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CLINICAL RESEARCH
Epidemiology, prevention, and health care policies

Autoantibodies against the chemokine receptor 3 predict cardiovascular risk

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Abstract

Background and Aims Chronic inflammation and autoimmunity contribute to cardiovascular (CV) disease. Recently, autoantibodies (aAbs) against the CXC-motif-chemokine receptor 3 (CXCR3), a G protein-coupled receptor with a key role in atherosclerosis, have been identified. The role of anti-CXCR3 aAbs for CV risk and disease is unclear.

Methods Anti-CXCR3 aAbs were quantified by a commercially available enzyme-linked immunosorbent assay in 5000 participants (availability: 97.1%) of the population-based Gutenberg Health Study with extensive clinical phenotyping. Regression analyses were carried out to identify determinants of anti-CXCR3 aAbs and relevance for clinical outcome (i.e. all-cause mortality, cardiac death, heart failure, and major adverse cardiac events comprising incident coronary artery disease, myocardial infarction, and cardiac death). Last, immunization with CXCR3 and passive transfer of aAbs were performed in ApoE$^{(-/-)}$ mice for preclinical validation.

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Results The analysis sample included 4195 individuals (48% female, mean age 55.5 ± 11 years) after exclusion of individuals with autoimmune disease, immunomodulatory medication, acute infection, and history of cancer. Independent of age, sex, renal function, and traditional CV risk factors, increasing concentrations of anti-CXCR3 aAbs translated into higher intima–media thickness, left ventricular mass, and N-terminal pro-B-type natriuretic peptide. Adjusted for age and sex, anti-CXCR3 aAbs above the 75th percentile predicted all-cause death [hazard ratio (HR) (95% confidence interval) 1.25 (1.02, 1.52), $P = .029$], driven by excess cardiac mortality [HR 2.51 (1.21, 5.22), $P = .014$]. A trend towards a higher risk for major adverse cardiac events [HR 1.42 (1.0, 2.0), $P = .05$] along with increased risk of incident heart failure [HR per standard deviation increase of anti-CXCR3 aAbs: 1.26 (1.02, 1.56), $P = .03$] may contribute to this observation. Targeted proteomics revealed a molecular signature of anti-CXCR3 aAbs reflecting immune cell activation and cytokine–cytokine receptor interactions associated with an ongoing T helper cell 1 response. Finally, ApoE$^{(-/-)}$ mice immunized against CXCR3 displayed increased anti-CXCR3 aAbs and exhibited a higher burden of atherosclerosis compared to non-immunized controls, correlating with concentrations of anti-CXCR3 aAbs in the passive transfer model.

Conclusions In individuals free of autoimmune disease, anti-CXCR3 aAbs were abundant, related to CV end-organ damage, and predicted all-cause death as well as cardiac morbidity and mortality in conjunction with the acceleration of experimental atherosclerosis.

Structured Graphical Abstract

Key Question

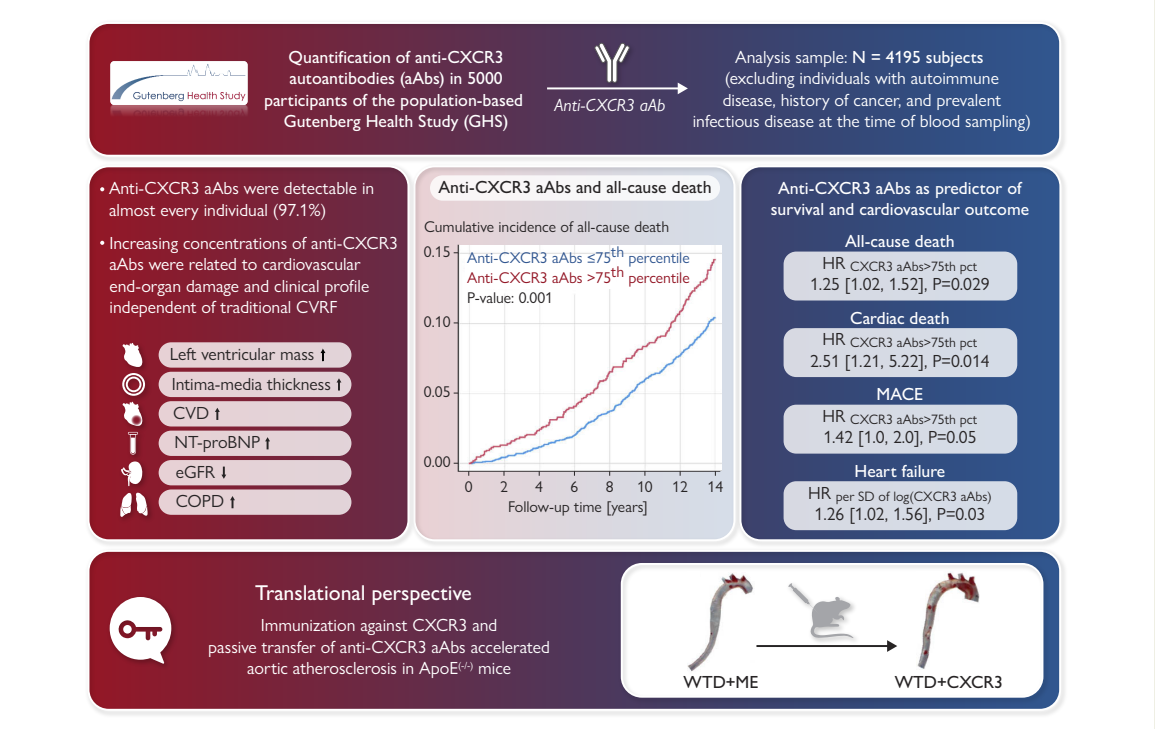
Are autoantibodies (aAbs) directed against the CXC-motif-chemokine receptor 3 (CXCR3), a G-protein coupled receptor (GPCR), of clinical relevance in the general population?

Key Finding

This translational study demonstrated that anti-CXCR3 aAbs are abundant in the general population. Increased concentrations of anti-CXCR3 aAbs were associated with traits of cardiac and vascular end organ damage, and predicted cardiovascular outcomes and all-cause death.

Take Home Message

In the general population, aAbs directed against CXCR3 have been identified as novel biomarkers indicating cardiovascular morbidity and mortality beyond established risk factors.



The schematic overview over the present study including description of the analysis sample, cross-sectional analyses, and data on clinical outcome, expressed by hazard ratios derived from Cox regression analysis with corresponding 95% confidence intervals and *P*-values, as well as experimental

data on anti-CXCR3 autoantibodies and murine atherosclerosis. aAbs, autoantibodies; ApoE, apolipoprotein E; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; CVRF, cardiovascular risk factors; CXCR3, CXC-motif-chemokine receptor 3; eGFR, estimated glomerular filtration rate; HR, hazard ratio; MACE, major adverse cardiac events (comprising coronary artery disease, myocardial infarction, and cardiac death); ME, membrane extract; NT-proBNP, N-terminal pro-B-type natriuretic peptide; pct, percentile; SD, standard deviation; WTD, western-type diet.

Keywords Autoimmunity • Autoantibodies • G protein-coupled receptor • Chemokine receptor • Cardiovascular disease

Introduction

Chronic systemic inflammation is a harbinger for the development and progression of cardiovascular disease (CVD)[1–4] and is linked to autoimmunity through the dysregulation of immune responses to self-antigens.[5,6] Autoantibodies (aAbs) targeting antigens of proteins with pivotal importance for the cardiovascular (CV) system have been implicated in the multifaceted aetiology of CVD, particularly heart failure (HF).[7,8] Inflammation often plays a double-edged role in CVD, as evidenced by the inflammatory cascade following myocardial infarction: immediate immune activation promotes infarct healing through angiogenesis, while prolonged inflammation drives maladaptive ischaemic myocardial remodelling.[9]

Similarly, cardiac aAbs, particularly those that agonistically activate the β1-adrenoreceptor (β1-AR), have been detected at varying concentrations both in the general population and individuals with HF.[8] While considerable evidence suggests a causal role of anti-β1-AR aAbs in the development of dilated cardiomyopathy (DCM), there are conflicting signals regarding myocardial recovery,[10] underscoring the complexity of the relationship between autoimmunity and CVD, which remains poorly understood to date. Recently, aAbs directed against the CXC-motif-chemokine receptor 3 (CXCR3) have been identified as modulators of the receptor[11–13] with potential agonistic activity.[11] A member of the G protein-coupled receptor (GPCR) superfamily, CXCR3 regulates chemotaxis of T helper 1 (Th1) effector cells into inflamed tissues[14] and is increasingly recognized as a mediator of tissue inflammation in CVD.[15,16] The Th1 lineage cytokine interferon-γ is considered the major proatherogenic cytokine, promoting local expression of adhesion molecules, cytokines, and chemokines such as CXCL9, CXCL10, and CXCL11 and of their receptor CXCR3.[15,16] Preclinical evidence suggests a key role of the CXCR3–CXCL10 axis in the development of atherosclerotic lesions through modulation of the local Th1/Th2 balance,[17] and CXCR3 signalling has been linked to the transition from left ventricular (LV) hypertrophy to HF.[18] However, data on the distribution, determinants, and association of anti-CXCR3 aAbs with CVD in the population are not yet available.

