Original Article

CXCL16 Haplotypes in Patients with Human Carotid Atherosclerosis: Preliminary Results

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Aim: Chemokine CXC ligand 16 (CXCL16) has chemoattractive, adhesive and scavenging properties and may play a role in the formation of atherosclerotic lesions. However, studies of CXCL16 polymorphisms in patients with atherosclerosis are scarce. The missense polymorphisms I123T and A181V are potentially important factors in the regulation of presentation and shedding of the CXCL16 chemokine domain. The aim of this study was to analyze the association between I123T and A181V polymorphism haplotypes and the accumulation of carotid plaque as well as the effect of the haplotype on the CXCL16 mRNA expression in carotid plaques in patients with advanced atherosclerosis. Additionally, we performed a bioinformatic prediction analysis of the impact of CXCL16 protein sequence variation on CXCL16-CXCR6 interactions and analyzed the soluble CXCL16 plasma levels according to the CXCL16 haplotype.

Methods: This study evaluated a total of 733 participants, including 283 controls and 450 patients with carotid atherosclerosis (CA) undergoing endarterectomy. Analyses of the polymorphisms and the gene expression were performed using real-time PCR. The soluble CXCL16 levels were measured with ELISA.

Results: The missense allele haplotype, T123V181, was found to be significantly and independently associated with the occurrence of CA plaque (OR=1.27;1.02-1.57, $p=0.03$). This haplotype was predicted to significantly change the CXCL16-CXCR6 interaction, compared to I123A181. Neither the CXCL16 mRNA expression in the human plaques nor the soluble CXCL16 plasma levels differed according to the haplotype.

Conclusions: These results indicate that the CXCL16 T123V181 haplotype is a moderate genetic risk factor for the development of carotid plaque. Further functional and replication studies are needed to clarify the mechanisms by which this combination of alleles promotes advanced CA and validate its impact on disease progression.

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Key words: CXCL16, CXCR6, Haplotype, Gene expression, Carotid plaque

Introduction

Atherosclerosis is a chronic inflammatory disease

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involving both innate and adaptive immune responses during all phases of disease development¹). The chemokine CXCL16, a.k.a SR-PSOX, has primarily been identified to be a scavenger receptor that facilitates the uptake of oxidized low-density lipoproteins (ox-LDL) and phosphatidylserine and is therefore involved in macrophage-to-foam cell transformation²). CXCL16 is expressed in macrophages^{3, 4}), dendritic cells^{5, 6}), endothelial cells⁷) and vascular smooth muscle cells^{7, 8}).

Soluble CXCL16 (sol-CXCL16) is a typical chemokine that acts as an attractant for cells expressing the CXCR6 receptor 6 (CXCR6)⁹. Sol-CXCL16 represents the extracellular chemokine domain of the transmembrane CXCL16, which undergoes cleavage by the metalloproteinase ADAM 10¹⁰.

Recently, several studies have suggested the proatherogenic role of CXCL16, and it has been shown that the expression of CXCL16 is enriched in the atherosclerotic plaques of humans and murine experimental models of atherosclerosis^{11, 12}. The absence of the CXCR6 gene attenuates atherosclerosis and decreases the percentage of CXCR6+ T-cells within the aortic wall, thus indicating the proatherogenic role of the CXCL16-CXCR6 axis¹³. Despite this evidence, a recent rodent study suggested the atheroprotective effect of CXCL16¹⁴, and the plasma sol-CXCL16 levels have been reported to be decreased in patients with coronary atherosclerosis¹⁵. These data thus raise questions regarding the exact role of CXCL16 in atherosclerosis.

Evidence of the association between CXCL16 gene polymorphisms and cardiovascular phenotypes is scarce¹⁶⁻¹⁸. In a recent study, the CXCL16 A181V polymorphism itself was not found to be associated with CHD among Indian Asian men and women¹⁸. In addition, it has been reported that the transmembrane CXCL16 T123V181 variant does not promote the adhesion of CXCR6+ cells¹⁸. The missense polymorphisms I123T (I142T) and A181V (A200V) have the highest frequencies of rare alleles among all missense variations in the CXCL16 gene. Both polymorphisms are located in the gene sequence coding for the mucin-like stalk domain of transmembrane CXCL16. Therefore, these two polymorphisms are potentially important factors for the regulation of presentation and shedding of the CXCL16 chemokine domain. However, to date, no CXCL16 polymorphisms have been analyzed in association with the accumulation of carotid plaque.

