Complement component C4 structural variation and quantitative traits contribute to sex-biased vulnerability in systemic sclerosis

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INTRODUCTION

Systemic sclerosis (SSc) is a chronic immune-mediated inflammatory disease (IMID) more frequently observed in women (female/male ratio ~3.8–11.5) that affects the connective tissue and is associated with considerable morbidity and mortality. The heterogeneous clinical manifestations of SSc are characterized by functional and structural vasculopathy, fibrosis of the skin and internal organs, in addition to inflammatory and immunological alterations like auto-antibody production. The individual genetic background, together with environmental risk factors and epigenetics factors, play an important role in the pathogenesis of the disease.

A recent genome-wide association study (GWAS) has identified new genes and pathways implicated in the development and progression of SSc. Similar to other IMIDs, these genetic variations account for a limited portion of estimated heritability, making clear that additional genetic variants remain to be found with the potential to bring novel insights into disease etiology and pathogenesis. In this sense, structural variants not captured by GWAS, such as copy number (CN) polymorphisms, which have been implicated in the etiology of several diseases, could contribute substantially to the genetic risk of SSc. Several CN variants in immunological genes have been found to be associated with autoimmune diseases, although technical limitations and the complexity of CN polymorphisms have reduced the impact of their analysis in understanding autoimmunity.

The complement system plays an important role in innate immunity and forms a bridge to the adaptive immune response. Functional abnormalities in the complement system have been widely described in rheumatic diseases, such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), and to a lesser extent in SSc. Furthermore, genetic variability in several complement components may contribute to the development of inflammatory and autoimmune diseases.
Complement component 4 (C4), encoded by two closely linked, highly polymorphic genes C4A and C4B within the major histocompatibility complex (MHC) class III region on chromosome 6, is an important protein in the classical and lectin complement activation pathways, which are major effectors for controlling microbial infections and for promoting clearance of apoptotic cells and soluble immune complexes. C4A and C4B encode proteins with distinct affinities for their molecular targets and present variability in genomic copy number (CN)24 and length. The long and comparable to published results28 but varied substantially with distinct allelic variability. Haplotype frequencies were found to be similar in both cohorts. We determined nine CNs that was suggested by logistic-regression analysis (b(C4A:sex:HERV-K) = 0.14, P = 2.1 × 10⁻⁵). While higher total C4 CN provide protection, strong deviations from the 1:1 ratio of C4A and C4B CNs are of higher risk like e.g., four copies of C4A and zero copies of C4B (Fig. 1b). We found evidence for a sexual dimorphism for C4A and C4B but not for HERV-K CN. Stratified analysis showed C4A to be more protective in men (b(C4A:sex) = -0.49, P = 1.7 × 10⁻⁹ vs b(C4A:sex) = -0.27, P = 6.5 × 10⁻¹²) while C4B showed statistical evidence to be protective only in women (b(C4B:sex) = -0.22, P = 1.1 × 10⁻¹⁰ vs b(C4B:sex) = -0.09, P = 0.23) (Table 1). Indeed, C4B CNs of two or higher seem to augment the risk for men (Fig. 1c). Logistic regression with interaction terms confirmed our results for the C4A:sex interaction, while the significance of the C4B:sex interaction was only suggestive (b(C4B:sex) = -0.17, P = 0.0034, b(C4B:sex) = 0.13, P = 0.065) (Supplementary Table 3). For total C4 CN, we did not find a significant sex bias (b(C4:sex) = -0.1, P = 0.074). We calculated the statistical power for C4B association in males to be 0.41 and for the interaction of C4B:sex to be 0.33. HERV-K copies generally augment the risk for SSc (Fig. 1d and Table 1). The protection afforded by C4A and C4B CNs is affected by HERV-K copies in a slightly different way. Protection associated with each C4B copy is affected more strongly by HERV-K (b(C4B:HERV-K) = 0.085, P = 0.053) in comparison to C4A (b(C4A:HERV-K) = 0.042, P = 0.03). A logistic-regression analysis with separate terms for the long and the short forms of C4A and C4B confirmed that the short forms confer more protection than the long forms (Table 1).

C4 haplotype diversity and its correlation with classical HLA alleles

The complex genetic variation of C4A and C4B, which consists of many haplotypes with different numbers of C4A and C4B genes, was recently made accessible for analysis in large cohorts.26 We imputed 29 C4 haplotypes independently in both cohorts. Haplotype frequencies were found to be similar in both cohorts and comparable to published results28 but varied substantially across countries (Supplementary Fig. 2 and Supplementary Table 2A). We determined nine C4 haplotypes to have a moderate to strong correlation (r² > 0.4) to at least one classical HLA allele (Supplementary Dataset 1). Correlation to classical HLA alleles associated to SSc on a genome-wide level was found to be small (r² < 0.3) for most haplotypes with the exceptions of HLA-B*08:01 and HLA-C*07:01 which had a strong correlation (r² = 0.82 and 0.67, respectively) with the B5 haplotype and HLA-C*16:01 with ALB5 (H21118B) of 0.63 (Supplementary Dataset 1).

