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Clinical research

Association of RANTES G-403A gene polymorphism with increased risk of coronary arteriosclerosis

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KEYWORDS
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Aims Polymorphisms in the RANTES (G-403A), monocyte chemoattractant protein-1 (MCP-1; A-2518G), stromal cell-derived factor-1β (SDF-1β; G801A), and C–C chemokine receptor-5 (CCR5; Δ32) genes have been associated with functional effects. These chemokines have been implicated in leucocyte recruitment to arterial lesions. In a case-control study, we explored relations between these polymorphisms and coronary artery disease (CAD), with respect to angiographic abnormalities and acute coronary syndromes (ACS).

Methods and Results The LUdwigshafen Risk and Cardiovascular health (LURIC) cohort was genotyped by RFLP-PCR. Based on coronary angiography, individuals were sub-divided into CAD cases ($n = 2694$) and controls ($n = 530$). RANTES-403 genotype frequencies were significantly different in cases and controls ($\chi^2 = 4.17,\ p = 0.041$), as were A allele carrier frequencies (36.01% vs. 30.19%, OR = 1.30 [95%-CI = 1.06–1.60], $p = 0.010$). By multivariate analysis, RANTES A-403 retained significant association with CAD ($\chi^2 = 8.40,\ p = 0.0038$). RANTES A-403 was associated with increased ACS prevalence (OR = 1.36 [95%-CI = 1.08–1.71], $p = 0.0073$). MCP-1 G-2518, SDF-1β A801, and CCR5 Δ32 were not associated with CAD.

Conclusions RANTES A-403 was associated with CAD independently from conventional risk factors and CRP or fibrinogen as inflammatory biomarkers. The association was enhanced in smokers and ACS, conditions where platelet activation and inflammation predominate. RANTES A-403 may increase genetic susceptibility to CAD.

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Introduction

Arteriosclerosis is a multi-factorial condition which is determined both by environmental and genetic factors, and involves a strong inflammatory component.[1] Cell adhesion and signalling interactions between circulating leucocytes and the endothelium are key events in the recruitment of leucocytes to inflammatory foci. While beneficial in defense against infection and cancer, interactions between leucocytes and endothelium initiate arterial lesion formation. In response to chemoattractant signals, monocytes and T-cells adhere to inflamed endothelium, transmigrate into the sub-endothelial space, recognise intra-lesional antigens such as oxidised low-density lipoproteins (ox-LDL), and acquire an activated phenotype in the vessel wall.[2]

Chemoattractant peptides (chemokines) regulate leucocyte trafficking in inflammatory diseases.[3] The C—C chemokine Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), and stromal cell-derived factor-1β (SDF-1β) chemokines have been implicated in atherogenesis. RANTES is a potent chemoattractant for monocytes, lymphocytes, eosinophils, and basophils. RANTES expression has been demonstrated in T-cells in human atherosclerotic plaques,[4] as well as in lymphocytes, macrophages, endothelial cells, and vascular smooth muscle cells in transplant-associated arteriosclerosis.[5] Mice deficient in the RANTES gene show impaired T-cell and monocyte recruitment to inflammatory sites.[6] MCP-1 is a potent chemoattractant for monocytes and memory T-cells, and an activator for monocytes/macrophages within arterial lesions.[7] Mice deficient in either the MCP-1 or in the C—C chemokine receptor (CCR)-2 gene that encode the main receptor for MCP-1 show reduced arterial lesion formation when crossed with arteriosclerosis-prone mice deficient in the LDL receptor gene.[8,9] These findings support a central role for MCP-1 in atherogenesis. SDF-1β is a potent platelet agonist that has been implicated in lymphocyte arrest on inflamed endothelium.[10]

Recently, common polymorphisms in the RANTES, MCP-1, SDF-1β, and CCR5 genes have been associated with functional and biological effects. The A-403 variant in the promoter region of the RANTES gene resulted in up to 8-fold increased constitutive transcriptional activity after transient transfection of the human mast-cell line HMC-1 and the T-cell line Jurkat with reporter vectors driven by either the mutant or the reference RANTES promoter.[11] Clinical associations of RANTES A-403 with inflammatory diseases such as atopic dermatitis,[11,12] asthma,[12] polymyalgia rheumatica,[13] sarcoidosis,[14] diabetic nephropathy,[15] as well as susceptibility to HIV infection and disease progression[16] have been reported.