Aim

The aim of the present study was to analyze the association between the CXCL16 I123T and A181V polymorphism haplotypes and the presence of carotid atherosclerotic plaque and assess the effects of the haplotypes on the CXCL16 mRNA expression in carotid plaques in patients with advanced atherosclerosis undergoing carotid endarterectomy. Additionally, we examined the possible effects of these two non-synonymous polymorphisms at the protein level by performing a bioinformatic prediction analysis of the

impact of CXCL16 protein sequence variation on CXCL16-CXCR6 interactions and by quantifying the soluble CXCL16 levels in the plasma of different CXCL16 haplotypes carriers.

Methods

Patients and Controls

A total of 450 patients undergoing carotid endarterectomy and 283 control subjects were enrolled in this study. All participants were unrelated Caucasians of European descent from Serbia, and the ethnicity of the study population was homogeneous (Serbian). Patients recruited from the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, Belgrade, Serbia (2008-2009) included those with evidence of carotid plaque in the internal carotid artery (ICA) and/or common carotid artery (CCA) who were consecutively admitted for carotid endarterectomy. In all enrolled patients, a complete medical history was collected, including smoking and drinking habits, diagnoses of hypertension, diabetes mellitus, coronary heart disease and peripheral arterial occlusive disease and drug treatment (antihypertensive drugs, oral hypoglycemic drugs, insulin). The exclusion criteria for all patients were as follows: a history of previous carotid endarterectomy (possible restenosis), carotid kinking, carotid aneurysm, tumors, chronic inflammatory and autoimmune diseases and renal failure.

Two hundred and eighty-three (283) control subjects with no evidence of carotid atherosclerosis, cerebrovascular or cardiovascular disease, chronic inflammatory disease, renal failure or diabetes mellitus were recruited from the Occupational Medical Center, VINČA Institute of Nuclear Sciences, Belgrade, Serbia during annual medical check-ups, including clinical, ultrasound and ECG examinations.

The study protocol was approved by the ethics committees of the participating medical centers. Each participant provided their written informed consent to participate in this study.

Ultrasound Assessment of the Carotid Arteries

The ultrasound assessments of the bilateral carotid arteries were performed using a high-resolution B-mode ultrasound Acuson AntaresTM system (Siemens, Munich, Germany) and HDL 3500 linear transducer (5 to 12 MHz, Philips ATL, Bothell, WA). The degree of carotid stenosis was calculated according to the North American Symptomatic Carotid Endarterectomy Trial method (NASCET)¹⁹. The highest peak systolic velocity (PSV) and end-diastolic

velocity (EDV) as well as the ICA/CCA ratio were recorded. All patients included in this study had > 70% stenosis, according to the NASCET analysis. Carotid atherosclerosis was defined as the presence of atherosclerotic plaque in the internal or common carotid artery. Atherosclerotic plaque was defined as focal widening relative to that of adjacent segments, as evidenced by protrusion into the lumen and/or localized roughness with increased echogenicity. The intra-class correlation coefficients for inter-rater and intra-rater reliability for the carotid ultrasound measurements were 0.916 and 0.968, respectively.

A Duplex ultrasound analysis was conducted in order to classify the plaques into different types in terms of hypoechogenicity (dominantly echolucent) and hyperechogenicity (dominantly echogenic) according to the Gray-Weale criteria²⁰. The time interval between the ultrasonographic imaging examinations and the collection of tissue samples was approximately two weeks.

Collection of Carotid Atherosclerotic Specimens

Carotid plaque tissue specimens ($n=40$) were obtained from patients consecutively undergoing carotid endarterectomy (CEA). Immediately after being removed from the artery, each tissue sample was snap frozen in liquid nitrogen and stored at -70°C until the extraction of RNA followed by the CXCL16 and CXCR6 gene expression analysis.

Biochemical Measurements

All biochemical analyses were performed according to standard laboratory procedures.

