Identification of CSK as a systemic sclerosis genetic risk factor through Genome Wide Association Study follow-up

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Systemic sclerosis (SSc) is complex autoimmune disease affecting the connective tissue; influenced by genetic and environmental components. Recently, we performed the first successful genome-wide association study (GWAS) of SSc. Here, we perform a large replication study to better dissect the genetic component of SSc. We selected 768 polymorphisms from the previous GWAS and genotyped them in seven replication cohorts from Europe. Overall significance was calculated for replicated significant SNPs by meta-analysis of the replication cohorts and replication-GWAS cohorts (3237 cases and 6097 controls). Six SNPs in regions not previously associated with SSc were selected for validation in another five independent cohorts, up to a total of 5270 SSc patients and 8326 controls. We found evidence for replication and overall genome-wide significance for one novel SSc genetic risk locus: CSK [P-value = 5.04 \times 10^{-12}, odds ratio (OR) = 1.20]. Additionally, we found suggestive association in the loci PSD3 (P-value = 3.18 \times 10^{-7}, OR = 1.36) and NFKB1 (P-value = 1.03 \times 10^{-6}, OR = 1.14). Additionally, we strengthened the evidence for previously confirmed associations. This study significantly increases the number of known putative genetic risk factors for SSc, including the genes CSK, PSD3 and NFKB1, and further confirms six previously described ones.

INTRODUCTION
Systemic sclerosis (scleroderma, SSc) is an autoimmune disease characterized by vascular damage, altered immune responses and extensive fibrosis of skin and internal organs (1), in which common genetic factors play an essential role, similar to most complex autoimmune diseases (2,3). So far, only a limited number of genes explaining little of the genetic variance present have been found in SSc (4,5).

In other autoimmune complex diseases for which extensive follow-up studies have been performed, such as inflammatory bowel disease and systemic lupus erythematosus (SLE), ~90 and 35 associated genes, respectively, have been identified (6,7). Therefore, it is expected that a number of risk factors for SSc are still to be defined. To date, only two genome-wide association studies (GWAS) have been published in populations of European ancestry in SSc (8,9). This calls for further replication studies and meta-analysis of SSc.

Due to the high rate of type 1 errors inherent to the GWAS technique, a number of strategies can be followed to discern truly associated genes from false positives in the tier 2 range of associations (ranging $P$-values from $10^{-3}$ to $5 \times 10^{-8}$), e.g. biological pathway analysis, meta-GWAS analysis or genetic interaction analysis. Another approach is to select the most strongly associated genetic variants from GWAS, where most true associations are harbored, by just accepting a small proportion of false negatives left out of the replication study. In this study, we performed the latter approach.

SSc is a clinically heterogeneous disease with a wide range of clinical manifestations (3); patients can be classified according to the severity of skin or organ involvement of the disease (10), or according to the presence or absence of several highly disease-specific auto-antibodies which are almost mutually exclusive in individual patients (1). Each of these disease phenotypes has proven to have specific genetic associations (11–17). In order to determine more of the genetic component of this profoundly disabling disease, such considerations need to be taken into account when selecting a battery of genetic variants to further test in other populations.

Considering the above, we followed the strategy of replication and validation of 768 genetic associations selected from our previous GWAS of SSc under different criteria in seven independent cohorts of European ancestry from five European countries, and subsequently performed a meta-analysis including a total of 5270 SSc patients and 8326 healthy controls.

RESULTS
After quality filtering the replication stage data, 720 out of the 768 selected SNPs were analyzed. Mantel–Haenszel meta-analysis was performed for the three replication cohorts and for those cohorts together with the four GWAS cohorts. Six SNPs were selected for the validation stage in five independent cohorts. The overall process followed in the present study is summarized in Figure 1.

The genotyping success call rate was 99.88 and 97.99% in the replication and validation stages, respectively. Table 1 shows the statistics for the six SNPs selected for validation, while Table 2 shows previously described associations with SSc. Figure 2 shows meta-analysis association results of GWAS and replication cohorts for all 720 SNPs. Figure 3 shows relative risk for each of the three novel associations found in this study, for each population analyzed separately and for the meta-analysis. Detailed analysis and results for each associated region can be found below.