The G-2518 variant in the distal regulatory region of the MCP-1 gene resulted in increased MCP-1 expression in human peripheral blood mononuclear cells (PBMC) by ~2-fold upon IL-1 stimulation.[17] An association between MCP-1 G-2518 homozygosity and coronary artery disease (CAD) was also reported recently.[18]

The A801 variant in the 3′ untranslated region of the SDF-1β gene has been associated with genetic restriction of AIDS pathogenesis,[19] as well as with enhanced $CD34^+$ progenitor cell mobilisation from the bone marrow.[20] Although it has been proposed that the A801 variant may enhance SDF-1β bioavailability,[19] available data on its effect on SDF-1β expression are conflicting.[21–23]

The Δ32 deletion mutation in the gene encoding CCR5, a receptor for RANTES, results in a frameshift and premature termination of transcript translation, preventing expression of the receptor molecule on cell surfaces.[24] Associations of CCR5 Δ32 with resistance to HIV infection,[24] reduced severity of asthma[25] and rheumatoid arthritis,[26] as well as decreased risk of premature myocardial infarction have been reported.[27]

These findings suggest functional gene variants in chemokines and chemokine receptors may affect genetic susceptibility to inflammatory diseases, most likely by modulating leucocyte recruitment and activation in inflammatory foci. We hypothesised that functional polymorphisms in the RANTES, MCP-1, SDF-1β, and CCR5 genes influence the development of coronary artery lesions. To test this hypothesis, we genotyped a large cohort studied by coronary angiography, and we correlated genotypic and angiographic findings. A significant association with angiographically detectable coronary artery lesions was found for RANTES G-403A, but not for the other candidate gene polymorphisms.

Methods

Study population

We genotyped the LUdwigshafen Risk and Cardiovascular health (LURIC) cohort of 3316 individuals who underwent coronary angiography because of chest pain or non-invasive tests consistent with myocardial ischaemia.[28] All individuals were Caucasians born in Germany from parents of German ancestry. Three thousand two hundred and twenty four subjects were included in the analysis after exclusion of 92 individuals due to incomplete angiographic data ($n = 49$), clinical evidence of arterial disease including stroke, carotid artery stenosis and severe peripheral artery disease (defined by a history of intermittent claudication, angiographic documentation of lumen obstruction, aneurysm of the abdominal aorta, or a history of peripheral arterial intervention for atherosclerotic disease) in patients with normal coronary angiograms ($n = 39$), a history of myocardial infarction in patients with normal coronary angiograms ($n = 2$), or missing genetic data ($n = 2$).

Angiographic classification

Angiographic criteria defining CAD cases vs. controls were as follows: (1) lumen reduction $\geqslant 20\%$ on one or more major epicardial coronary arteries; (2) lumen reduction 10—19% in three or more (out of 15) coronary artery segments. Mild coronary lesions were defined as

Table 1 Clinical characteristics of controls and cases, and their associations with CAD (univariate analysis)

Characteristic	*n*	Controls, $n = 530$	Cases, $n = 2694$	*p*-value
Age: years	3224	56.9 ± 11.9	63.8 ± 9.9	< 0.0001
Male gender, (%)	3224	51.3	73.9	< 0.0001
Smoker, (%)	3224	46.4	67.7	< 0.0001
Arterial hypertension, (%)	3224	42.1	61.9	< 0.0001
Type 2 diabetes, (%)	3127	7.5	22.5	< 0.0001
Hypercholesterolaemia, (%)	3224	36.6	68.4	< 0.0001
Hypertriglyceridaemia, (%)	3223	39.1	48.8	< 0.0001
Low HDL cholesterol, (%)	3223	44.3	55.2	< 0.0001
Waist-to-hip ratio	3184	0.93 ± 0.08	0.97 ± 0.07	< 0.0001
Fibrinogen: mg/dL	3221	347.5 ± 74.8	406.4 ± 109.9	< 0.0001
C reactive protein: mg/L	3218	335 (295–392)	388 (327–463)	< 0.0001

Nominal variables (definitions: see "Methods") are percentages. Age, WHR, and fibrinogen are mean values ± SD; CRP are median values (25–75 percentiles). Missing data in controls for diabetes ($n = 8$), WHR ($n = 5$), and fibrinogen ($n = 1$). Missing data in cases for diabetes ($n = 89$), TG ($n = 1$), HDL-C ($n = 1$), WHR ($n = 35$), fibrinogen ($n = 2$), and CRP ($n = 6$).