Genetic Analysis

Genomic DNA was isolated from whole-blood samples collected with EDTA using the standardized BloodPrep[®] DNA Chemistry isolation kit (Applied Biosystems, Forester City, CA) on the AB PRISM[™] 6100 Nucleic Acid Prep Station (Applied Biosystems, Forester City, CA). The CXCL16 I123T (rs1050998) and A181V (rs2277680) polymorphisms were detected via real-time PCR (AB 7500, Applied Biosystems, US) (each PCR reaction contained 120 ng of DNA) using the TaqMan[®] SNP Genotyping Assay (C_8718197_20 for the I123T polymorphism and C_15885167_10 for the A181V polymorphism) purchased from and tested by Applied Biosystems (Applied Biosystems, Forester City, CA).

Quantitative Real-Time Reverse Transcription PCR (Real Time RT-PCR)

Total RNA was isolated from frozen plaque tis-

sue segments with the largest plaque burden using TRIZOL reagent (Life Technologies), according to the manufacturer's instructions. The quality and quantity of RNA samples were assessed on a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). Of the 40 human carotid plaques collected, total RNA from 26 tissue specimens had an OD 260/280 of >1.8, and these 26 total RNA samples were converted to cDNA, in which one microgram of total RNA was treated with DNase I (Fermentas, Lithuania) followed by reverse transcription using the First strand cDNA synthesis kit with the oligo-dT18 primer (Fermentas, Lithuania) according to the manufacturer's instructions. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR system (AB, Foster City, CA). The CXCL16 and CXCR6 gene expression was detected using the TaqMan[®] gene expression assays Hs01055223_g1 and Hs01890898_s1 (AB, Foster City, CA), respectively. The internal reference, 18s rRNA, was detected using the TaqMan[®] Gene Expression Assay Hs99999901_s1 (AB, Foster City, CA). All samples were run in duplicate. The relative levels of CXCL16 mRNA were calculated according to the comparative Ct method²¹, and the THESIAS software program was used to assess the haplotype effects of the I123T and A181V polymorphisms on the relative CXCL16 gene expression. Differences in the CXCL16 and CXCR6 mRNA expression levels between the hypoechogenic and hyperechogenic plaques were tested using the REST 2009 software package, which is suitable for comparing the relative gene expression levels between two sample groups, with quick and reliable results for gene expression analyses.

ELISA Quantification of the Soluble CXCL16 Levels

Peripheral blood samples were obtained with EDTA as an anticoagulant and centrifuged for 15 minutes at $1,000 \times g$ within 30 minutes of collection. The plasma samples were then aliquoted and stored at -20°C until use. For quantification of the soluble CXCL16 levels in the plasma samples, we used the Quantikine[®] Human CXCL16 Immunoassay (R&D Systems, Minneapolis, MN, USA) and performed a quantitative sandwich enzyme immunoassay, according to the manufacturer's instructions. The samples and standards were assayed in duplicate. The optical density was determined using a VICTOR³ (PerkinElmer, Waltham, MA, USA) microplate reader set to 450 nm (wavelength correction at 540 nm). Each sample's average optical density (the average of duplicate readings) was used to determine the soluble

CXCL16 plasma concentration (ng/ml) based on a standard curve generated according to a five-parameter logistic (5-PL) curve-fit (<http://www.myassays.com/five-parameter-logistic-curve.assay>).

Information Spectrum Method (ISM) Analysis

The potential effects of CXCL16 sequence variations (I123T and A181V) on CXCL16-CXCR6 interactions were assessed according to the information spectrum method (ISM), an established sequence analysis technique²². The amino acid sequence was transformed into a numerical sequence in order to create an information spectrum (IS) for each of the analyzed proteins, and mathematical filtering was performed by multiplying the conjugate complex Fourier transformation by the Fourier transformation of the target signal to distinguish the common spectral characteristics of two sequences. The result of this multiplication procedure is the cross-spectral (CS) density function. The prominent peak in the CS function denotes the common frequency component of the explored proteins. According to the ISM concept, amino acid sequence variations affecting the amplitude at this characteristic frequency will affect protein-protein interactions. The information spectrum method (ISM) analysis was performed at the Center for Multidisciplinary Research, VINČA Institute of Nuclear Sciences, Belgrade, Serbia.

