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Brief report: A regulatory variant in *CCR6* is associated with anti-topoisomerase positive systemic sclerosis susceptibility

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ABSTRACT

Introduction. Systemic sclerosis (SSc) is a rare autoimmune disease (AID) with a complex genetic etiology. Evidence for a shared pathogenesis across AIDs is given by the well-known pleiotropism of autoimmune genes. Polymorphisms of the *CCR6* gene, encoding chemokine receptor 6, a surface marker for Th17 cells, were reproducibly found to be associated with Rheumatoid Arthritis (RA) susceptibility, and the causal variant was recently identified.

Objective. To investigate whether *CCR6* polymorphisms are associated with SSc.

Methods. Twelve Tag SNPs of *CCR6*, including rs3093023 for which convincing association was reported with RA, were genotyped in a total of 2,411 SSc patients and 7,084 healthy individuals from three European populations (France, Italy, Germany). Meta-analyses were performed to assess whether an association exists between *CCR6* polymorphisms and SSc or its main subtypes. Direct sequencing was performed to detect the functional dinucleotide found in RA.

Results. The combined analyses revealed an association between the rs10946216 SNP and SSc susceptibility: P_{adj}=0.026, OR=1.13 (95%CI 1.05-1.21). The rs3093023 A and rs10946216 T alleles were in high linkage disequilibrium (LD) and were both found to confer susceptibility to the anti-topoisomerase-positive SSc subset: $P_{adj}=1.5x10^{-3}$, OR=1.27 (95%CI 1.13-1.42), and $P_{adj}=9.0x10^{-5}$, OR=1.32 (95%CI 1.17-1.48), respectively. Direct sequencing in 78 individuals supports the hypothesis that the regulatory dinucleotide could be the causal variant.

Conclusions. Our study establishes *CCR6* as a new susceptibility factor for anti-topoisomerase-positive SSc patients, in a European Caucasian population, confirming the sharing of autoimmune risk alleles by SSc and RA. It also suggests a potential role of the IL-17 pathway in SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a chronic systemic disease with a complex pathogenesis characterized by early vascular alterations and activation of the immune system with autoimmune features preceding the deposition of extracellular matrix leading to systemic fibrosis. Although it is frequently characterized as an autoimmune disease (AID), the mechanisms underlying the early inflammatory phase, involving both T and B cells, remain poorly understood.

In recent years, numerous genetic factors underlying disease susceptibility to SSc have been identified, mainly through association studies using candidate gene approaches and a few genomewide association studies (GWAS) (1, 2). The vast majority of these susceptibility loci belong to pathways leading to auto-immune responses or inflammation and are involved in antigen processing (*MHC*), innate immunity (*IRF5*), T-cell differentiation and/or activation (*STAT4*, *TNFSF4, CD226*), and signaling (*TNFAIP3*, *TNIP1*, *PTPN22*, *BANK1*, *BLK*, *CD247*) (1). Most of these susceptibility loci have also been identified in other AIDs, highlighting the existence of a genetic overlap between SSc and other AIDs and the concept of shared autoimmunity. In particular, several loci including *PTPN22*, *STAT4*, *TNFAIP3*, have been reported to be associated with rheumatoid arthritis (RA) (3).

A large meta-analysis of GWAS studies of RA patients positive for RA-typical auto-antibodies and of European descent confirmed associations of four loci implicated in other AIDs, including rs3093023, located in the *CCR6* gene region (4). A concurrent GWAS study also identified a strong association between the *CCR6* locus and RA in a Japanese population (5). The association was replicated in two independent Japanese replication cohorts (5). In addition, a functional triallelic dinucleotide polymorphism of *CCR*6 (*CCR6DNP*) was recently discovered (5). Indeed, this *CCR6DNP* polymorphism was correlated with the quantity of CCR6 mRNA expression, was associated with IL-17 detection in the sera of RA patients and

exhibited effects on gene transcription, suggesting this variant to be the causal one (5). The same *CCR6DNP* variant was also associated with susceptibility to several other AIDs including Graves' disease and Crohn's disease (5).

