Impact of single nucleotide polymorphisms in the VEGFR2 gene on endothelial cell activation under non-uniform shear stress

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Abstract. Single nucleotide polymorphisms (SNPs) in vascular endothelial growth factor receptor 2 (VEGFR2) are associated with coronary artery disease, hypertension and myocardial infarction. However, their association with atherosclerosis remains to be fully elucidated. The purpose of the present study was to determine whether SNPs are involved in atherogenesis, by analyzing their impact on human umbilical vein endothelial cells (HUVECs) under laminar and non-uniform shear stress in a well-established *in vitro* model that simulates shear stress-induced proatherogenic processes at vessel bifurcations. All experiments were performed using freshly isolated HUVECs. Three SNPs in the *VEGFR2* gene (rs1870377 T>A, rs2071559 A>G and rs2305948 C>T) were genotyped and the expression levels of VEGFR2 were semi-quantitatively determined using western blotting. Subsequently, the HUVECs were seeded in bifurcating flow-through cell culture slides and flow (9.6 ml/min) was applied for 19 h, including tumor necrosis factor-α stimulation during the final 2 h of flow. The protein expression levels of VCAM-1, E-selectin and VEGFR2 and the adhesion of THP-1 cells were analyzed in laminar and non-uniform shear stress regions. Data were analyzed for associations with the respective SNPs. The total expression of VEGFR2 was significantly lower under non-uniform shear stress than under laminar shear stress conditions, independent of the genotype. The expression of VEGFR2 between the different shear stress patterns was not significantly altered by the different SNPs. The expression levels of VCAM-1 and E-selectin were lower in the A/A genotype compared with those in other genotypes in rs1870377 T>A and rs2071559 A>G. In conclusion, the results suggested that SNPs within the *VEGFR2* gene have a significant impact on shear stress-related endothelial activation.

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Abbreviations: CAD, coronary artery disease; CVD, cardiovascular disease; EC, endothelial cell; HUVECs, human umbilical vein endothelial cells; HWE, Hardy-Weinberg equilibrium; MI, myocardial infarction; NF-κB, nuclear factor-κB; PECAM-1, platelet endothelial cell adhesion molecule-1; PI3K, phosphoinositide 3-kinase; SNP, single nucleotide polymorphism; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VEGFR2, vascular endothelial growth factor 2

Key words: vascular endothelial growth factor receptor 2, single nucleotide polymorphism, endothelial dysfunction, atherosclerosis, shear stress, adhesion molecules

Introduction

The early stage of atherosclerotic plaque formation is characterized by endothelial dysfunction and inflammation. It has been shown that plaque development occurs at sites with disturbed blood flow, resulting in non-uniform shear stress acting on the endothelium (1). Hemodynamic forces at arterial bifurcations and curvatures have a marked impact on inflammatory gene expression in endothelial cells (ECs) (2,3). In addition, several risk factors for cardiovascular disease (CVD), including hypertension, diabetes mellitus, hypercholesterolemia and smoking, contribute to injury and inflammation of the endothelium.

Non-uniform shear stress induces endothelial dysfunction characterized by the expression of inflammatory cytokines and chemokines, increased endothelial permeability and leukocyte recruitment, and elevated expression of adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (4,5). By contrast, laminar shear stress in straight vessel segments induces laminar cell alignment and a quiescent phenotype in ECs, which prevents EC activation, and thus has an atheroprotective effect (6).

For several years it has been known that ECs express receptors to sense the hemodynamic status. Accordingly, the endothelium acts as a dynamic interface between hemodynamic

factors and the vascular wall. A mechanosensory complex on the EC surface is composed of platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial cadherin (VE-cadherin) and vascular endothelial growth factor receptor 2 (VEGFR2) transducing the physical properties of flow into an equivalent intracellular signal (7).

VEGFR2 is one of the three VEGF receptors, and its activation affects vascular permeability in addition to the proliferation, migration, differentiation and survival of ECs (8,9). Studies using VEGFR2-knockout mice have revealed that this receptor is indispensable for the development and formation of a blood vessel network, leading to fetal lethality in these mice (9,10). The activation of VEGFR2 triggers the conformational activation of integrins followed by the stimulation of nuclear factor-κB (NF-κB) (11), a transcription factor responsible not only for adaptation to flow but also for the *de novo* synthesis of adhesion molecules, including VCAM-1, E-selectin and ICAM-1. Therefore, the expression of VEGFR2 regulates endothelial activation by influencing the expression of adhesion molecules, leading to enhanced leukocyte adhesion that contributes to atherosclerotic plaque formation. Increased plasma levels of VEGF, which activates VEGFR2, are associated with the occurrence of atherosclerosis and coronary artery disease (CAD) (12,13). In our previous study, it was shown that the knockdown of VEGFR2 in human umbilical vein endothelial cells (HUVECs) decreased the expression of adhesion molecules and consequently reduced monocyte adhesion in regions of non-uniform shear stress. This effect was mediated by inhibition of the translocation of NF-κB from the cytoplasm to the nucleus (14).

The heritability of CVD is estimated to be 50-60% (15). In this context, single nucleotide polymorphisms (SNPs) have been a focus of interest for several years (16-19). Regarding VEGFR2, which consists of 1,356 amino acids, several hundreds of SNPs have been identified and are reported to be associated with the angiographic severity of atherosclerosis in patients with CAD (15). In particular, a study examining two independent populations showed that three SNPs in *VEGFR2*, rs2305948 C>T, rs1870377 T>A and rs2071559 A>G, are associated with an increased risk of CAD (20), most likely due to their role in blood vessel formation (10,21). Associations have been reported between rs2071559 A>G and CVD; the G/G genotype is more frequent than other genotypes in patients with type 2 diabetes and myocardial infarction (MI) (22), although the G allele showed a protective effect against CVD by increasing high-density lipoprotein cholesterol in an Asian population (23). Therefore, evidence suggests that SNPs in the VEGF/VEGFR2 system may be involved in the development of atherosclerosis. Furthermore, SNPs in PECAM-1, another member of the above-mentioned mechanosensory complex, have been shown to be associated with MI (24), atherosclerosis (25), ischemic stroke (26) and monocyte adhesion to endothelial cells (27).

Although the sensitivity of VEGFR2 to shear stress is well described, no information exists on the impact of the SNPs in VEGFR2 on shear stress-induced endothelial dysfunction on a molecular basis.

In terms of the present study, it was hypothesized that SNPs in VEGFR2 alter the expression of genes that are associated with endothelial cell activation (i.e., VCAM-1 and E-selectin) under different shear stress conditions. To test this hypothesis, the molecular responses in ECs to laminar and non-uniform shear stress were analyzed using a well-characterized *in vitro* flow chamber model. This model mimics hemodynamic properties at vessel bifurcations comparable to physiological conditions (4).