Novel SSc genetic associations
After the replication stage (genotyping of 768 SNPs selected from GWAS data), six SNPs were selected for validation with GWAS and replication combined Mantel–Haenszel $P$-value ($P_{\text{MHH}}$) lower than $1 \times 10^{-3}$ (in either the complete set of patients or its subphenotypes) and located in regions not previously associated with SSc. The SNPs in NFKB1, PSD3, ACADS and CSK were selected for their association with overall SSc, while those in IPO5/FARP1 and ADAMTS17 were selected for their association with the anti-topoisomerase autoantibody (ATA)-positive subgroup of patients. Out of the six SNPs, we were able to validate the association in one of them at the GWAS level ($P < 5 \times 10^{-8}$) and in another two at a suggestive level of association ($5 \times 10^{-8} < P < 5 \times 10^{-6}$).

The GWAS level association in the meta-analysis of all 11 analyzed cohorts was observed for single nucleotide
polymorphisms (SNP) rs1378942 located in an intron of the CSK gene in chromosome 15 [PMH value = 5.04 × 10^{-12}, odds ratio (OR) = 1.202 (1.14–1.27)] with SSc (Table 1). Furthermore, we observed a suggestive overall level of association in two of the selected SNPs in all GWAS, replication and validation cohorts. One of them was located in the PDS3 gene, rs10096702 [PMH value = 3.18 × 10^{-7}, OR = 1.363 (1.21–1.54)], while the other was in the NFKB1 gene, rs1598859 [PMH value = 1.03 × 10^{-5}, OR = 1.140 (1.08–1.20)] (Table 1). Interestingly, we were only able to validate associations with the overall disease, but none of the subphenotype specific ones.

Previously reported SSc genetic associations

Associations with SSc previously reported were found for STAT4 (rs10168266, PMH value = 1.81 × 10^{-7}, OR = 1.234), CD247 (rs2056626, PMH value = 1.14 × 10^{-8}, OR = 0.832), TNFSF4 (rs4916334, PMH value = 1.00 × 10^{-5}, OR = 1.150 (1.11–1.19)), TNFAIP3 (rs2220926, PMH value = 2.29 × 10^{-6}, OR = 1.463) and NPSO3/IRF5 (rs4728142, PMH value = 4.74 × 10^{-10}, OR = 1.216; and rs10488631, PMH value = 1.58 × 10^{-18}, OR = 1.513), further confirming their role in SSc (Table 2). Since several SNPs within both NPSO3/IRF5 and IRF8 genomic regions were selected, a conditional logistic regression analysis was performed to determine the independent signals of both loci (Table 3). While these associations found within each previously reported region spanned across the whole disease, SNP rs11642873 in IRF8 was significantly associated with lcSSc (PMH value = 2.30 × 10^{-9}, OR = 0.730) and only marginally with dcSSc (PMH value = 3.77 × 10^{-2}, OR = 0.874).

DISCUSSION

Through a large replication study, designed using previously published GWAS data, we have been able to identify novel genetic associations with SSc (Table 1), and to provide further evidence for previously reported associations (Table 2). In the present study, a genetic variant within the CSK region, selected for replication because it was associated with SSc in the US cohort, has been identified as susceptibility factor for this disease. Furthermore, two other suggestive associations have been found in PDS3 and NFKB1 genetic variants, which were included in this study because they reached statistical significance in the European cohort. Further investigation will be required to further clarify the potential role of these two signals in SSc genetic predisposition.

CSK (c-src tyrosine kinase) is known to phosphorylate a tyrosine at the C-terminus of src kinases leading to their inactivation (18,19). In turn, src kinases are involved in fibrosis through their regulation of FAK (19,20), which is necessary for transmission of integrin signaling upon adhesion of fibroblasts to the extracellular matrix [and thus, their differentiation into myofibroblasts (21)] and has been involved in experimental pulmonary fibrosis (20), a major hallmark of SSc. Indeed, it has been demonstrated that either incubation of fibroblasts with Csk inhibitors or overexpression of Csk lead to a decreased expression of COLIA1, COLIA2 and FNI, which are key components of the fibrotic process (22). Thus, genetics variants in CSK can be affecting its expression or functionality in a way that src kinases are not inhibited, which in turn will contribute to the fibrosis in SSc. Furthermore, the CSK variant rs1378938, which is in relatively high linkage disequilibrium (LD) with the SSc-associated rs1378942 reported here (r^2 = 0.72 in the CEU population of the HapMap project), has been recently associated with celiac disease (23). Consequently, it is likely that CSK may represent another common autoimmunity risk factor. Further studies in related autoimmune disorders, such as SLE and rheumatoid arthritis (RA), may be performed to draw firm conclusions about this hypothesis.

