Common CCR5-del32 Frameshift Mutation Associated With Serum Levels of Inflammatory Markers and Cardiovascular Disease Risk in the Bruneck Population

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Background and Purpose—Atherosclerosis is a progressive inflammatory disease and can develop in large arteries such as carotid and femoral arteries or medium-sized muscular arteries of the heart. Previous predominantly experimental studies suggested an important role of chemokines in the development of atherosclerosis. The main aim of this study was to examine potential effect of the CCR5-del32 mutation on systemic inflammation, intima-media thickness in carotid and femoral arteries, and on the indices of cardiovascular disease.

Methods—In the present study, we have examined the association of a common functional 32-bp frameshift deletion mutation in a chemokine receptor (CCR5) in relation to inflammation and atherosclerosis. CCR5 is a G protein-coupled receptor involved in inflammatory response and regulation of leukocytes activation and migration. Genetic screening of this mutation was carried out on a well-known and previously described cohort of Bruneck (n=826) using polymerase chain reaction.

Results—Screening was successful in 810 subjects of whom 7 were homozygous, 102 were heterozygous, and 701 were normal. The mutation was associated with significantly lower levels of C-reactive protein in a dose-dependent manner. Moreover, CCR5-del32 was associated with a significantly lower carotid intima-media thickness in the common carotid artery (del32/del32, 837±8 μm; wt/del32, 909±21 μm; wt/wt, 958±8 μm; P=0.007 after multivariable adjustment). Furthermore, incident cardiovascular disease (1995 to 2005) was markedly reduced in del32 homozygotes and heterozygotes subjects compared with wild-type homozygotes (del32/del32 0%, wt/del32 7.8%, wt/wt 14.8%, P=0.020). Findings equally applied to coronary artery and cerebrovascular disease.

Conclusions—The chemokine receptor CCR5-del32 frameshift mutation is associated with low levels of C-reactive protein, decreased intima-media thickness, and cardiovascular disease risk. These findings are consistent with the hypothesis that the chemokine receptor CCR5 is involved in the mediation of low-grade systemic inflammation and may play a role in human atherosclerosis and cardiovascular disease. (Stroke. 2008;39:1972-1978.)

Key Words: atherosclerosis ■ chemokine receptors ■ genetics ■ IMT ■ inflammation

Atherosclerosis is a progressive inflammatory disease and can be described as a generalized disease of the arterial intima media, which is characterized by vascular inflammation, endothelial dysfunction, and accumulation of immune cells and lipids within the intima of the vessel wall. Carotid intima-media thickness (IMT) is heritable and is predictive of future vascular events; it is thus suitable for genetic association studies of atherosclerosis. We have previously shown that in the Bruneck cohort, presence of chronic infections and levels of different markers of inflammation amplified the risk of atherosclerosis development in the carotid arteries, which is consistent with other independent published data.

The chemoattractant cytokines or chemokines and their receptors constitute a large family of mediators of inflammation produced in acute and chronic inflammation with the specific responsibility of mobilizing and activating white blood cells, macrophages, or T-cells. Chemokines are 8 to 16 kDa soluble proteins produced by leukocytes either constitutively or after induction by exogenous irritants or endogenous mediators such as interleukin-1 and tumor necrosis factor-α and exert their effects locally in a paracrine or
autocrine fashion using a variety of cell surface receptors such as CCR2, CCR3, CCR4, and CCR5. Binding of chemokines to their receptors lead to the initiation of a G-protein-dependent intracellular signal, which results in cell chemotaxis of leukocytes toward the source of the chemokine usually along a chemokine concentration gradient. 

Different studies have demonstrated that chemokine inhibitors block chemokine receptors and can prevent this chemotaxis occurring. CCR5, similar to other chemokine receptors, is a serpentine 7 transmembrane-spanning α-helices structure with an extracellular N-terminal segment involved in chemokine binding and a cytoplasmic C-terminal tail involved in G protein signaling. CCR5 is expressed on the peripheral blood-derived dendritic cells, macrophages, lymphocytes, endothelial cells, and vascular smooth muscle cells and mediates the activities of its ligands RANTES, eotaxin, and macrophage inflammatory proteins (MIP-1α, MIP-1β). Moreover, CCR5 and its ligands, ie, MIP-1β and RANTES, were detected in smooth muscle cells and macrophages of the atherosclerotic plaque.