Materials and methods

Determination of SNPs in HUVECs. Umbilical cords were collected at the Department of Obstetrics and Gynecology, Erlangen University Hospital, Comprehensive Cancer Center Erlangen-EMN, Friedrich-Alexander University Erlangen-Nürnberg (FAU; Erlangen, Germany), which was approved by the Ethics Committee of the Medical Department of the FAU (case no. 246-13B). The HUVECs were isolated from the freshly collected umbilical cords using standard techniques and cultured in endothelial cell growth medium (ECGM; Promo Cell) at 37˚C in a humidified 7.5% CO_2 atmosphere. In all experiments, HUVECs at passages 1-2 were used.

Genomic DNA was extracted from 400,000-500,000 cells using the Promega® DNA-Isolation kit (Promega Corporation). All DNA samples were genotyped for the three selected SNPs in *VEGFR2*: rs1870377 T>A, rs2305948 C>T and rs2071559 A>G using TaqMan® SNP genotyping assays (Thermo Fisher Scientific, Inc.; assay nos. rs1870377: C-11895315_20; rs2305948: C-22271999-20, and rs2071559: C-15869271-10) with a reaction volume of 5 μl, on the QuantStudio™ 12K Flex Real-Time PCR system following the manufacturer's protocol. The thermocycling steps were programmed as follows: Pre-read stage at 60˚C for 30 sec; hold stage at 95˚C for 20 sec; 50 cycles of PCR stages at 95˚C for 3 sec and at 60˚C for 30 sec; post-read stage at 25˚C for 30 sec. The primers are listed in Table I. TaqMan® Genotyper software (Thermo Fisher Scientific, Inc.) was used for accurate genotype calling and visualization of the data.

The linkage disequilibrium was analyzed to determine whether the frequencies of the three SNPs were related to each other by utilizing a web-based analysis tool (https://ldlink.nci.nih.gov/; LDpair, LDpop) (28,29), using genome-wide association study (GWAS) data from the European population as the reference.

Expression levels of VEGFR2 in HUVECs. The basal expression level of VEGFR2 was determined semi-quantitatively. The HUVECs were harvested to extract total protein for a bicinchoninic acid protein assay (Thermo Fisher Scientific, Inc.) and 30-60 ng protein was used for western blotting. The samples were separated on 8% SDS-PAGE gels and transferred onto a PVDF membrane (Macherey-Nagel) via semi-dry blotting. The membranes were blocked with 5% skim milk (Bio-Rad Laboratories, Inc.) in Tris-buffered saline with 0.1% Tween (TBS-T) or 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and stained with either anti-VEGFR2 antibody (cat. no. sc-505, 1:500, Santa Cruz Biotechnology, Inc.) in 2.5% skim milk, or anti-vinculin antibody (cat. no. sc-25336, 1:500, Santa Cruz Biotechnology, Inc.) in TBS-T at 4˚C overnight. The secondary antibodies anti-rabbit (cat. no. RPN4301, 1:10,000) and anti-mouse (cat. no. NX931, 1:50,000) coupled to horseradish peroxidase (Amersham; GE Healthcare Life Sciences) were incubated for 2 h at room temperature. The signals were

Table I. TaqMan assay probes for analysis of vascular endothelial growth factor receptor 2 SNPs.

NCBI RefSNP ID	SNP	Mutation	Context sequence
rs1870377 T>A	Exon 11	Q472H Glu Q (CAA)> His H (CAT)	5'-GGTATGGGTTTGTCACTGAGACAGC[A/T] TGGCTATAAGAAAGAGATAACAGCG-3'
rs2305948 C>T	Exon 7	I297VVal V (GTA)> Ile I (ATA)	5'-TACAATCCTTGGTCACTCCGGGTTA[C/T] ACCATCTATAGTTAAGGTGCTCAAA-3'
rs2071559 A>G	Intron	-	5'-GAAAACGCACTTGCCCAGTTCGCCA[A/G] CATTCCCGCTATTTCCCAAAATATT-3'

Context sequence refers to the TaqMan probe binding the DNA. SNP, single nucleotide polymorphism.

detected with ECL-Prime (GE Healthcare Life Sciences) using ChemiDoc XRS (Bio-Rad Laboratories, Inc.). The expression levels of VEGFR2 were semi-quantitatively analyzed using ImageLab® 5.1 software (Bio-Rad Laboratories, Inc.) with vinculin as a loading control.

Flow experiments. The HUVECs were seeded at $7x10^5$/ ml into bifurcation flow-through slides (Integrated BioDiagnostics) and grown until confluence. The cell monolayer was perfused with medium for 19 h, at a flow rate of 9.6 ml/min, corresponding to a laminar shear stress of 10.2-10.8 dyne/cm^2 in the straight main channel and a non-laminar shear stress of ~6.3 dyne/cm^2 to ~0.5 dyne/cm^2 in the channel distal to the bifurcation (4). The cells were stimulated with tumor necrosis factor (TNF)-α (2.5 ng/ml; Miltenyi Biotec) for the final 2 h of flow.

Immunofluorescent staining. Following the flow experiment, the protein expression of VCAM-1, E-selectin and VEGFR2 was determined by immunocytochemical staining. The HUVECs were fixed with 4% formalin (Roth), permeabilized with 0.2% Triton X-100 (Sigma; Merck KGaA) in PBS, and blocked with 5% horse serum (Gibco; Thermo Fisher Scientific, Inc.) in PBS. Staining was performed with anti-VCAM-1 (cat no. BBA5, CloneBBIG-V1, 1:100; R&D Systems, Inc.), anti-E-selectin (cat. no. BBA16, Clone BBIG-E4, 1:100; R&D Systems, Inc.) and anti-VEGFR2 (cat. no. sc-505, N-931; 1:100; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h and visualized using Alexa Fluor 488-conjugated anti-mouse IgG (cat. no. A11001) or anti-rabbit IgG (cat. no. A11008) for 45 min at room temperature (1:500 in PBS; Molecular Probes; Thermo Fisher Scientific, Inc.).

To determine the average protein expression in the laminar and non-uniform shear stress regions within every slide, six visual fields at x200 magnification (0.33 mm^2 each) were selected from the laminar shear stress region and eight visual fields from the non-uniform shear stress regions (Fig. S1) (4). Digital images were captured using an inverted fluorescent microscope (Olympus) with image processing software (NIS Elements® 3.2; Nikon). For every image, a threshold was set to define the positive fluorescent signal intensity of the entire image. Analyses were performed using ImageJ® 1.48v software, and the corrected total cell fluorescence (CTCF) was calculated as follows: CTCF = integrated density - (area of selected cell x mean fluorescence of background) (30).