There is a general need to identify autoimmune mechanisms contributing to CVD.[19] Therefore, this study aimed to characterize the seroprevalence of anti-CXCR3 aAbs in a large prospective cohort study of a population-representative sample and to investigate clinical determinants of anti-CXCR3 aAb levels and their relation to CV risk and disease.

Methods

Study design and population

Data from the Gutenberg Health Study (GHS), a large population-based, prospective, single-centre cohort study in mid-western Germany, were analysed. The study was approved by the local ethics committee [Medical Association Rhine-Hesse, Germany, reference number 837.020.07(5555)] and data safety commissioner before study initiation. All procedures were performed in accordance with the principles of the Declaration of Helsinki as well as the tenets of Good Clinical and Epidemiological Practice. Participants provided written informed consent before study enrolment. Details on the study design were published previously.[20]

Data assessment

During the baseline visit at the study centre, all participants underwent deep clinical phenotyping following predefined standard operating procedures. Investigations included assessment of prevalent CV risk factors (CVRF), CVD, and non-CVD as well as medication via computer-assisted personal interviews, venous blood sampling, anthropometry, echocardiography, and vascular structure measurement including intima–media thickness. The methodology of vascular structure measurement in the GHS has been described elsewhere.[21] A detailed description of the acquisition of echocardiographic measures and CVRF is provided in Supplementary data online. Anti-CXCR3 IgG aAbs were quantified from serum by a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (CellTrend, Luckenwalde, Germany). Further information on the characteristics of the aAb assay is provided in Supplementary data online, *Tables S1–S3*. Concentrations of humoral N-terminal pro-B-type natriuretic peptide (NT-proBNP; Elecsys proBNP II assay, ECLIA, Roche Diagnostics, Mannheim, Germany) and C-reactive protein (Abbott Diagnostic, Wiesbaden, Germany) were measured with commercially available assays. Estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation.[22] Data on CXCR3 expression in human samples were obtained by RNA sequencing using two publicly available human data sets: (i) human stable and unstable atherosclerotic plaques of carotid arteries (GEO accession number GSE120521[23]) and (ii) early and advanced human atherosclerotic lesions of carotid arteries (GSE28829[24]).

Proteomic profiling by targeted immuno-polymerase chain reaction

Proximity extension assay technology (Olink Proteomics, Uppsala, Sweden)[25] with quantification of DNA amplicons by real-time polymerase chain reaction (PCR) (Fluidigm BioMark HF platform) was used for high-throughput proteomic analysis. In total, 92 biomarkers representing systemic inflammation (Olink Target 96 Inflammation panel) were measured. A full list of proteins is provided in Supplementary data online, *Table S4*. Relative protein expression was expressed in normalized expression values (NPX), where each unit increase translates into a doubling of absolute circulating protein concentration. Protein expression levels with non-normal distribution were transformed to approximate normality by square root or natural logarithmic transformation. A detailed description of methodology and quality control procedures has been published recently.[26]

Clinical endpoints

Information on all-cause and CV mortality was obtained via death certificates with standardized coding acquired by the state registration office. Data on other clinical endpoints (i.e. coronary artery disease, HF, myocardial infarction, and stroke) were retrieved periodically by computer-assisted personal or telephone interview and additionally continuously by integration of medical records. Subsequently, endpoints were adjudicated by an independent physician-led endpoint committee.

Validation in the animal model: active immunization and passive transfer

To validate the clinical finding, B6.ApoE knockout (KO) female mice (B6.129P2-Apoe$^{tm1Unc/J}$, Charles River) were used as a proatherogenic murine model for immunization against CXCR3. Mice were fed with western-type diet for 90 days until sacrifice, followed by removal of the aorta and histological analysis for quantification of atherosclerotic burden.

In the active immunization model, mice aged 6–7 weeks were randomly divided into two groups (CXCR3 and control) with $n = 8$ in each group. Immunization against CXCR3 was done by subcutaneous injection of 200 µg membrane extract (ME) of CXCR3 overexpressing cells with complete Freund's adjuvant (CFA) followed by a booster injection of 200 µg CXCR3-ME with incomplete Freund's adjuvant (IFA) after another 3 weeks. Control mice received 200 µg ME with CFA and 200 µg ME with IFA without CXCR3 overexpression. Membrane extract–embedded CXCR3 was used to maintain the native conformation of CXCR3 and to induce natural anti-CXCR3 aAbs as recently performed for another GPCR.[27] At Days 1, 17, 30, and 83, venous post-diet blood samples were obtained and centrifuged at 4500 × *g* for 3 min at 4°C.

In the passive transfer model, mice at the age of 6–7 weeks were randomly divided into three groups, namely mice injected with 200 µg IgG ($n = 17$) derived from human donors with different anti-CXCR3 aAb levels and CV risk profiles, control mice injected with NaCl 0.9% ($n = 8$) at Days 1, 17, 30, and 83, and control mice that were not injected ($n = 14$). The mice treated with IgG and NaCl 0.9% were fed with western-type diet for 90 days, whereas the mice that were not injected were fed with a chow diet. At Day 90, venous post-diet blood samples were obtained and centrifuged at 4500 × *g* for 3 min at 4°C. Human IgG was isolated from sera by protein G sepharose chromatography in 20 mM phosphate buffer (pH 7.0) using ÄKTA start. IgG was eluted with 0.1 M glycine/HCl (pH 2.7), and the pH was neutralized with 1 M Tris-HCl (pH 9.0).

Anti-CXCR3-IgG aAbs were quantified from serum by a commercially available, murine-specific sandwich ELISA according to the manufacturer's instructions (CellTrend, Luckenwalde, Germany); measurements are presented as optical density, as a standard was not available. All animal experiments were approved by the German animal studies committee of Schleswig-Holstein, and animals were held and handled according to institutional and federal guidelines (animal licence AZ 103-8/16). Criteria for inclusion and exclusion were specified and set before the study according to the animal proposal. No animal was excluded after randomization. All experiments were performed blinded. Detailed information on the induction of atherosclerosis and histological analyses is provided in Supplementary data online.

Statistical analysis

The analysis sample was stratified into quartiles of concentrations of anti-CXCR3 aAbs for characterization, and further methods for descriptive analysis are provided in Supplementary data online. To investigate clinical determinants of anti-CXCR3 aAb concentrations, linear regression analyses with log-transformed anti-CXCR3 aAbs as dependent variable were carried out. Additionally, the association of medication with log-transformed anti-CXCR3 aAbs was analysed by linear multivariable regression with four-digit anatomical therapeutic chemical (ATC, classification system) coded medication classes (in classes with $n \geq 10$ individuals) as independent variables, subjected to Bonferroni correction with a significance threshold of 0.0005 (0.05/number of tests). To further characterize the influence of anti-CXCR3 aAbs on continuous and categorical CV and non-CV traits, multivariable regression analysis was performed with log-transformed anti-CXCR3 aAbs as independent variable and adjustment for age, sex, renal function as expressed by eGFR, as well as traditional CVRF, and comorbidities. Cardiovascular disease was defined as composite variable, comprising atrial fibrillation, coronary artery disease, HF, history of myocardial infarction or stroke, and peripheral artery disease. The composite variable inflammatory disease comprised atopic dermatitis, osteoarthritis, asthma, allergic rhinitis, and upper respiratory infection < 14 days without residual systemic inflammation as evidenced by C-reactive protein < 10 mg/L at baseline blood sampling. Logistic regression analysis was carried out to characterize the relationship of anti-CXCR3 aAbs with history of stroke or myocardial infarction.

The predictive value of anti-CXCR3 aAbs for study endpoints was investigated in time-to-event analysis. First, cumulative incidence plots were generated for all outcome variables according to quartiles of concentrations of anti-CXCR3 aAbs. For all-cause mortality, Cox regression and the log-rank test were used to compare quartiles. For all other endpoints, subdistribution hazard models by Fine and Gray with consideration of death as competing risk and Gray's test were utilized to compare quartiles. To account for potential confounders or mediators, two models were calculated for each endpoint. The first model had adjustment for age and sex, while the second model additionally introduced eGFR and traditional CVRF as adjustment covariates. Outcome analysis was also carried out in continuous fashion per standard deviation (SD) increase of log-transformed anti-CXCR3 aAb concentrations as independent variable. Due to the explorative nature of this analysis, a threshold for statistical significance was not defined, and *P*-values were rather interpreted as continuous measure of statistical evidence. R Version 4.2.0 (http://www.r-project.org) and GraphPad Prism 7 software were used for statistical analysis. More details on statistical methods of this work are provided in Supplementary data online.

Bioinformatic characterization of anti-CXCR3 autoantibodies

To identify molecular determinants of anti-CXCR3 aAbs, a supervised machine learning approach was applied to evaluate proteins measured with the immuno-PCR panel on inflammation (see above) according to their relationship with the anti-CXCR3 aAb concentration. Specifically, least absolute shrinkage and selection operator (LASSO) regularized regression was computed, with anti-CXCR3 aAbs as dependent variable and 95 covariates comprising 92 proteins reflecting systemic inflammation, age, sex, and eGFR. Further information on the bioinformatics analyses and the databases used for this work can be found in Supplementary data online.

