3′
β-Actin	(s) 5′-CCT GGC ACC CAG CAC AAT-3′
	(a) 5′-GCC GAT CCA CAC GGA GTA CTT-3′
	(p) 5′-ATC AAG ATC ATT GCT CCT CCT GAG CGC A-3′

MSRI, macrophage scavenger receptor I; *LOX1*, lectin-like oxidized low-density lipoprotein (LDL) receptor 1; *ApoE*, apolipoprotein E; *ABCA1*, ATP-binding cassette sub-family A member 1; *LDLR*, low-density lipoprotein receptor; *MIP-1α*, macrophage inflammatory protein-1α; *IL*-6, interleukin-6.

Single nucleotide polymorphism genotyping

DNA was extracted from whole blood using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). The –22 674T → C single nucleotide polymorphism (SNP; rs2151916) was genotyped by melting curve analysis in a homogeneous fluorescent-based PCR assay.[10,11] The DNA was amplified using the primers 5′-CAAAGGATGGAATGAATCAAGGGAATTA-3′ (900 nmol/L) and 5′-TGAAAGAGCACAAGGCCACAAATGT-3′ (100 nmol/L). The reaction mixture consisted of 4 mmol/L $MgCl_2$, 1× PCR puffer (Solis BioDyne, Tartu, Estonia), 250 nmol/L dNTP, 0.05 U/μL Taq polymerase (Solis BioDyne), 0.5 mg/mL BSA (New England Biolabs, Ipswich, MA, USA) primers and 100 nmol/L FAM/TAMRA-labelled probe 5′-TTGTGCT-GGGTCAAATCACTGCT-3′ in a final volume of 12.5 μL. Amplification and subsequent melting curve analysis were performed in a 384-well format using an ABI 7900 sequence detector by denaturing (95°C, 3 min), amplification for 45 cycles (95°C, 20 s; 62.5°C, 30 s; 72°C, 60 s), elongation of the final product (72°C, 7 min), denaturing of the PCR product (95°C, 3 min), annealing of the probe (30°C, 2.5 min) and slowly heating the mixture at a ramp rate of 10% to 90°C while measuring the fluorescence in the sample. Genotypes were assigned according the melting points of the T allele (67°C) and the C allele (59°C).

Statistical analysis

All data are given as the mean±SD unless stated otherwise. Statistical significance was calculated using SPSS software version 11.5 (SPSS, Chicago, IL, USA) using the Mann–Whitney–White, Kruskal–Wallis and Chi-squared tests. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Coronary angiography revealed that 79 of 119 patients included in the study had stenoses in at least one major epicardial artery (≥ 30% narrowing) and were thus classified as 'CHD patients', whereas 40 individuals had normal coronary arteriograms and were classified as controls (no CHD). Characteristics of the study groups are given in Table 1. As expected, there were significantly more males and patients with diabetes and hypertension in the CHD patient group than in the control group. In addition, CHD patients were older and were more often treated with nitrates, aspirin and statins. Low-density lipoprotein (LDL)–cholesterol was significantly lower but very low-density lipoprotein (VLDL)–cholesterol was significantly higher in CHD patients compared with controls (Table 3). Small but significant differences were also observed for leucocyte count and serum creatinine concentrations. Even though there was a trend towards higher C-reactive protein levels in CHD patients compared with controls, the difference was not statistically significant. One could speculate that this lack of significance was due, in part, to the relatively high variability of individual values and the medications being taken by patients.

mRNA expression in circulating monocytes

Quantitative gene expression was investigated in circulating $CD14^+$ blood monocytes from each of the 119 patients included in the study. Using TaqMan RT-PCR, we measured the mRNA expression of 11 individual genes involved in cholesterol uptake (*SR-A*, *SR-B*, *LOX1*, *CD36*, *LDLR*), reverse cholesterol transport (*ApoE*, *ABCA1*), inflammation (*TNF-α*, *MIP-1α*, *IL-6*) and procoagulatory activity (*tissue factor*). We then analysed the means and range of mRNA expression levels for each of the genes in the complete study group, because it was hitherto unknown whether and at what level the genes were expressed in isolated human monocytes. We found that mean mRNA levels were distributed over a wide range: *LOX1*, *tissue factor* and *LDLR* had the lowest expression ($< 1 \times 10^3$ copies/10^6 copies *β-actin*) and were deemed to be not expressed at a relevant level. Copy numbers of the remaining eight genes ranged between $1 \pm 1 \times 10^3$ copies/10^6 copies *β-actin* for IL-6 and $112 \pm 52 \times 10^3$ copies/10^6 copies *β-actin* for *CD36*. Next, we tested for differences in expression levels between CHD patients and controls. The major finding of the present study was that the expression of *CD36*, a receptor for oxidized LDL uptake, was significantly higher in CHD patients than controls (median 111×10^3 *vs* 96×10^3 copies/10^6 copies *β-actin*, respectively; $P < 0.05$). There were no signficant differences detected between the CHD patient and control groups for the other genes included in the present study (Fig. 1).