Adhesion assay. Adhesion assays were performed as described in previous studies (5,31). THP-1 monocytic cells (American Type Culture Collection; cat. no. TIB-202) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/l glutamine (Biochrom AG), 100 U/ml penicillin and 100 µg/ml streptomycin (both antibiotics from Gibco; Thermo Fisher Scientific, Inc.). The THP-1 cells used for adhesion assays were in passages 5-25. Following TNF-α stimulation (2.5 ng/ml), the HUVECs were perfused with 7,500,000 THP-1 monocytic cells for 1 h at 37˚C in ECGM. Subsequently, any non-adhering THP-1 cells were flushed away with ECGM. The HUVECs with adhering THP-1 cells were fixed with 4% formalin, treated with 0.2% Triton X-100 in PBS and stained with hematoxylin and eosin, which stains nuclei dark blue and the cytoplasm light red. The distinction between HUVECs and THP-1 cells was clear using this staining (Fig. 1B). For every slide, six visual fields at x200 magnification (0.33 mm^2) were selected from the laminar shear stress region and eight visual fields were selected from the non-uniform shear stress regions. Digital images were captured using an inverted microscope (Olympus) with image processing software and the numbers of THP-1 cells per visual field were counted (NIS Elements® 3.2; Nikon).

Statistical analysis. Statistical analyses were conducted using SigmaPlot® 12.3 (Systat Software, Inc). The Hardy-Weinberg equilibrium (HWE) of the occurrence and distribution in sex for three SNPs were assessed using Fisher's exact test or the χ^2 test, respectively. Differences between the laminar and non-uniform shear stress regions were analyzed using the Wilcoxon Signed Rank test. Among the three genotype groups, differences in adhesion molecule expression, the expression of VEGFR2 and the number of adhered THP-1 cells were compared using Kruskal-Wallis one-way ANOVA on ranks with a post hoc test (Bonferroni). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the observed HUVECs. The HUVECs were collected from 113 individuals, comprising 61 men (54%) and 52 women (46%). The distribution of genotypes regarding the three SNPs was in accordance with the Hardy-Weinberg equilibrium, and the allele frequencies were similar to those in former studies (Fig. S2). There was no difference in the

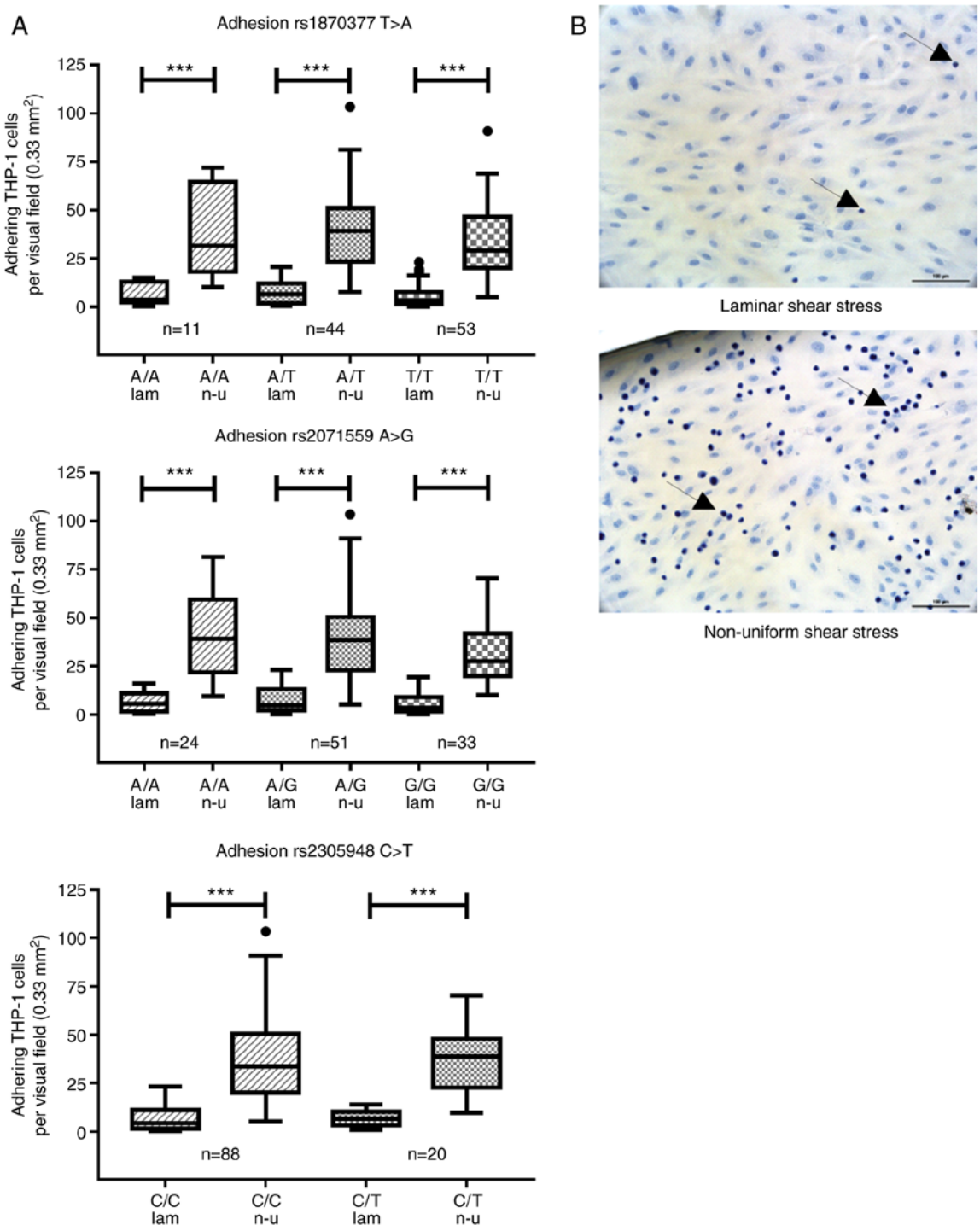

Figure 1. THP-1 monocytic cell adhesion to flow-activated endothelial cells under laminar and non-uniform shear stress. Human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h and perfused for an additional 1 h with 7,500,000 THP-1 monocytic cells. Cells were fixed and stained with hematoxylin and eosin. Data are expressed as the median with 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles (Tukey-style). (A) Results of adhesion assays in laminar and non-uniform shear stress regions in rs1870377 T>A; rs2071559 A>G and rs2305948 C>T, measured by Wilcoxon Signed Rank test. ***P<0.001. (B) Representative images of THP-1 adhesion in regions of laminar and non-uniform shear stress; images were captured at x200 magnification; the arrows point to THP-1 monocytic cells (scale bar, 100 μm). lam, laminar; n-u, non-uniform.

ratio of men to women among genotypes for all SNPs. No significant correlations were observed between any of the genotypes and being male, which is a cardiovascular risk factor (Table II). The homozygous T/T genotype in rs2305948 C>T was excluded from further analyses due to its sample size being too small (Fig. S2 and Table II). Even in a T allele dominant model, comparing the C/C genotype with the C/T + T/T genotypes revealed no significant differences in the protein expression of VEGFR2, VCAM-1 or E-selectin (data not shown).