NFKB has been extensively described to participate in and control the inflammatory process and thus, its role in the development of autoinflammatory disorders is widely accepted (24,25). The gene NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cell 1) encodes a 105 kDa protein which can undergo cotranslational processing by the 26S proteasome to produce a 50 kDa protein. The 105 kDa protein is a Rel protein-specific transcription inhibitor and the 50 kDa protein is a DNA-binding subunit of the NFkB protein complex. SNP rs1598859, located in an intron of NFKB1, has been identified in this study as a risk genetic factor for SSc. This variant, or any other in the same haplotype block, could be affecting the expression or the function of NFKB1, altering the inflammatory response, and thus participating in the development and course of the disease. Indeed, an ATTG in/del in the promoter of NFKB1 has been recently
Table 1. Novel genetic associations found in this study with SSc

<table>
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<tr>
<th>Phenotype</th>
<th>Chr.</th>
<th>Gene</th>
<th>SNP</th>
<th>Base pair(^a)</th>
<th>Location</th>
<th>Change(^b)</th>
<th>Stage</th>
<th>Sample size</th>
<th>MAF</th>
<th>P-value(^c)</th>
<th>OR(^d)</th>
<th>CI (95%)</th>
<th>Breslow–Day P-value(^e)</th>
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<td>C/T</td>
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<td>5187</td>
<td>0.358</td>
<td>3.12 × 10(^{-6})</td>
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<td></td>
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<td>A/G</td>
<td>GWAS</td>
<td>2357</td>
<td>5187</td>
<td>0.044</td>
<td>1.05 × 10(^{-5})</td>
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<td>GWAS</td>
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<td>T/G</td>
<td>GWAS</td>
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<td>GWAS + Rep</td>
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<td>6097</td>
<td>0.175</td>
<td>6.07 × 10(^{-7})</td>
<td>1.416</td>
<td>1.23–1.62</td>
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</table>

Chr., chromosome; MAF, minor allele frequency; OR, odds ratio; CI, confidence intervals. GWAS, genome-wide association study; Rep, replication stage (768 SNPs); Val, validation stage (6 SNPs).

\(^a\) All genomic positions are referent to genome build 36.

\(^b\) Minor allele first.

\(^c\) Mantel–Haenszel meta-analysis of the cohorts involved in the corresponding stage of the analysis (see Materials and Methods). \(^d\) All ORs are for the minor allele.
Table 2. Previously reported associations with SSc found in this study

<table>
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<tr>
<th>Chr.</th>
<th>Gene</th>
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<th>Location</th>
<th>Change</th>
<th>Sample size</th>
<th>MAF</th>
<th>P-value</th>
<th>OR</th>
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<td>G/T</td>
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<td>0.78–0.89</td>
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<td>A/G</td>
<td>3237 6097</td>
<td>0.336</td>
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<td>2372 4395</td>
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<td>0.730</td>
<td>0.72–0.86</td>
<td>8.28 x 10^-1</td>
</tr>
</tbody>
</table>

Chr., chromosome; MAF, minor allele frequency; OR, odds ratio; CI, confidence intervals.

a All genomic positions are referent to genome build 36.

b Minor allele first.

c Mantel–Haenszel meta-analysis of GWAS and GoldenGate cohorts.

d All ORs are for the minor allele.

e TAT4 SNP rs10168266 was not genotyped in the European GWAS cohorts, nor could its genotype could be imputed.