Table 3 Laboratory values

Variable	No CHD ($n = 40$)	CHD ($n = 79$)	P value
Total cholesterol (mmol/L)	5.2 ± 1.1	4.9 ± 1.0	NS
LDL–cholesterol (mmol/L)	3.4 ± 0.9	3.1 ± 0.9	$P < 0.05$
HDL–cholesterol (mmol/L)	1.24 ± 0.36	1.11 ± 0.35	NS
VLDL–cholesterol (mmol/L)	0.54 ± 0.23	0.67 ± 0.36	$P < 0.02$
Triglycerides (mmol/L)	1.36 ± 0.56	1.62 ± 0.83	NS
ApoAI (μmol/L)	48.2 ± 9.6	45.3 ± 8.2	NS
ApoB (μmol/L)	2.02 ± 0.42	1.97 ± 0.40	NS
Glucose (mmol/L)	5.23 ± 0.61	5.95 ± 1.78	NS
Lp(a) (mmol/L)	0.21 ± 0.25	0.36 ± 0.48	NS
Fibrinogen (g/L)	3.24 ± 0.83	3.53 ± 0.77	NS
hsCRP (mg/L)	5.2 ± 8.8	9.3 ± 19.5	NS
Leucocytes ($\times 10^9$/L)	6.1 ± 1.4	6.9 ± 1.8	$P < 0.05$
Creatinine (μmol/L)	91 ± 81	103 ± 81	$P < 0.02$
ACE (μkat/L)	522 ± 302	467 ± 283	NS

LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; Apo, apolipoprotein; Lp(a), lipoprotein (a); hsCRP, high sensitive C-reactive protein; ACE, angiotensin-converting enzyme.

CD36–22 674T → C polymorphism (rs2151916) and gene expression

Recent data have suggested that the T allele of a T → C polymorphism (rs2151916) located only 14 bp upstream of the upstream transcriptional start (UTS) site (–22 674 bp from ATG) may decrease expression levels of *CD36*.[11,12] The polymorphism was in Hardy–Weinberg equilibrium with an allele frequency of 59% for the T allele. Median expression levels of this polymorphism in the present study were 110×10^3 copies/10^6 copies *β-actin* in subjects with the T/T genotype, 102×10^3 copies/10^6 copies *β-actin* in subjects with the T/C genotype and 113×10^3 copies/10^6 copies *β-actin* in subjects with the C/C genotype. There was no significant difference between the three groups (Kruskal–Wallis analysis). We also calculated the frequency of carriers of the T allele in the CHD patient and control groups. Although the T allele was slightly more frequent in CHD patients (88%) than in controls (76%), the difference did not reach statistical significance (Chi-squared test).

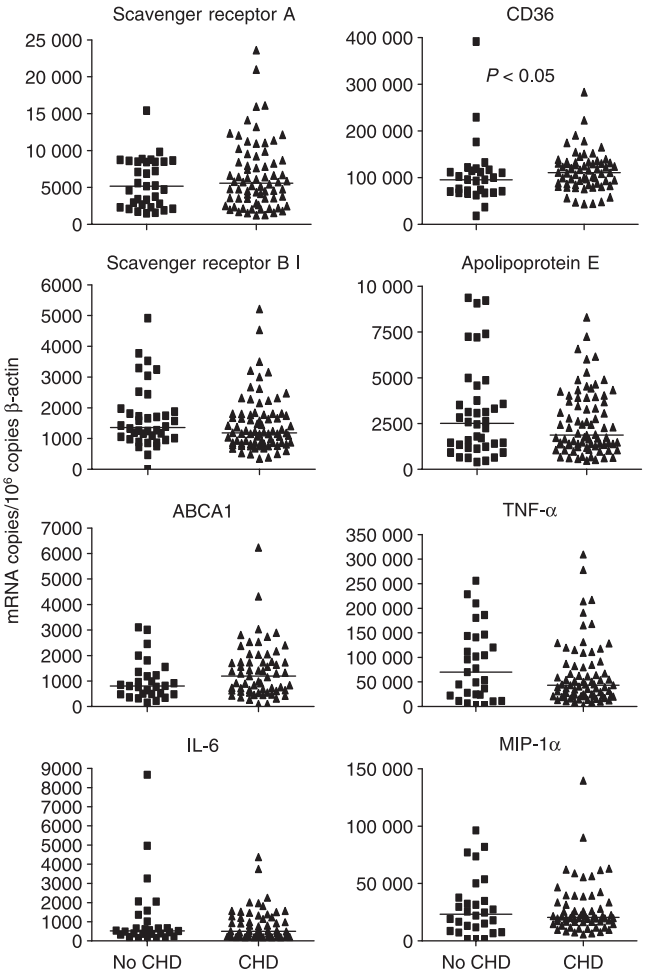

Fig. 1 mRNA expression levels of atherosclerosis-related genes in $CD14^+$ monocytes isolated from patients with and without coronary heart disease (CHD). Expression levels were normalized to 10^6 copies *β-actin*. Data are given as individual data points and median mRNA expression levels for each group. A statistically significant difference between CHD patients and controls was observed for *CD36* only ($P < 0.05$). *ABCA1*, ATP-binding cassette sub-family A member 1; *IL-6*, interleukin-6; *TNF-α*, tumour necrosis factor-α; *MIP-1α*, macrophage inflammatory protein-1α.