A naturally occurring CCR5 deletion mutation in the CCR5 gene, ie, CCR5-del32, was recognized in various human populations, which results in a truncated nonfunctional receptor lacking the last 3 transmembrane domains (TM5–7). In the homozygous state, the receptor is eliminated from the cell surface due to this mutation. Moreover, in the heterozygous state, the deleted genotype leads to a decreased surface receptor amount by 20% to 30% of the wild-type concentrations through endoplasmic reticulum-associated protein degradation. This mutation was shown in patients to cause resistance or reduce inflammatory damage to viruses, including HIV and hepatitis C, and to correlate with less severe rheumatoid arthritis. Altogether, important characteristics of CCR5 make it an ideal candidate for genetics studies in atherosclerosis.

The main aim of the present study is to examine potential effects of the CCR5-del32 on low-grade systemic inflammation, carotid and femoral artery IMT, and cardiovascular disease risk.

Materials and Methods

Study Subjects

The Bruneck study is a prospective population-based survey of the epidemiology pathogenesis of atherosclerosis. The study protocol was approved by the appropriate ethics committees, and all study subjects gave their written informed consent. At the 1990 baseline, the study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck 40 to 79 years old (125 women and 125 men in the fifth to eighth decades each). A total of 93.6% participated with data assessment completed in 919 subjects. During 1990 and the reevaluation in 1995, a subgroup of 63 individuals died or moved away. In the remaining population, follow-up was 96.5% complete (n=826). Blood specimens for DNA extraction were drawn as part of the follow-up in 1995. No adequate polymerase chain reaction products were obtainable from 16 samples, so 810 individuals formed the current study population. Follow-up for cardiovascular disease between 1995 and 2005 was 100% complete.

Clinical History and Examination

Current and past smoking status was assessed in each subject. Regular alcohol consumption was quantified in terms of grams per day. Hypertension was defined as blood pressure (mean of 3 measurements) ≥140/90 mm Hg or the use of antihypertensive drugs. Diabetes mellitus was coded as present for subjects with fasting glucose levels ≥140 mg/dL and/or a 2-hour value ≥200 mg/dL (World Health Organization definition). Impaired glucose tolerance was defined by a fasting glucose <140 mg/dL and a 2-hour value ≥140 and ≤200 mg/dL. Obesity was defined by a body mass index >30 kg/m². Chronic infection was assessed by an extensive screening procedure as detailed previously. Information on severe acute infection of bacterial origin, including pneumonia, pyelonephritis, peritonitis, diverticulitis, and sepsis, was collected from a detailed self-reported medical history, medical records provided by the general practitioners, death certificates, and reviews of the Bruneck Hospital databases, which is the only hospital in the district.

The cardiovascular end point comprised all incident cases of myocardial infarction (fatal and nonfatal), ischemic stroke (fatal and nonfatal), transient ischemic attack, new-onset symptomatic peripheral artery disease, and any revascularization procedure (n=112). Myocardial infarction was deemed confirmed when World Health Organization criteria for definite disease status were met. Stroke and transient ischemic attack were classified according to the criteria of the National Survey of Stroke. The diagnosis of symptomatic peripheral arterial disease required a positive response to the Rose questionnaire (typical claudication) with the vascular nature of complaints confirmed by standard procedures (ankle–brachial pressure index or angiography) or an acute peripheral artery occlusion requiring revascularization. All other revascularization procedures (angioplasty and surgery) were carefully recorded. Ascertainment of events or procedures did not rely on hospital discharge codes or the patient’s self-report but on a careful review of medical records provided by the general practitioners and files of the Bruneck Hospital and the extensive clinical and laboratory examinations performed as part of the study protocols. A major advantage of our study is that virtually all subjects living in the area were referred to the local Bruneck Hospital and that the network existing between the hospital and general practitioners allowed the retrieval of virtually all medical information on people living in the area.

