No Major Host Genetic Risk Factor Contributed to A(H1N1)2009 Influenza Severity

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¶ Membership in the CIBERESP Cases and Controls in Pandemic Influenza Working Group is provided in the acknowledgments.

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Abstract

While most patients affected by the influenza A(H1N1) pandemic experienced mild symptoms, a small fraction required hospitalization, often without concomitant factors that could explain such a severe course. We hypothesize that host genetic factors could contribute to aggravate the disease. To test this hypothesis, we compared the allele frequencies of 547,296 genome-wide single nucleotide polymorphisms (SNPs) between 49 severe and 107 mild confirmed influenza A cases, as well as against a general population sample of 549 individuals. When comparing severe vs. mild influenza A cases, only one SNP was close to the conventional p = 5x10^-8. This SNP, rs28454025, sits in an intron of the GSK233 gene, which is involved in a neural development, but seems not to have any connections with immunological or inflammatory functions. Indirectly, a previous association reported with CD55 was replicated. Although sample sizes are low, we show that the statistical power in our design was sufficient to detect highly-penetrant, quasi-Mendelian genetic

Citation: Garcia-Etxebarria K, Bracho MA, Galán JC, Pumarola T, Castilla J, Ortiz de Lejarazu R, et al. (2015) No Major Host Genetic Risk Factor Contributed to A(H1N1)2009 Influenza Severity. PLoS ONE 10(9): e0135983. doi:10.1371/journal.pone.0135983

Editor: Hiroshi Nishiura, The University of Tokyo, JAPAN

Received: June 15, 2015

Accepted: July 28, 2015

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Data Availability Statement: The primary data used in association analyses, namely allele frequencies in cases and controls, have been deposited in the GWAS Central repository as study HGVST1832 (http://www.gwascentral.org/study/HGVST1832) and in the figshare repository (http://dx.doi.org/10.6084/m9.figshare.1528227).

Funding: Fundig for this work came from Instituto de Salud Carlos III (Madrid, Spain; www.isciii.es) grant GR09/0032. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
factors. Hence, and assuming that rs28454025 is likely to be a false positive, no major genetic factor was detected that could explain poor influenza A course.

**Introduction**

In 2009, the influenza A(H1N1)2009 pandemic swept the globe. Some of its features caused concern, such as a higher mortality risk in infants and children than in seasonal influenza epidemics, and activity peaks out of the cold season. Although it followed a mild course in most patients, in others it was much more aggressive for then unknown reasons. In Spain, a collection of severe, hospitalized patients was compared to a series of controls (defined as confirmed but mild influenza cases that were treated ambulatorily), and a number of sociodemographic and health risk factors were identified [1]. Yet, 37.2% of the severe cases showed no clinical or sociodemographic risk factor for severe influenza.

Host genetic factors may be a contributor to influenza severity. Two genealogy studies in Utah and Iceland clearly demonstrated familial aggregation of the risk of influenza-associated death [2]. Albright et al. [3] compiled 4855 deaths due to influenza from a Utah database between 1904 and 2004, and observed that the relative risk for relatives dying of influenza was larger than for spouses. Thus, on top of the risk due to cohabitation and shared sociodemographic and environmental factors, the authors concluded that the risk of dying from influenza is heritable. On the contrary, in the 1918 influenza epidemic in Iceland [4], the 455 deaths showed no increased risk for the cases’ relatives when compared to spouse’s relatives. Notice that the sample in the Icelandic study was an order of magnitude smaller than the Utah sample, and its statistical power was presumably smaller.

Several studies have tackled human genetic variation in relation to influenza. A group of candidate genes includes the pro-inflammatory cytokines and chemokines. Morales-García et al. [5] compared Mexican influenza A patients and matching, co-inhabiting controls and found that single nucleotide polymorphisms (SNPs) in the TNF and LTA genes contributed to developing the disease, with an OR as high as 27 (95% CI 3–1248) for rs361525/C3 AA in TNF. This allele was also found to be associated with influenza A in Greek patients, and in particular with those developing pneumonia (OR 3.74, 95% CI 1.06–13.25) [6]. However, it was not associated with fatal influenza (regardless of type, and accumulated over 10 influenza seasons) in 105 US children and young adults [7]. Another candidate gene is the interferon-inducible transmembrane protein IFITM3, which was shown to be essential against the influenza A virus in mice in vivo. A sample of 53 severe UK patients showed higher frequencies of the minor allele in an IFITM3 SNP, namely rs12252, compared to population databases comprising 3,000–9,000 individuals [8]. Moreover, genetic variation around this SNP revealed the footprints of recent, positive natural selection in Europeans but not in Asians or Africans [8].