<50% lumen narrowing and moderate to severe coronary artery stenoses as ⩾ 50% lumen narrowing.

Clinical data

Clinical characteristics of cases and controls are shown in Table 1. Diabetes was defined according to the new ADA and WHO criteria[29,30] by presence of one or more of the following criteria: (1) treatment with hypoglycaemic agents; (2) two diagnostic tests showing high blood glucose levels (fasting plasma glucose ⩾ 126 mg/dL and/or 2-h plasma glucose ⩾ 200 mg/dL during an oral glucose tolerance test according to the WHO criteria[30]); (3) history of diabetes confirmed by at least one diagnostic test performed during the study. Subjects ($n = 97$) with a single high blood glucose level (no confirmatory test) were not assigned an unequivocal diabetes status. Hypertension was defined as a supine systolic blood pressure ⩾ 140 mm Hg and/or diastolic pressure ⩾ 90 mm Hg (average from three consecutive measurements after 10 min of rest). Hypercholesterolaemia was defined as fasting cholesterol ⩾ 240 mg/dL or treatment with lipid-lowering drugs. High triglycerides (TG) were ⩾ 150 mg/mL. Low HDL-cholesterol (HDL-C) was <35 mg/mL in males and < 45 mg/mL in females. Fibrinogen was measured by the STA fibrinogen/STA Stago Kit (Stago Diagnostica/Roche). C-reactive protein (CRP) was measured using a mAb nephelometric assay (N LATEX CRP mono; Dade Behring). Among the 3224 individuals included in the analysis, data sampling was incomplete with respect to TG and HDL-C in 1 subject each, and to fibrinogen, CRP, and waist-to-hip ratio (WHR) in 3, 6, and 40 patients, respectively.

Acute coronary syndromes

In addition to an analysis of the presence of any angiographic lesion (CAD) as the primary endpoint, the prevalence of acute coronary syndromes (ACS) was analysed as a secondary endpoint reflecting functionally active lesions resulting in major coronary events in CAD patients. ACS were defined as unstable angina or acute myocardial infarction, with or without ST-segment elevation, in accordance with the Joint European Society of Cardiology/American College of Cardiology Committee.[31]

Genotyping

Genotyping of RANTES G-403A, MCP-1 A-2518G, and SDF-1β G801A polymorphisms was performed by restriction fragment length polymorphism (RFLP)-PCR as described,[13,17,19] with a minor modification for RANTES G-403A. This modification consisted of the change of a single base (capital letter, underlined) within the 3' primer (5′-gttcctgcttattcattacagatcGta-3′) to create a new restriction digestion site for better discrimination of the PCR fragments. Because genotype frequencies of RANTES-403 did not comply with Hardy–Weinberg equilibrium (HWE) proportions in the control group (see: "Results"), genotyping was repeated twice in this group using higher concentrations of restriction enzyme to rule out partial digestion, and using an improved method for RFLP with a modified 5′ primer (5′-caatgcccagctcagatcaactgcctc-3′). Genotyping of CCR5 Δ32 was performed by PCR, as described.[24] Technicians and investigators were blinded with respect to case-control status. Two investigators scored gels independently and unclear positions were repeated. Genotype results were typed twice into data files to avoid errors in recording.

Statistical analysis

Power calculations with a pre-set level of significance ($\alpha = 0.05$) had been calculated for equal numbers of cases and controls according to Fleiss et al.,[32] in the LURIC project (see LURIC study design,[28] Appendix II). For an unequal number of cases ($n = 2694$) and controls ($n = 530$), the power to detect a 6% or 7% difference in genotype/allele frequencies for a two-sided test with a level of significance of $p = 0.05$ was 74% or 86%, respectively. Data are shown as frequencies or means ± standard deviation (SD). CRP did not have a normal standard distribution and was log-transformed for analysis. The JMP program (version 5; SAS Institute, Cary, NC) was used for statistical analysis. Frequencies were compared with the Pearson χ^2-test; means were com-