On top of the primary composite cerebrovascular disease end point, we performed analyses focusing on coronary artery disease (CAD: fatal and nonfatal myocardial infarction and coronary revascularization, including percutaneous coronary intervention and coronary artery bypass grafting), peripheral artery disease (PAD: cases of new-onset symptomatic peripheral artery disease and peripheral artery revascularization), stroke (fatal and nonfatal ischemic stroke), and a composite cerebrovascular end point (stroke/TIA/CEA: fatal and nonfatal ischemic stroke, transient ischemic attacks, and carotid endarterectomy).

Laboratory Methods

Blood samples were drawn after an overnight fast and 12 hours’ abstinence from smoking. In subjects with acute infection, blood drawing was delayed for >6 weeks. High-density lipoprotein (HDL) cholesterol was determined enzymatically (CHOD-PAP method; Merck; coefficient of variation 2.2% to 2.4%). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula (fasting glucose/200 mg/dL, triglyceride ≥4.52 mmol/L in whom it was directly measured. Markers of inflammation were measured with commercial assays as follows: high-sensitivity C-reactive protein (CRP) and α1-antitrypsin (Date Behring), procollagen (ProCa-S 2-step-assay; Brahms, Henningsdorf/Berlin, Germany), and fibrinogen according to the method of Clauss.

Genetic Analysis of the CCR5 Gene

Genotyping was performed following a methodology previously described. In summary, polymerase chain reaction contained REDTaq polymerase enzyme (0.75 U; Sigma), 25 to 50 ng of genomic DNA, primers (10 pmol each), and it was performed in a total volume of 20 μL. To conduct the genotyping, the polymerase chain reaction products were electrophoresed on a 2% agarose gel containing 1% ethidium bromide and visualized on an ultraviolet transilluminator.
Using this method, the normal (wild-type) allele (A) produces one fragment of 189 bp, but the deleted allele (B) produces a fragment of 157 bp. One positive and one negative control were included in each set of reaction to avoid potential misgenotyping.

Scanning Protocol
The ultrasound protocol involves the scanning of the right and left common carotid arteries and of the femoral arteries 40 mm proximal and 10 mm distal to the bifurcation into the superficial and deep branches. Scanning was performed with a 10-MHz imaging and a 5-MHz Doppler probe.5–7 IMT was measured at plaque-free sections of the far wall of the femoral artery and common carotid artery (intraclass coefficient of variation, 7.9%; n = 100) as the distance between the lumen–intima and media–adventitia interfaces.5–7 In the analysis, the mean maximum IMT of the left and right arteries was entered as the number of CCR5-del32 alleles (additive model) and multiplicative changes in inflammation markers calculated per CCR5-del copy.

Statistical Analysis
Data were analyzed using SPSS (version 12.0.1). Continuous variables were presented as means±SD or medians (interquartile range) and dichotomous variables as absolute numbers (percentages; Tables 1 through 3). General linear models were used to determine relationships between CCR5 genotypes (wild-type CCR5, heterozygous CCR5-del32, homozygous CCR5-del32) and markers of inflammation. Levels of variables with a markedly skewed distribution were log-transformed to satisfy the assumption of normality and constant variance of residuals. Analyses were adjusted for age and sex. Associations between CCR5 genotypes and IMT, acute and chronic infections, and 10-year risk of cardiovascular disease and death (1995 to 2005) were analyzed by means of general linear models, logistic regression analysis, and Cox proportional hazard models. Proportional hazard assumptions were satisfied. Base models were adjusted for age and sex and multivariable models for age, sex, social status, diabetes, impaired glucose tolerance, smoking status, alcohol consumption (g/d), LDL and HDL cholesterol, ferritin concentration, hypertension, and body mass index. All Cox models were additionally adjusted for baseline cardiovascular disease. Differential associations in subgroups (gene×risk factor interactions) were analyzed by inclusion of appropriate interaction terms. All probability values