A genome-wide association study (GWAS) indicated the complement regulatory protein CD55 as a possible candidate for severe A influenza [9]; indeed, genotype rs2564978’T’/T in CD55 showed an OR = 1.75 (P = 0.011) in 177 severe vs. 248 mild Chinese A(H1N1) cases [9]. Finally, a GWAS was also performed in 91 severe Mexican patients vs. 98 exposed but asymptomatic controls [10], by using an array designed for genes related with cardiovascular diseases. Four genome regions were identified as putative candidates to contribute to infectivity and/or severity; these regions contained genes for the immunoglobulin-related FCGR2A, the RPA interacting protein, and the complement-binding C1QBP protein, with ORs up to 2.63.
The goal of the present study is to explore the existence of major genetic determinants of influenza A(H1N1) severity by comparing the genotypes of a dense array of genomewide SNPs in 49 severe and 107 mild influenza patients from Spain, and in a general population sample of 549 individuals. To the best of our knowledge, this is the first attempt to analyze genetic factors associated to influenza infection in a population of European ancestry.

**Material and Methods**

**Samples**

Cases were defined as confirmed influenza A(H1N1)2009 patients who had to be admitted to a hospital. Genotypes were successfully obtained for 49 cases (27 from the Hospital Clínico de Valladolid, 17 from Hospital Ramón y Cajal, Madrid, and 5 from Hospital Virgen del Camino, Pamplona, all in Spain; 27 were women). Controls were also confirmed influenza patients with mild symptoms who received ambulatory assistance; they were recruited as part of the Spanish Influenza Case-Control Study cohort during the 2009–2010 first epidemic season of the new virus. 107 controls (56 women) were successfully genotyped: 28 originated from the PIDIRAC primary care influenza network in Catalonia, and 79 from the Red Centinela and Hospital Clínico in Valencia. Additionally, a sample from the general population was used in some comparisons. It consisted of 549 DNA samples from subjects that self-reported having at least two generations of ancestors born in Spain and without personal or familiar history of chronic diseases. These individuals were obtained from the CeGen-PRB2-ISCIII project, and consisted of unrelated healthy adult individuals collected from diverse geographic locations of Spain by Fundacion Publica Galega de Medicina Xeno, and with appropriate written informed consent was obtained from all participants in this study, which was approved by the Clinical Research Ethics Committee-Parc de Salut Mar, Barcelona.

**Influenza A virus detection and sample genotyping**

Samples were divided into two aliquots to extract DNA and RNA separately. To extract DNA, samples were first incubated for an hour with a lysis buffer plus proteinase K to digest the cells membranes, followed by a standard phenol-chloroform purification and ethanol precipitation. RNA was extracted by incubating first with TRIzol reagent (phenol/guanidine isothiocyanate), then adding chloroform followed by a centrifugation step to recover the aqueous phase. We then incubated this with isopropanol for an hour followed by centrifugation and ethanol wash. Finally, the RNA pellet was diluted in RNase-free water. The WHO RT-PCR protocol [11] was followed to confirm infection by influenza A (H1N1)2009 virus. Samples were genotyped with the Affymetrix Axiom Genome-Wide Human CEU Array, which contains 587,353 genome-wide SNPs and indels.

**Statistical analyses**

Genotypes were called with the Affymetrix Power Tools 1.14.4 provided by the manufacturer; all samples were analyzed together to avoid array biases. A total of 156 samples (49 cases of influenza A and 107 mild cases) passed the quality controls suggested by the manufacturer. In addition, SNPs that failed in more than the 5% of samples were discarded, with 547,296 SNPs remaining. Allele frequencies have been deposited in GWAS Central as study HGVST1832 (http://www.gwascentral.org/study/HGVST1832) and in the figshare repository (http://dx.doi.org/10.6084/m9.figshare.1528227)

A genome-wide association study between severe and mild influenza cases was carried out using the program Plink 1.07 [12]. In addition, 549 population samples from Galicia (NW
Spain) were used as controls. The significance threshold for multiple test correction was set at \(5 \times 10^{-8}\), as suggested by [13].