pared with the two-tailed unpaired Student's *t*-test or ANOVA test. Gene counting method estimated allele frequencies, and the χ^2 analysis was used to test for deviations of genotype proportions from HWE. The Armitage's test for trend in proportions (does not assume HWE) was used for statistical analysis of genotype and allele carrier frequencies according to Sasieni,[33] as proposed by Xu et al.[34] In addition, the variance of differences in allele frequencies was determined using the method by Schaid and Jacobsen.[35] Odds ratios for genotype analysis were calculated according to Sasieni.[33] Correction for multiple testing was performed by Bonferroni adjustment. The Cochran–Mantel–Haenszel method was used to test association of RANTES A-403 with CAD after stratification for cigarette smoking, age, and gender. The independent contribution of RANTES A-403 to CAD was determined by nominal logistic analysis, with CAD as the dependent variable, using three different models with increasingly conservative character: (1) conventional coronary risk factors including age, male gender, smoking, hypertension, diabetes, hypercholesterolaemia, low HDL-C, high TG, and WHR were entered as co-variates; (2) in addition to those traditional risk factors, CRP was entered as a co-variate; (3) in addition to traditional risk factors and CRP, fibrinogen was also entered as a co-variate. Age, WHR, CRP, and fibrinogen were analysed as continuous variables that satisfied the assumption of linearity. This assumption was checked by: (1) sub-dividing each continuous variable into five sub-groups, corresponding to the each 20th percentiles of the distribution; (2) calculating the logits of CAD probabilities for each sub-group; (3) analysing the linearity of the logit vs. the sub-divided variable by simple linear regression (age, $r^2 = 0.95$; WHR, $r^2 = 0.91$; CRP, $r^2 = 0.99$; fibrinogen, $r^2 = 0.97$). To test the influence of gene–gene interaction of MCP-1 A-2518G, SDF-1β G801A, and CCR5 Δ32 polymorphisms with RANTES G-403A and CAD, a multivariate model with interaction terms (MCP-1 A-2518G*RANTES G-403A, SDF-1β G801A*RANTES G-403A, and CCR5 Δ32*RANTES G-403A) was used. We adopted a significance level of $p < 0.05$ for this exploratory study.

Results

Angiographic results

Based on coronary angiographic criteria, 3224 subjects of the LURIC cohort included in the analysis were subdivided into cases $(n = 2694)$ with abnormal angiograms and controls $(n = 530)$ with normal angiograms. Among cases, the majority $(n = 2231)$ showed moderate to severe lumen reduction ($\geqslant 50\%$) in one $(n = 620)$, two $(n = 623)$, or three $(n = 988)$ major epicardial coronary arteries. A minority of cases presented with 0-vessel disease (<50% stenosis; $n = 463$) defined as mild angiographic abnormalities with lumen reduction of 20–49% of at least one major coronary artery $(n = 336)$ or minor narrowings (10–19%) of $\geqslant 3$ coronary artery segments $(n = 127)$.

Associations between clinical characteristics and CAD

Patients with CAD presented with the typical changes in the cardiovascular risk profile when compared to subjects without CAD (all $p < 0.0001$; Table 1).

Genotypic data and HWE

Genotype frequencies for RANTES-403 complied with HWE both in the study population as a whole and in cases (data not shown), but deviated from it in controls $(\chi^2 = 4.49,\ p = 0.034)$. The true Type-I error rate (δ) and the fraction of maximum discrepancy with respect to the HWE model (f, where HWE: $f = 0$; maximum discrepancy: $f = 1.0$), calculated according to Schaid and Jacobsen,[35] were modest $(\delta = 0.00239,\ f = 0.015)$. Odds ratios for genotype analysis were calculated according to

Table 2 Clinical characteristics of controls and cases in carriers (AG + AA) and non-carriers (GG) of RANTES A-403