Results

Sample characteristics

Concentrations of anti-CXCR3 aAbs were available in 4857 of the first 5000 study participants. After exclusion of individuals with insufficient amount of serum to detect anti-CXCR3 aAbs ($n = 143$), recent upper respiratory tract infection and residual systemic inflammation evidenced by C-reactive protein > 10 mg/L at baseline ($n = 63$), history of cancer diagnosed within 5 years prior to enrolment ($n = 168$), intake of immunomodulatory medication ($n = 265$), and autoimmune disease ($n = 246$, Supplementary data online, *Figure S1*), the analysis sample included 4195 individuals (see Supplementary data online, *Figure S2*). The sample had a mean age of 55.5 ± 11.0 years with 48% females. The median concentration of anti-CXCR aAbs was 6.4 U/mL [interquartile range (IQR) 4.5–10.2]. Concentrations modestly increased with age, as presented in *Table 1*. The overall distribution is shown in Supplementary data online, *Figure S3*. Individuals above the 75th percentile of anti-CXCR3 aAb concentrations had a more pronounced CV risk profile compared with other individuals, as evidenced by the higher prevalence of arterial hypertension, diabetes mellitus, family history of myocardial infarction or stroke, and obesity. The prevalence of overt CVD was also higher in these individuals, mainly driven by a higher burden of HF, coronary artery disease, history of myocardial infarction or stroke, and peripheral artery disease. Detailed characteristics of the

Table 1 Age- and sex-specific distribution of anti-CXCR3 autoantibodies in the study sample

	Age (years)	No. of individuals	Mean (SD)	Median (IQR)	5th/95th percentile
Men	35–44	356	9.73 (12.8)	6.12 (4.32/8.44)	2.88/34.9
	45–54	670	10.3 (11.5)	6.52 (4.76/10.9)	3.09/30.9
	55–64	590	10.9 (14.5)	6.28 (4.33/10.3)	2.96/42.4
	65–74	565	11.0 (14.4)	6.62 (4.64/10.9)	2.94/32.3
Women	35–44	488	9.47 (10.7)	6.51 (4.58/9.90)	2.87/27.4
	45–54	484	10.1 (12.7)	6.36 (4.49/9.55)	3.12/28.9
	55–64	534	10.3 (13.6)	6.23 (4.37/9.53)	2.92/33.4
	65–74	508	11.2 (14.7)	6.69 (4.60/10.8)	2.76/39.7
Total	35–44	844	9.58 (11.6)	6.27 (4.43/9.24)	2.88/30.4
	45–54	1154	10.2 (12.0)	6.45 (4.62/10.3)	3.09/30.5
	55–64	1124	10.6 (14.0)	6.25 (4.34/10.0)	2.95/36.9
	65–74	1073	11.1 (14.5)	6.63 (4.61/10.8)	2.85/35.2

No., number; IQR, interquartile range; SD, standard deviation.

analysis sample are provided in *Table 2* and Supplementary data online, *Table S5*.

After exclusion of 3206 individuals with CVRF, CVD, and non-CVD associated with systemic inflammation, a reference sample comprising 989 individuals was defined (for derivation of the sample and its characteristics, see Supplementary data online, *Figure S4* and *Table S6*). The median concentration of anti-CXCR3 aAbs in the reference sample was 6.16 U/mL (IQR 4.3 U/mL; 9.38 U/mL).

The age- and sex-specific distribution of anti-CXCR3 aAb concentrations is displayed in Supplementary data online, *Table S7*. The mean time from blood sampling to quantification of anti-CXCR3 aAbs in the analysis sample was 12 ± 0.4 years.

Determinants of anti-CXCR3 autoantibody concentrations

When adjusting for sex, higher age was associated with marginally higher concentrations of log-transformed anti-CXCR3 aAbs [β-estimate (β) per 10-year increment 0.03 and corresponding 95% confidence interval (CI), 0.01; 0.05, $P = .004$], whereas sex itself did not influence concentrations. Other variables were evaluated as potential determinants in individual regression models while controlling for age and sex: higher body mass index, glycated haemoglobin (HbA_{1c}), and concentrations of triglycerides were among the continuous CV risk characteristics modestly associated with higher concentrations of anti-CXCR3 aAbs, while levels of HDL demonstrated an inverse relationship. Systolic and diastolic blood pressure showed no association with anti-CXCR3 aAbs, nor did C-reactive protein. Further analysis revealed eGFR as predictor of anti-CXCR3 aAb concentrations. Prevalent CVD, chronic obstructive pulmonary disease (COPD), and inflammatory disease were associated with higher concentrations of anti-CXCR3 aAbs (*Figure 1*; Supplementary data online, *Table S8*). Controlling for continuous CVRF, comorbidities, and medication, CVD [$\beta = 0.135$ (0.057, 0.213), $P < .001$] remained the strongest predictor of higher anti-CXCR3 aAb concentrations. Suggestive evidence for the relation between several medication classes and anti-CXCR3 aAbs was found, although not meeting the Bonferroni-corrected significance threshold (see Supplementary data online, *Figure S5*).

The observed relationship between CVD and anti-CXCR3 aAbs was further investigated in regression analyses in which CV end-organ damage was considered as dependent variable and anti-CXCR3 aAbs as independent variable (*Table 3*). Higher concentrations of anti-CXCR3 aAbs translated into higher intima–media thickness [$\beta_{\text{per SD increase in log(anti-CXCR3 aAbs)}} = 0.0039$ (0.00076, 0.007), $P = .015$] and LV mass [$\beta = 0.382$ (0.09, 0.67), $P = .01$]. Lending further evidence for an association with CVD, anti-CXCR3 aAbs also predicted NT-proBNP as circulating biomarker of haemodynamic stress [$\beta = 0.041$ (0.007, 0.0076), $P = .018$]. Controlling for renal function and traditional CVRF did not substantially alter these results. No association was observed for LV ejection fraction (EF) and E/E′ ratio, reflecting systolic and diastolic cardiac function. Concentrations of anti-CXCR3 aAbs displayed an association with the history of stroke but not myocardial infarction (see Supplementary data online, *Table S9*). Furthermore, a strong association of anti-CXCR3 aAbs with chronic kidney disease was found independent of traditional CVRF [odds ratio per SD log(anti-CXCR3 aAbs) = 1.23 (1.06, 1.43), $P = .006$, Supplementary data online, *Table S9*], along with a decrease in eGFR of −0.76 mL/min/1.73m^2 (−1.09, −0.42, $P < .001$) per SD increase in log(anti-CXCR3 aAbs) (*Table 3*).

Anti-CXCR3 autoantibodies and clinical outcome

The relevance of anti-CXCR3 aAbs for all-cause mortality was investigated over a median follow-up time of 13.7 years (IQR 13.2, 14.0) with 450 events. Cardiovascular endpoints were investigated in a 5-year follow-up period and comprised cardiac death ($n = 28$ events), myocardial infarction ($n = 46$ events), stroke ($n = 51$ events), major adverse cardiac events (MACE, comprising incident coronary artery disease, myocardial infarction, or cardiac death, $n = 142$ events), and HF ($n = 65$ events). Individuals above the 75th percentile of anti-CXCR3 aAb concentrations showed an elevated risk for all-cause death ($P = .001$), cardiac death ($P = .003$), and MACE ($P = .004$), as compared with those