Statistical Analysis

The allele frequencies and genotype distributions were estimated based on the gene counting method and compared between the analyzed groups using the chi-square (χ^2) test. The square (χ^2) test was also used to estimate the degree of deviation from Hardy-Weinberg equilibrium. Normally distributed and skewed continuous variables were compared using the unpaired t-test and nonparametric Mann-Whitney *U* test, respectively. All statistical analyses were conducted using the Statistica Version 5 software package (StatSoft Inc., 1997), Thesias software package²³⁻²⁴ (www.genecanvas.org) and REST 2009 software package²⁵. The Thesias program implements the stochastic EM (expectation maximization) algorithm for the haplotype analysis²⁴, thereby allowing for the simultaneous estimation of haplotype frequencies and haplotype effects on the phenotype of interest. For haplotype-phenotype associations, the software package performs the likelihood ratio test (LRT). In cases of quantitative phenotypes, the linear formulation is used to link the haplotype to a phenotype under the assumption of additive effects²⁴, and the expected phenotypic mean for one dose of each haplotype is

given with the corresponding 95% confidence interval. The REST 2009 is a relative expression software tool that uses the pairwise randomization and bootstrapping technique to provide 95% confidence intervals for the gene expression ratios, without normal or symmetrical distribution assumptions²⁵. The REST results are represented as the mean fold induction with the standard error²⁵. In all tests, differences with a two-tailed alpha probability (*p*) value of <0.05 were considered to be statistically significant.

Results

Characteristics of the Study Population

The biochemical parameters and clinical characteristics of the patients and control subjects are presented in **Table 1**. In comparison to the controls, the patients were significantly older, had higher BMI values and higher numbers of smokers, hypertensive individuals and statin users. They also had significantly higher triglyceride (TG) levels, but lower total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) serum levels, than the controls. Meanwhile, the gender distribution and levels of low-density lipoprotein cholesterol (LDL-C) did not differ significantly between the two groups.

Haplotype Analysis of the I123T and A181V Polymorphisms

Both of the investigated polymorphisms were in Hardy-Weinberg equilibrium (HWE) in the control and patient groups. We found a strong linkage disequilibrium between the investigated polymorphisms ($D' = 1$; $r^2 = 0.98$); therefore, there were only two inferred haplotypes (out of four possible). Plausible haplotypes with their frequencies in the control and patient groups are shown in **Table 2**. The results obtained with the Thesias software program showed that the T123V181 haplotype was significantly and independently associated with the occurrence of CA plaque, when compared to the most frequent (referent) I123A181 haplotype (**Table 2**). For the observed haplotype effect, the study power was 45% for a given sample size (number of cases, $n = 450$) at a significance level of 0.05²⁶.

In addition, we analyzed the haplotype effects on different ultrasonographically defined plaque phenotypes (hypoechoic vs. hyperechoic). Consequently, there were no significant associations between the T123V181 haplotype and the hypoechoic plaque phenotype compared to the I123A181 referent haplotype (OR=0.78 95%CI (0.56-1.09), $p = 0.14$, adjusted for gender, family history of atherosclerosis

Table 1. Characteristics of the controls and the patients with carotid plaque (CP)

Parameter	Controls <i>n</i> = 283	CP <i>n</i> = 450	<i>p</i> value
Age (years)	56.5 ± 10.1	62 ± 9.7	< 0.001 [§]
BMI (kg/m ²)	25.1 ± 3.2	26.6 ± 3.3	< 0.001
Males, <i>n</i> (%)	153 (54)	268 (60)	0.13
Smoking (%)	57.2	66.7	0.01
Hypertension (%)	32.2	73.9	< 0.001
TC (mmol/L)	6.16 ± 1.5	5.8 ± 1.2	< 0.001
LDLC (mmol/L)	3.85 ± 1.2	3.7 ± 1.1	0.12
HDLc (mmol/L)	1.24 ± 0.3	1.1 ± 0.3	< 0.001 [§]
TG (mmol/L)	1.87 ± 1.6	2.3 ± 2.6	< 0.001 [§]
C-reactive protein	N/A	7.4 ± 14.9	N/A
Statin treatment (%)	0	21.2	< 0.001
Diabetes mellitus (%)	0	23.5	N/A

The values are presented as the mean ± SD for age, BMI, total cholesterol (TC), low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLc) and triglycerides (TG). Pearson's Chi-square test was used to assess categorical variables. Student's *t*-test was used to compare the values of normally distributed continuous variables between the controls and the patients with carotid plaque. [§] - The Mann-Whitney *U* test was used to compare the values of skewed continuous variables between the controls and the patients; N/A-not applicable; in all tests; *p* values of < 0.05 were considered to be statistically significant.