The association between C4 copy number variants and SSc is modified by sex and HERV-K

We found a higher C4 CN to be protective for SSc (Fig. 1a and Table 1). Less than four copies of C4 were found in 28.6% of SSc patients, and 2.8% had less than three copies. Interestingly, less than four copies of C4 were found in 28% of female and 32% of male patients. In a simple additive model C4A, C4B and HERV-K CNs exhibited 1.8-fold variation in their relative risk of SSc (95% confidence interval (95% CI), [1.51–2.33]; P = 1.04 × 10⁻¹⁴). Logistic-regression analysis estimated a rather small difference between the protection afforded by each copy of C4A (odds ratio (OR) = 0.73; 95% CI = 0.69–0.78) or C4B (OR = 0.82; 95% CI = 0.77–0.87) (Table 1). We replicated our calculation in the second cohort and performed a meta-analysis, showing consistent results (Table 1).

The number of subjects in the first cohort permits the simple additive model to be expanded to a more complex one investigating the predictors that influence each other. We found evidence for three two-way interactions: an interaction of C4A with C4B, a sexual dimorphism of C4A and C4B, and an interaction of HERV-K CN with C4A and C4B (Supplementary Table 3). The full model log(risk) ~ b(C4A) + b(C4B) + b(HERV-K) + b(C4A:sex) + b(C4B:sex) + b(C4A:HERV-K) + b(C4B:HERV-K) predicted 7.7-fold variation in the relative risk of SSc. The following analyses have been derived from this complex model and its calculated coefficients.
Fig. 1  C4 and HERV-K copy numbers and Systemic Sclerosis risk. a depicts relative systemic sclerosis (SSc) risk vs total C4 copy number stratified by C4A CN. The SSc risk score is calculated per individual as the sum of effect sizes (betas) multiplied with the design matrix. Betas of C4A, C4B and C4A:C4B were taken from the most complex model “d” (see “Methods”). Crosses are calculated as average relative risk per rounded C4 CN +/− 2 standard deviations (y axis). Linear regression lines are colored by C4A CN and drawn to visualize the interaction effect of C4A and C4B. The y axis contains a color code to aid a comparison with (b). b depicts the relative SSc risk of combinations of C4A and C4B CNs. Relative risk is calculated like in (a). Outer circles are drawn according to population frequency ranges of each C4A, C4B combination and highlight more common combinations. Diagonal dotted lines help to identify combinations of equal total C4 CN. c depicts relative SSc risk in male individuals vs total C4 CN stratified by C4B CN. Relative risk is calculated like in (a) using effect sizes of C4A, C4B, C4A:C4B, Sex:C4A, and Sex:C4B. Crosses are calculated as average relative risk per rounded C4 CN +/− 2 standard deviations (y axis). Cubic regression lines are colored by C4B CN and drawn to visualize the interaction effect of C4A and C4B. d depicts relative SSc risk vs total C4 CN stratified by HERV-K CN. Relative risk is calculated like in (a) using effect sizes of C4A, C4B, C4A:C4B, HERV-K:C4A, and HERV-K:C4B. Crosses are calculated as average relative risk per rounded C4 CN +/− 2 standard deviations (y axis). Linear regression lines are colored by HERV-K CN.

(AS, AL, BS, BL) instead of C4A plus C4B plus total HERV-K CNs. Interestingly, expression models were best if CNs of C4B were included in the model of C4A and vice versa. About 21% (27%) of expression variance of C4A (C4B) can be attributed to C4A and C4B CNs (Supplementary Table 4B, C). This seems to be the upper bound of C4 expression variance explained by C4 genetics as C4A and C4B CN and eQTLs together could not explain more than 40% of expression variance albeit with only 12 (C4B: 13) additional SNPs (Supplementary Tables 4B, C). C4 eQTLs seem to integrate C4 and HERV-K CN information. Indeed, copy numbers of C4AShort, C4ALong, C4BShort, and C4BLong can be predicted well ($r^2_{CAAL} = 0.5$, $r^2_{CAAS} = 0.58$, $r^2_{CABL} = 0.54$, $r^2_{CABS} = 0.67$, all $P < 2.2 \times 10^{-16}$) using C4 eQTLs forward selected to explain C4A or C4B expression variance.

Blood serum concentrations of C4 protein were well correlated with C4 CNs ($r_{Pearson} = 0.25$, $P = 1.3 \times 10^{-12}$, Fig. 2b). Regression analysis determined that C4 and HERV-K CNs, sex, age, and SSc explained about 12% of C4 serum concentration variance ($P = 1.4 \times 10^{-20}$) with HERV-K copies again weakening C4 levels ($b_{HERV-K} = -0.03$, $P = 9 \times 10^{-7}$) (Fig. 2b and Supplementary Table 5). CN-corrected serum C4 levels determined men to have more C4 protein than women ($b_{male} = 0.04$, $P = 4.6 \times 10^{-4}$) independent of disease (Fig. 2e and Supplementary Table 5). Individuals with
SSc had less CN-corrected C4 protein than healthy subjects independent of sex ($b_{\text{sex}} = -0.02$, $P = 0.012$).