Table 2 Clinical characteristics by quartiles of anti-CXCR3 autoantibodies

	1st quartile: anti-CXCR3 aAbs ≤ 4.50 U/mL ($n = 1047$)	2nd quartile: anti-CXCR3 aAbs > 4.50, ≤6.40 U/mL ($n = 1054$)	3rd quartile:anti-CXCR3 aAbs > 6.40, ≤10.1 U/mL ($n = 1031$)	4th quartile: anti-CXCR3 aAbs > 10.1 U/mL ($n = 1063$)	Overall analysis sample ($n = 4195$)
Anti-CXCR3-autoantibodies (U/mL)	3.63 (3.09/4.07)	5.38 (4.94/5.89)	7.76 (7.02/8.73)	15.9 (12.25/27.31)	6.4 (4.5/10.22)
Age (years)	55.2 ± 10.7	55.0 ± 10.9	55.2 ± 11.2	56.5 ± 11.0	55.5 ± 11.0
Female sex (%) (*n*)	48.5 (508)	47.0 (495)	49.9 (514)	46.8% (497)	48.0 (2014)
Traditional cardiovascular risk factors, % (*n*)					
Arterial hypertension	52.2 (547)	50.7 (534)	48.4 (499)	54.7 (581)	51.5 (2161)
Diabetes	8.7 (91)	9.0 (95)	9.0 (93)	11.7 (124)	9.6 (403)
Dyslipidaemia	34.2 (358)	35.9 (378)	36.1 (372)	34.9 (370)	35.3 (1478)
Family history of myocardial infarction or stroke	22.7 (238)	22.7 (239)	22.6 (233)	23.9 (254)	23.0 (964)
Obesity	23.2 (243)	23.1 (243)	24.4 (252)	26.8 (285)	24.4 (1023)
Smoking	20.6 (215)	19.3 (203)	18.8 (194)	18.4 (195)	19.3 (807)
Comorbidities, % (*n*)					
Allergic rhinitis	15.2 (159)	16.4 (173)	17.5 (180)	17.2 (182)	16.6 (694)
Asthma	1.4 (15)	2.3 (24)	2.1 (22)	3.2 (34)	2.3 (95)
Osteoarthritis	3.2 (33)	3.1 (33)	5.1 (53)	5.9 (63)	4.3 (182)
Atopic dermatitis	1.9 (20)	1.6 (17)	1.9 (20)	1.9 (20)	1.8 (77)
Cardiovascular disease	9.7 (101)	11.2 (117)	13.4 (137)	15.8 (168)	12.5 (523)
Atrial fibrillation	1.6 (17)	3.1 (32)	3.0 (31)	3.2 (34)	2.7 (114)
Coronary artery disease	4.1 (42)	4.1 (43)	4.9 (50)	4.9 (51)	4.5 (186)
Heart failure	4.4 (35)	3.3 (27)	4.2 (33)	7.9 (67)	5.0 (162)
History of myocardial infarction	2.8 (29)	2.6 (27)	3.1 (32)	4.3 (45)	3.2 (133)
History of stroke	1.1 (11)	1.1 (12)	2.3 (24)	2.6 (28)	1.8 (75)
Peripheral artery disease	3.2 (33)	3.6 (38)	4.2 (43)	4.5 (48)	3.9 (162)
Chronic kidney disease	2.5 (26)	3.5 (37)	2.9 (30)	5.5 (58)	3.6 (151)
Chronic liver disease	0.5 (5)	0.9 (9)	0.7 (7)	0.7 (7)	0.7 (28)
Chronic obstructive pulmonary disease	3.2 (33)	4.0 (42)	4.3 (44)	5.7 (61)	4.3 (180)
Clinical parameters					
Left ventricular ejection fraction (%)	63.9 ± 6.4	64.0 ± 6.6	63.9 ± 6.5	64.0 ± 7.2	64.0 ± 6.7
E/E′ ratio	7.05 (5.87/8.72)	6.97 (5.67/8.69)	6.95 (5.64/8.47)	6.85 (5.62/8.63)	6.97 (5.71/8.64)

Continued

Table 2 *Continued*

	1st quartile: anti-CXCR3 aAbs ≤ 4.50 U/mL ($n = 1047$)	2nd quartile: anti-CXCR3 aAbs > 4.50, ≤6.40 U/mL ($n = 1054$)	3rd quartile:anti-CXCR3 aAbs > 6.40, ≤10.1 U/mL ($n = 1031$)	4th quartile: anti-CXCR3 aAbs > 10.1 U/mL ($n = 1063$)	Overall analysis sample ($n = 4195$)
Left ventricular mass index ($g/m^{2.7}$)	35.4 (30.0/42.3)	36.1 (30.7/42.4)	35.9 (30.4/42.6)	36.9 (30.8/44.1)	36.0 (30.5/42.9)
Carotid intima–media thickness (mm)	0.65 ± 0.13	0.65 ± 0.12	0.65 ± 0.13	0.67 ± 0.13	0.65 ± 0.13
Laboratory parameters					
C-reactive protein (mg/L)	1.60 (1.00/3.10)	1.60 (0.50/2.90)	1.70 (0.50/3.10)	1.80 (0.50/3.40)	1.60 (0.50/3.20)
Estimated glomerular filtration rate (mL/min/1.73 m^2)	86.75 ± 13.19	85.78 ± 13.69	85.04 ± 12.98	83.50 ± 14.72	85.26 ± 13.71
NT-proBNP (pg/mL)	57.54 (27.19/115.93)	58.83 (27.21/114.65)	62.33 (29.46/123.23)	66.65 (29.07/144.98)	61.10 (27.84/123.37)

Sample characteristics at the time of baseline examination according to quartiles of concentrations of anti-CXCR3 autoantibodies are provided. Data are presented as mean (±standard deviation), median (interquartile range), or relative and absolute frequencies. Chronic kidney disease was defined as estimated glomerular filtration rate < 60 mL/min/1.73 m^2.
aAbs, autoantibodies; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

below (*Figure 2*; Supplementary data online, *Figures S6 and S7*). In detail, Cox regression analysis revealed that individuals with concentration above the 75th percentile of anti-CXCR3 aAbs displayed a 25% increased relative risk for death from any cause under adjustment for age and sex [hazard ratio (HR) 1.25 (1.02, 1.52), $P = .029$]. The relationship of all-cause mortality with anti-CXCR3 aAbs seemed to be, at least partly, driven by a 2.4-fold increased risk for cardiac death [HR 2.4 (1.17, 4.92), $P = .017$]. In line with this finding, there was a trend towards a higher risk for MACE [HR 1.42 (1.0, 2.0), $P = .05$] under adjustment for age and sex. No relevant associations were found for myocardial infarction or stroke when analysed separately, given the low number of events. Further analysis identified incidental HF as a potential driver of increased cardiac mortality [$HR_{\text{per SD increase in log(anti-CXCR3 aAbs)}}$ 1.26 (1.02, 1.56), $P = .03$, Supplementary data online, *Figure S8*]. The results of the time-to-event analysis are displayed for log-transformed concentrations of anti-CXCR3 aAbs as independent variable in Supplementary data online, *Figure S9*. Adjustment for renal function and risk profile did not alter the HR substantially (*Table 4*; Supplementary data online, *Table S10*). Results were not relevantly altered after correcting for C-reactive protein or time from blood draw until analysis (see Supplementary data online, *Tables S11 and S12*).

Molecular determinants of anti-CXCR3 autoantibodies

Supervised machine learning identified 36 proteins as determinants of anti-CXCR3 aAbs, which were ranked according to variable importance as shown in *Figure 3A*. Out of the selected proteins, 12 were negatively, and 23 were positively associated. The three proteins of highest variable importance were tumour necrosis factor receptor superfamily member 9 (TNFRSF9 or CD137), a costimulatory receptor that is expressed by activated T cells, in particular by regulatory T cells (Treg), signalling lymphocytic activation molecule (SLAMF1), which is involved in the regulation of the Th1/Th2 balance, and the translational repressor eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), a substrate of the mechanistic target of rapamycin (mTOR) signalling pathway. Notably, albeit to a lesser extent, also the CXCR3 ligand CXCL11 and the Th1 cytokine interleukin (IL)-18 and its receptor showed a positive association with levels of anti-CXCR3 aAbs. The three most important proteins with inverse relation to anti-CXCR3 aAbs were Delta and Notch-like epidermal growth factor-related receptor (DNER), IL-20 receptor subunit alpha (IL-20RA), and fibroblast growth factor 21 (FGF21). Full results of the analysis are listed in Supplementary data online, *Table S13*. Weak but consistently positive correlations of anti-CXCR3 aAbs with the relative expression of CXCL9, CXCL10, and CXCL11 were identified (see Supplementary data online, *Figure S10*).

A protein–protein interaction network was generated for the selected proteins, highlighting the top cluster cytokine–cytokine receptor interaction (*Figure 3B*). Finally, pathway and process enrichment analysis revealed cytokine–cytokine receptor interactions, and pathways related to leucocyte activation and chemotaxis reflecting cellular inflammation as strongest related biological clusters (*Figure 3C*).

Immunization against CXCR3 accelerates atherosclerosis in ApoE knockout mice

During a 90-day course of feeding with western-type diet (*Figure 4A*), mice immunized against CXCR3 displayed increasing concentrations of anti-CXCR3 aAbs which were detectable from Day 17 after immunization, reaching a maximum at Day 30 and declining hereafter