Table 2. Haplotype effects of the CXCL16 I123T and A181V polymorphisms on the incidence of carotid plaque (CP)

Haplotype	Controls (frequency)	CP (frequency)	OR [§] (± 95% CI)	<i>p</i> value
I123A181	0.579137	0.510922	referent haplotype	
I123V181	0.000000	0.006068	N/A	
T123A181	0.001799	0.000000	N/A	
T123V181	0.419065	0.483010	1.27 (1.02-1.57)	0.03

OR[§] - odds ratio adjusted to age, BMI, hypertension status, smoking status, HDLC, TG, diabetes mellitus and use of statins. CI - confidence interval; N/A-not applicable.

Table 3. Haplotype effects of the I123T and A181V polymorphisms on the carotid plaque phenotype: hyperechogenic and hypoechogenic

Haplotype	hyperchogenic (frequency)	hypoechogenic (frequency)	Crude OR (± 95% CI)	<i>p</i> values	OR [§] (± 95% CI)	<i>p</i> values
I123A181	0.481367	0.586197	referent haplotype		referent haplotype	
I123V181	0.020673	0.009957	N/A		N/A	
T123A181	0.020673	0.009957	N/A		N/A	
T123V181	0.477286	0.393889	0.73 (0.53-1.01)	0.057	0.78 (0.56 - 1.09)	0.14

OR - odds ratio. [§] - adjusted to gender, family history of atherosclerosis and use of antihypertensive drugs. CI - confidence interval; N/A-not applicable.

and use of antihypertensive drugs) (Table 3). Detailed clinical data for the patients with hypoechogenic and hyperechogenic plaques are available in the supplemental table (Supplemental Table 1).

Information Spectrum Method (ISM) Analysis of Transmembrane CXCL16-CXCR6 Interactions

The information spectra (IS) of the transmembrane CXCL16 and CXCR6 proteins were calculated,

Fig. 1. Information spectrum method analysis of the transmembrane CXCL16 and CXCR6 interaction

a) The IS of transmembrane CXCL16 and CXCR6 proteins was calculated, and, according to cross-spectral (CS) filtering, a frequency of 0.429 was found to be characteristic for their interaction. Frequencies for the Fourier transformation of the sequence of electron-ion interaction potential values corresponding to the amino-acid sequence of proteins are placed on the abscissa axis. The lowest frequency is 0.0 and the highest is 0.5. The amplitudes, presented in arbitrary units corresponding to each frequency component in the information spectrum, are placed on the inordinate axis. b) Relative amplitude changes associated with the characteristic Fourier frequency (0.429) of four sequence variants of transmembrane CXCL16 proteins corresponding to the four CXCL16 haplotypes.

and, according to cross-spectral filtering, a frequency of 0.429 was found to be characteristic for their interaction (**Fig. 1a**). The amplitudes at this characteristic Fourier frequency (0.429) for the four sequence variants of transmembrane CXCL16 proteins corresponding to the four haplotypes were determined. The ΔA (0.429) value of the T123V181 was -7.10%, in comparison to the wild-type haplotype, I123A181 (**Fig. 1b**). The most prominent amplitude deviation was detected for the T123A181 haplotype ($\Delta A = -13.10\%$); however, based on the experimental results, the estimated frequency of this haplotype in the controls was only 0.001799.

Relative mRNA Expression of CXCL16 in the Carotid Plaques According to the Haplotype

We next analyzed the relative CXCL16 mRNA levels in the carotid atherosclerotic plaques ($n=20$), according to the inferred CXCL16 haplotype. The relative levels of CXCL16 mRNA were calculated using the comparative Ct method and analyzed according to the CXCL16 haplotype using the Thesias software package. Since the Thesias software program chooses the referent haplotype according to the highest frequency in the study group, we selected 20 of 26 tissue samples in order to retain the genotype frequencies in such a way that the wild-type, I123A181, haplotype remained the referent haplotype. Consequently, the

CXCL16 mRNA expression did not differ significantly according to the haplotype ($p=0.76$) (**Fig. 2a**).