Table 1. Demographic Characteristics and Vascular Risk Factors According to CCR5 Genotype in the Bruneck Study (n=810)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CCR5 Genotype</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>wt/wt (n=701)</td>
</tr>
<tr>
<td>Age, years</td>
<td>62.9±11.1</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>350 (49.9)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>337 (48.1)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>148.1±20.3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>87.0±9.1</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>58.5±16.2</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>145.4±38.3</td>
</tr>
<tr>
<td>Low HDL, n (%)*</td>
<td>134 (19.1)</td>
</tr>
<tr>
<td>High LDL, n (%)*</td>
<td>186 (26.5)</td>
</tr>
<tr>
<td>Alcohol intake, g/d</td>
<td>23.4±40.0</td>
</tr>
<tr>
<td>Current/exsmoker, n (%)</td>
<td>323 (46.1)</td>
</tr>
<tr>
<td>Diabetes/IGT, n (%)</td>
<td>140 (20.0)</td>
</tr>
<tr>
<td>Obesity, n (%)</td>
<td>62 (8.8)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.7±3.9</td>
</tr>
<tr>
<td>Low social status, n (%)</td>
<td>432 (61.6)</td>
</tr>
<tr>
<td>Ferritin, µg/L</td>
<td>136.6±169.9</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD or n (%).
*Low HDL was defined by an HDL cholesterol level <40 mg/dL in men and <50 mg/dL in women (National Cholesterol Education Panel), high LDL was defined by a LDL cholesterol level >160 mg/dL (National Cholesterol Education Panel). IGT indicates impaired glucose tolerance. No significant difference was observed for any of these characteristics and risk factors across the CCR5 genotype (analyses adjusted for age and sex).

Table 2. Associations Between CCR5 Genotype and Levels of Inflammation Marker in the Bruneck Study (n=810)

<table>
<thead>
<tr>
<th>Inflammation Marker</th>
<th>CCR5 Genotype</th>
<th>Multiplicative Change per CCR5-del32 Copy</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt/wt (n=701)</td>
<td>wt/CCR5del32 (n=102)</td>
<td>CCR5-del32/CCR5-del32 (n=7)</td>
</tr>
<tr>
<td>High-sensitivity CRP, mg/L</td>
<td>1.8 (0.9–3.4)</td>
<td>1.4 (0.7–2.4)</td>
<td>0.6 (0.4–2.3)</td>
</tr>
<tr>
<td>α1-Antitrypsin, mg/dL</td>
<td>197 (179–220)</td>
<td>189 (172–213)</td>
<td>188 (145–215)</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>276 (246–316)</td>
<td>272 (245–303)</td>
<td>260 (213–352)</td>
</tr>
<tr>
<td>Procalcitonin, pg/mL</td>
<td>24.4 (17.6–34.4)</td>
<td>21.4 (16.2–32.2)</td>
<td>20.4 (14.0–30.8)</td>
</tr>
</tbody>
</table>

Data are expressed as medians (interquartile range). P values were derived from general linear models adjusted for age and sex. CCR5 genotype was entered as the number of CCR5-del32 alleles (additive model) and multiplicative changes in inflammation markers calculated per CCR5-del copy.
were 2-sided and a probability value <0.05 was considered statistically significant.

**Results**

Genotyping was performed on all the DNA samples that were available from the Bruneck cohort. Genotype was determined successfully on 810 of 826 subjects (98.1% success rate). The 3 genotypes (ie, normal wild type, del32 heterozygous, and del32 homozygous) were present in 701, 102, and 7 cases, respectively (frequencies of 86.5%, 12.6%, and 0.9%, respectively). The assumption of Hardy-Weinberg equilibrium was satisfied. Demographic characteristics and vascular risk factors according to CCR5 genotype is presented in Table 1. No significant differences for any of these variables across the CCR5 genotype were observed.

Presence of del32 mutation in CCR5 was associated with lower levels of inflammatory markers (CRP, α1-antitrypsin, fibrinogen, and procalcitonin) in a dose-dependent manner (Table 2). These associations were statistically significant for CRP, α1-antitrypsin, and procalcitonin after adjustment for age and gender. In particular, CRP levels were substantially lower in del32 heterozygous carriers and homozygous subjects (Table 2). It should be noted that the mean and median of high-sensitivity CRPs were 3.4 and 1.7, respectively (range, 0.1 to 133.0), which is similar to other European populations but lower than data recently published as normative values for the United States.27

The risk of severe acute and of chronic infections was not significantly enhanced in carriers of the CCR5-del32 mutation (Table 3).