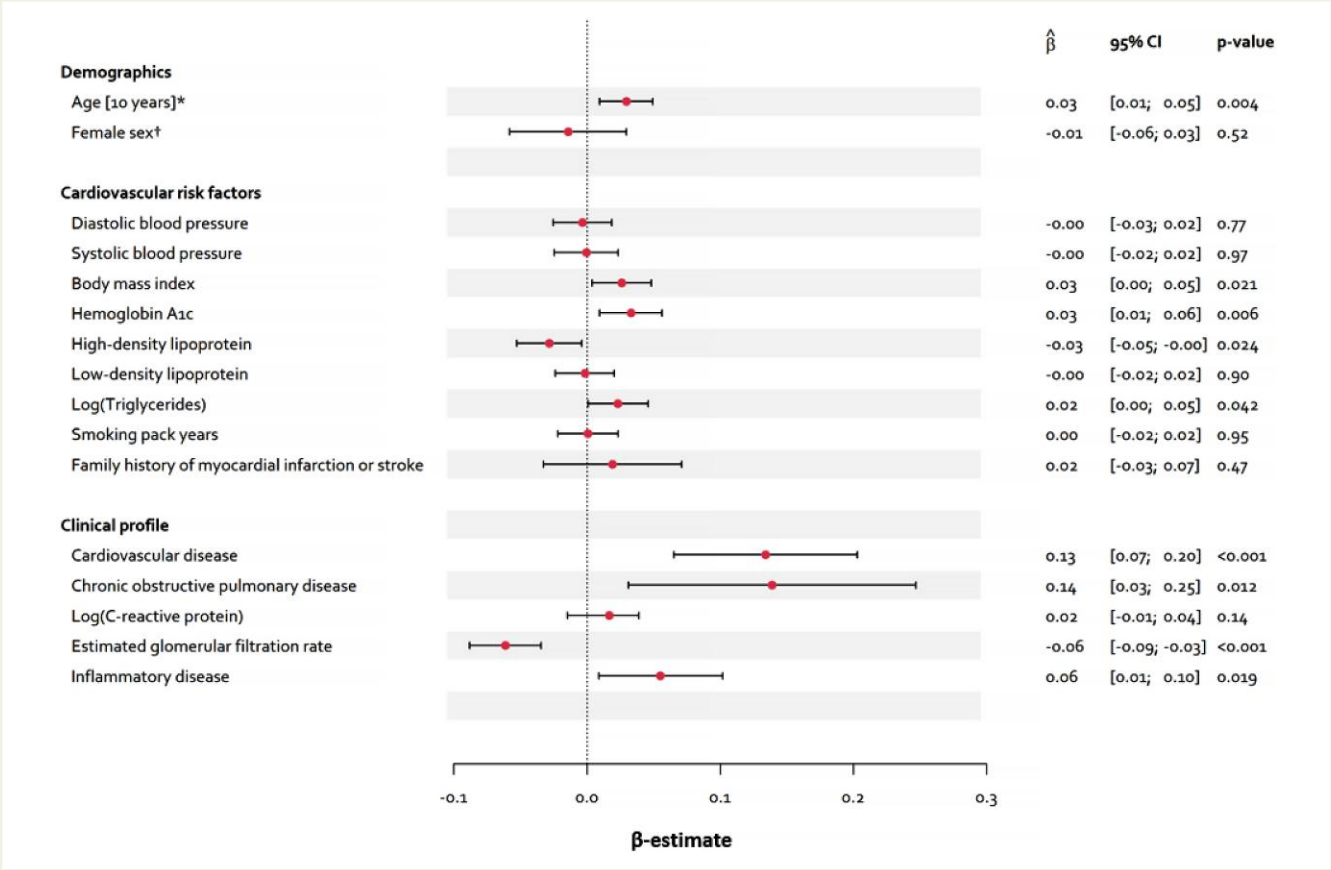


Figure 1 Clinical determinants of anti-CXCR3 autoantibodies. *Adjustment for sex. †Adjustment for age. Presented are estimates of multivariable regression analyses with anti-CXCR3 autoantibodies as dependent variable and continuous or categorical clinical traits as independent variable, expressed by β-estimate and corresponding 95% confidence interval. Continuous traits were analysed per standard deviation. Cardiovascular disease comprised prevalent atrial fibrillation, coronary artery disease, heart failure, history of myocardial infarction and/or stroke, and peripheral artery disease. Inflammatory disease comprised osteoarthritis, asthma, allergic rhinitis, upper respiratory tract infection < 14 days prior baseline examination and C-reactive protein < 10 mg/L at baseline, and atopic dermatitis.

(*Figure 4B*). Such induction of anti-CXCR3 aAbs was not observed in mice of the control group. Notably, mice immunized against CXCR3 showed a significantly greater atherosclerotic burden in the aorta and aortic root compared to controls ($P < .05$), as quantified by histological analysis using Oil Red O (ORO) staining after sacrifice at Day 90 (*Figure 4C* and *D*). In line with these results, total fat content within the plaque was greater in immunized mice (*Figure 4D*), whereas no significant differences in plaque composition (i.e. plaque content, plaque area, and collagen content) were found between immunized mice and controls (see Supplementary data online, *Figure S11*). Of note, immunized mice displayed significantly lower total cholesterol and triglyceride concentrations compared to controls (see Supplementary data online, *Figure S12*). Immunized mice also displayed a higher granulocyte-to-lymphocyte ratio (see Supplementary data online, *Figure S13*), along with increased pulmonary CXCR3 expression and lung infiltration as compared to controls, indicating a systemic effect of CXCR3 immunization (see Supplementary data online, *Figure S14*).

In the passive immunization model (*Figure 4E*), concentrations of anti-CXCR3 aAbs positively correlated with the degree of aortic atherosclerotic burden (*Figure 4F*).

Lastly, CXCR3 gene expression analysis of two independent human data sets demonstrated an increased CXCR3 expression in human unstable and advanced atherosclerotic plaques of carotid arteries (*Figure 4G*).

Discussion

The present study demonstrated that aAbs directed against CXCR3 can be determined and quantified in a large sample from the general population free of overt autoimmune disease. It provided first evidence that anti-CXCR3 aAbs are associated with the presence of CVRF, changes in CV structure and function, as well as with the presence of CV, pulmonary, renal, and inflammatory disease. Higher levels of anti-CXCR3 aAbs translated to increased overall mortality over the long-term follow-up to 14 years, particularly to an ~2.5-fold elevated risk for cardiac mortality, along with a higher risk of new-onset HF and MACE (*Structured Graphical Abstract*). Increased susceptibility to atherosclerosis through the presence of anti-CXCR3 aAbs was experimentally confirmed, as active immunization against CXCR3 and passive transfer of anti-CXCR3 aAbs resulted in a higher aortic plaque burden in ApoE KO mice. In line with this, RNA sequencing revealed higher CXCR3 gene expression in human unstable or advanced atherosclerotic plaques compared with stable or early lesions. Thus, anti-CXCR3 aAbs could represent both a biomarker and mediator of

Table 3 Relationship of anti-CXCR3 autoantibodies with end-organ damage

Dependent variables	Single regression models adjusted for age and sex		Additional adjustment for renal function and traditional CVRF	
	β-estimate per SD increase in log(anti-CXCR3 aAbs) (95% CI)	*P*-value	β-estimate per SD increase in log(anti-CXCR3 aAbs) (95% CI)	*P*-value
Cardiac and vascular measures of end-organ damage				
Carotid intima–media thickness (mm)	0.00388 (0.000761, 0.007)	.015	0.0038 (0.00073, 0.00691)	.015
E/E′ ratio	−0.0599 (−0.131, 0.0113)	.099	−0.065 (−0.134, 0.00434)	.066
Left ventricular ejection fraction (%)	0.0753 (−0.125, 0.275)	.46	0.0947 (−0.106, 0.295)	.35
Left ventricular mass ($g/m^{2.7}$)	0.382 (0.0904, 0.673)	.01	0.338 (0.0682, 0.608)	.014
Circulating biomarkers of end-organ damage				
log(NT-proBNP)	0.0412 (0.00695, 0.0755)	.018	0.0346 (0.0002, 0.0689)	.049
Estimated glomerular filtration rate ($mL/min/1.73\ m^2$)	−0.763 (−1.1, −0.429)	<.001	−0.756 (−1.09, −0.422)	<.001

Presented are estimates of multivariable regression analyses with continuous measures of end-organ damage as dependent variable, and anti-CXCR3 autoantibodies as independent variable, expressed by β-estimate and corresponding 95% confidence interval (CI). Each line represents a separate model. Renal function was expressed by estimated glomerular filtration rate. Traditional cardiovascular risk factors comprised arterial hypertension, diabetes mellitus, dyslipidaemia, family history of myocardial infarction or stroke, obesity, and smoking.
aAbs, autoantibodies; NT-proBNP, N-terminal pro-B-type natriuretic peptide; CVRF, cardiovascular risk factors.

atherosclerosis. The observed molecular associations suggest that anti-CXCR3 aAbs reflect chronic Th1-driven cellular inflammation which can be considered an inflammatory CVRF independent of general surrogates of systemic inflammation such as C-reactive protein.