Relative mRNA Expression of CXCL16 and CXCR6 in the Carotid Plaques

We then compared the relative expression levels of CXCL16 and CXCR6 mRNA in the carotid atherosclerotic plaques *ex vivo* between the ultrasonographically defined plaque phenotypes (hypoechogenic and hyperchogenic). The relative expression levels of CXCL16 and CXCR6 mRNA were not significantly different between the hypoechogenic ($n=10$) and hyperchogenic ($n=16$) carotid plaques (CXCL16: mean factor=1.030, S.E. range=0.344-3.352, $p=0.92$; CXCR6: mean factor=1.686, S.E. range=0.544-4.546, $p=0.13$) (**Fig. 2b**).

We also analyzed the correlation between the CXCL16 and CXCR6 mRNA levels in the 26 carotid plaque tissue specimens. Spearman's rank order correlation test showed a significant correlation between the mRNA levels of CXCL16 and CXCR6 ($r=0.45$, $p<0.05$) (**Fig. 2c**).

Effects of the CXCL16 Haplotypes on the Soluble CXCL16 Levels

In order to test the effects of the two investigated CXCL16 haplotypes on the production of soluble CXCL16, we analyzed the soluble CXCL16 plasma

Fig. 2. Relative mRNA expression of CXCL16 and CXCR6 and the correlation of these parameters in the carotid plaques *ex vivo*

a) Relative CXCL16 mRNA expression according to the haplotype. The cDNA samples obtained from 20 endarterectomized human carotid plaques were used as templates in real-time PCR to quantify the CXCL16 and housekeeping 18s rRNA gene expression levels. The relative expression of CXCL16 normalized to that of the housekeeping gene was calculated using the comparative Ct method. The effect of the CXCL16 T123V181 haplotype on the CXCL16 mRNA expression was evaluated in comparison with the referent haplotype, I123A181, using the Thesias software program. Thesias software program calculates the expected phenotypic mean of one dose of each haplotype, with the corresponding 95% confidence interval. For the most frequent haplotype, this value represents the intercept of the linear model. For all other haplotypes, this value corresponds to the sum of the intercept and the corresponding regression parameter. The 95% confidence interval of the estimate is provided as well as the p -value obtained with the χ^2 test of the nullity of the estimate. The CXCL16 mRNA levels determined according to the haplotype were not significantly different in the atherosclerotic plaques *ex vivo* ($p=0.76$).

b) Relative mRNA expression levels of CXCL16 and CXCR6 in the hypoechogenic vs. hyperechogenic carotid atherosclerotic plaques. The cDNA samples obtained from 26 endarterectomized human carotid plaques were used as templates in real-time PCR to quantify the CXCL16, CXCR6 and housekeeping 18s rRNA gene expression levels. The Ct values of CXCL16, CXCR6 and 18s rRNA were entered into the REST software program. Consequently, the relative expression levels of CXCL16 and CXCR6 mRNA were not significantly different between the hypoechogenic ($n=10$) and hyperechogenic ($n=16$) carotid plaques *ex vivo*.

c) Correlation between the CXCL16 and CXCR6 mRNA levels in the carotid plaques *ex vivo*. The cDNA samples obtained from 26 endarterectomized human carotid plaques were used as a template in real-time PCR of the CXCL16, CXCR6 and 18s mRNA gene expression. The relative levels of CXCL16 and CXCR6 mRNA were standardized against that of the housekeeping gene and calculated using the $2^{-\Delta\Delta C_t}$ method. Spearman's rank order correlation test showed a significant correlation between the mRNA levels of CXCL16 and CXCR6 ($r=0.45$, $p<0.05$).

levels in the control subjects ($n=29$) using the Thesias software program. Consequently, there were no significant differences in the plasma CXCL16 levels between the two inferred haplotypes I123A181 and T123V181 (mean for one dose of each haplotype and its 95%CI: 1.19 ng/ml (95%CI 1.09-1.28) vs. 1.27 ng/ml (95%CI 1.18-1.37), respectively, $p=0.24$).