**CCR5-del32 and Lower Incident of Cerebrovascular Disease**

Incident cardiovascular disease (CVD; 1995 to 2005) was significantly reduced in del32 homozygotes and heterozygotes compared with wild-type homozygotes (del32/del32=0%, wt/del32=7.8%, wt/wt=14.8%; P=0.020 after multivariable adjustment; Table 3; see also the Figure; cumulative hazard curves of incident cardiovascular disease in a different genotype). The multivariable hazard ratio associated with each copy of the CCR5-del32 allele (additive model) was 0.42 (0.21 to 0.85) and remained virtually unchanged when additionally controlling the analysis for cardiovascular drug therapy (platelet agents, antihypertensive drugs, statin and hormone replacement therapy; hazard ratio=0.38; range, 0.19 to 0.78; multivariable P=0.008). All analyses were adjusted for baseline CVD status. Exclusion of 70 subjects who had experienced CVD before 1995 would result in virtually the same findings (eg, hazard ratio=0.40; range, 0.18 to 0.89; multivariable P=0.026).

CCR5-del32 mutation was associated with a significantly lower carotid IMT in the common carotid artery (del32/del32, 837±8 μm; wt/del32, 909±21 μm; wt/wt, 958±8 μm;
del32 homozygotes had substantial protection against CVD (1995 to 2005), including myocardial infarction, ischemic stroke, TIA, new-onset symptomatic PAD, and revascularization procedures compared with the wild type (Table 3). Findings equally applied to CAD and CVD. We have also observed that overall mortality tended to be lower in CCR5-del32 than other genotypic subgroups (Table 3), which points to the possible importance of this deletion in the human life cycle.

CCR5-del32 and Its Effect on Inflammatory Markers and Intima-Media Thickness

Data from the Bruneck study cohort5,6,25 clearly indicate that individuals with the CCR5-del32 genotype have significantly lower levels of different inflammatory markers, especially CRP (Table 2). These associations followed a linear trend with the least level of the inflammatory markers observed in the individuals with homozygous CCR5-del32 state (homozygous<heterozygous<wild type). Although contribution of CCR5-del32 to the overall variability of CRP was low (as is true for all other genetic variants affecting CRP levels), differences in CRP levels between carriers of CCR5-del32 and noncarriers were substantial with homozygous exhibiting levels 3 times lower than individuals with the wild-type CCR5.

Previous studies have demonstrated that serum levels of inflammatory factors such as CRP28 reflect the inflammatory state of individuals, and lower levels of these markers correlate with the lesser extent and severity of the CAD and atherosclerosis in general.29 Moreover, lower blood levels of inflammatory proteins such as CRP could themselves attenuate accumulation of monocytes in the atherogenic arterial wall.30 Similarly, in the present study, we observed a significant association not only between the CCR5-del32 mutation and reduced levels of inflammatory markers (Table 2), but also between this mutation and carotid artery IMT (Table 3), an index of early atherosclerosis and powerful predictor of vascular risk.2,5,6,10,25 Both of these effects are consistent with the gene dosage effect.

Clinical Studies Support the Role of CCR5 in Atherogenesis

Only few studies have yet examined the association of CCR5-del32 with the risk of myocardial infarction or coronary atherosclerosis. Gonzalez et al reported an inverse association between the del32 allele and myocardial infarction (MI) in a case-control study involving 214 male patients with MI and 360 healthy control subjects (OR, 0.42 [95% CI, 0.23 to 0.77]). In this study, none of the patients with MI was homozygous for the variant.31 Similar findings were obtained by Pai et al in an 8-year follow-up study of 248 incident cases of nonfatal MI and fatal coronary heart disease and matched control subjects.32 Additionally, Szalai et al found that among 318 Hungarian patients undergoing coronary artery bypass surgery, the numbers of homozygotes for CCR5-del32 were significantly lower than expected compared with 320 control subjects.33

