No evidence of association of *ATP8B4* F436L missense variant in a large systemic sclerosis cohort

Elena López-Isac[1*], Lara Bossini-Castillo[1], Ana B Palma[1], Shervin Assassi[2], Carmen P Simeón[3],

Norberto Ortego-Centeno[4], Esther Vicente[5], Carlos Tolosa[6], Manel Rubio-Rivas[7], José A Román-Ivorra[8], the Spanish Scleroderma Group[9], Lorenzo Beretta[10], Gianluca Moroncini[11], Nicolas Hunzelmann[12], Jörg HW Distler[13], Gabriella Riekemasten[14], Jeska de Vries-Bouwstra[15], Alexandre E. Voskuyl[16], Timothy RDJ Radstake[17], Ariane Herrick[18], Christopher P. Denton[19], Carmen Fonseca[19], Maureen D. Mayes[2], Javier Martín[1*]

1. Institute of Parasitology and Biomedicine López-Neyra, IPBLN-CSIC, Granada, Spain.
2. The University of Texas Health Science Center–Houston, Houston, USA.
3. Department of Internal Medicine, Valle de Hebrón Hospital, Barcelona, Spain.
4. Department of Internal Medicine, Clinic University Hospital, Granada, Spain.
5. Department of Rheumatology, Hospital La Princesa, Madrid, Spain.
6. Department of Internal Medicine, Hospital Parc Tauli, Sabadell, Spain.
7. Autoimmune Diseases Unit, Bellvitge University Hospital, Barcelona, Spain.
8. Department of Rheumatology, Hospital Universitari i Politecnic La Fe, Valencia, Spain.
9. See supplementary note.
10. Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan Italy.
11. Clinica Medica, Department of Clinical and Molecular Science, Università Politecnica delle Marche and Ospedali Riuniti, Ancona, Italy.
12. Department of Dermatology, University of Cologne, Cologne, Germany.
13. Department of Internal Medicine, Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany.
14. Clinic of Rheumatology, University of Lübeck, Lübeck , Germany.
15. Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.
16. Department of Rheumatology, VU University Medical Center, Amsterdam, The Netherlands.
17. Department of Rheumatology & Clinical Immunology, Laboratory of Translational Immunology, department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands.
18. Centre for Musculoskeletal Research and NIHR Manchester Musculoskeletal Biomedical Research Unit, The University of Manchester, Manchester Academic Health Science Centre, Manchester, UK.
19. Centre for Rheumatology, Royal Free and University College Medical School, London, United Kingdom.

*Correspondence to:

Javier Martin MD, PhD

Instituto de Parasitología y Biomedicina López-Neyra, IPBLN-CSIC. Parque Tecnológico Ciencias de la Salud. Avenida del Conocimiento s/n 18016-Armilla (Granada), Spain.

E-mail: martin@ipb.csic.es

Tel: 34 958181669

Fax: 34 958181632

Elena López-Isac

Instituto de Parasitología y Biomedicina López-Neyra, IPBLN-CSIC. Parque Tecnológico Ciencias de la Salud. Avenida del Conocimiento s/n 18016-Armilla (Granada), Spain.

E-mail: elenalopezisac@ipb.csic.es

Tel: 958181621

Fax: 958181632

Key words: systemic sclerosis, replication study, *ATP8B4*

Over the past seven years our knowledge of the systemic sclerosis (SSc) genetic component has considerably increased, mainly thanks to large genetic studies including genome wide association studies (GWASs) and Immunochip analysis (1-3). However, there is still a large portion of the SSc heritability that remains unexplained, as for most complex traits (4). One of the hypotheses proposed to explain the missing heritability for complex diseases involves rare and low-frequency variants. These types of genetic variations are not well covered by GWAS, which are mainly focused on common variants. However next-generation sequencing technologies, like whole exome sequencing (WES), have rapidly overcome this issue. In this regard, Gao *et al*. performed for the first time WES on SSc and reported a novel gene, *ATP8B4*, as a risk factor for the disease (5). They suggested a missense rare variant (F436L [rs55687265]) as a potential causal variant for the association signal in *ATP8B4*. Therefore our aim was to further evaluate the reported signal of association taking advantage of our large SSc cohorts.

ATP8B4 rs55687265 rare variant was genotyped in six independent case-control sets of European ancestry reaching a total of 7,426 SSc patients and 13,087 healthy controls (Suppl. Table 1). All SSc patients fulfilled previously described classification criteria for the disease (3). First, we performed association analyses to test whether *ATP8B4* rs55687265 was associated with SSc susceptibility in each of the cohorts included in the present study (Supp Methods). A trend towards association was observed for the Spanish case-control set (P_{value} = 0.071, OR = 1.58) (Table 1). However, we did not observe any suggestive or significant signal of association for the remaining cohorts. We also observed opposite-direction allelic effects across the different case-control sets. The meta-analysis combining all the sample sets, which was performed using an inverse variance fixed effects model, showed no significant association with the disease (P_{meta} = 0.484, OR = 1.07) (Table 1 and

3

Supp Methods). Thus, according to our results, we did not find statistically significant differences of the *ATP8B4* rs55687265*C allele frequency between the SSc patients and controls enrolled in the study. It is noteworthy that the impact of rare variants in the development of autoimmune diseases (ADs) remains an unanswered and controversial question (6). Moreover, high throughput DNA sequencing technologies, such as WES, are substantially affected by technical artifacts, which may lead to type I errors (7). This issue becomes especially important when cases and controls have not been exactly sequenced in the same way, which leads to excessive false positive findings in detecting rare variant associations (7-9). The present study highlights the importance of validation of WES results with other sequencing methods as well as replication of the new associations in independent studies in order to detect actual disease-causing mutations.