Very recently, a meta-analysis of GWAS strongly confirmed the association of RA with the *CCR6* locus in the Japanese population and provided strong evidence for some shared genetic risk factors for RA between populations of European and Japanese ancestry (6).

The *CCR6* gene encodes C-C motif chemokine receptor 6, i. e. the receptor for CCL20 and a surface marker for IL-17 producing T helper type 17 (Th17) cells (7). It is believed that CCL20/CCR6 signalling plays a role in the recruitment of immature dendritic cells and their precursors, but also Th17 cells, into sites of potential antigen entry (7). Various disease models for SLE and RA have been linked to infiltration of Th17 cells, most of them reporting CCR6 as a key factor for Th17 infiltration to the target tissues (8). In SSc, scarce data have been reported but an animal model suggested that IL-17 might play a role in the development of skin fibrosis (9).

Given the evidence of shared common autoimmune genes between RA and SSc, together with the increasing data showing that Th17 cells may be critical in AIDs, the current study sought to investigate the association of *CCR6* polymorphisms with SSc.

PATIENTS AND METHODS

Study population

In total, 2,411 SSc patients and 7,084 healthy unrelated ethnically matched individuals from three European populations were included (France: SSc cases 1,082; controls 3,084; Italy: SSc cases 680; controls 1,895; Germany: SSc cases 649; controls 2,105). Detailed phenotypic assessment was carried out for all SSc patients, as previously described (2, 10). For all patients with SSc, we determined LeRoy's cutaneous subtype and carried out a phenotypic assessment. All patients were tested for antinuclear antibodies and their putative specificity. Anticentromere antibodies were determined on the basis of their distinctive immunofluorescence pattern. Anti-topoisomerase I antibodies were determined by counterimmunoelectrophoresis. The study was approved by local institutional review boards, and written informed consent was obtained from all subjects. SSc patients known as having associated RA were excluded prior to analysis in order to avoid bias due to a possible excess of the risk alleles attributable to these patients, as previously described (10).

SNP selection and genotyping

DNA samples from SSc patients and controls were genotyped for 12 TagSNPs of *CCR6* (6q27): rs11575083, rs10946216, rs3093010, rs2071171, rs3093007, rs3093012, rs1855025, rs3093009, rs3798315, rs3093006, rs9459883, rs3093023. We also included available genome-wide data from 1984 French, 291 Italian, and 993 German controls from the Three cities cohort (3C), HYPERGENES (www.hypergenes.eu) and KORA F4 studies, since some of the *CCR6* TagSNPs were included in the chips used for these projects (2). Rs3093023 is known to be associated with RA (4). In addition, in European Caucasian populations, this SNP appears to be in strong LD ($r^2 > 0.80$) with two other RA-associated SNPs: rs3093024

and the rs963334 SNP (part of the *CCR6DNP*) both found to be associated with RA by Kochi et al (5). Genotyping was performed using a competitive allele-specific polymerase chain reaction system (KASPar Genotyping, Kbioscience, Hoddeston, UK), as previously described (10).

Sequencing of the *CCR6DNP* polymorphism. To further investigate the potential causal variant, we sought to determine whether rs3093023 could tag the recently identified functional *CCR6DNP*. We randomly selected 78 individuals from the French cohort (42 SSc patients and 36 controls) according to their genotypes for rs3093023 in order to include individuals exhibiting the 3 genotypes (17 were A/A, 36 were G/A, and 25 were G/G). Genomic DNA was extracted from blood samples (Macherey-Nagel) and was amplified for direct sequencing by PCR using the following forward and reverse sequences: 5'-CAACCACCTTTGAAAGAGCAG-3' and 5'-CCCTTGTTCATCCCAACCT-3'. DNA products of 207 base-pairs were purified using a Multiscreen PCR filter plate (Millipore) and then directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Statistical analyses. Statistical analyses were conducted using the recommendations published in Arthritis and Rheumatism (11), and included power calculation and haplotype analysis. Tests for conformity with Hardy-Weinberg equilibrium (HWE) were performed using a standard chi-square test (1 degree of freedom). All odds ratios (ORs) are provided with their 95% confidence intervals (95% CI). Individual association analyses of *CCR6* SNPs with SSc were performed by comparing cases and controls with Fisher's exact test on allelic distribution. The same procedure was applied to subgroups stratified according to SSc phenotype. We applied a conservative Bonferroni correction for multiple testing, that took into account both the number of phenotypic subsets and the number of tag SNPs tested (n=22). An adjusted p-value <0.05 was considered as statistically significant.