The results of the linkage analyses revealed that there was no disequilibrium among the three analyzed SNPs compared with the GWAS data of a European population; therefore, these SNPs were considered as independent variables (28,29).

Table II. SNP characteristics.

SNP	Genotype (n)	Sex, n (%)		P-value[a]	Allele frequency	Global MAF[b]
		Male	Female			
rs1870377 T>A	AA (11)	5 (45.5)	6 (54.5)	0.790		
	AT (44)	24 (54.5)	20 (45.5)		A=0.297	A=0.212
	TT (58)	33 (56.9)	25 (43.1)		T=0.703	
rs2071559 A>G	AA (33)	20 (60.6)	13 (39.4)			
	AG (53)	25 (47.2)	28 (52.8)	0.235	A=0.536	A=0.499
	GG (26)	17 (65.4)	9 (34.6)		G=0.464	
rs2305948 C>T	CC (90)	51 (56.7)	39 (43.3)			
	CT (21)	10 (47.6)	11 (52.4)	0.612[c]	C=0.885	T=0.153
	TT (1)	1 (100)	0 (0)		T=0.115	

No significant correlations were observed between genotype and male sex, a known cardiovascular risk factor. Statistical analysis was performed using 2x3 Fisher's exact test or χ^2 test. P<0.05 was considered to indicate a statistically significant difference. [a]Fisher's exact test; http://vassarstats.net/fisher2x3.html; [b]Global MAFs refer the data established by the 1000 Genomes Project (https://www.ncbi.nlm.nih.gov/projects/SNP/). [c]χ^2 test between CC and CT. SNP, single nucleotide polymorphism; MAFs, minor allele frequencies.

Basal expression of VEGFR2 is not affected by SNPs in VEGFR2. The basal expression levels of VEGFR2 under static conditions were semi-quantitatively evaluated by western blotting. The results revealed no significant differences among the three genotypes, although the highest median expression of VEGFR2 was observed in A/A in rs2071559 A>G compared with that in the other two genotypes (Fig. 2; one-way ANOVA on ranks 0.096).

Flow-induced expression of VEGFR2 is not affected by SNPs. The expression levels of VEGFR2 were evaluated in HUVECs cultured under non-uniform shear stress and laminar shear stress for 19 h. The collective VEGFR2 expression data from samples with any genotype are shown in Fig. 3, grouped according to flow type (Fig. 3A). In regions of non-uniform shear stress, the HUVECs expressed significantly lower levels of VEGFR2 compared with that in regions of atheroprotective laminar shear stress (P<0.001; Fig. 3A), independent of the genotypes of any the SNPs. There was no significant difference in expression of VEGFR2 under either laminar or non-uniform shear stress among the three genotypes of any the SNPs (Fig. 4).

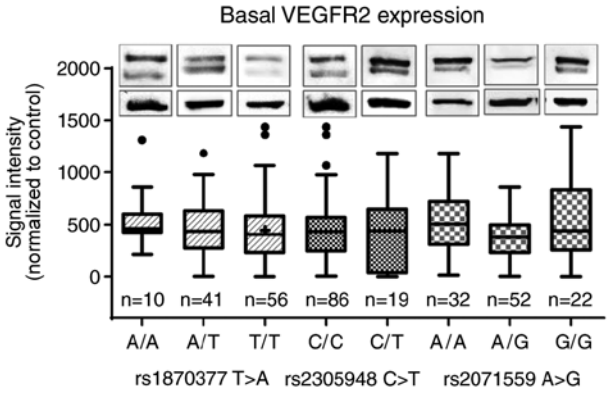

Figure 2. Basal expression of VEGFR2. Human umbilical vein endothelial cells were cultured under static conditions. Cells were harvested for protein measurement and semidry western blotting was performed for semi-quantitative analyses of VEGFR2, with vinculin as a loading control. Representative western blot images show the expression of VEGFR2 (upper double-band) and vinculin (lower band) of different donors. Data are expressed as the median with 25th and 75th percentiles (Tukey-style). Comparison between genotypes of one SNP was measured by one-way ANOVA on Ranks (rs1870377 T>A, rs2071559 A>G) or the Mann-Whitney Rank Sum test (rs2305948 C>T). VEGFR2, vascular endothelial growth factor receptor 2

Expression of endothelial cell activation markers is affected by SNPs in VEGFR2. The HUVECs were cultured under non-uniform or laminar shear stress conditions and stimulated with TNF-α. The protein expression levels of adhesion molecules (i.e., VCAM-1 and E-selectin, markers for endothelial activation) were quantified by means of fluorescent immunocytochemical staining (Fig. 5). As expected, the expression of VCAM-1 was significantly higher in the non-uniform regions compared with that in laminar shear stress regions in all datasets analyzed using the Wilcoxon Signed Rank test.

In rs1870377 T>A, significant differences were observed between the different genotypes: Under laminar shear stress, the highest median expression of VCAM-1 was observed in T/T, followed by a lower expression in T/A (67% of T/T), and the lowest expression in A/A (18% of T/T) genotypes. This showed an increased expression of VCAM-1 with increasing content of the T allele (one-way ANOVA on Ranks 0.052). This trend was strengthened by non-uniform shear stress conditions; the expression of VCAM-1 was significantly lower in the A/A genotype (74% reduction) than that in the T/T genotype (one-way ANOVA on Ranks 0.023), with intermediate expression in the heterozygous A/T (40% reduction) genotype (Fig. 6A). A tendency of increased median expression of VCAM-1 with increasing G-content was observed in rs2071559 A>G, but the differences were not statistically significant (52% reduction in A/A under laminar flow pattern and 63% under non-uniform shear stress; Fig. 7A). Furthermore, no significant differences in VCAM-1 were observed among the different genotypes in rs2305948 C>T (data not shown).