The observed link between anti-CXCR3 aAbs and CV outcome in the general population is a novel finding, as previous analyses mainly focused on the role of this aAb in autoimmune or infectious diseases. In individuals with systemic sclerosis, anti-CXCR3 aAbs were identified for the first time to reflect pulmonary outcome (i.e. interstitial lung disease) and correlated with the prevalence of obstructive pulmonary disease, i.e. COPD, in the present, population-based study. However, in patients with systemic sclerosis, anti-CXCR3 aAbs did not correlate with LVEF or estimated systolic pulmonary arterial pressure.[11] Recently, Cabral-Marques *et al.*[13] demonstrated higher concentrations of anti-GPCR aAbs in patients with COVID-19 compared to healthy controls. In this study, anti-CXCR3 aAbs, among other GPCR aAbs, were the strongest predictors of disease severity and mortality, illustrating that autoimmunity against CXCR3 might be triggered by systemic inflammation. There is evidence for potential agonistic receptor activation by anti-CXCR3 aAbs, as IgG fractions from systemic sclerosis patients with high concentrations of anti-CXCR3 aAbs induced directional migration of $CD4^+$ T cells, which was attenuated by addition of CXCR3-specific blockers.[11] In CXCR3-immunized mice, we also found increased pulmonary CXCR3 expression and inflammation, indicating a systemic effect of CXCR3 immunization. These data correspond to the previous role of anti-CXCR3 aAbs as biomarker for enhanced inflammation in autoimmune and non-autoimmune (viral-induced) diseases.[11,13] In addition, increased expression of CXCR3 in human carotid plaques could illustrate the influx of $CXCR3^+$ monocytes and T cells into plaques, a hypothesis which merits further study. Interestingly, a decrease in murine total cholesterol and triglycerides was observed when lipid plasma levels between immunized mice and controls were compared, supporting cholesterol-independent effects of anti-CXCR3 aAbs. Corresponding to the 'lipid paradox' in inflammatory disease, where reductions of cholesterol and triglycerides have been described even in the presence of a markedly elevated CV risk[30], the higher fat content in the plaques of immunized mice could reflect increased uptake in macrophages or by fat adhering to activated endothelial cells and adhesion molecules. Passive transfer of anti-CXCR3 aAbs in ApoE KO mice and the identified correlations between the anti-CXCR3 aAb levels and plaque burden further support a role for anti-CXCR3 aAbs in atherosclerosis progression. The present work underscores previous data, where the CXCR3–CXCL10 axis played an important role in Th1 differentiation, migration, and maintenance of prolonged Th1 responses, thus driving development and progression of atherosclerotic plaques.[31] In line with this, antagonizing CXCR3 in $LDLr^{(-/-)}$ mice mitigated aortic plaque formation, and genetic KO of CXCR3 reduced atherosclerosis burden in ApoE KO mice, which was associated with a lower prevalence of $CD4^+$ T cells in atherosclerotic lesions.[32] The mechanistic significance of Th1-driven immunity in CVD is also supported by the proteomic signature of anti-CXCR3 aAbs identified in the present study, which is suggestive of an ongoing Th1-driven inflammatory response. Notably, the most prominent determinant of anti-CXCR3 aAbs, CD137, is predominately expressed by activated Treg,[33] which are considered to play an important role in controlling the progression of atherosclerosis[32] and to contribute to tissue repair and cardiac remodelling after myocardial infarction.[34] Atherosclerosis features autoimmune properties even in the absence of autoimmune disease, and several antibodies have been implicated in increased CV risk.[35,36] Thus, potentially agonistic activation of CXCR3 by anti-CXCR3 aAbs could promote accelerated atherosclerosis by mediating the recruitment of Th1 and other proinflammatory cells into atherosclerotic lesions. This is consistent with our findings, in which anti-CXCR3 aAbs were associated with CVD, intima–media thickness and history of stroke. However, the association with incident MACE was only moderate, which could be

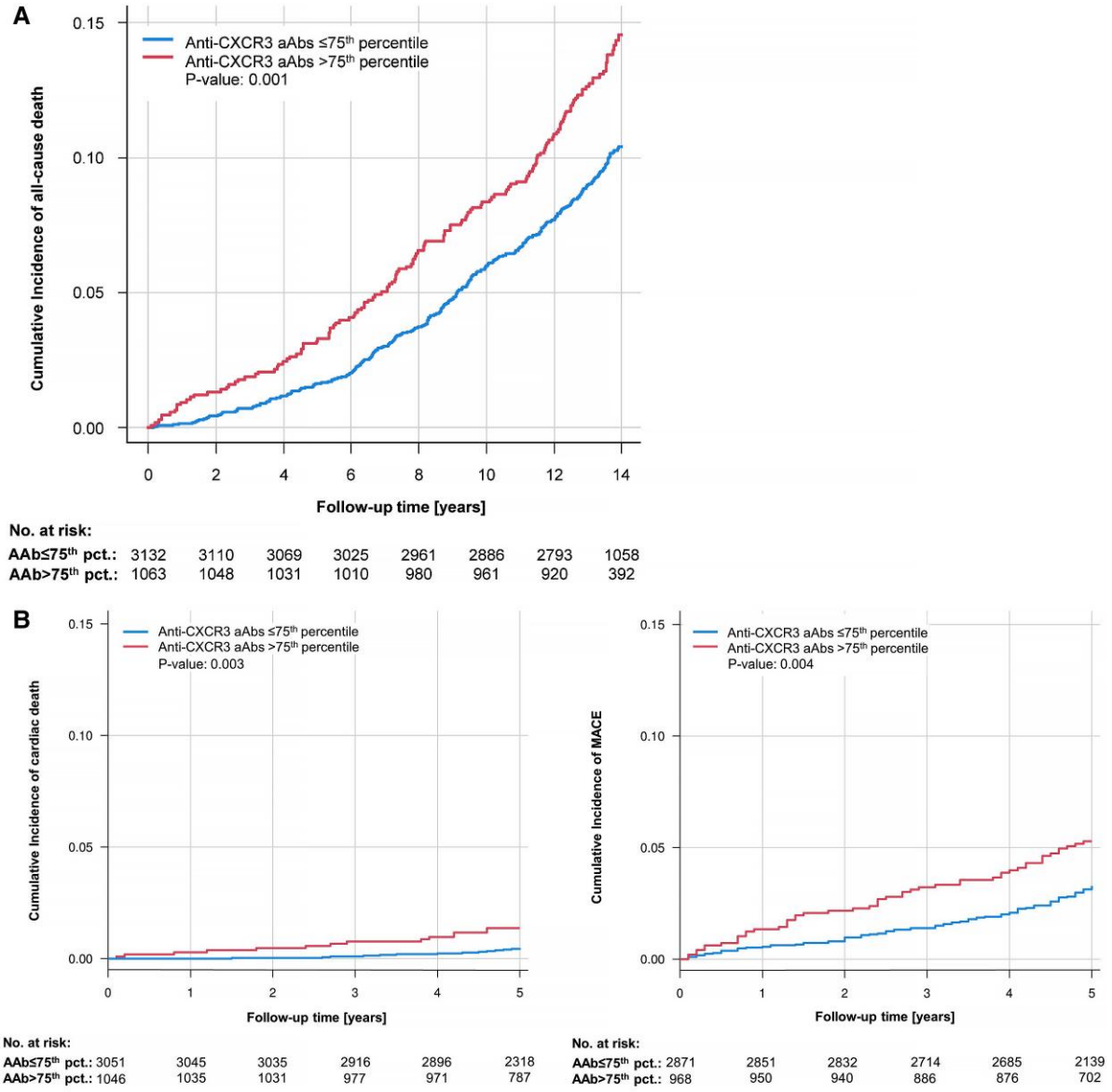


Figure 2 Association between concentrations of anti-CXCR3 autoantibodies and outcome. (*A*) All-cause mortality. Cumulative incidence of all-cause death during a mean follow-up of 12.9 years by concentrations of anti-CXCR3 autoantibodies above and below the 75th percentile. Log-rank test was used to assess inter-group differences. No., number; pct, percentile. (*B*) Cardiac death and major adverse cardiac events. Left bar: cumulative incidence of cardiac death during a mean follow-up of 4.85 years by concentrations of anti-CXCR3 autoantibodies above and below the 75th percentile. Gray's test was used to assess inter-group differences. No., number; pct, percentile. Right bar: cumulative incidence of major adverse cardiac events during a mean follow-up of 4.79 years by concentrations of anti-CXCR3 autoantibodies above and below the 75th percentile. Gray's test was used to assess inter-group differences. MACE, major adverse cardiac events (comprising incident coronary artery disease, myocardial infarction, or cardiac death); No., number; pct, percentile.

explained by the low event number after 5 years in the population sample but also by the biased signalling of CXCR3 by different ligands (i.e. CXCL10 vs. CXCL11) that promote inflammation on the one hand and inhibit it on the other.[16] Apart from $CD4^+$ T cells, CXCR3 is also expressed by vascular smooth muscle cells, which proliferate when CXCR3 is activated by CXCL10.[37] Thus, also direct effects of anti-CXCR3 aAbs on vascular cells driving atherosclerotic processes and increasing intima–media thickness are conceivable.