Meta-analysis of CCR6 rs3093023 and rs10946216 SNPs. The Breslow-Day method was applied to calculate the homogeneity of ORs between the three cohorts. The combined data for the three populations were subsequently analyzed by calculating the pooled ORs using a Cochran-Mantel-Haenszel test for stratified analysis.

Power calculation. Power calculation was assessed using the R version 2.15.2 software. Taking into account the expected frequency of the *CCR6* rs3093023 minor allele (40.6%) in the general population, the combined set of 2,411 SSc cases and 7,084 controls provided a power of 98.0% to detect an association between SSc and these variants with an OR of 1.3, at the 5% significance level.

RESULTS

Association of *CCR6* polymorphisms with SSc and its main subtypes

Twelve *CCR6* polymorphisms were genotyped in three European populations (French, Italian, and German). The average genotyping call rate was higher than 98% in all three cohorts. Of note, the number of controls differs between the various tested SNPs according to their availability in the chips for the cohorts that were made available by collaborators. Because of our large experience and previous studies using these samples, we were confident with the homogeneity of the 3 cohorts of different geographical origin but of same ethnicity. Therefore, we directly performed combined analyses in order the get the largest power. Homogeneity of the cohorts was confirmed by the Breslow–Day test showing no evidence of interpopulation heterogeneity. We thus conducted a meta-analysis using the Mantel-Haenszel test under fixed effects. All SNPs studied in the *CCR6* gene region were in HWE in Caucasian controls. We found an association between the *CCR6* rs10946216 polymorphism and susceptibility to SSc in the pooled analysis (Table 1). To investigate the possible association of the *CCR6* polymorphisms with clinical features, we stratified the patients according to the

main SSc subtypes. We observed a significant increase in the frequency of the *CCR6* rs3093023 A and rs10946216 T alleles in the anti-topoisomerase positive SSc subset compared with controls: P_{adj}= $1.5x10^{-3}$, OR = 1.27, 95% CI [1.13-1.42] for rs3093023, and P_{adj}=$9.0x10^{-5}$, OR 1.32, 95% CI [1.17-1.48] for rs10946216 (Table 1 and Figure 1). In addition, intra-cohort analyses found that the *CCR6* rs3093023 A and rs10946216 T allele frequencies were significantly increased in anti-topoisomerase positive SSc compared with anti-topoisomerase negative SSc: 50.9% vs 45.3%, p=0.0009, OR=1.25 (95% CI 1.09 to 1.42) for rs3093023, and 51.9% vs 46.5%, p=0.0014, OR=1.24 (95% CI 1.08 to 1.41) for rs10946216. No association was observed with anti-topoisomerase negative SSc patients for both rs3093023 (P=0.74, OR=1.01, 95%CI 0.94-1.10) and rs10946216 (P=0.13, OR=1.07, 95%CI 0.98-1.16) SNPs. The allele distribution of the 2 SNPs in each of the cohorts is detailed in the Supplementary Tables 1 and 2. No association was detected between the *CCR6* rs11575083, rs3093010, rs2071171, rs3093007, rs3093012, rs1855025, rs3093009, rs3798315, rs3093006, rs9459883 SNPs and any of the subtypes investigated.