Despite that only about a third of SSc patients have less than four copies of C4, we found significant downregulation of C4 expression ($\rho_{\text{female}} = 0.001$, $\rho_{\text{male}} = 0.003$) and C4 protein levels ($P = 0.004$) in SSc patients compared to healthy controls (Fig. 2c, d).

C4 genetics can explain a part of the SSc association to the MHC region

We performed conditional association analysis for genetic markers across the MHC genomic region. Conditioning on C4A, C4B, and HERV-K CN or on a risk score calculated using the complex C4 CN interaction model derived above, showed an impact on residual association levels limited to the vicinity of the C4 gene (Supplementary Fig. 3B, C). In addition, we calculated a C4 risk score recently proposed for SLE and SjS based only on CNs and eQTLs alone can explain more of the SSc association to the MHC region without being implicated in SSc pathogenesis. We therefore asked if SSc association with the MHC could be explained by C4 eQTLs selected to explain C4A and C4B expression variance in the second dataset. Using expression-model eQTL SNPs, selected in the second cohort to explain expression variance, as co-factors in the first cohort to determine residual genetic association with SSc again rendered most MHC association with SSc nonsignificant ($P > 5 \times 10^{-8}$), except for the peaks shown in Fig. 3c centered on HLA-DBP1 and HLA-DPB1.

Remaining MHC signal after conditioning on C4 genetics highlights HLA-DRB1 and HLA-DPB1

Having attributed most SSc association within the MHC to C4 genetics, we investigated which classical HLA alleles and HLA amino acids (AA) demonstrated C4-independent association to SSc. Conditioning on expression derived independent C4 eQTLs results in residual significance ($P < 5 \times 10^{-8}$) for classical alleles and AAs of HLA-DRB1, HLA-DPB1, HLA-DPA1 and HLA-DQA1, HLA-DQB1, and HLA-B (Supplementary Dataset 2A, B). Forward selection to derive independent residual signals marked HLA classical alleles for HLA-DRB1 ($*07:01$, $*11:03$, $*11:04$, $*13:01$), HLA-DPB1 ($*13:01$, $*26:01$, $*40:01$, $*06:01$), HLA-DQA1 ($*04:01$), and HLA-DQB1*$05:01$.

We tested if classical alleles and AAs of HLA-DRB1 and HLA-DPB1 alone could explain residual C4-independent association. We found that 9 HLA classical alleles (HLA-DRB1: $*11:04$, $*08:01$, $*07:01$, $*13:01$, $*11:03$ and HLA-DPB1: $*13:01$, $*26:01$, $*40:01$, $*06:01$) together with 16 expression-model-derived independent C4 eQTLs can explain almost all associations of SSc with the MHC region (Supplementary Fig. 5B). The same is true for AAs of HLA-DRB1 and HLA-DPB1, which can also explain residual C4-independent association with eight independent AAs (HLA-

### Table 1. Logistic-regression analysis for total C4, C4A, C4B, and HERV-K copy numbers.

| Model | Model terms | 1st cohort (N = 26,633) | 2nd cohort (N = 857) | Meta-analysis |
|-------|-------------|--------------------------|---------------------|--------------|
|       |             | Beta | s.e. | P   | Beta | s.e. | P   | Beta | s.e. | P   |
| a: all | total C4   | $-0.23$ | $0.03$ | $6.3E-17$ | $-0.20$ | $0.13$ | $0.12$ | $-0.23$ | $0.03$ | $1.9E-17$ |
|       | HERV-K     | $0.12$ | $0.02$ | $2.9E-10$ | $0.16$ | $0.10$ | $0.10$ | $0.12$ | $0.02$ | $7.9E-11$ |
| b: all | C4A        | $-0.31$ | $0.03$ | $3.7E-19$ | $-0.36$ | $0.18$ | $0.04$ | $-0.31$ | $0.03$ | $4.7E-20$ |
|       | C4B        | $-0.20$ | $0.03$ | $7.0E-11$ | $-0.14$ | $0.14$ | $0.33$ | $-0.19$ | $0.03$ | $4.6E-11$ |
|       | HERV-K     | $0.16$ | $0.02$ | $2.6E-13$ | $0.23$ | $0.11$ | $0.04$ | $0.16$ | $0.02$ | $3.4E-14$ |
| b: female | C4A   | $-0.27$ | $0.04$ | $6.5E-12$ | $-0.81$ | $0.33$ | $0.01$ | $-0.28$ | $0.04$ | $2.6E-12$ |
|       | C4B        | $-0.22$ | $0.03$ | $1.1E-10$ | $-0.10$ | $0.27$ | $0.70$ | $-0.22$ | $0.03$ | $2.3E-13$ |
|       | HERV-K     | $0.15$ | $0.02$ | $1.4E-10$ | $0.41$ | $0.19$ | $0.03$ | $0.15$ | $0.02$ | $1.5E-14$ |
| b: male | C4A       | $-0.49$ | $0.08$ | $1.7E-09$ | $-1.19$ | $0.48$ | $0.01$ | $-0.51$ | $0.08$ | $1.1E-10$ |
|       | C4B        | $-0.09$ | $0.07$ | $2.3E-01$ | $-0.73$ | $0.43$ | $0.09$ | $-0.11$ | $0.07$ | $1.2E-01$ |
|       | HERV-K     | $0.18$ | $0.05$ | $2.8E-04$ | $0.66$ | $0.28$ | $0.02$ | $0.19$ | $0.05$ | $7.5E-05$ |
| c: all | C4AShort   | $-0.29$ | $0.18$ | $1.0E-01$ | $-0.43$ | $0.32$ | $0.18$ | $-0.32$ | $0.16$ | $3.9E-02$ |
|       | C4ALong    | $-0.16$ | $0.02$ | $2.0E-10$ | $-0.11$ | $0.18$ | $0.55$ | $-0.16$ | $0.02$ | $1.1E-15$ |
|       | C4BShort   | $-0.20$ | $0.03$ | $1.4E-10$ | $-0.12$ | $0.28$ | $0.67$ | $-0.20$ | $0.03$ | $2.5E-11$ |
|       | C4BLong    | $-0.04$ | $0.03$ | $1.8E-01$ | $0.09$ | $0.15$ | $0.56$ | $-0.04$ | $0.03$ | $2.3E-01$ |