f IRF8 SNP rs11642873 association was confined to lcSSc and these are the statistical shown in the table rather than global SSc as in the rest of the SNPs.
Figure 2. The Manhattan plot of the GWAS and replication cohorts meta-analysis. Grey dots represent $P$-values of SNPs which are genotyped only in the GWAS cohorts in the previous study by Radstake et al. (8). Other color dots represent $P_{MHI}$ values of the 720 SNPs which were in both the GWAS and the replication cohorts (Mantel–Haenszel meta-analysis). Gene names in black have been previously reported as genetic risk factors for SSC, while gene names in red are novel ones.
Dieude et al. (29) found the SNP rs5029939 in TNFAIP3 associated with SSc, which showed stronger association in the dcSSc and ATA+ subgroups of the disease, although the association remained significant in the opposing lcSSc and ACA+ subgroups. In the present study, rs2230926 (in total LD with rs5029939 in the HapMap CEU population) has shown GWAS level association with the global SSc, and no sign of heterogeneity in the SSc subgroups was detected (Table 2). Taking both studies together, the association of TNFAIP3 is most likely due to an effect on SSc susceptibility overall rather than any of its sub-phenotypes. The previously reported fluctuating level of significance is probably due to different population composition and cohort size (1656 cases and 1311 controls in the study by Dieude et al. (29) and 3237 cases and 6097 controls in the present study). SNP rs2230926 is located in exon 3 of TNFAIP3, and encodes for a phenylalanine to cysteine change at residue 127 which has been demonstrated to alter the function of the protein, which makes it a functionally associated genetic variant with SLE (35).

Association in the TNPO3/IRF5 region has been detected previously in SSc (8,33,34) and SLE (36,37). The three independently associated polymorphisms have been detected in SLE: an in/del in exon 6 which change the expression level of IRF5 (tagged by rs10488631 in our study), a variant in exon 1B which affects splicing of this exon (not tagged in our study) and a variant which disrupts a polyA signal site (tagged by rs4728142 in our study). Our findings in the present study are consistent with associations found in SLE. We detect two of the three independent variants (Table 3): the in/del and the polyA signal, yet the variant in exon 1B was not captured in this study. This observation adds more evidence for a similar genetic component of SSc and SLE. Nevertheless, further research will be needed in order to determine whether the variant in exon 1B, affecting splicing of the gene, also has a major role in SSc.

IRF8 has been recently described as a risk factor for lcSSc (30) and has also been associated with the risk of multiple sclerosis (38). Of all the SNPs selected for replication in the present study, only rs11642873 remained independently significant (Table 3), and its association was found, as in the previous study, exclusively confined to lcSSc. Further fine-mapping and functional studies are necessary in order to determine the role of genetic variants in IRF8 in the pathogenesis of SSc and autoimmunity.

In summary, by the analysis of 720 genetic variants, selected from GWAS data’s ‘grey zone’ of association, we describe three new genetic risk factors for SSc (CSK, PSD3 and NFkB1) and confirm five previously reported associations (CD247, STAT4, TNFAIP3, TNPO3/IRF5, TNFSF4 and IRF8). Also in the case of TNPO3/IRF5, we clarify the nature of the association with SSc which is similar to that found in SLE, thus highlighting the similarities of the genetic component for both diseases.

MATERIALS AND METHODS

Populations

The four populations used in the previous SSc GWAS have been previously described (8). Replication and validation
cohorts from Spain (two independent cohorts), Germany, Netherlands, Italy (two independent cohorts) and the UK for a total of 2913 cases and 3139 controls were recruited from hospitals and blood banks from each of these countries (numbers before quality control filters). Key features of these populations can be found in Supplementary Material, Table S1. This study was approved by the local ethics committees of the participating hospitals. All of the SSc patients participating in this study met American College of Rheumatology criteria (39) and were classified in the disease subtypes according to LeRoy and Medsger (10). All individuals in this study were of European ancestry [either self-reported and/or principal component analysis (PCA) determined, see below] and gave written informed consent.

Samples in this study included those in the initial discovery GWAS cohorts as well as those in the replication cohorts in the work by Radstake et al. (8). In the present study, the previous replication cohorts were genotyped for 768 additional SNPs.

Combination of GWAS and replication cohorts genotyped in this study resulted in a total of 5270 cases and 8326 controls (after QC was applied), which gave us a statistical power of 100% to detect an OR of 1.3 with a minor allele frequency of 0.2. Other power calculations can be found in Supplementary Material, Table S2.

**Study design**

**SNP selection.** First, we aimed to select the putative SSc-associated genetic variants that did not reach genome-wide significance in our initial GWAS (8). We filtered GWAS data as previously published. We excluded from our analysis the extended HLA region on chromosome six, since association of this region with SSc is well known, and was not the focus of the present study. Since there is growing evidence for the existence of genetic heterogeneity in SSc, we included the clinical subtypes of the disease (i.e. lcSSc and dcSSc) and the presence of the two most common auto-antibodies (i.e. ACA and ATA) in our selection criteria (1,3,10). Because of the differences between the US and European samples in the PCA, we decided to select the SNPs for each set separately, lending greater weight to the European cohorts (it should be noted that all our replication cohorts were of European countries).