***CCR6* haplotype analysis and LD relationship in healthy controls**

The two associated SNPs rs3093023 and rs10946216 were in strong LD (r^2 ≥0.80) in the combined European Caucasian control population (Figure 2). Two of the haplotypes formed of these 2 SNPs had a frequency greater than 5% in controls. The AT haplotype containing the minor alleles of the rs3093023 and rs10946216 SNPs was more frequent in SSc cases than among controls in the combined cohort (46 vs 44%, p=0.0096). Moreover, the AT haplotype was also found to be significantly associated with anti-topoisomerase I positivity (50 vs 44%, p=$6.9x10^{-6}$) (Supplementary table 3).

Sequencing of the *CCR6DNP* polymorphism. The direct sequencing of *CCR6DNP* in the sample of 78 individuals for whom the rs3093023 genotype was known revealed 5 genotypes, among which the *CCR6DNP* TG/TG genotype (Supplementary Figure 1). This TG/TG genotype was correlated with the rs3093023 A/A genotype in 94.1% of cases. We subsequently considered that the rs3093023 A allele could tag the *CCR6DNP* TG allele in the entire genotyping cohort.

DISCUSSION

To our knowledge, the present study, using a very large European Caucasian population, is the first to report an association between *CCR6* polymorphisms and susceptibility to SSc. Moreover, the most remarkable association was observed with the anti-topoisomerase antibody positive subset, for both the rs3090323 and rs10946216 SNPs: OR =1.27 [1.13-1.42], $P_{adj}=1.5x10^{-3}$, and OR = 1.32 [1.17-1.48], $P_{adj}=9.0x10^{-5}$, respectively. Intra-cohort analyses further support this preferential association with the anti-topoisomerase positive subtype, suggesting that the risk variant could contribute to a disease specific phenotype, and may be considered as a specific marker of severe disease, since anti-topoisomerase antibody positivity is known to be associated with more severe disease, both for skin diffusion and organ involvements (12). The association of *CCR6* risk alleles with disease-specific autoantibody production has also been reported in RA. Indeed, different patterns of allelic associations between ACPA-positive RA and ACPA-negative disease have been described at several loci, among which *CCR6* (13).

It is noteworthy that this *CCR6* association has not been found in GWAS in SSc, which may be related to an underestimation of low to moderate effect sizes that may not have reached genome-wide significance, but with true effects. Furthermore, phenotypic and genotypic heterogeneity may also explain some false negative results in GWAS. Although the splitting

of subjects into distinct phenotypic subtypes may decrease the power to identify potential associated loci, we believe that it is a major step in the genetic analysis of complex traits. It is also important to point out that the comparison of SSc patients with a particular disease subset with patients without this trait (case-case analysis) is more relevant for biomarker development than comparison of patient with the disease manifestation to unaffected controls (case-control analysis). The limitation for rare diseases is the possibility to collect large samples allowing to analyze relevant subsets of patients, as illustrated in the herein work in which independent cohorts were mainly suggestive of association in the anti-topoisomerase positive subset (Supplementary Tables 1 and 2) but the meta-analysis revealed significant association.

The strong LD observed between the rs10946216 and rs3090323 *CCR6* SNPs in our control population raised the question of the causal variant. Both of these SNPs are located in non-coding regions. To further address this question, we sought to determine whether the functional triallelic dinucleotide polymorphism *CCR6DNP* identified by Kochi et al was present in our SSc cohort, and whether this SNP was in LD with the rs3093023 SNP for which an association was identified in our association study. The very strong correlation between the *CCR6DNP* TG allele and the *CCR6* rs3093023 A allele supports that this functional variant could be the causal variant responsible for the association with the anti-topoisomerase I positive SSc subset.

In SSc, the exact mechanisms underlying the early inflammatory stage of disease and leading to the ultimate stage of fibrosis remain largely unknown. Evidence for a T-cell-driven autoimmune response is supported by the histological examination of the skin of SSc patients during the inflammatory phase, marked by the presence of mononuclear cell infiltrates containing T cells preceding the development of fibrosis. Several lines of evidence point to a role of Th17 cells and of Th17-derived cytokines in the early stage of disease pathogenesis (9,

14). Indeed, increased frequencies of circulating Th17 cells have been detected in patients with SSc (9). Elevated serum levels of IL-17A and increased IL-17A expression in peripheral blood lymphocytes and skin lesions have been found in SSc patients (14). Moreover, IL-17 has been found to enhance the proliferation of human fibroblasts *in vitro*, and has demonstrated pro-fibrotic effects in a murine bleomycin-induced skin fibrosis model (9). However, it has been suggested that while IL-17+ cells directly promote inflammation they may have inhibitory effect on human myofibroblasts (14). Altogether, these data suggest that IL-17 blocking agents could represent interesting therapeutic targets in SSc, as supported by recent findings in other rheumatic diseases (15).