Discussion

The main result of the present study is the finding of an independent association between the CXCL16 T123V181 haplotype and the presence of CA plaque. Although the importance of CXCL16 with respect to the pathogenesis of atherosclerosis is strongly supported by previous research, only a small number of studies have investigated the associations between CXCL16 gene variations and this disease. The first study of the effects of CXCL16 gene variations on the incidence of cardiovascular disease investigated the A181V polymorphism because this polymorphism has been suggested to influence the cleavage site within the transmembrane CXCL16, which is recognized by certain cell membrane sheddases, such as like ADAM17¹⁶, thus affecting the levels of soluble CXCL16. The role of CXCL16 in the onset of cardiovascular pathology was first documented when the levels of its soluble form were found to be positively and independently associated with acute coronary syndrome²⁷. The A181V polymorphism was subsequently investigated in a few different populations in association with coronary artery disease¹⁶⁻¹⁸, but not carotid atherosclerosis. In one of these studies, a significant association between the V181V genotype and a high degree of coronary artery stenosis was found in Caucasian CAD patients¹⁶. In the present study, the missense allele haplotype, T123V181, was identified to be significantly more common in the patients than in the controls. All patients included in this study had an advanced phenotype of carotid atherosclerosis, with a high degree of carotid artery stenosis (>70%). Therefore, our results are in agreement with those of Lundberg *et al.*¹⁶. However, since this study had limited statistical power for the observed association, we consider our findings to be preliminary.

The novelty of this study is the investigation of a potential association between CXCL16 haplotypes and the CXCL16 gene expression levels in human atherosclerotic plaques. Consequently, no significant effects of the haplotypes on the CXCL16 mRNA expression were detected. We also analyzed the soluble CXCL16 levels and found no significant associations between the CXCL16 plasma levels and the I123T

and A181V haplotypes in the healthy subjects. This finding is in accordance with the results of a previous functional *in vitro* study¹⁸. Keeping in mind that only a few studies, including ours, have investigated the effects of CXCL16 genetic variations on the CXCL16 mRNA and protein expression, further research is required.

Since the missense haplotype did not affect the plaque CXCL16 mRNA levels in this study, there may be other ways in which the combination of I123T and A181V alleles plays a role in the onset of advanced CA. The predictive bioinformatic analysis of the haplotype effects on the transmembrane CXCL16-CXCR6 interactions revealed that each of the three possible haplotypes had different effects on the interaction, compared to that observed for the wild-type allele. Given that the I123V181 and T123A181 haplotypes were practically absent in the study population, we suggest that the T123V181 haplotype has the ability to significantly change the ligand-receptor interaction, compared to the wild-type I123A181. Petit *et al.* showed that, unlike I123A181 CXCL16, the T123V181 CXCL16 variant is unable to promote adhesion by binding to CXCR6 on CXCR6-expressing cells, both *in vitro* and *ex vivo*, while the roles of T123V181 CXCL16 in chemotaxis and oxLDL scavenging are not affected¹⁸. At the protein level, the findings of Petit *et al.* demonstrated different effects of the two investigated CXCL16 haplotypes on the transmembrane CXCL16-CXCR6 interaction, as predicted by the current bioinformatic analysis. With respect to the study by Petit, our results highlight the relevance of the ISM analysis as a useful bioinformatic tool for predicting gene haplotypes causing significant changes in protein-protein interactions.

Soluble CXCL16 diminishes the adhesion of transmembrane CXCL16 to CXCR6-expressing cells²⁸ and influences intracellular signaling pathways, possibly enhancing aortic smooth muscle cell proliferation²⁹, thus stabilizing atherosclerotic lesions, making them less vulnerable. In this study, there were no significant associations between the T123V181 haplotype and the plaque phenotypes, although the frequency of T123V181 was higher in the patients with hyperechoic plaques than in those with hypoechoic plaques. In addition, we found no significant differences in the expression levels of CXCL16 and CXCR6 mRNA between the hypoechoic and hyperechoic plaques. Given that the effects of CXCL16 on cell adhesion, proliferation and migration are complex and may be either destructive or protective, we can only describe the CXCL16 polymorphisms as having a moderate effect on CA. Still, we

cannot be sure whether the T123V181 haplotype is significantly more frequent in patients with atherosclerosis because it carries a risk for this phenotype or because it is protective in a way that stabilizes the disease course, as suggested by the trend in the higher frequency of this haplotype in the patients with hyper-echogenic plaques.