We selected all SNPs with a Mantel–Haenszel, λ corrected P-value lower than 10⁻³ from the European cohorts (i.e. Netherlands, Germany and Spain) meta-analysis. We also selected all SNPs with a Mantel–Haenszel λ-corrected P-value lower than 10⁻³ in the lcSSc, dcSSc, ACA + and ATA + subgroups. We also considered the possibility that SNPs associated in one of the three populations could have been filtered out in the others due to any criteria, so we included all SNPs with a corrected P-value lower than 10⁻⁴ in any of the three populations before merging the data sets. This gave a total of 676 SNPs selected. In order to use all the genotyping capacity of the chosen platform (in which 768 SNPs could be analyzed), and taking into consideration that the first 676 SNPs were selected only from the European panel, we also included the 92 previously non-described independent signals from the US panel that showed the most significant P-values. The overall strategy followed in the present study can be found in Figure 1.

**Replication stage.** In this stage, we aimed to confirm selected associations from GWAS data on case/control cohorts from Spain, Holland and Italy up to a total of 880 cases and 910 controls. All individuals in these cohorts were genotyped for the 768 selected SNPs and association analyses were performed.

**Validation stage.** At this point, we selected the top SNPs from the meta-analysis of the pervious stages (those genotyped in
GWAS and GoldenGate platforms) and further tested their association in larger cohorts from Spain, Germany, Italy and the UK up to a total of 2033 cases and 2229 controls. All SNPs with a meta-analysis P-value $< 1 \times 10^{-5}$ either in the disease or any of its subphenotypes (i.e. lcSSc, dcSSc, ACA+ or ATA+) and not previously reported to be associated with SSC were selected for validation, comprising a total of six SNPs. Finally, we performed meta-analyses for all associated SNPs combining the GWAS and all replication cohorts.

Genotyping methods
The replication cohort was genotyped for 768 SNPs, selected from the GWAS analysis as described above, using a custom Illumina GoldenGate array run on the Illumina iScan system. The validation stage was genotyped using TaqMan genotyping assays from Applied Biosystems.

Genotype imputation of data
As described in Radstake et al. (8), the different cohorts were genotyped using different genotyping arrays (the European cohorts were genotyped mostly with the Illumina HumanHap 370k array, whereas the US cohort was genotyped with the Illumina 550k array). As a consequence, it was possible that some SNPs were present in one but not in the other platform. To prevent this, a genotype imputation was performed in all the GWAS cohorts to obtain a full overlap between platforms. Imputation was performed with IMPUTE software 1.00 as previously described (40), using as reference panels the CEU and TSI HapMap populations.

Data analysis
All data were analyzed using Plink software version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (41). LD patterns among SNPs were also calculated with the $r^2$ statistic using HaploView software (42). Manhattan plots were generated using HelixTree SNP Variation Suite 7 (http://www.goldenhelix.com/SNP_Variation/HelixTree/index.html). In order to avoid any position discrepancies, all base-pair locations for the analyzed genetic variations correspond to those reported in the genome build 36, as these were the positions used in the original GWAS.

All data were quality filtered using the following criteria: Hardy–Weinberg equilibrium P-value $> 0.001$ on controls of any of the populations analyzed separately, minor allele frequency $> 0.01$, success call rate per individual $> 0.95$ and per SNP $> 0.95$. After applying quality control, remaining SNPs were statistically analyzed using the Chi-squared case/control approach in 1433 cases and 1644 controls. The meta-analysis of the different cohorts was conducted using the Mantel–Haenszel test to calculate a pooled OR, and the 95% confidence interval for the OR was estimated using a random effect model. Heterogeneity between cohorts was tested using the Breslow–Day test ($P$-values $< 0.05$ were considered statistically significant); nevertheless, we did not rule out any association in the basis of OR heterogeneity. Combined $P$-values for the Mantel–Haenszel tests were calculated as implemented in Plink. The independence of effects of SNPs in the same genetic region was tested by multiple logistic regression analysis, conditioning each SNP to all others, as implemented in the Plink software. Population stratification was assessed by PCA as previously described (43). All individuals who deviated more than four standard deviations from the centroid of their population were removed as outliers.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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