Characteristics	Controls, (*n*)		Cases, (*n*)	
	GG ($n = 370$)	AG + AA ($n = 160$)	GG ($n = 1724$)	AG + AA ($n = 970$)
Age: years, (*n*)	57.1 ± 11.6 (370)	56.5 ± 12.6 (160)	63.5 ± 10.1 (1724)	64.3 ± 9.5 (970)
Male gender, % (*n*)	51.1 (189)	51.9 (83)	75.1 (1294)	71.9 (697)
Smoker, % (*n*)	48.8 (179)	41.9 (67)	68.8 (1187)	65.8 (638)
Arterial hypertension, % (*n*)	42.7 (158)	40.6 (65)	61.9 (1068)	61.7 (599)
Type 2 diabetes, % (*n*)	6.0 (22)	10.8 (17)	21.8 (364)	23.7 (222)
Hypercholesterolemia, % (*n*)	37.8 (140)	33.7 (54)	68.7 (1184)	68.0 (660)
Hypertriglyceridemia, % (*n*)	39.5 (146)	38.1 (61)	50.0 (861)	46.6 (452)
Low HDL cholesterol, % (*n*)	43.2 (160)	46.9 (75)	55.5 (957)	54.6 (530)
Waist to hip ratio (*n*)	0.93 ± 0.08 (367)	0.93 ± 0.08 (158)	0.97 ± 0.07 (1707)	0.96 ± 0.08 (952)
Fibrinogen, mg/dL (*n*)	344.7 ± 73.2 (369)	353.7 ± 78.1 (160)	403.5 ± 108.6 (1722)	411.5 ± 112.0 (970)
CRP, mg/L [25–75 percentiles] (*n*)	335 [294–390] (370)	338 [297–396] (160)	384 [326–460] (1720)	393 [332–469] (968)

Age, WHR, and fibrinogen are mean values ± SD; CRP are median values [25–75 percentiles]. Missing data in carriers of the A allele for diabetes ($n = 2$) and WHR ($n = 2$) in controls, and for diabetes ($n = 33$), WHR ($n = 18$), and CRP ($n = 2$) in cases. Missing data in non-carriers of the A allele for diabetes ($n = 6$), WHR ($n = 3$), and fibrinogen ($n = 1$) in controls, and for diabetes ($n = 56$), TG ($n = 1$), low HDL-C ($n = 1$), WHR ($n = 17$), fibrinogen ($n = 2$), and CRP ($n = 4$) in cases.

Table 3 Genotype, allele, and allele carrier frequencies of RANTES A-403 in controls and cases

Group	Genotypes frequencies, % (*n*)						A allele frequencies, %				A allele carrier frequencies, %			
	GG	AG	AA	χ^2	*p*	OR		Z	*p*	OR (95% CI)		χ^2	*p*	OR (95% CI)
Controls	69.8 (370)	25.8 (137)	4.3 (23)	4.17	0.041	1.14	17.3	2.04	0.041	1.20 (1.01–1.43)	30.2	6.58	0.010	1.30 (1.06–1.60)
Cases	64.0 (1724)	32.0 (862)	4.0 (108)				20.0				36.0			

(Calculation of OR, see [33]).

Sasieni.[33] Genotype frequencies for MCP-1-2518, SDF-1β 801, and CCR5 Δ32 were in HWE in both cases and controls (data not shown).

Association between RANTES A-403 and coronary risk factors

Clinical characteristics of carriers (AG/AA) and non-carriers (GG) of the RANTES A-403 allele were comparable ($p > 0.05$; Table 2). In the study population as a whole, only plasma fibrinogen was correlated with RANTES-403 genotype and allele carrier frequencies (F ratio $= 3.47$, $p = 0.0310$ and $t = -2.59$, $p = 0.0098$, respectively).

Association between RANTES A-403 and CAD

Genotype and allele frequencies of RANTES-403, as well as allele carrier frequencies of RANTES A-403, were significantly different between cases and controls (Table 3). The analysis of allelic frequencies using the method by Schaid and Jacobsen[35] that corrects for deviations from HWE yielded $Z = 2.04$ $(p = 0.041)$. For comparison, assuming a HWE model would yield $Z_{HWE} = 2.06$ $(p = 0.040)$, consistent with a minor difference between the HWE and non-HWE models for this analysis. After subdivision of cases based on coronary lesion severity, allele carrier frequencies of RANTES A-403 were significantly increased both in cases with moderate to severe lesions ($\geqslant 50\%$) and in those with mild lesions ($<50\%$), as compared to controls (Table 4). Data stratification for gender and age revealed no significant effect of these parameters on the association between RANTES A-403 and CAD (data not shown). After data stratification for cigarette smoking, the significant association between RANTES A-403 and CAD was slightly enhanced (genotype and A allele carrier frequencies: $\chi^2 = 9.41$, $p = 0.0090$ and $\chi^2 = 8.20$, $p = 0.0042$, respectively).