The high seroprevalence of anti-CXCR3 aAbs identified in the present study corroborates the hypothesis of natural anti-GPCR aAbs forming the 'antibodiom' that has recently been proposed.[38] It defines physiological autoimmunity against GPCRs that maintains homeostasis and that is ultimately dysregulated by or contributes to chronic systemic inflammation in the course of autoimmune and CVDs.[12,38] Previously, our group identified signatures of aAbs directed against GPCR, such as CXCR3, in both healthy donors and diseased individuals; remarkably, the anti-GPCR-specific aAb signature varied by sex, age, and concomitant autoimmune disease but was not entirely dependent on the presence of autoimmune disease.[12] Determinants of anti-CXCR3 aAbs such as high body mass index, HbA_{1c}, and triglyceride

Table 4 Relationship of anti-CXCR3 autoantibodies with clinical outcome

	Adjustment for age and sex		Additional adjustment for renal function and traditional cardiovascular risk factors	
	Hazard ratio anti-CXCR3 aAbs > 75th percentile (95% CI)	*P*-value	Hazard ratio anti-CXCR3 aAbs > 75th percentile (95% CI)	*P*-value
All-cause death	1.25 (1.02; 1.52)	.029	1.21 (0.99; 1.48)	.063
Cardiac death	2.51 (1.21; 5.22)	.014	2.4 (1.17; 4.92)	.017
Heart failure	1.58 (0.95; 2.62)	.079	1.52 (0.91; 2.52)	.11
Major adverse cardiac events	1.42 (1.0; 2.0)	.05	1.38 (0.97; 1.98)	.078
Myocardial infarction	1.31 (0.70; 2.45)	.40	1.35 (0.71; 2.56)	.35
Stroke	0.89 (0.47; 1.67)	.70	0.88 (0.47; 1.65)	.69

Cox proportional hazard models with competing risk analysis for death and adjustment for potential confounders or mediators were analysed. Results are presented as hazard ratio and corresponding 95% confidence interval (CI). Major adverse cardiac events (MACE) comprised incident coronary artery disease, myocardial infarction, or cardiac death. Renal function was expressed by estimated glomerular filtration rate. Traditional cardiovascular risk factors comprised arterial hypertension, diabetes mellitus, dyslipidaemia, family history of myocardial infarction or stroke, obesity, and smoking.
aAbs, autoantibodies; CVRF, cardiovascular risk factors.

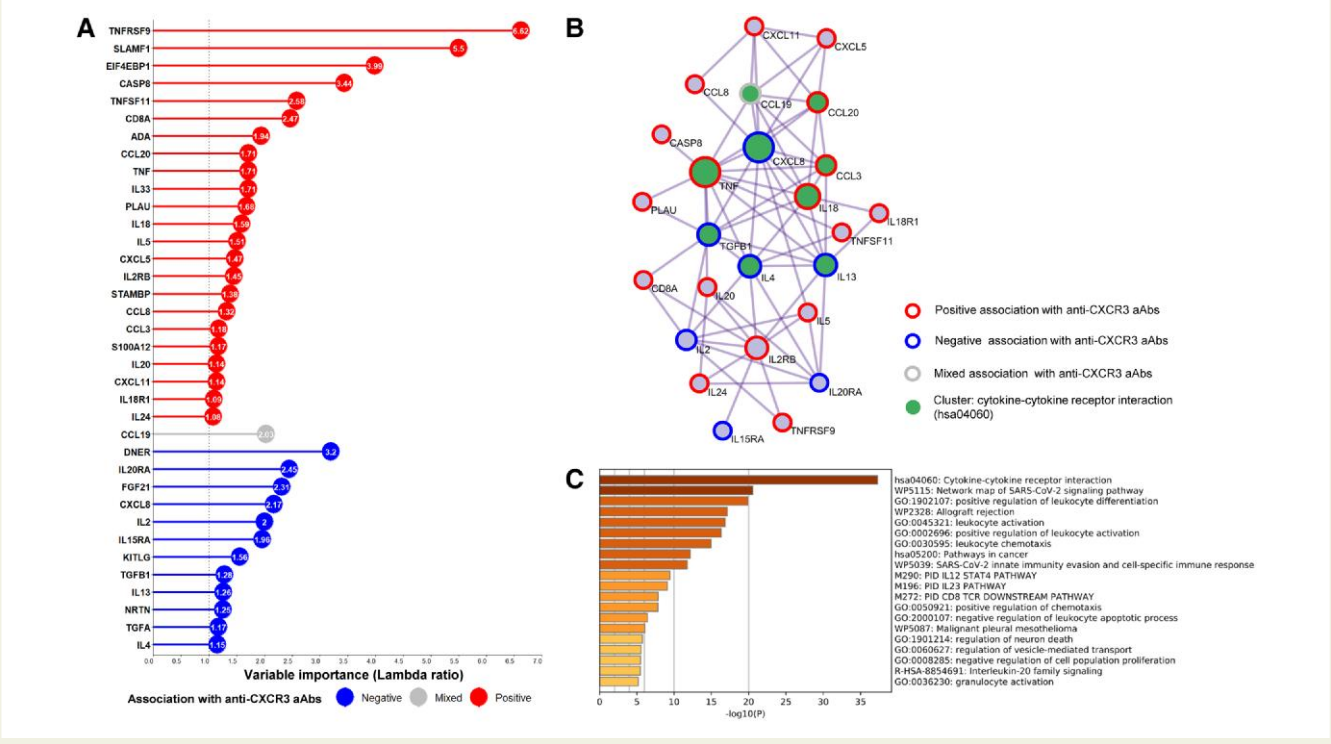


Figure 3 Molecular determinants of anti-CXCR3 autoantibodies identified by a machine learning approach. (*A*) Importance and direction of association of proteins selected by regularized regression. Proteins selected by least absolute shrinkage and selection operator graded according to variable importance (i.e. lambda ratio) and direction of association. Fractional polynomials were applied to account for non-linear relationships. Mixed association describes that transformation factors and direction do not indicate a negative nor positive association. The model yielded a coefficient of variation (R^2) of 0.053 (10-fold cross-validated R^2: 0.025). (*B*) Protein–protein interaction network comprising selected proteins. Depicted is the protein–protein interaction network based on protein–protein interaction enrichment analysis conducted with Metascape.[28] STRING, BioGrid, OmniPath, and InWeb_IM constituted the databases used for analysis. Molecular Complex Detection algorithm[28] was applied and identified cytokine–cytokine interaction (GO term hsa04060) as functional cluster. Finally, the network was modeled with Cytoscape.[29] Proteins related to this cluster are marked in green. Dot size reflects number of protein–protein interactions, and circle color identifies direction of association. Date of Metascape query: 28 April 2022. (*C*) Pathway and process enrichment analysis. Presented are the results of pathway and process enrichment analysis conducted with Metascape[28] using KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, Cell Type Signatures, CORUM, TRRUST, DisGeNET, PaGenBase, Transcription Factor Targets, WikiPathways, PANTHER Pathway, and COVID as ontology sources. Input variables were the proteins selected by regularized regression. *P*-values were calculated based on the accumulative hypergeometric distribution.[28] Date of Metascape query: 28 April 2022. A full legend of protein names is provided in Supplemental *Table S4*. aAbs, autoantibodies.

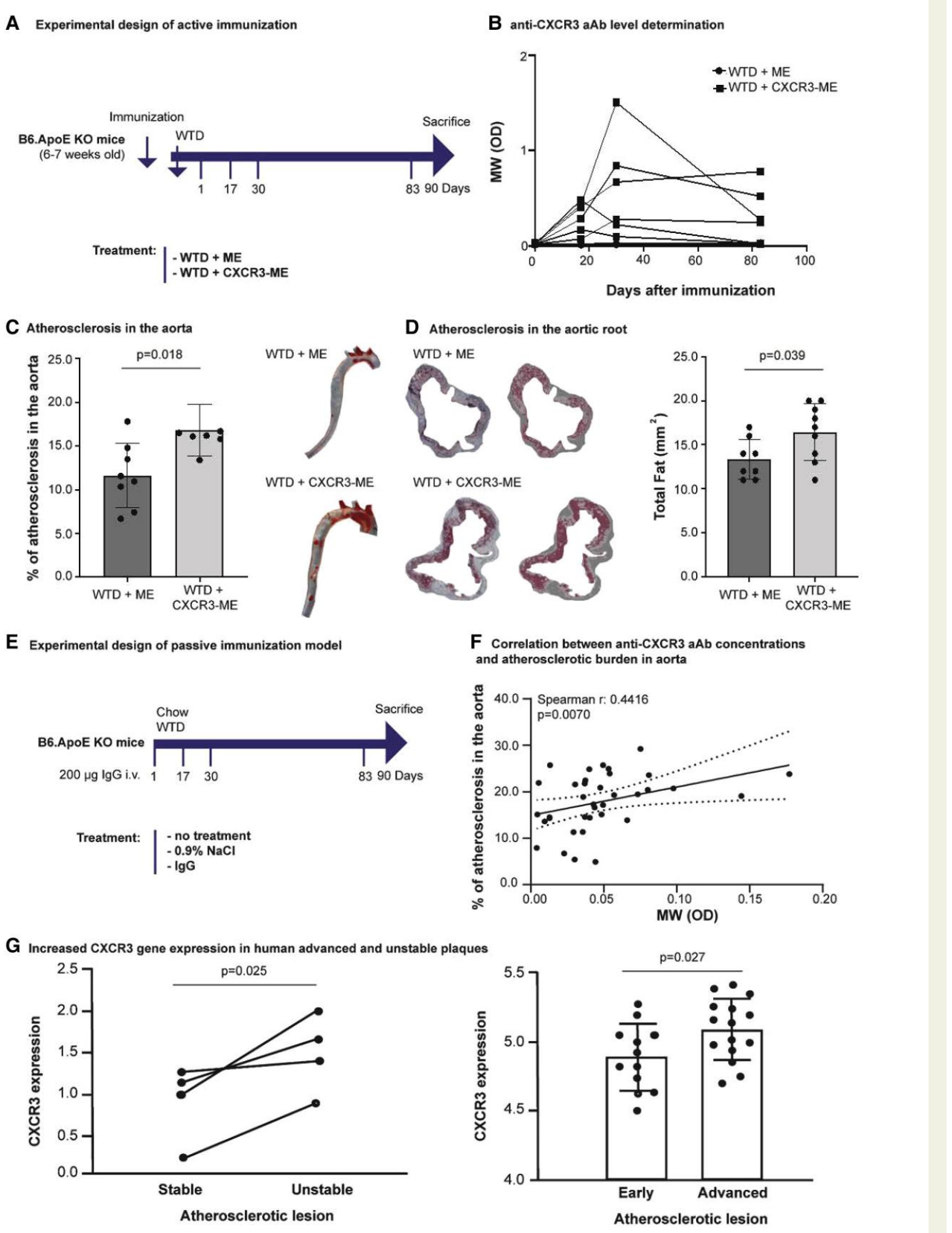


Figure 4 Active and passive immunization of ApoE knockout mice against CXCR3 and quantification of atherosclerosis burden. ApoE$^{-/-}$ C57BL6 mice were either immunized against CXCR3 by subcutaneous injection of 200 µg CXCR3 membrane extract with complete Freund's adjuvant followed by a second injection of 200 µg CXCR3-membrane extract with incomplete Freund's adjuvant at 9–10 weeks of age (treatment group, $n = 8$) or