The present study demonstrates the association of the most severe SSc subset, anti-topoisomerase positive patients, with a regulatory variant of the *CCR6* gene, which appears as a new susceptibility gene for SSc and this specific subtype. This also raises the question of the potential role of Th17 cells in SSc and opens new clues for treatments.

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TABLE AND FIGURES

Table 1. Pooled analysis of the CCR6 rs3093023 and rs10946216 SNPs in the combined Caucasian populations (French, Italian, German) in an additive recessive model

SNP	1/2	Subgroup	N	Genotype 1/1	1/2	2/2	MAF(%)	P-value	P_{adj}*	OR (95%CI)
Rs3093023	A/G	SSc	2366	511 (21.6)	1176 (49.7)	679 (28.7)	46.5	0.07		1.06[0.99-1.14]
		LcSSc	1471	311(21.1)	730 (49.6)	430 (29.2)	46.0	0.36		1.04[0.96-1.13]
		SSc ACA+	870	182 (20.9)	422 (48.5)	266 (30.6)	45.2	0.89		1.01[0.91-1.11]
		DcSSc	709	165 (23.3)	349 (49.2)	195 (27.5)	47.9	0.034		1.13[1.01-1.26]
		SSc ATA+	**648**	**161 (24.9)**	**337 (52.0)**	**150 (23.2)**	**50.9**	**$6.6x10^{-5}$**	**$1.5x10^{-3}$**	**1.27[1.13-1.42]**
		SSc-FA	810	183 (22.6)	407 (50.3)	220 (27.2)	47.7	0.04		1.12 [1.01-1.24]
		SSc plus other AID	275	70 (25.5)	135(49.1)	70 (25.5)	50.0	0.02		1.23[1.03-1.46]
		Controls	6912	1360 (19.7)	3495 (50.6)	2057 (29.8)	45.0	NA	NA	NA
Rs10946216	T/C	SSc	2351	528 (22.5)	1186 (50.5)	637 (27.1)	47.5	0.0012	0.026	1.13[1.05-1.21]
		LcSSc	1457	318 (21.8)	738 (50.7)	401 (27.5)	47.2	0.035		1.10[1.01-1.19]
		SSc ACA+	860	186 (21.6)	431 (50.1)	243 (28.3)	46.7	0.21		1.07 [0.96-1.19]
		DcSSc	710	169 (23.8)	354 (49.9)	187 (26.3)	48.7	0.006	0.13	1.17 [1.05-1.31]
		SSc ATA+	**649**	**166 (25.6)**	**341 (52.5)**	**142 (21.9)**	**51.9**	**$4.1x10^{-6}$**	**$9.0x10^{-5}$**	**1.32 [1.17-1.48]**
		SSc-FA	805	189 (23.5)	405 (50.3)	211 (26.2)	48.6	0.005	0.11	1.17 [1.05-1.30]
		SSc plus other AID	268	69 (25.8)	136 (50.8)	63 (23.5)	51.1	0.0049	0.11	1.29[1.08-1.54]
		Controls	5017	1015 (20.2)	2491 (49.7)	1511 (30.1)	45.1	NA	NA	NA

*p Value <0.05 adjusted after Bonferroni's correction for multiple comparisons. ½ minor allele/major allele; ACA, anti-centromere antibodies; AID: autoimmune disease (other than RA); ATA: anti-topoisomerase I antibodies; DcSSc, diffuse cutaneous subtype; FA, fibrosing alveolitis; LcSSc, limited cutaneous subtype; SSc, systemic sclerosis;