RANTES A-403 is an independent predictor of CAD

Genotype and allele frequencies of RANTES A-403 retained significant association with CAD, independent of conventional risk factors, in a multivariate model with conventional coronary risk factors entered as co-variates (first model; Table 5). The association was maintained when CRP was entered as a co-variate in addition to conventional risk factors (second model; $\chi^2 = 6.65$, $p = 0.036$ and $\chi^2 = 6.47$, $p = 0.011$ for genotype and allele frequencies, respectively), or when CRP and fibrinogen were both entered as additional co-variates (third model; $\chi^2 = 6.19$, $p = 0.045$ and $\chi^2 = 6.02$, $p = 0.014$ for genotype and allele frequencies, respectively).

Association between RANTES A-403 and ACS

Among CAD cases, ACS occurred in 1032 patients, whereas 1662 patients did not suffer from ACS. Genotype frequencies of RANTES-403 were significantly different

Table 4 Genotype and allele frequencies of RANTES A-403 with respect to CAD severity

Group	Genotypes frequencies, % (*n*)						A allele carrier frequencies, %			
	GG	AG	AA	χ^2	*p*	OR		χ^2	*p*	OR (95% CI)
Controls	69.8 (370)	25.8 (137)	4.3 (23)				30.2			
CAD < 50%	61.6 (285)	33.7 (156)	4.7 (22)	5.67	0.017	1.24	38.4	7.50	0.006	1.44 (1.10−1.90)
CAD ⩾ 50%	64.5 (1439)	31.6 (706)	3.9 (86)	3.16	0.075	1.11	35.5	5.35	0.021	1.27 (1.03−1.57)

Data are shown for subsets with mild (lumen reduction: <50%) or moderate to severe CAD (lumen reduction: ⩾ 50%)(Calculation of OR, see [33]).

Table 5 Nominal logistic fit, with CAD as the dependent variable (first model; see "Methods")

	Genotype analysis				A allele carrier analysis			
Source of variation	DF	R^2	L-R χ^2	*p* value	DF	R^2	L-R χ^2	*p* value
Multivariate model (n = 3086)								
All variables	11	0.24	665.9	<0.0001	10	0.24	665.6	<0.0001
Age (yr)	1		156.1	<0.0001	1		156.2	<0.0001
Gender	1		60.8	<0.0001	1		60.8	<0.0001
Smoker	1		61.7	<0.0001	1		61.7	<0.0001
Arterial hypertension	1		23.6	<0.0001	1		23.6	<0.0001
Type 2 diabetes	1		23.6	<0.0001	1		23.6	<0.0001
Waist to hip ratio	1		1.98	0.16	1		2.03	0.15
Hypercholesterolemia	1		153.1	<0.0001	1		153.2	<0.0001
Hypertriglyceridemia	1		0.43	0.51	1		0.44	0.51
Low HDL cholesterol	1		21.2	<0.0001	1		21.2	<0.0001
RANTES-403 polymorphism	2(#)		8.64	0.013	1(*)		8.40	0.0038

Type of analysis: (#)3-class (GG = 0, AG = 1, and AA = 2); (*)2-class (GG = 0 and AG + AA = 1). Due to incomplete data in a small number of subjects (see "Methods"), the multivariate model included 3086 subjects with complete data.

between cases with ACS and controls ($\chi^2 = 5.17$, OR = 1.19, $p = 0.023$), but not between cases without ACS and controls ($\chi^2 = 2.69$, OR = 1.10, $p = 0.10$). Similarly, allele carrier frequencies of RANTES A-403 were significantly increased in cases with ACS compared with controls (37.02% vs. 30.19%; $\chi^2 = 7.20$, OR = 1.36 [95%-CI = 1.08−1.71], $p = 0.0073$), even after Bonferroni adjustment for multiple testing. In contrast, A allele carrier frequencies were only slightly increased in cases without ACS (35.38%; $\chi^2 = 4.82$, OR = 1.27 [95%-CI = 1.02−1.57], $p = 0.028$).