DISCUSSION

We have studied individual mRNA expression levels of 11 different atherosclerosis-related genes in monocytes isolated from 119 angiographically characterized patients with and without CHD. The main finding of the present study was that the mRNA expression of *CD36* in circulating monocytes was increased in angiographically assessed CHD patients compared with controls. The mRNA expression of the 10 other candidate genes of atherosclerosis included in the present study was either not detectable in monocytes or showed no significant differences between the two groups. CD36 is a scavenger receptor, present on the cell surface of monocytes and macrophages. It is responsible for approximately 50% of the binding activity of oxidized LDL.[13,14] Thus, high *CD36* expression could subsequently lead to an increased uptake of oxidized LDL and foam cell formation. Using a mouse model, it was shown that *CD36*-deficient animals had atherosclerotic lesions that were 76% smaller on the *ApoE*-deficient background.[15] Very recently, the same group, using bone marrow transplantation of *CD36*-deficient marrow into *ApoE*-deficient mice, demonstrated that the absence of macrophage-derived CD36 was protective against atherosclerosis.[16] Because most macrophages in the vessel wall originate from bone marrow-derived monocytes, the expression level of *CD36* mRNA in human monocytes may reflect the expression level in macrophages, similar to the findings in mice. Previous studies in cultivated cells have shown that *CD36* expression was further upregulated during the initial phase of maturation of monocytes into macrophages.[17] Owing to the paucity of monocytes isolated using the present study protocol, we have not investigated *CD36* expression levels during maturation of monocytes into macrophages.

It has been demonstrated previously that incubation of isolated monocytes with statins *in vitro* reduces *CD36* expression.[18,19] However, this does not explain the difference in *CD36* expression observed in the present study, because significantly more patients in the CHD patient group were on statins and still had increased *CD36* expression levels compared with he controls. Similarly, it has been shown that aspirin increases *CD36* expression in the monocyte cell line THP-1;[20] however, a subanalysis in the study could not confirm this effect in isolated circulating monocytes. Because there were significant differences in the numbers of male and female subjects in the groups with and without CHD, we also tested gender as a potential confounder of expression levels, but found no significant effect. Thus, it appears that increased expression of *CD36* is specific for CHD and is independent of the potentially confounding factors investigated.

Recent data from genetic association studies suggest that genetic variation may regulate expression levels of *CD36*.[11,12] A remarkable structural feature of the *CD36* gene is the presence of two alternative and independent promoters and first exons separated by approximately 13 kb.[21] It was suggested that the T allele of a –22 674T → C polymorphism (rs2151916) located only 14 bp upstream of the UTS site may decrease expression levels of *CD36*; however, this has not been proven experimentally. The T allele determines the position of a binding site for the transcriptional repressor Gfi-1B, whereas the C allele determines its absence.[11,22] However, *CD36* expression levels were independent of the –22 674T → C genotype in the present study population. The –22 674T → C polymorphism is in complete linkage disequilibrium (LD) with a –33 137 A → G polymorphism (rs1984112). The *CD36* gene consists of two LD blocks that could be tagged by the –33 137 A → G and four other polymorphisms. One particular haplotype, linked with the –33 137A allele was associated with increased cardiovascular risk.[11] Although not significant, our data also show a comparable trend. Thus, the –22 674T → C polymorphism may be associated with cardiovascular risk, but our data do not support an association of the polymorphism with *CD36* expression levels.

It is also know that *CD36* expression in monocyte/macrophages is upregulated by inflammatory mediators, such as macrophage colony stimulating factor (M-CSF) or IL-4.[23] In addition, expression of *CD36* has been shown to be upregulated by mildly and moderately oxidized LDL.[24] Thus, in light of these studies, we speculate that higher expression of *CD36* in monocytes from patients with CHD may be an indicator of an increased burden of oxidized LDL and inflammation in these patients. One caveat of the present data is that we investigated 11 different candidate genes in a relatively small cohort of 119 individuals. In this context, it should be kept in mind

that significant differences may also be due to multiple testing and will need replication in further studies.

In conclusion, we have investigated an association of mRNA levels of candidate genes of atherosclerosis in circulating monocytes from patients with CHD. Our results indicate that increased *CD36* mRNA expression in circulating monocytes may be a marker of CHD burden.

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