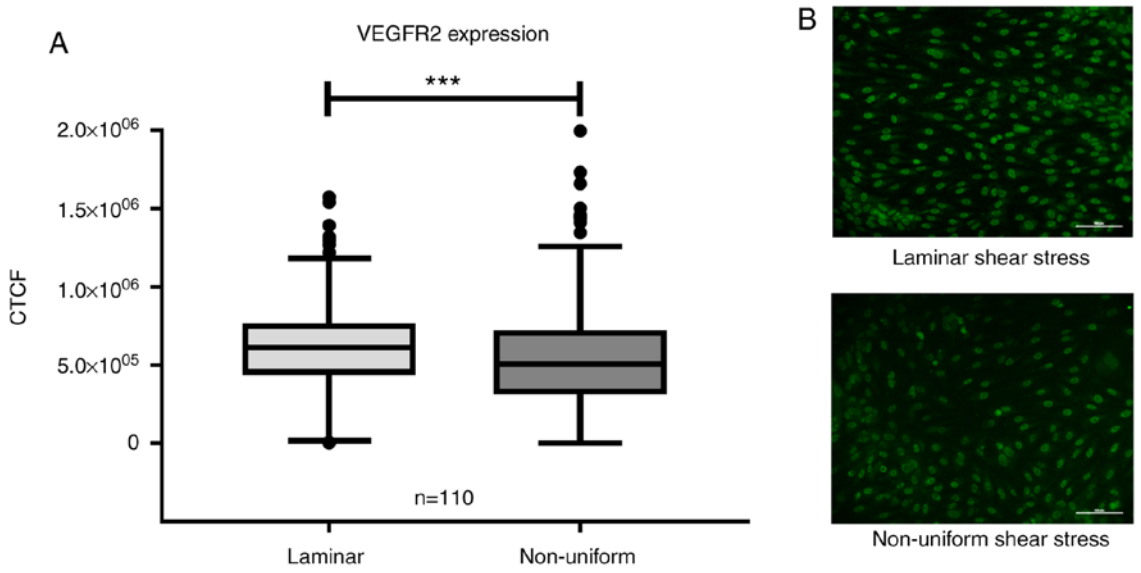

Figure 3. Expression of VEGFR2 under flow conditions. (A) Expression of VEGFR2 under laminar and non-uniform shear stress conditions, independently of the genotype; human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h. Cells were fixed and stained for VEGFR2; data are expressed as the median with 25th and 75th percentiles (Tukey-style); ***P<0.001. Differences between groups were measured using Wilcoxon Signed Rank Test. (B) Representative images of VEGFR2 staining of regions with laminar and non-uniform shear stress (scale bar, 100 μm). VEGFR2, vascular endothelial growth factor receptor 2; CTCF, corrected total cell fluorescence.

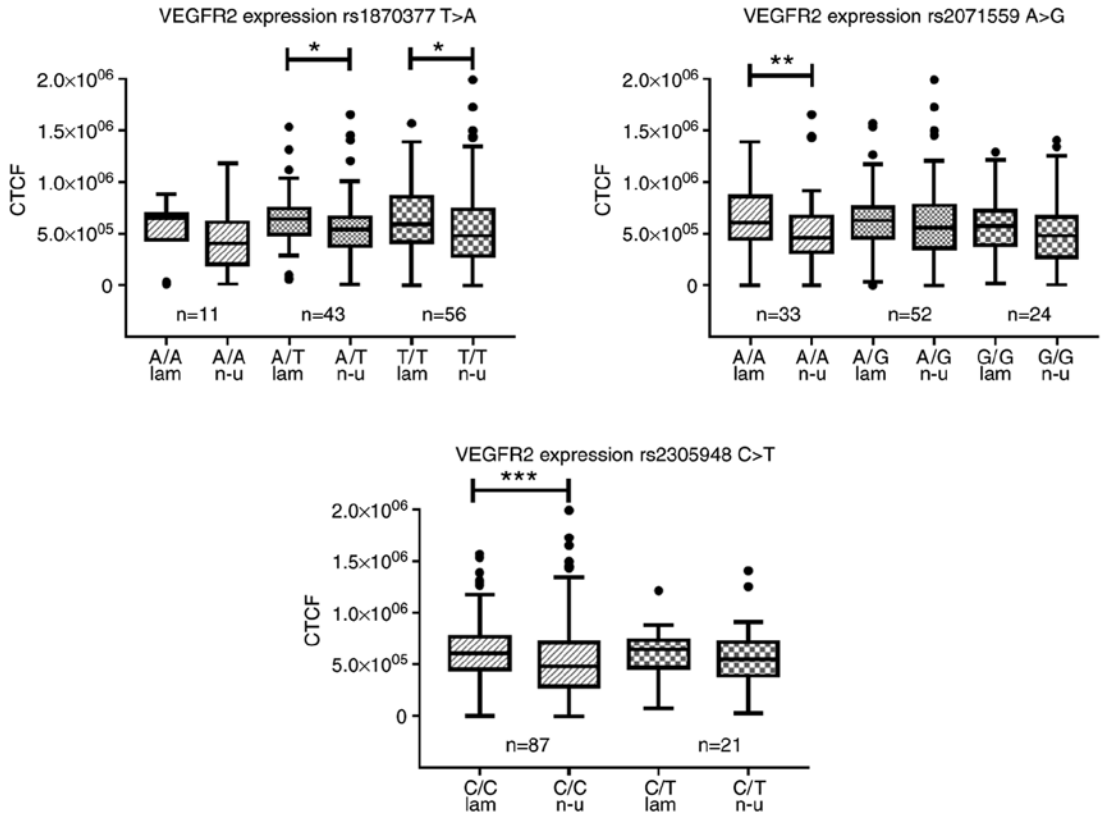

Figure 4. Expression of VEGFR2 under different flow conditions. Human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h. Cells were fixed and stained for VEGFR2. Data are expressed as the median with 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles (Tukey-style); *P<0.05, **P<0.01, ***P<0.001. No significant differences, measured by Kruskal-Wallis one-way ANOVA on Ranks, were observed under atheroprotective or atheroprone shear stress in any of the analyzed SNPs. VEGFR2, vascular endothelial growth factor receptor 2; CTCF, corrected total cell fluorescence; lam, laminar; n-u, non-uniform.

Similarly, significant flow-dependent differences were observed in the expression of E-selectin. With the exception of the A/A genotype of rs1870377 T>A, laminar shear stress regions showed a significant reduction in E-selectin compared with non-uniform shear stress regions (Fig. 5). Comparable to the expression of VCAM-1, the lowest expression of E-selectin