Figure 1. Association of *CCR6* rs3093023 and rs10946216 with systemic sclerosis (SSc): meta-analysis of the combined European Caucasian populations

Forest plots showing the results of the Mantel–Haenszel meta-analysis of *CCR6* rs3093023 and rs10946216 in patients with SSc (A) and in the subset of SSc patients with antibodies against topoisomerase I (ATA+) (B)

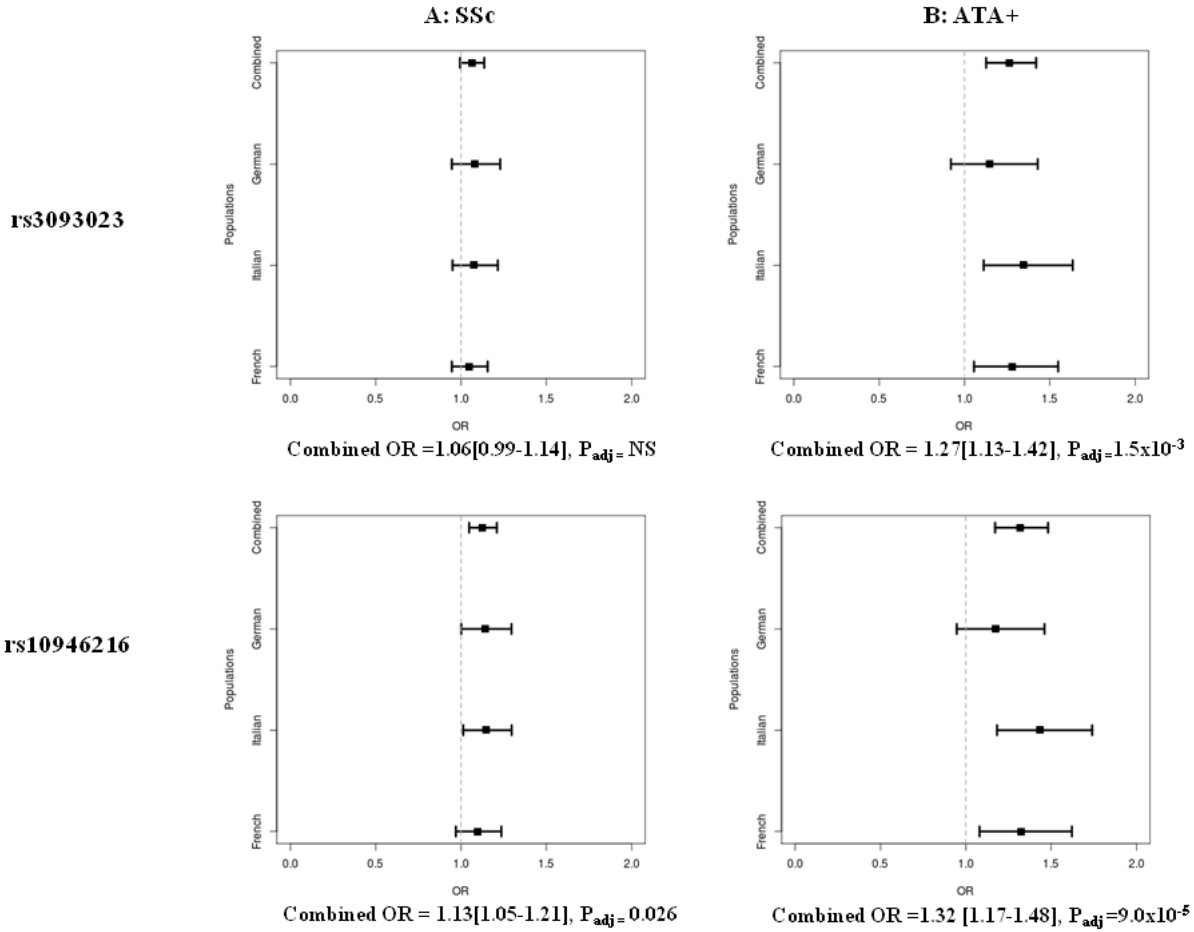
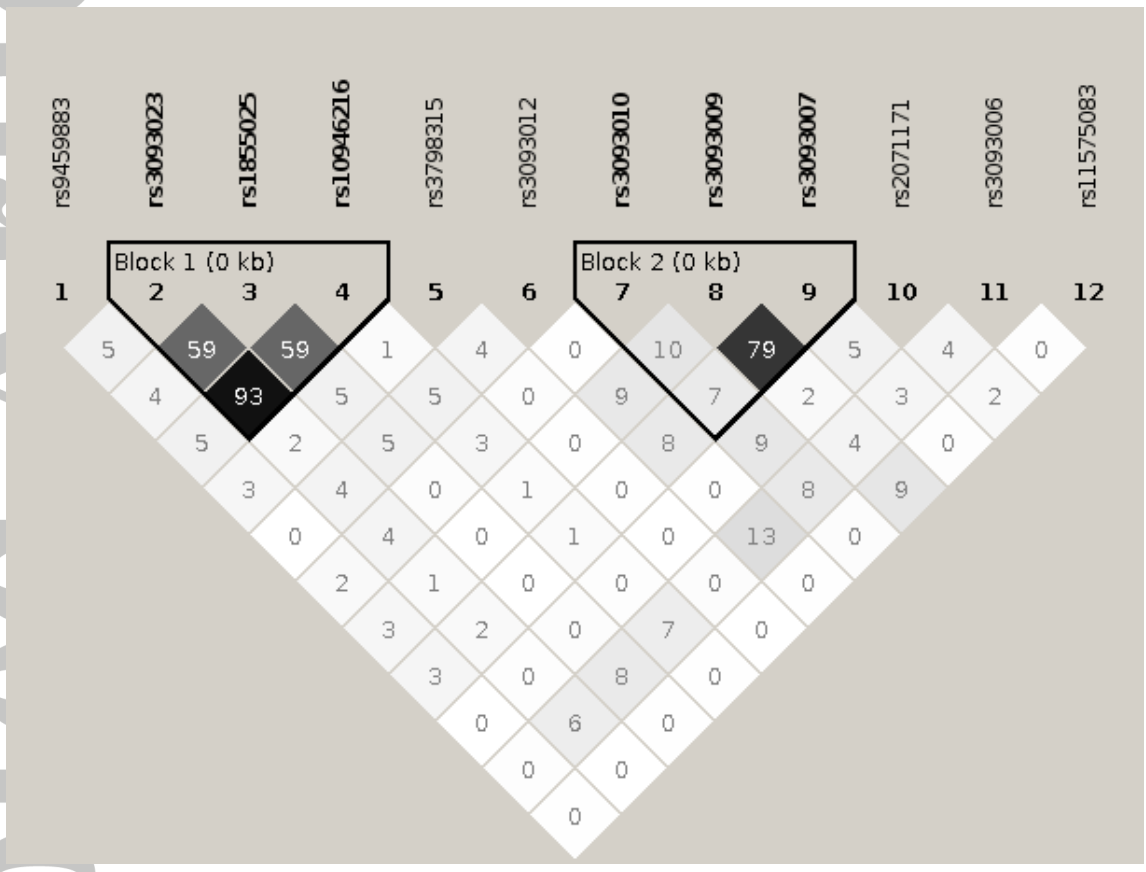
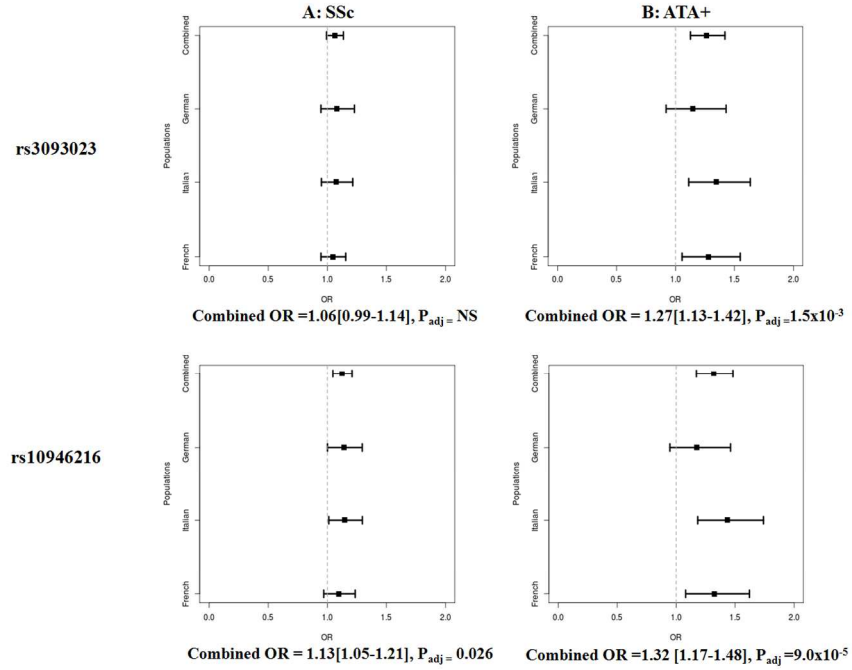