In conclusion, this study could not replicate the association of *ATP8B4* rs55687265. However, the possible role of this gene cannot be discarded, since the present report did not attempt to evaluate whether other rare or common variants were associated with SSc susceptibility.

ACKNOWLEDGMENTS

REFERENCES

1.   Radstake TR, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, Palomino-Morales R, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. Nat Genet. 2010;42:426-9.

2.   Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet. 2011;7:e1002091.

3.   Mayes MD, Bossini-Castillo L, Gorlova O, Martin JE, Zhou X, Chen WV, et al. Immunochip analysis identifies multiple susceptibility loci for systemic sclerosis. Am J Hum Genet. 2014;94:47-61.

4.   Bossini-Castillo L, Lopez-Isac E, Martin J. Immunogenetics of systemic sclerosis: Defining heritability, functional variants and shared-autoimmunity pathways. J Autoimmun. 2015:53-65.

5.   Gao L, Emond MJ, Louie T, Cheadle C, Berger AE, Rafaels N, et al. Identification of Rare Variants in ATP8B4 as a Risk Factor for Systemic Sclerosis by Whole-Exome Sequencing. Arthritis Rheumatol. 2016;68:191-200.

6.   Hunt KA, Mistry V, Bockett NA, Ahmad T, Ban M, Barker JN, et al. Negligible impact of rare autoimmune-locus coding-region variants on missing heritability. Nature. 2013;498:232-5.

7.   Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17:333-51.

8.   Hu YJ, Liao P, Johnston HR, Allen AS, Satten GA. Testing Rare-Variant Association without Calling Genotypes Allows for Systematic Differences in Sequencing between Cases and Controls. PLoS Genet. 2016;12:e1006040.

9.   Johnston HR, Hu Y, Cutler DJ. Population genetics identifies challenges in analyzing rare variants. Genet Epidemiol. 2015;39:145-8.

Table 1. Association analysis of *ATP8B4* F436L variant in six independent cohorts and meta-analysis.

Cohort	Minor/Major	Cases/Controls	MAF Cases	MAF Controls	*P*-value	OR* [CI 95%]
Spain	C/G	2,056/2,718	0.008	0.005	0.071	1.58 [0.96-2.61]
Germany	C/G	909/486	0.019	0.022	0.613	0.87 [0.50-1.50]
The Netherlands	C/G	435/783	0.013	0.007	0.185	1.78 [0.75-4.20]
Italy	C/G	1,114/980	0.006	0.011	0.096	0.56 [0.28-1.12]
UK	C/G	1,456/5,272	0.012	0.011	0.532	1.13 [0.77-1.67]
USA	C/G	1,456/2,848	0.015	0.015	0.991	1.00 [0.69-1.45]
Meta-analysis**	C/G	7,426/13,087			0.484	1.07 [0.88-1.31]

*Odds ratio for the minor allele.

**Q heterogeneity value = 0.17; I = 35.73

CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.

SUPPLEMENTARY MATERIAL

Supplementary methods

1. Genotyping methods

The genotyping of *ATP8B4* rs55687265 was performed with TaqMan SNP genotyping technology (assay ID: AHI1051) in a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Genotyping call rate was > 95%. All the samples were genotyped by TaqMan assay, with the exception of the UK and USA control cohorts. For these two control sets, genotyping data were obtained from previously published studies (WTCCC and Lung Health Study, respectively) (1-2).

2. Data analysis

All the statistical analyses were carried out with PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/). Association test for the six cohorts were performed in each population by 2x2 contingency tables and χ2 test. *P*-values lower than 0.05 were considered as statistically significant. The meta-analysis was performed with inverse-variance method under a fixed-effects model. Heterogeneity of the ORs across cohorts was assessed using Cochran's Q test. None of the included control sets showed significant deviation from Hardy Weinberg Equilibrium (HWE) (HWE *P*-values < 0.01 were considered to show significant deviation from equilibrium). The statistical power of the combined analysis is shown in Supp. Table 2 and was calculated according to Power Calculator for Genetic Studies 2006 software under an additive model (3).

Supplementary tables

Supp. Table 1. Study cohorts

COHORT	Patients with SSc	Controls
Spain	2,056	2,718
Germany	909	486
The Netherlands	435	783
Italy	1,114	980
UK	1,456	5,272
USA	1,456	2,848
TOTAL	**7,426**	**13,087**

Supp. Table 2. Overall statistical power of the study for a statistical significance *P*-value = 0.05.