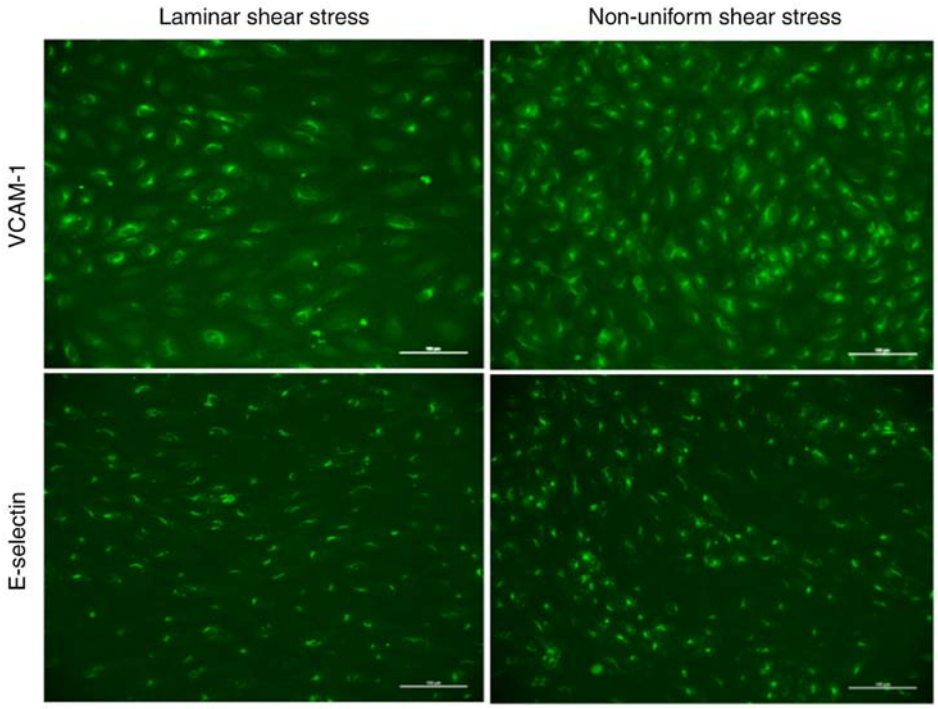

Figure 5. Expression of VCAM-1 and E-selectin under different shear stress conditions. Human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h. Cells were fixed and stained for VCAM-1 or E-selectin. Representative images show VCAM-1 and E-selectin staining of regions with laminar and non-uniform shear stress; images were captured at x200 magnification (scale bar, 100 μm). VCAM-1, vascular cell adhesion molecule-1.

in rs1870377 T>A was in the A/A genotype (~70% reduction) under laminar and non-uniform shear stress conditions, although the difference was not statistically significant (Fig. 6B). In rs2071559 A>G, the A/A genotype showed similar expression to VCAM-1, with the lowest expression of E-selectin under laminar (~60% reduction) and non-uniform (57% reduction) shear stress conditions (one-way ANOVA on Ranks, 0.093/0.121) compared with that in the G/G genotype (Fig. 7B). No significant differences were observed in rs2305948 C>T (data not shown).

SNPs do not influence the adhesion of THP-1 monocytic cells to an endothelial cell monolayer under flow conditions. The adhesion of THP-1 monocytic cells was analyzed in an *in vitro* flow model after 2 h of TNF-α stimulation. As expected, the adhesion of THP-1 cells significantly increased in regions of non-uniform shear stress in all analyzed SNPs compared with that in atheroprotective laminar shear stress conditions, although no significant differences in THP-1 adhesion were observed among the distinct genotypes in any of the SNPs (Fig. 1).

Discussion

The aim of the present study was to investigate the association between SNPs in VEGFR2 and atherogenesis on a cellular basis. To date, only a small number of studies have investigated a possible influence of SNPs in VEGFR2 on the occurrence of atherosclerosis or associated complications, such as myocardial infarction or stroke. No previous studies have addressed the cellular mechanism of the first step of atherogenesis in endothelial dysfunction. Therefore, the present study performed flow simulation experiments, in which regions of high laminar shear stress and non-uniform shear stress were readily differentiated within an *in vitro* flow chamber model. The region of laminar shear stress has a strictly laminar flow pattern, and these regions were identified throughout the straight main channel, whereas regions of non-uniform shear stress were located distal to the bifurcation, characterized by a reduced flow rate and perturbed shear pattern with a steep shear stress gradient in a direction transversal to the flow (4).

Quiescent growth factor receptors in the absence of their ligands are preferentially localized on the plasma membrane (32,33). Ligand binding and receptor activation in the majority of cases leads to receptor internalization (34). Growth factor receptors, such as VEGFR2, are either recycled back to the plasma membrane or degraded, via ubiquitinylation by c-Cbl (35), following signal transduction (36). Only 60% of VEGFR2 molecules are located at the plasma membrane, with the remaining 40% localized in endosomes (37). The process of internalization may be critical for protecting VEGFR2 against shedding (38). The internalized VEGFR2 interacts with the transcription factor Sp1, which is known to be implicated in angiogenesis. It has also been shown that nuclear VEGFR2 is phosphorylated in proliferating cells. Furthermore, hypoxic stimulation increases the nuclear localization of total VEGFR2, whereas phosphorylated VEGFR2 remains predominantly perinuclear (39-41). Additionally, Domingues *et al* showed that nuclear VEGFR2 can act as a transcription factor regulating its own expression (42).

The phosphorylation and subsequent activation of VEGFR2 by fluid shear stress are dependent on PECAM-1 and VE-cadherin via several adapter molecules (43). Shay-Salit *et al* observed a rapid nuclear translocation of VEGFR2 following the onset of laminar flow, in addition to a quiescent mechano-