Lack of association between MCP-1 G-2518, SDF-1β A801, CCR5 Δ32, and CAD

Genotype, allele, and allele carrier frequencies of MCP-1-2518, SDF-1β 801, and CCR5-32 were not significantly different in cases and controls (Table 6). In disagreement with a previous report,[27] CCR5 Δ32 was not correlated with premature myocardial infarction (data not shown). No significant gene−gene interaction of the four polymorphisms with respect to CAD risk was observed (data not shown).

Table 6 Genotype, allele, and allele carrier frequencies of MCP-1 A-2518G, SDF-1β G801A, and CCR5 Δ32 gene polymorphisms in controls and cases

		Genotype frequencies, *n* (%)			Allele 2* frequencies, %	Allele 2* carrier frequencies, %
Polymorphism	Group	Genotype 1−1*	Genotype 1−2*	Genotype 2−2*		
MCP-1 A-2518G	Controls	288 (54.5)	207 (39.2)	33 (6.2)	25.8	45.4
	Cases	1423 (52.8)	1068 (39.7)	202 (7.5)	27.3	47.2
SDF-1β G808A	Controls	350 (66.2)	159 (30.1)	20 (3.8)	18.8	33.8
	Cases	1709 (63.5)	885 (32.9)	96 (3.6)	20.0	36.5
CCR5 Δ32	Controls	423 (80.1)	99 (18.7)	6 (1.1)	10.5	19.9
	Cases	2093 (78.1)	553 (20.6)	35 (1.3)	11.6	21.9

(*) 1, frequent allele and 2, rare allele. Missing data for MCP-1 A-2518G: *n* = 2 controls and 1 case; SDF-1β G808A: *n* = 1 control and four cases; CCR5 Δ32: *n* = 2 controls and 13 cases. $p > 0.10$ in cases vs. controls for each polymorphism with respect to differences in genotype, allele, and allele carrier frequencies between cases and controls.

Discussion

Gene polymorphisms that modify expression and/or bioavailability of chemokines and their cellular receptors may affect leucocyte trafficking in inflammatory diseases, including arteriosclerosis. In our case-control study of $n = 3224$ subjects, RANTES A-403, but not the other three gene variants studied, was significantly associated with CAD. Carriers of the A allele were at increased risk of developing angiographically detectable coronary lesions compared with individuals homozygous for the G allele. The association was stronger in patients with mild (<50%) coronary lesions ($p = 0.017$) and in those with ACS ($p = 0.023$). After Bonferroni adjustment for multiple testing of the four gene polymorphisms, the frequency of carriers of the A allele remained significantly increased in CAD patients. The association between RANTES A-403 and CAD was enhanced in cigarette smokers. Finally, RANTES A-403 remained significantly associated with CAD after multivariate adjustment for conventional risk factors and for CRP and fibrinogen, two biomarkers of systemic inflammation associated with CAD.[36,37]

Thus, we were able to replicate recent results from Hungary showing an increased frequency of the RANTES A-403 allele in patients undergoing coronary artery bypass surgery.[18] However, the statistical significance in this smaller cohort ($n = 628$) was borderline ($p = 0.052$; OR = 1.33 [95%-CI = 1.00–1.77]). Of note, this odds ratio is closely comparable to the one obtained in the present study. Obviously, replicating results in independent cohorts is crucial for establishing a genetic association.

The biological mechanism underlying this genetic association most likely is increased gene expression from the RANTES A-403 promoter variant.[11] RANTES expression is differentially regulated in many tissues. Several putative *cis*-acting elements have been described in the RANTES promoter region.[38] The A-403 variant introduces a new consensus binding site for the GATA transcription factor family. This variant has been associated with enhanced RANTES promoter activity in human T-cell and mast cell lines, which express GATA binding proteins, but not in epithelial cells, which do not express these proteins.[11] Because megakaryocytes constitutively express both RANTES and GATA binding proteins,[39] RANTES production and release by platelets might be enhanced in carriers of the A-403 allele. RANTES stored in platelet secretory vesicles is released upon platelet activation and immobilised on the surface of inflamed endothelium,[40–42] where it promotes shear-resistant monocyte arrest and *trans*-endothelial migration,[40,43–45] while generating a critical signal for chemokine production by monocytes.[46] RANTES deposition on the luminal surface of carotid arteries has been demonstrated in apolipoprotein E-deficient mice with early atherosclerotic lesions in vivo, as well as in human atherectomy samples.[40] The RANTES antagonist Met-RANTES inhibited monocyte recruitment on carotid endothelium, neointima formation and macrophage accumulation after arterial injury, as well as atherosclerosis progression in uninjured arteries in apolipoprotein E-deficient mice.[40,46,47] These findings suggest an important role for RANTES in vascular disease, potentially explaining the enhanced association between RANTES A-403 and CAD in conditions characterised by platelet activation, such as smoking and ACS.