	OR = 1.2	OR = 1.5	OR = 1.8	OR = 2.2	OR = 2.5	OR = 2.8
rs55687265	43%	99%	100%	100%	100%	100%

Supplementary note

Spanish Scleroderma Group

Raquel Ríos and **Jose Luis Callejas**, Unidad de Enfermedades Sistémicas Autoinmunes, Department of Internal Medicine, Hospital Clínico Universitario San Cecilio, Granada; **José Antonio Vargas Hitos**, Department of Internal Medicine, Hospital Virgen de las Nieves, Granada; **Rosa García Portales**, Department of Rheumatology, Hospital Virgen de la Victoria, Málaga; **María Teresa Camps**, Department of Internal Medicine, Hospital Carlos Haya, Málaga; **Antonio Fernández-Nebro**, Department of Rheumatology, Hospital Carlos Haya, Málaga; **María F. González-Escribano**, Department of Immunology, Hospital Virgen del Rocío, Sevilla; **Francisco José García-Hernández** and **Mª Jesús Castillo**, Department of Internal Medicine, Hospital Virgen del Rocío, Sevilla; **Mª Ángeles Aguirre** and **Inmaculada Gómez-Gracia**, Department of Rheumatology, Hospital Reina Sofía/IMIBIC, Córdoba; **Patricia Carreira,** Department of Rheumatology. Hospital Universitario 12 de Octubre, Madrid; **Benjamín Fernández-Gutiérrez** and **Luis Rodríguez-Rodríguez**, Department of Rheumatology, Hospital Clínico San Carlos, Madrid; **José Luis Andreu** and **Mónica Fernández de Castro**, Department of Rheumatology, Hospital Puerta de Hierro Majadahonda, Madrid; **Paloma García de la Peña**, Department of Rheumatology, Hospital Madrid Norte Sanchinarro, Madrid; **Francisco Javier López-Longo** and **Lina Martínez**, Department of Rheumatology, Hospital General Universitario Gregorio Marañón, Madrid; **Vicente Fonollosa**, **Alfredo Guillén**, Department of Internal Medicine, Hospital Valle de Hebrón, Barcelona; **Gerard Espinosa**, Department of Internal Medicine, Hospital Clinic, Barcelona; **Iván Castellví,** Department of Rheumatology, Hospital de la Santa Creu i Sant Pau, Barcelona; **Anna Pros**, Department of Rheumatology, Hospital Del Mar, Barcelona; **Mónica Rodríguez Carballeira**, Department of Internal

Medicine, Hospital Universitari Mútua Terrasa, Barcelona; **Francisco Javier Narváez**, Department of Rheumatology, Hospital Universitari de Bellvitge, Barcelona; **Vera Ortiz-Santamaría**, Department of Rheumatology, Hospital General de Granollers, Granollers; **Ana Belén Madroñero,** Department of Internal Medicine, Hospital General San Jorge, Huesca. **Miguel Ángel González-Gay**, Epidemiology, Genetics and Atherosclerosis Research Group on Systemic Inflammatory Diseases, DIVAL, University of Cantabria, Santander; **Bernardino Díaz** and **Luis Trapiella**, Department of Internal Medicine, Hospital Central de Asturias, Oviedo; **Mayka Freire** and **Adrián Sousa**, Unidad de Trombosis y Vasculitis, Department of Internal Medicine, Hospital Xeral-Complexo Hospitalario Universitario de Vigo, Vigo; **María Victoria Egurbide**, Department of Internal Medicine, Hospital Universitario Cruces, Barakaldo; **Patricia Fanlo Mateo**, Department of Internal Medicine Hospital Virgen del Camino, Pamplona; **Luis Sáez-Comet**, Unidad de Enfermedades Autoinmunes Sistémicas, Department of Internal Medicine, Hospital Universitario Miguel Servet, Zaragoza; **Federico Díaz** and **Vanesa Hernández**, Department of Rheumatology, Hospital Universitario de Canarias, Tenerife; **Emma Beltrán**, Department of Rheumatology, Hospital General Universitario de Valencia, Valencia; **Elena Grau**, Department of Rheumatology, Hospital Universitario y Politécnico La Fe, Valencia; **Juan José Alegre Sancho**, Department of Rheumatology, Hospital del Doctor Peset, Valencia; **Francisco J. Blanco García** and **Natividad Oreiro**, Department of Rheumatology, INIBIC-Hospital Universitario A Coruña, La Coruña.

SUPPLEMENTARY REFERENCES

1.   Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet. 2010;42:508-14.

2.   Hansel NN, Ruczinski I, Rafaels N, Sin DD, Daley D, Malinina A, et al. Genome-wide study identifies two loci associated with lung function decline in mild to moderate COPD. Hum Genet. 2013;132:79-90.

3.   Skol AD, Scott LJ, Abecasis GR, Boehnke M. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet. 2006;38:209-13.