Depicted are beta values from the logistic-regression analysis of three different models (blocks of rows, see "Methods"). All models contained sex and five genetic principal components as co-variables. Logistic-regression analysis for the first cohort additionally contained cohort as co-variable. Model b was also calculated separately for females and males. Models contain copy numbers as calculated from the imputed C4 alleles per individual as dosages.
DISCUSSION

In this study, we found a strong association of low C4 and high HERV-K CN with SSc in two independent SSc datasets and their meta-analysis, supporting the protective role of C4 copies in IMIDs. C4A gene copies were slightly more protective than C4B as has been shown in SLE and SjS28 but our data suggest a complex interaction of C4A and C4B CNs that has to be evaluated in the context of HERV-K copies and sex. We found that in SSc, an equal number of C4A and C4B gene copies grants more protection than (strongly) imbalanced numbers which we found to be a risk for SSc (Fig. 1b). Our results might differ from recent observations in SLE and SjS where C4A and C4B copies have been described to act in an additive way, but the authors did not describe the interaction with HERV-K copies in detail.

Our results showed a sexual dimorphism with respect to the protection afforded by C4A and C4B. While in female individuals, C4A copies grant slightly more protection than C4B copies, our data suggest that in male individuals only C4A confers protection while we did not observe a strong effect for C4B. In male individuals, C4B might therefore function like a null allele with respect to protection from SSc as higher CNs of C4B are associated to higher SSc risk (Fig. 1c). However, as the power of our study to detect significant C4B signals in males was limited, and the sex:C4B interaction was only of suggestive significance, we cannot rule out that C4B has a protective effect in males. While C4 alleles have been described to act more strongly in men, no distinction was made between C4A and C4B activity in SLE or SjS in a recent study of similar size28. It has been described that activated C4A bonds preferably with protein antigens, such as immune complexes, while activated C4B reacts rapidly with carbohydrate antigens, such as bacterial cell walls30. This could partly explain the greater susceptibility to and severity of infections reported in men and the higher incidence of autoimmune diseases in women31,32. In addition, low C4B CNs have been associated to cardiovascular disease33 where the incidence in women is usually

Fig. 2  

C4 expression and C4 protein concentrations in whole blood. a depicts residualized total C4 expression levels by total C4 copy number (CN) stratified by HERV-K CN. C4 expression is calculated as the sum of C4A and C4B expression as obtained by RNA-Sequencing. The residualized expression has been calculated by regressing out 20 (18) principal components for controls and cases, respectively. Data has been grouped by rounded C4 and HERV-K CN dosage. b depicts normalized C4 protein levels in plasma by total C4 CN stratified by HERV-K CN. C4 protein levels have been normalized across 10+ laboratory sites. c depicts residualized total C4 expression levels (like in a) for SSc and controls, stratified by sex. Significant comparisons are highlighted by asterisk (*P < 0.05, **P < 0.01, ***P < 0.001). d depicts normalized C4 protein levels (like in b) for SSc and controls, stratified by sex. Significant comparisons are highlighted by asterisk (*P < 0.05, **P < 0.01, ***P < 0.001). e depicts normalized C4 protein levels in blood from 119 adult men (blue) and 447 adult women (red) as a function of age with locally estimated scatterplot smoothing (LOESS). Protein levels are normalized to the number of C4 gene copies in an individual’s genome. All boxplot are drawn with default settings in R 4.0.3: lines are defined as first, second and third quartile (Q1, Q2, Q3), whiskers depict the most extreme data points within Q1–3 interquartile range (IQR), and Q3 + 1.5 IQR. Boxplot notches are defined as 95% confidence interval of the median.