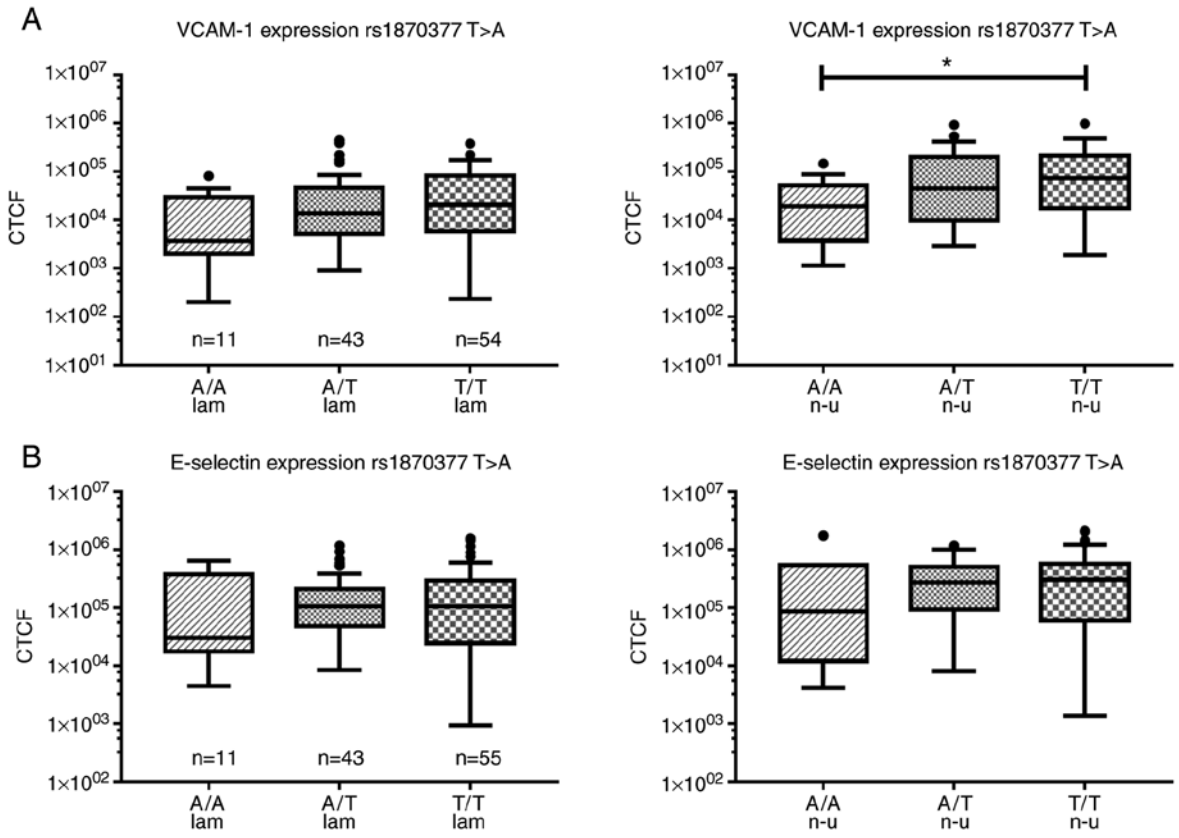

Figure 6. VCAM-1 and E-selectin in rs1870377 T>A. Expression of (A) VCAM-1 and (B) E-selectin in rs1870377 T>A. Human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h. Cells were fixed and stained for VCAM-1 or E-selectin. Significant differences in the expression of VCAM-1 were observed between A/A and T/T genotypes under non-uniform shear stress, measured by Kruskal-Wallis one-way ANOVA on Ranks (0.023) with a post-hoc test (Bonferroni). Data are expressed as the median with 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles (Tukey-style). *P<0.05. VCAM-1, vascular cell adhesion molecule-1; CTCF, corrected total cell fluorescence; lam, laminar; n-u, non-uniform.

sensory complex during sustained laminar flow conditions (44). In the present study, the results obtained by immunofluorescence in the model of different flow conditions revealed an overall decreased expression of VEGFR2 in regions of non-uniform shear stress, regardless of genotype. However, whether the receptor was degraded or internalized was not elucidated.

Rs1870377 T>A and rs2305948 C>T are located in the extracellular region at the third and fifth Ig domain. This affects the primary protein structure and its ligand binding properties due to polymorphisms of the amino acids (Table II). An increased risk of CVD for carriers of the A/A genotype of rs1870377 T>A has been demonstrated in case-control studies, arguing that the SNP impairs VEGF binding efficiency to its receptor due to conformational changes of VEGFR2 (20).

Rs2071559 A>G is located at the VEGFR2 promotor region and leads to structural alterations of the binding site for the transcription factor E2F, which possibly leads to alterations in the expression of VEGFR2. Wang *et al* hypothesized that the polymorphisms lead to a conformational change, thereby influencing the binding affinity of VEGFR2 to VEGF, leading to dysfunctional signal transduction (20). However, the present study did not analyze VEGF receptor binding. In the population examined, the basal expression of VEGFR2 in HUVECs showed no significant differences in any of the SNPs. A comparison of the basal expression of VEGFR2 in *in vitro* endothelial cells with an *in vivo* study of serum levels in patients should be considered with caution.

Kariž and Petrovič showed a higher risk for myocardial infarction (MI) for a genotype with reduced levels of VEGFR2 (i.e., G/G of rs2071559 A>G) (22) and others have suggested that the reduced expression of VEGFR2 is associated with a higher risk profile for CVD (20), which is in line with the observation in the present study of reduced expression of VEGFR2 under non-uniform shear stress conditions. The results indicated that a decreased signal of VEGFR2 may be one factor underlying the pathologic conditions of non-uniform shear stress.

In further experimental settings, the present study analyzed the expression of adhesion molecules, in addition to THP-1 monocyte adhesion, focusing on the different genotypes within the VEGFR2 SNPs. Our previous study described the direct impact of the expression of VEGFR2 on the expression of adhesion molecules in HUVECs under non-uniform shear stress (14). A direct link between polymorphisms in VEGFR2 and the activation and expression of adhesion molecules has not been discussed in former studies. However, VEGFR2 is associated with adhesion molecule expression, as stimulation of VEGFR2 can induce the phosphoinositide 3-kinase (PI3K)-Akt and NF-κB pathway (11,45,46), which in turn leads to the *de novo* synthesis of molecules, including VCAM-1 and E-selectin.

From the results of the present study, the presence of endothelial dysfunction in an *in vitro* bifurcation model was confirmed; the A/A genotype for SNP rs1807377 T>A was identified as having a significantly lower adhesion molecule expression. Therefore, this genotype may attenuate endothelial