Genetic Variations in the Chemokines and Chemokine Receptors in Atherosclerosis

Carotid IMT is heritable, and different genetic studies have provided clues in the pathogenesis of atherosclerosis.1,10
Variants in chemokine–chemokine receptor pathways seem to be one of these genetic factors. Recently, genetic variants of monocyte chemotactic protein-1 have been shown to be related to IMT, CAD, occult ischemia, and MI. In particular, the monocyte chemotactic protein-1 (−927C) allele was associated with increased IMT in the additive model (0.040 mm for each C allele; \( P = 0.001 \)). Another polymorphism (−2578 G) in the same gene has been incrimented in prevalent MI in the Framingham Heart Study. RANTES polymorphisms (−28G, −403A) were also shown to be associated with CAD, cardiac events, and all-cause mortality.\(^{3,3,5}\) Polymorphism in chemokine receptor CCR2 (V64I) is associated with a higher prevalence of MI before age 65 years.\(^{3,7}\) However, data are conflicting regarding this rare allele.\(^{3,1}\) On the other hand, the CX3CR1-M280 allele is associated with a markedly reduced risk of CAD and acute coronary syndrome and improved endothelium-dependent vasodilation.\(^{3,8}\)

**Animal Models and In Vitro Studies Support the Role of CCR5 in Atherogenesis**

Recently, Bursill et al\(^{14}\) carried out intravenous injection of an adenoviral 35 kDa soluble protein (antinflamatory protein) that binds to and inactivates nearly all of the CC-chemokine receptors (including CCR5) in ApoE-knockout mice. They showed that this inactivation can reduce atherosclerosis by inhibiting CC-CK-induced macrophage recruitment in athroerosclerosis. Similarly, using gene transfer of vaccinia 35 kDa protein in a rabbit model of vein graft stenosis, this protein was shown to be an efficient factor in reducing the accumulation of macrophages and neointima formation in this model after 2 and 4 weeks, respectively.\(^{1,6}\)

Moreover, in a recent study on ApoE−/−, CCR5−/− mice fed a high-fat diet, Brautersreuther et al showed that these animals were protected against plaque formation in the aortic root and thoracoabdominal aortas sustained over 22 weeks of a high-fat diet with reduction in lesional macrophage content, aortic CD4, and Th1-related Tim3 expression.\(^{3,9}\)

Using in vitro experiments on human vascular endothelial cells and human primary macrophages, Veillard et al\(^{40}\) have demonstrated that statins reduce chemokine (monocyte chemotactic protein-1, MIP-1α, and MIP-1β) and chemokine receptor expressions (CCR1, CCR2, CCR4, and CCR5) in human endothelial cells and macrophages.

**Possible Mechanisms Involving CCR5 in the Development of Atherosclerosis**

Chemokines, in addition to their action as chemotactictracs, also activate phagocytic cells, which in turn would release inflammatory mediators and reactive oxygen species such as H\(_2\)O\(_2\).\(^{4,1,2}\) Accumulation of H\(_2\)O\(_2\) in the environment of immune cells would then promote in particular an increase in CCR5 expression on the cell surface of monocytes through transcription factor NF-KB\(^{4,1,2}\) leading to a greater response to the chemokines MIP-1α, MIP-1β, and RANTES.

**Limitations**

Our study lacks statistical power for defining whether the nonsignificant association of the CCR5-del32 mutation with PAD and femoral artery IMT is a true finding reflecting differential effects in various vascular territories or if it arises by chance. Moreover, observational studies even if prospective and well designed can on their own not infer causality for the associations obtained.

**Conclusion**

Effect of chemokines and chemokine receptors as important determinants of inflammation and atherosclerosis is so far mainly based on animal and experimental data. Our study is the first prospective large-scale epidemiological investigation suggesting an association between a common functional mutation in CCR5 and carotid IMT and cardiovascular disease. It lends support to the importance of inflammation and chemokine/chemokine receptors, particularly in human atherosclerosis. The findings are of potential clinical significance in view that specific pharmacological blockage of CCR5 is practicable and currently being tested in patients with HIV.

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**Disclosures**

None.

**References**


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