Conclusion

In the present study, the CXCL16 T123V181 haplotype was significantly more frequent in the patients with CA plaques than in the controls. Although this study has limited statistical power to prove an association between the T123V181 haplotype and the accumulation of carotid plaque, it represents one of two studies performed in Caucasians to date. Only a small number of association studies have investigated these polymorphisms, and the lack of additional functional data represents the limits for drawing definitive conclusions regarding the current results. Therefore, our findings should be replicated in a larger number of carotid plaque specimens using a detailed histological classification.

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Conflicts of Interest Statement

The authors declare that they have no conflicts of interest.

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Supplemental Table 1. Characteristics of the CA patients with a hyperechogenic and hypoechogenic plaques

Overall	Hyperechogenic <i>n</i> = 245	Hypoechogenic <i>n</i> = 104	<i>t</i> -test <i>p</i>
Age, (years)	61.8 ± 9.3	62.2 ± 7.9	0.56
BMI (kg/m ²)	26.1 ± 2.9	26.9 ± 3.8	0.17
Males, <i>n</i> (%)	142 (58)	76 (73)	0.01 [§]
Smoking (%)	73.6	69.2	0.41 [§]
Hypertension (%)	88.4	89.4	0.79 [§]
Stenosis (%)	90.2 ± 7.8	89.9 ± 9.0	0.92 [#]
TC (mmol/L)	5.8 ± 1.2	5.7 ± 1.2	0.26
LDLC (mmol/L)	3.7 ± 1.0	3.6 ± 1.1	0.19
HDLc (mmol/L)	1.2 ± 0.3	1.1 ± 0.4	0.38
TG (mmol/L)	1.9 ± 0.9	1.9 ± 1.1	0.54 [#]
Hyperlipidemic subjects (%)	21.1	27.9	0.17 [§]
Glucose (mmol/L)	6.7 ± 2.2	7.2 ± 3.1	0.09 [#]
Factor VIII (g/L)	105.6 ± 31.7	101.8 ± 15.5	0.81
Factor VII (g/L)	97.3 ± 41.6	99.7 ± 34.3	0.59 [#]
DDIM (μg/L)	174.6 ± 165.3	222.3 ± 272.4	0.12 [#]
Fibrinogen (g/L)	5.3 ± 1.7	5.2 ± 1.7	0.94
vWf (IU/ml)	159.4 ± 84.9	168.9 ± 72.4	0.45 [#]
Antithrombin III (g/L)	98.8 ± 16.1	97.7 ± 20.8	0.70
Protein C (g/L)	125.6 ± 25.7	123.0 ± 27.5	0.56
Apolipoprotein A1 (g/L)	1.7 ± 0.5	1.6 ± 0.4	0.56
Apolipoprotein B (g/L)	1.2 ± 0.3	1.1 ± 0.3	0.13
Lp(a) (mg/dl)	38.1 ± 41.8	31.1 ± 35.5	0.11 [#]
Apolipoprotein A-II (mg/L)	345.4 ± 65.3	325.3 ± 56.9	0.11
ApoE (mg/L)	45.2 ± 14.4	42.7 ± 11.6	0.36
CRP (mg/L)	7.5 ± 15.8	4.0 ± 5.7	0.27 [#]
IL6 (pg/ml)	6.1 ± 12.3	5.4 ± 11.4	0.73 [#]
White blood cells (10 ⁹ /L)	7.4 ± 1.8	7.9 ± 2.0	0.20
Urea (mmol/L)	6.9 ± 2.7	7.0 ± 2.4	0.79 [#]
LDH (U/L)	351.7 ± 79.3	362.7 ± 80.7	0.49
Family history of atherosclerosis (%)	6.2	17.3	<0.01 [§]
Coronary heart disease (%)	36.8	40.4	0.53 [§]
Diabetes mellitus (%)	21.5	24.0	0.60 [§]
Peripheral arterial occlusive disease (%)	15.8	16.4	0.89 [§]
Antihypertensive therapy (%)	30.6	56.7	<0.01 [§]
Statin treatment (%)	22.6	21.6	0.84 [§]

The values for continuous variables are presented as the mean ± SD. *p*- Students' *t*-test was used to compare values between the CA patients with hyperechogenic and hypoechogenic plaques. [#] - Mann-Whitney *U* test. [§] - Fisher's exact two-tailed test was used for categorical variables.