DBR1: 37SY, 58A, 74RL, 96E, and HLA-DPB1: 8, 76I, 91H, 96x) (Fig. 3d).

To complement our analysis, we repeated our search for residual C4-independent association to SSc, this time conditioning on the C4 genetic signal which was not derived from the expression dataset but from the first dataset as ten independent C4 eQTL signals as described. Repeating the forward selection of AAs or classical HLA alleles conditioning on the ten dataset-derived independent C4 eQTLs signals resulted in four independent AAs from HLA-DRB1 (9F, 76I, 91H, 96x) or five independent alleles (HLA-DRB1: *11.04 and HLA-DPB1: *13.01, *26.01, *28.01, *30.01) (Supplementary Fig. 4D, E) supporting the role of HLA-DRB1 and HLA-DPB1 as a C4-independent association.

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lower than in men\textsuperscript{34}. Interestingly, in a recent paper studying the female-biased expression in human skin, several genes from the complement activation pathway were identified as a molecular signature and in genome-wide co-expression networks\textsuperscript{35}. Sexual dimorphism has been extensively reported in vascular physiology and pathophysiology\textsuperscript{36}, where women more commonly develop microvascular dysfunction, and in autoimmune-related interstitial lung diseases, where young women are most commonly affected\textsuperscript{37}. All of these clinical manifestations, for which the role of C4 is yet to be elucidated, are hallmarks of SSc.

Our data confirm that C4 and HERV-K CNs are strong predictors of C4 expression levels in blood and other tissues\textsuperscript{29}. While the major site of C4 expression is the liver\textsuperscript{38}, it has been shown that whole blood can be used with some caution as a surrogate tissue for quantitative trait analysis\textsuperscript{39–41}. In addition, local complement production by bone-marrow-derived monocytes and macrophages can restore humoral response in c4 deficient mice\textsuperscript{42}. Interestingly C4A and C4B expression models both profit strongly from the other gene’s CN as a predictor, which supports the genetic interaction between them suggested in this study. Furthermore, the distinction between the long and the short forms of C4: AS, AL, BS, and BL as expression predictors instead of C4A, C4B, and HERV-K CNs alone, greatly favors the accuracy of the expression model. This suggests that HERV-K acts specifically on the gene where it is located, to suppress its expression, which is supported by studies in brain and serum\textsuperscript{32,29,43}. C4A and C4B CNs were able to explain about 20% of C4A and C4B expression variance, which is clearly lower than the ability of C4 eQTLs, which could explain \( \approx 40\% \). Although we most likely over-fitted the expression data, SNPs seem to be the superior instrument in

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**Fig. 3** MHC region conditional association with systemic sclerosis. Association is calculated in the first dataset (\( N = 26,633 \)) using logistic regression with cohort, genetic background (PC1-5), and sex as covariates and depicted as position (GRCh38) by significance (Manhattan plot) in gray if no additional covariates were used. The dotted line represents the genome-wide significance cutoff \( P = 5 \times 10^{-8} \). \( \textbf{a} \) Manhattan plot with marked C4 eQTLs obtained from the GTEx v8 database. \( \textbf{b} \) Manhattan plot with additional conditioning on ten independent C4 eQTLs, obtained by forward selection in the first dataset, depicted in blue. The arrow marks the position of HLA-DPB1. \( \textbf{c} \) Manhattan plot with additional conditioning on 16 independent C4 eQTLs (obtained by forward selection to explain expression variance in the second dataset (\( N = 857 \)) depicted in blue. The arrows mark the positions of HLA-DPB1 and HLA-DRB1. \( \textbf{d} \) Manhattan plot with additional conditioning on 16 independent C4 eQTLs (obtained by forward selection to explain expression variance in the second dataset) and 8 independent amino acids of DRB1 and DPB1 (obtained by forward selection in the first dataset conditioning on 16 independent C4 eQTLs) depicted in blue.
predicting C4 expression as they can integrate CN as well as classical eQTL signals. This finding might help to bring C4 genetics to the clinic in the form of simple genetic tests. The interconnect- edness of C4 CN and eQTLs is further supported by C4 eQTLs being able to predict C4 CNs (AS, AL, BS, BL) with coefficients of determination of \( r^2 > 0.5 \).

C4 and HERV-K CNs are strong predictors of C4 protein levels in blood serum\(^4\). Interestingly, it was reported that men had on average 27% more C4 protein per C4 CN than women and that this bias is stronger during reproductive years\(^29\). While we found the difference between men and women to be smaller; men had on average 17% more C4 protein per C4 CN than women; we think our dataset confirms this finding and its timeframe (Fig. 2f) reinforcing the role of C4 in the differential susceptibility between men and women observed in SSc. The deficiency of C4 may trigger an inappropriate clearance of apoptotic debris and stimulate chronic activation of myeloid cells. Also, it results in a defect to eliminate autoreactive B-cell clones, and a higher tendency to form self-reactive germinal centers\(^23\) and has been previously associated with more severe SLE with earlier disease onset\(^44\). We also observed that patients with SSc have lower C4 serum levels than unaffected individuals even after correcting for C4 gene CN, suggesting that hypocomplementemia in SSc is not simply due to C4 genetics but also reflects disease effects on background complement levels\(^45\).