Continued

Figure 4 Continued
received 200 µg membrane extract/complete Freund's adjuvant followed by 200 µg membrane extract/incomplete Freund's adjuvant (control group, $n = 8$). Mice were fed with western-type diet for 90 days until organ harvesting (*A*). Anti-CXCR3 autoantibodies were quantified on four time points (d1, d17, d30, and d83; *B*) from murine serum with a commercially available, murine-specific sandwich enzyme-linked immunosorbent assay; results of the measurements are presented as optical density. Aortas and hearts were removed and fixed in 4% paraformaldehyde solution for histological analysis. After cleaning and removal of the adventitia, whole aortas were stained using Oil Red O to detect atherosclerotic deposits (*C*). Lesions were quantified in the aortic root and entire aorta. The ratio of Oil Red O-positive lesions in each animal was defined as the percentage of the lesion area normalized to the total area of the aorta (*D*). Groups were compared using unpaired Student's *t*-test (two-sided $P < .05$). ApoE$^{-/-}$ C57BL6 mice at the age of 6–7 weeks were randomly divided into three groups, namely mice injected with 200 µg IgG ($n = 17$) derived from human donors with different anti-CXCR3 autoantibody levels and cardiovascular risk profiles, control mice injected with NaCl 0.9% ($n = 8$) at Days 1, 17, 30, and 83, and control mice that were not injected ($n = 14$). The mice that were treated with IgG and NaCl 0.9% were fed with western-type diet for 90 days, whereas the mice that were not treated were fed with chow diet. At Day 90, mice were sacrificed (*E*). Anti-CXCR3 autoantibodies were quantified at d90 from murine sera with a commercially available, murine-specific sandwich enzyme-linked immunosorbent assay; results of the measurements are presented as optical density. Atherosclerosis in the aorta was analysed as described for (*C*) and (*D*). Correlation between anti-CXCR3 autoantibodies and atherosclerosis in the aorta was analysed using Spearman correlation analysis (*F*). CXCR3 gene expression in human arteriosclerotic plaques in the carotid arteries was analysed in stable and unstable plaques (GEO accession number GSE120521) as well as in early and advanced arteriosclerotic lesions (GSE28829). Groups were compared using paired Student's *t*-test for stable vs. unstable plaques and unpaired Student's *t*-test for early vs. advanced plaques (two-sided $P < .05$) (*G*). aAb, autoantibody; ApoE apolipoprotein E; IgG, immunoglobulin G; KO, knockout; ME, membrane extract; MW, molecular weight; NaCl, sodium chloride; OD, optical densitiy; WTD, western type diet.

concentrations suggest that lifestyle could affect the levels of anti-CXCR3 aAbs.

Autoantibodies directed against cardiac structural antigens (e.g. myosin or troponin) and against GPCRs such as β_1-AR have been identified in healthy control subjects with a markedly lower prevalence than in patients with HF, particularly DCM.[8] This is complemented by experimental evidence in rats, where immunization against the second extracellular β_1-AR loop led to the development of a progressive DCM phenotype.[39] In a study analysing myocardial tissue samples obtained at the time of cardiac transplantation or LV assist device implantation, anti-cardiac aAbs predicted disease onset within 5 years in healthy relatives of DCM patients[40] and were present in >70% of patients with end-stage HF regardless of aetiology.[41] Notably, the presence of activated complement components was detected in areas where aAbs were found, suggesting a potential role of cardiac autoimmunity as driver of progressive HF.[41] Besides IgG, IgM antibodies have been implicated in the development and progression of atherosclerosis.[42]

The present study demonstrated that anti-CXCR3 aAbs predicted both HF and cardiac death. This may suggest a pathophysiology at least partially independent of coronary artery disease, because the association was not primarily driven by events involving myocardial infarction. Indeed, higher concentrations of anti-CXCR3 aAbs were associated with greater LV mass and higher concentrations of NT-proBNP. In the population-based Cardiovascular Health Study,[43] LV mass predicted the occurrence of HF independently of prior myocardial infarction. Here, combined phenotyping with NT-proBNP and LV mass provided a 'malignant LV phenotype' characterized by both increased LV mass and increased concentrations of NT-proBNP, portending an adverse CV prognosis.[44,45]

Preclinical studies suggested a possible role for CXCR3 in the transition from LV hypertrophy to overt HF, as increased levels of the CXCR3 ligand CXCL10 and an abundance of CD4$^+$ T cells were found in ventricular tissue isolated from a mouse model of pressure overload.[18] Similarly, CXCR3$^+$ Th1 cells have been shown to invade the heart in humans and mice under pressure overload conditions. Genetic deletion of CXCR3 reduced Th1 infiltration and prevented maladaptive cardiac remodelling.[46] Depletion of B cells in mice with non-ischaemic HF improved LV hypertrophy, preserved EF, and reduced expression of proinflammatory cytokines,[47] strengthening the evidence for aAb-driven mechanisms in HF. The present study confirms these complex relationships in humans: anti-CXCR3 aAbs were significantly related not only to LV mass but also to NT-proBNP, whereas no association with cardiac systolic and diastolic function nor blood pressure or the presence of hypertension was found. CXCR3-mediated recruitment of Th1 cells and Treg also regulated the extent of renal inflammation in a broad spectrum of renal diseases,[48,49] which may be reflected by renal function as an important determinant of anti-CXCR3 aAbs.

Strengths and limitations

This study has several strengths and limitations: a large and deeply phenotyped population-based cohort allowed careful selection of the analysis sample, excluding potential confounders such as autoimmune disease, history of cancer, or immunomodulatory drugs. Relations of aAb concentrations with the clinical profile, the extent of subclinical and clinical disease, and incident events could be comprehensively demonstrated. The use of animal data in a translational approach further strengthens the data, although CXCR3 ApoE double KO mice were not available and no sex differences were investigated for the present analysis. The protein signature identified with regularized regression focusing on systemic inflammation showed interesting correlations, although the results should be considered exploratory and no clear evidence for a causal role of autoimmunity against CXCR3 in CVD can be derived from these observations. The analysis with respect to incident stroke and myocardial infarction was limited by the low frequency of respective events.

Future directions

These results support the hypothesis that autoimmunity to CXCR3 may be an important biological regulator and contributes to CVD and other diseases beyond traditional CVRF.

Conclusions

In a large population-representative sample of individuals who had no autoimmune disease and no recent history of cancer, the universal

presence of aAbs against CXCR3 was demonstrated. Anti-CXCR3 aAbs were associated with the presence of CVRF, changes in CV structure, and presence of CV, pulmonary, renal, and inflammatory disease. Furthermore, higher levels of anti-CXCR3 aAbs resulted in increased all-cause mortality and were associated with a higher risk of cardiac death, HF, and MACE. In an experimental validation study, immunization against CXCR3 as well as passive transfer of anti-CXCR3 aAbs accelerated murine atherosclerosis.

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Supplementary data

Supplementary data are available at *European Heart Journal* online.

Declarations

Disclosure of Interest

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Data Availability

The R-code used for the analyses is available upon request. Based on the written consent of the study participants, the data will only be made available locally within the framework of the access procedure specified in the rules of procedure of the research consortium. Interested researchers should direct their enquiries to the Gutenberg Health Study Steering Committee via the coordinating principal investigator (contact via info@ghs-mainz.de).

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Ethical Approval

The Gutenberg Health Study was approved by the local ethics committee [Medical Association Rhine-Hesse, Germany, reference number 837.020.07(5555)] and data safety commissioner before study initiation. All procedures were performed in accordance with the principles of the Declaration of Helsinki as well as the tenets of Good Clinical and Epidemiological Practice. Participants provided written informed consent before study enrolment. All animal experiments were approved by the German animal studies committee of Schleswig-Holstein, and animals were held and handled according to institutional and federal guidelines (animal licence AZ 103-8/16).

Pre-registered Clinical Trial Number

None supplied.

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