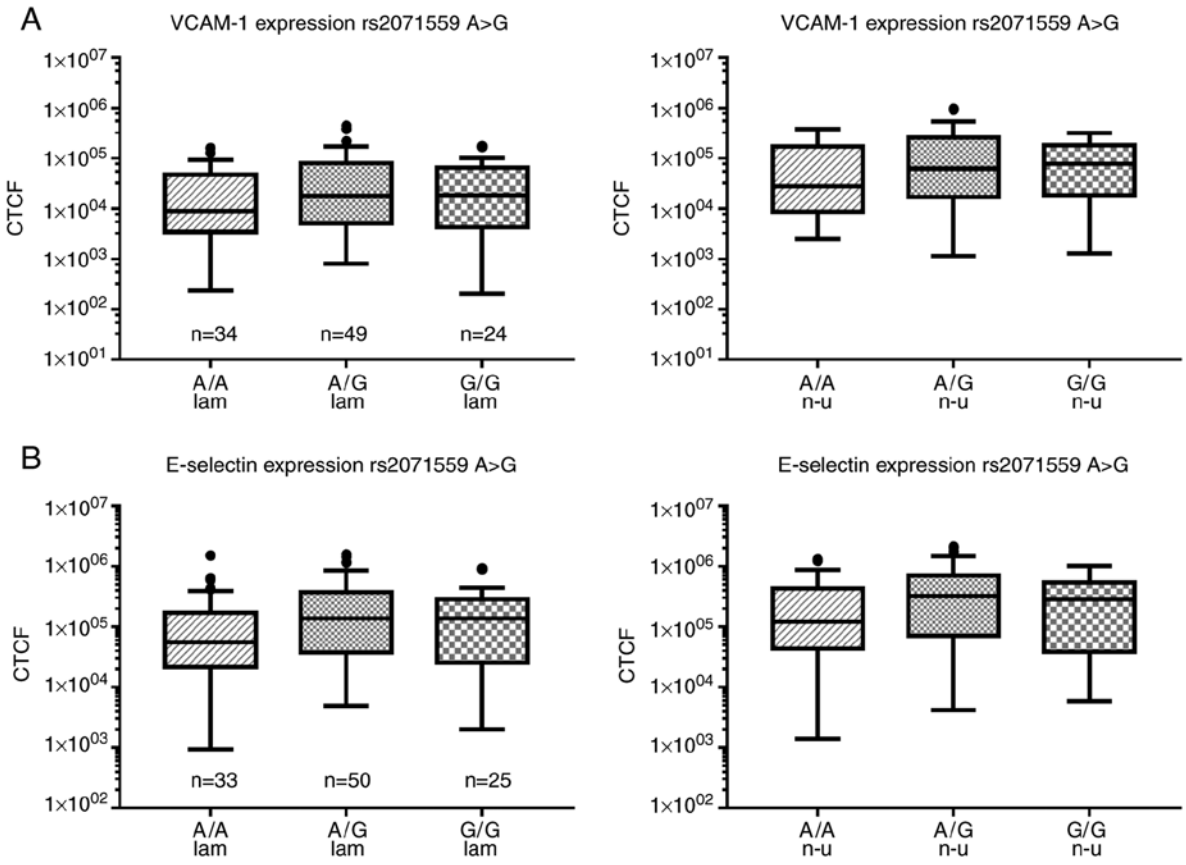

Figure 7. VCAM-1 and E-selectin in rs2071559 A>G. Expression of (A) VCAM-1 and (B) E-selectin in rs2071559 A>G. Human umbilical vein endothelial cells were exposed to flow for 17 h followed by tumor necrosis factor-α stimulation for 2 h. Cells were fixed and stained for VCAM-1 or E-selectin. Data are expressed as the median with 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles (Tukey-style). VCAM-1, vascular cell adhesion molecule-1; CTCF, corrected total cell fluorescence; lam, laminar; n-u, non-uniform.

dysfunction in atherosclerosis-prone regions and may have a protective effect against the development of atherosclerosis and CVD. This is in line with reports from two Chinese groups: In rs1870377 T>A, the A allele leads to reduced vulnerability to CVD, whereas the T allele may have the opposite effect (47,48), particularly when considering the haplotypes. However, contradictory results have also been reported in reports: A subpopulation of patients with diabetes mellitus type 2 had a reduced risk of CVD with the T allele, and another subpopulation of patients who smoke, as the classical risk factor for CVD, also had a significant reduction in risk of CVD with the T allele, compared with the in non-smokers (47,48). These contradictory results within one study indicate the difficulties in interpreting the results of SNP analyses for a disease like atherosclerosis with a wide variety of influencing factors, for example, genetic predisposition, comorbidity factors and lifestyle.

The G/G genotype of rs2071559 A>G showed increased median expression of VCAM-1 and E-selectin, leading to increased endothelial activation, thereby supporting the conclusion that the G allele of the SNP may be a risk allele for CVD. Karič and Petrovič found a significant correlation between MI and the G/G genotype in rs2071559 A>G in patients with type 2 diabetes, with a 1.6-fold higher risk of MI in G/G carriers (22). Therefore, the results from Karič and Petrovič and the present study do not agree with the results from Li *et al*, which showed a reduced risk of CVD for carriers of the G allele in rs2071559 A>G compared with carriers with the corresponding A allele (47), and those of Zhang *et al*, which found a negative correlation between rs2071559 A>G and intima-media thickness for G allele samples (21).

The third SNP analyzed in the present study was rs2305948 C>T. Again, the results from previous studies analyzing potential associations of this SNP with CVD are conflicting. Zhang *et al* reported that the T allele was associated with increased risk for stroke (21), and Li *et al* confirmed this from analyzing data of a Chinese population adjusted for cardiovascular risk factors (47). Additionally, Liu *et al* analyzed this SNP regarding the risk for CVD in a Han Chinese population. Without adjustment for additional risk factors, individuals with the C allele showed a significantly higher risk for CVD. However, following regression analyses for the individual risk factors, such as smoking, diabetes, hypertension or alcohol abuse, the C allele was shown to be protective against CVD in smoking and non-smoking patients. The individual analyses of patients with and without hypertension, and of patients with or without diabetes, also showed a protective effect of the C allele against CVD (48). As the T/T genotype was rare, with only one sample, it was not possible to compare the two divergent homozygous genotypes in the present study, where the most pronounced differences were expected. The comparison between C/C and C/T showed no significant difference in any of the experimental settings.

Regarding discrepancies between former studies and the present study, it is important to consider that the *in vitro* model

used in the present study simulates the early events of atherogenesis. This may not allow a direct comparison with the cited association studies in fully developed CVD having additional risk factors. Atherosclerosis and CVD are complex diseases with a large number of subtle contributing factors that may influence the severity or outcome in different patients. Considering that inherited factors, genetic markers and epigenetics influence the diverse regulation of VEGFR2 and endothelial cell homeostasis, the comparability of results in this field of research is impeded. Taken together, compared with most clinical case-control studies, the set of 113 samples in the present study was relatively small. Furthermore, due to the anonymization of the samples, information about the donors, including ethnic background, age of the mother, complications at birth, or any accompanying diseases, are missing. The majority of subjects in the present study were reportedly central Europeans. Compared with an East Asian population, a European population appears to have a higher frequency of T in rs1870377 T>A and C in rs2305948 C>T (49).

As the results of the present study are conflicting with those of former studies in a Chinese population (16,48), there may be variations in gene expression due to a variety of factors, including ethnicity, which affected the results obtained (50). This may explain the large variability within the population; and despite a large proportional reduction of adhesion molecule expression, statistical significance was not detected.

In conclusion, the present study provides new evidence that the three investigated SNPs may offer potential as novel risk markers for CVD and warrant further investigation in the pathogenesis of atherosclerosis and CVD. The A/A genotype of rs1870377 A>T appeared to be atheroprotective, whereas the G/G genotype of rs2071559 A>G may enhance endothelial dysfunction. The discrepant findings between the observations in the present study at a cellular level and those from clinical studies suggest that any genetic polymorphism would be strongly affected by additional risk factors for CVD, including type 2 diabetes, hypertension, hypercholesterolemia and smoking. In addition, SNPs may influence collateral development not only individually but also when acting together with other SNPs, through gene haplotype networks.

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Availability of data and materials

The datasets are available from the corresponding author upon reasonable request.

Authors' contributions

KU and BD conceived and designed the study. NMS and KU collected and analyzed the data. KU and NMS wrote the manuscript. MT and DRS substantively revised the researched articles and were involved in the interpretation of results. FMS provided the umbilical cords and contributed to the interpretation of results. SA revised the results from a clinicians view, thereby being involved in analyses and interpretation of the results in the specific professional context. SA, BD and MT critically revised the manuscript for important intellectual content. FP was involved in data collection and interpretation of the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The collection of human material and the study protocol were approved by the Ethics Committee of the Medical Department of the Friedrich-Alexander-Universität Erlangen-Nürnberg (case no. 246_13B). Written consent was obtained from all women prior to donation.

Patient consent for participation

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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