C4 expression was clearly down-regulated in SSc patients compared to healthy individuals, as were C4 protein levels, although to a lesser extent (Fig. 2c, d). This might be explained by the difficulty in standardizing C4 protein assays across 10+ laboratory sites but might also point to differential mRNA stability adding another layer of complexity yet to be analyzed. Indeed, while we observed clear disease-independent differences of C4 protein levels between men and women, there was no significant differential expression of C4 between healthy men and women, which suggests post-translational effects to play a role. In this line it has been proposed that IFN-gamma increases the stability of C3 and C4 mRNA\(^46\) and a recent expression analysis in SSc detected a strong IFN signature in a subset of patients\(^47\).

More than a third of more than 10,000 C4 eQTLs from the GTEx v8 database are associated with SSc (\( p_{\text{GWAS}} < 10^{-7} \)) and C4 eQTLs alone can be used to explain most of the association of SSc within the MHC region, further supporting their importance in SSc. Interestingly, C4A-specific eQTLs can explain more SSc association than C4B-specific eQTLs (Supplementary Fig. 4B, C), which supports a stronger role for C4A in SSc. While C4 eQTLs could in principle be associated with SSc by the strong linkage structure present in the MHC, our data suggest that C4 eQTLs, forward selected to explain expression variance in blood, can also explain most genetic association with the MHC. Both analyses raise the possibility that C4 genetics is indeed the main signal on chromosome 6 for SSc, as has been suggested for SLE and SJ\(S\)\(^28\), both rheumatic diseases that can co-occur with SSc.

C4-independent genetic association with SSc centers on two peaks (Fig. 3c), most of which can be explained by four AAs each of HLA-DPB1 and HLA-DRB1 (Fig. 3d). Interestingly, the AA positions for HLA-DPB1 and HLA-DRB1 overlap and all 8 AAs positions can be associated with four of five binding pockets described for class II HLA molecules\(^48\) likely interfering with (auto-) antigen binding. In addition, three of the HLA-DRB1 AA positions (37, 58, and 74) are close to sites (30, 60, and 74) which have been described to play a role in binding the consensus antigenic peptide of the topoisomerase I epitope, auto-antibodies to which define the ATA\(^+\) subgroup of SSc patients\(^49\). Furthermore, we found that the C4-independent genetic association with SSc can be explained by 10 independent classical HLA-Alleles instead of AAs, seven of which overlap with a model of nine independent HLA-Alleles recently described\(^50\), which supports the independence of C4 and HLA associations with SSc.

Our study has some limitations. First, the number of samples in the second dataset is very low in terms of GWAS. While we were able to replicate the association of C4A, C4B, and HERV-K CNs, replication of the most complex model was out of reach and needs to be the subject of further study. Second, we did not stratify the patients by clinical or serological subgroups. While our results on HLA-DRB1 AAs, being associated with SSc independently of C4 genetics, point towards anti-topoisomerase auto-antibodies and probably diffuse cutaneous SSc, the topic is too vast to explore in this study. Third, unfortunately, we could not distinguish C4A and C4B protein levels in serum, which would have been very useful, to further investigate the sexual dimorphism described. Fourth, the exact amino acid positions and classical alleles from the models calculated in our study might change in the future. New imputation reference panels might provide new associations that could influence the models as forward selection is sensitive to statistical fluctuations. Last, C4 forms a genetic module termed RCX with three genes: serine/threonine nuclear protein kinase \( R P \), steroid 21-hydroxylase \( C Y P 2 1 \), and extracellular matrix protein tenascin TN\(X\)\(^51\). Although we only assessed C4 CNs associated with SSc, we cannot discard the possibility that this module plays a role in disease susceptibility. Specifically, \( T N X \) is involved in the maintenance of collagen networks and tissue integrity\(^52\), as well as in TGFB activation and signaling, typical for fibrotic conditions such as SSc\(^53,54\).

Many rheumatic diseases, including SSc, could benefit from therapies directed toward the complement system. These are currently under active development and are not only focused on inhibitory mechanisms, but on activators or downstream activation fragments\(^55\). The inhibition of the complement pathway has proven challenging. Eculizumab, a C5 inhibitor, is a complement-targeting approved drug for a variety of vascular disorders and has recently been approved in kidney diseases\(^56\). Moreover, it has been studied in idiopathic inflammatory myopathies and SSc renal crisis, with promising results\(^56,57\). Our data suggest that C4 genetics in SSc, by affecting expression and C4 protein levels, plays an important role in mediating the genetic association in the MHC locus and might also be involved in the epidemiological sex bias of SSc. This highlights the contribution of the complement to the development of SSc and to autoimmune disorders in general, which could benefit from therapies directed towards the complement system. Our findings might help to bring C4 genetics to the clinic in the form of simple genetic tests.