Recently, RANTES A-403 has been associated with several inflammatory diseases.[11–15] Polymorphisms in other chemokine genes have also been associated with CAD. Fraktalkine and its receptor, CX3CR1, which are expressed in human plaques,[48] have been implicated in atherogenesis.[49] An association between the I249 variant of CX3CR1 and reduced endothelial dysfunction and CAD lesions was reported.[50] The loss-of-function mutation CX3CR1-M280 was associated with cardiovascular protection in Framingham participants.[51] The rare Val64Ile polymorphism in the CCR2 gene coding for the main MCP-1 receptor was associated with reduced coronary calcifications in one study,[52] and with premature myocardial infarction (but not coronary lesions) in another study.[53] As mentioned, MCP-1 G-2518 homozygosity was also associated with CAD.[18] However, this association was not replicated in the present study in a larger cohort, nor was the reported protection against premature myocardial infarction by CCR5 $\Delta 32$.[27]

A methodological aspect of the present study that deserves discussion, like in many case-control association studies, is the appropriateness of the control group. Because angiography is relatively insensitive for early lesions that do not impact on the vascular lumen, early lesions undetectable by angiography could not be ruled out in controls, nor was coronary microvascular dysfunction. Nevertheless, coronary angiography remains a gold standard for the diagnosis of CAD, and it is well known that the future incidence of cardiac events is low in subjects with normal coronary angiograms. Another issue that deserves consideration is the departure of RANTES-403 genotypes from HWE proportions in controls ($\chi^2 = 4.49$, $p = 0.034$), the number of AA homozygous subjects being higher than expected. Apparent violations of HWE might arise because of errors in genotyping. A literature survey found that 16 out of 133 single nucleotide polymorphisms (12%) from 75 case-control studies deviated from HWE.[34] Therefore, genotyping was repeated three times (with two different methods) to carefully rule out genotyping errors in controls. Another potential cause for deviations from HWE is ethnic diversity within the population. The LURIC study was restricted to native Germans of German ancestry living in the Rhine valley area (a region where >95% are Caucasians of German descent and where second and third-generation family members are often alive) in order to limit ethnic heterogeneity and to enhance access to family members. All other gene polymorphisms tested in this cohort in the present and previous studies were in HWE, also consistent with limited ethnic heterogeneity. Thus, no explanation could be found for the departure from HWE in controls. A comparison of our data with those from the Hungarian cohort[18] (complying with HWE) shows almost identical frequencies of control subjects homozygous for the frequent G allele (69.1% vs. 69.8% in LURIC) and, hence, of controls carrying the A allele.

Stated differently, frequencies of carriers of the A allele in controls, which significantly differed from those in cases in our study ($\chi^2 = 6.58$, $p = 0.010$), were closely comparable in the two studies. It also should be emphasised that this analysis, like the one of genotype frequencies, was performed using statistical tests that do not assume HWE.[33] Moreover, allelic frequencies were analysed using the test by Schaid and Jacobsen[35] that corrects for deviations from HWE. This test also revealed that the true Type-I error rate and the fraction of maximum discrepancy with the HWE model were modest, corroborating the validity of our statistical data.

In conclusion, the RANTES A-403 allele was associated with CAD independently from conventional cardiovascular risk factors and from CRP and fibrinogen, two biomarkers of vascular inflammation. The association was enhanced in smokers and ACS, two conditions that are accompanied by platelet activation and vascular inflammation. Our findings are consistent with biological effects of Met-RANTES in experimental models of arteriosclerosis.[40,46,47] Novel non-peptide, orally bioavailable RANTES antagonists that have been successfully tested in heart transplantation[54] and HIV models[55] might also prove effective in preventing cardiovascular disease.

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