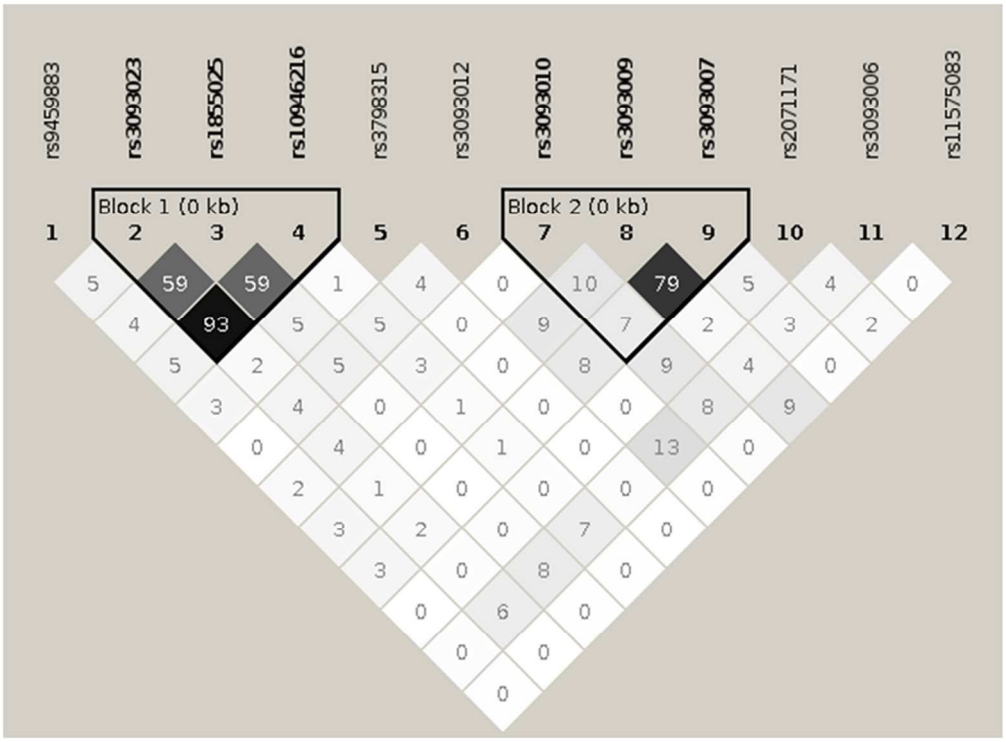
Figure 2. LD and haplotype block structure of the *CCR6* gene within healthy controls (combined population).

Blocks connecting pairs of single nucleotide polymorphisms (SNPs) are shaded according to the strength of the LD between the SNPs, from 0.0 (white) to 1.0 (black), as measured by r^2 values which are given as numerical values within each box.





514x354mm (300 x 300 DPI)



314x230mm (300 x 300 DPI)