METHODS

Patients

All patients fulfilled the classification criteria of the 2013 American College of Rheumatology (ACR) or The European League Against Rheumatism (EULAR) or the criteria proposed by LeRoy and Medsger for early SSc\(^58,59\). CSCC’s Ethics Committee approved the study and written informed consent was obtained in accordance with the Declaration of Helsinki.

Cohorts and datasets

We (re-)analyzed two independent cohorts of European descent: First cohort: genome-wide genotyped data from 14 independent epidemiological cohorts comprising a total of 28,179 unrelated individuals (9846 SSc patients and 18,333 healthy subjects) from 10 European countries\(^6\). To identify ancestry outliers -100,000 quality-filtered independent SNPs were selected from each case–control GWAS cohort. Principal component (PC) analysis was performed using PLINK v1.07. Samples showing >4 standard deviations from the cluster centroids of each cohort were considered outliers and removed from further analyses. The presence of relatives and/or duplicates was assessed by computing identity-by-descent (IBD) estimation using PLINK v1.07. An
individual from each pair of relatives (Pl_Hat > 0.45) or duplicates (Pl_Hat > 0.99) was removed. After exclusion of non-European samples, we recalculated genetic PCs using the merge of all imputed datasets, selecting ~100,000 independent markers using PLINK v1.9. Missing data values due to the different platforms used for genotyping were corrected by PLINK v1.9 (parameter –correct_for_missingness). We obtained informative principal components as the visualization of the first two PCs can be interpreted as a “map” of the European continent (Supplementary Fig. 1). Second cohort: this cohort included genome-wide genotyped data, whole blood expression data and blood serum C4 protein concentrations from 333 SSc patients and 524 healthy individuals from 9 European countries. This second cohort is a subset of a larger cohort of seven immune-mediated diseases plus (SPAPLUS analyzer)66. A corrective factor was calculated in order to normalize the data between the centers as described60. Potential non-genetic influences were regressed out for SSc and controls separately by 20 (SSc:18) PCs calculated from inter-sample expression correlation matrices.

C4 protein data

Human complement C4 serum data was obtained from the PRECISESADS consortium60 from a turbidimetric immunoassay method according to the manufacturer’s recommendations (SPAPLUS analyzer)66. A corrective factor was calculated in order to normalize the data between the centers as described60.

Imputation

SNPs. For both cohorts, we imputed SNPs from chromosome 6 using the TOPMed reference panel with default settings at https://imputation.biodatacatalog.nhlbi.nih.gov/67. Stringent QC measures were applied to both cohort’s pre-imputation as follows: SNPs with call rates < 0.98; minor allele frequencies (MAFs) < 0.01; and those that deviated from Hardy–Weinberg equilibrium (HWE; P < 0.001 in both case and control subjects) were filtered out from further analysis; samples with call rates < 0.95 were removed. Relatives and/or duplicated samples were removed. Post-imputation quality control included filtered for imputation quality (r^2 > 0.3), MAF > 0.05, and HWE, which resulted in 9,068 SSc patients and 17,565 healthy individuals for C4 haplotype imputation for the first cohort.

C4 haplotypes. A set of 7021 SNPs TOPMed imputed SNPs were selected as they were (a) imputed in all individuals in both cohorts and (b) overlapped the C4 CN reference panel. C4 haplotype imputation was carried separately for both cohorts using the software imputec429 and https://github.com/freeeseek/imputec4 and the reference panel downloaded from the dbGaP study accession: phs001992.v1.p1. Weighted imputation accuracy was calculated by multiplying r^2 Allele by Allele frequency in Supplementary Table 2B.

C4 copy numbers. Each C4 haplotype carries a specific number of C4 isotypes (C4A, C4B) and HERV-K elements (Supplementary Table 2C). We calculated total C4, C4A, C4B, and HERV-K CN dosages by multiplying the allele dosages of the structural haplotype by the number of copies of each C4 isotype and HERV-K that the haplotype contains. For instance, the haplotype AL-BL contains one C4A gene and one C4B gene and two HERV-K copies. The numbers of short and long forms of C4A and C4B (AL, AS, BL, BS) per haplotype are self-evident for 17 of 29 imputed haplotypes. For the remaining, long and short forms were inferred by the consensus that ~95% of C4A is present in the long form43,68-70. The haplotype AL-BS for instance can be coded as 0.95 AL, 0.05 AS, 0.05 BL, and 0.95 BS. CNs per haplotype can be found in Supplementary Table 2C.

Classical HLA alleles and HLA amino acids (AA). Data for the classical HLA alleles and AA variants were obtained from the first cohort by imputation using SNP2HLA71 and the reference panel from the Type 1 Diabetes Genetic Consortium72, described in ref. 50. After genotyping QC, all variants were imputed for each case–control dataset separately in the extended MHC region in chromosome 6. Imputed data were also filtered for 95% success call rate for alleles and amino acids, deviation from HWE considering a P value of <0.001 for SNPs in controls and 95% total call rate for individuals50.

Pearson correlation of C4 haplotypes and classical HLA Alleles

Was calculated among the C4 haplotype dosages and the allele dosages from the HLA imputation.

C4 copy number association analysis

Logistic-regression models from simple to complex were calculated (using the function glm in R 4.0.3) to assess the association of total C4 dosage and its isotype dosages with the disease. We included cohort, five genome-wide principal components (PCs) and sex as covariates, assuming their effects were not collinear:

(a) SSc ~ C4A + HERV-K + PC1-5 + cohort + sex
(b) SSc ~ C4A + C4B + HERV-K + PC1-5 + cohort + sex
(c) SSc ~ C4A_short + C4A_long + C4B_short + C4B_long + PC1-5 + cohort + sex

The number of subjects in our first cohort permits us to expand the simple additive model to a more complex one investigating the predictors that influence each other. We included three two-way interaction terms in the logistic-regression model:

(d) SSc ~ C4A + C4B + HERV-K + PC1-5 + cohort + sex + C4A:C4B + C4A:HERV-K + C4B:HERV-K + C4A:sex + C4B:sex

Meta-analysis was conducted with Metasoft73 using data from model a, b, and c from both datasets.

Power calculation

Power calculations in CNV studies are problematic because effect sizes and models of the association are based on approximations that may be unrealistic74.

C4. Power calculations for C4B in males was carried out using the GAS Power Calculator [https://csg.sph.umich.edu/abecasis/...
C4 gene expression analysis in whole blood

Using raw count data, we included disease, blood cell composition, and effective library size (calculated by EdgeR in R 4.0.3) in the final model. While cell type-specific expression changes between SSc and controls were found significant at a nominal level for most cell types, the direction of expression change coincided for all cell types. We decided to report only whole blood expression changes controlling for blood cell composition.

C4 protein blood serum analysis

We included disease, sex, age, AS, AL, BS, and BL in the final model. The significance for the difference between SSc and controls in men and women was calculated with both the Mann–Whitney test and a t test.

Residual association of genetic variants across the MHC region to SSc

We performed conditional association analysis for genetic markers across the MHC genomic region. The first dataset was analyzed. In all models, we included cohort, five genome-wide PCs and sex as basic covariates. Association analysis of MHC region variants was conditioned on the basic covariates plus:

(1) nothing;
(2) a risk score: $2.3 \times C4A \text{ CN } + C4B \text{ CN}$ as proposed;
(3) covariates from model “b”: $C4A \text{ CN } + C4B \text{ CN } + \text{HERV-K CN}$;
(4) covariates from the most complex model “d” described above;
(5) C4 (C4A or C4B or both) eQTLs from GTEx v8 (obtained by forward selection in the first dataset until no SNP had $p_{\text{SNP}} < 10^{-5}$, see Supplementary Tables 4B, 6 and 4C, 6);
(6) C4A-specific eQTLs from GTEx v8 (obtained by forward selection in the first dataset until no SNP had $p_{\text{SNP}} < 10^{-5}$). EQTLs were called C4A-specific if no C4B eQTL was reported in GTEx v8 with $P < 0.01$ for each SNP;
(7) C4B-specific eQTLs from GTEx v8 (obtained by forward selection in the first dataset until no SNP had $p_{\text{SNP}} < 10^{-5}$);
(8) expression-model SNPs (with $p_{\text{GWAS}} < 10^{-7}$) (obtained by forward selection in the second dataset as described above, see Supplementary Tables 4B, 7 and 4C, 7).

Residual, C4-independent, the association of the MHC region with SSc

After accounting for the contribution of C4 genetics with models “S” or “8” above, we sought to model residual, C4-independent, association of MHC SNPs with (a) forward selection of classical HLA alleles; (b) forward selection of classical HLA alleles of HLA-DRB1 and HLA-DPB1; (c) forward selection of AAs of HLA genes; (d) forward selection of AAs of HLA-DRB1 and HLA-DPB1. Forward selection was carried out until no more HLA allele or AA was found with $P < 5 \times 10^{-8}$.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

Summary statistics of the SSc meta-GWAS are available through the NHGRI-EBI GWAS Catalog (https://www.ebi.ac.uk/gwas/downloads/summary-statistics): GCST009131. Data from the PRECISESADS consortium are hosted by ELIXIR Luxembourg https://elixir-luxembourg.org/ and are available upon request. The access procedure is described on the data landing page (https://doi.org/10.17881/th9v-xt85). All other data are contained in the article file and its Supplementary Information.
CODE AVAILABILITY
All analysis has been performed with either the software described in “Methods” or within R 4.0.3. For logistic-regression analysis, the glm function of R was used.

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AUTHOR CONTRIBUTIONS

M.K., M.A.H., and J.M. contributed to the conception and study design. M.K. and M.A.H. contributed to data collection, QC, imputation, and data analysis. C.P.S.A., J.L.C., S.A., S.M.P., M.N., N.H., G.M., J.K.B.-V., G.O., A.B., A.H., C.T., Y.A., C.F., M.E.A-R., T.A.D.R., J.L.B., C.P.D., and M.D.M. contributed to GWAS and RNA-sequencing data collection. All co-authors made substantial contributions to data acquisition, data interpretation, and revised the work critically for important intellectual content.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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