Population stratification was estimated with two approaches: identity-by-state among individuals was computed with PLINK 1.07 and subsequently plotted with multidimensional scaling. Independently, a Bayesian approach to population stratification with Admixture 1.22 [14] was also used. Graphical representations of results were made using the R language [15].

**Results**

Population structure analysis using identity by state revealed a number of potential outliers: eight samples of severe influenza, 17 cases of mild influenza, and eight controls (Fig 1), which were removed from association analyses. ADMIXTURE analysis (S1 Fig) did not show any discernible pattern, and the optimal number of parental populations was \(K = 1\), implying that cases and controls can be considered as having been sampled from the same population.

The genome-wide association study between severe influenza A (41 cases) and mild influenza A (90 cases) revealed one SNP (rs28454025) slightly above multiple-testing significance level (unadjusted \(p = 5.595 \times 10^{-8}\); the OR could not be computed since minor allele frequencies were 0.1579 in cases but 0 in controls) as shown in Fig 2. This SNP is located in an intron of
the SGK223 gene (homolog of rat pragma of Rnd2), which seems to regulate neurite outgrowth.

In addition, 10 SNPs had $p < 10^{-5}$ (S1 Table). Three of these SNPs were in introns of NAA-LADL2 (N-acetylated alpha-linked acidic dipeptidase-like 2), at a maximum distance of 67.9 Kb between each other. This gene has been associated with, among other diseases, systemic lupus erythematosus[16] and Kawasaki’s disease[17], both autoimmune disorders. Two other SNPs were in introns of MAML2 (mastermind-like 2 (Drosophila)), a member of the Notch developmental pathway, and apparently unrelated to the immune function. We also found a 14-Kb block of 16 SNPs in the PARD3B gene (par-3 partitioning defective 3 homolog B (C. elegans)), involved in neurodevelopment) with $p < 10^{-3}$. The SNPs in this block had odd ratios between 2.1 and 2.9 and were ~1.4Mb downstream from CTLA4, a costimulatory molecule expressed by activated T cells that has been linked to a number of autoimmune diseases.

Next, we considered a subset of SNPs that were in candidate genes that had been previously associated with influenza A severity [8–10]. Unfortunately, none of the SNPs that had been previously associated were in the array we genotyped; therefore, we selected the SNPs that were
within 100 Kb of the previously associated SNPs. Note that we applied a hypothesis-specific Bonferroni correction, taking into account the number of proxy SNPs (ranging from 2 to 52) we found for each previously associated SNP. IFITM3 is the gene that has been most consistently associated with response to influenza A [8]; and, in particular, the rs12252 SNP. Our array contained 27 SNPs in the vicinity, and only one, namely rs4131943, had a nominally significant association with influenza A severity (p = 2.1×10^{-3}, OR = 2.60 95% CI 1.40–4.83); note that the hypothesis-specific number of tests was 27, and that the multiple testing correction would yield p = 0.0567. For CD55 [9], within 100 Kb of the reported SNP, only two SNPs were available in our array, one of which, rs2564978, was significantly associated with influenza severity (p = 0.00638, OR = 7.11, 95% CI 1.4–36). Out of 11 SNPs in the vicinity of FCGR2A [10], only one had a nominally significant association with influenza severity (rs7551957, p = 0.0288), which did not survive Bonferroni correction. For the SNPs in the RPAIN-C1QBP region, out of 52 SNPs in the vicinity, only two had p<0.05, with the smallest value (p = 0.0203, corresponding to rs28447573, which was not the closest to any of the two previously associated SNPs) far from surviving Bonferroni correction. In summary, we indirectly replicated the association for CD55, that for IFITM3 was close to significance, and we could not indirectly replicate the previous associations for FCGR2A, RPAIN, and C1QBP.

We also compared the two sets of influenza patients to the general population (S2–S4 Tables) and, given the larger sample size of the control population, more SNPs yielded significant association tests (Fig 3). In the GWAS of severe influenza A against the general population 34 SNPs had p<5×10^{-8} and were in Hardy-Weinberg equilibrium (p > 0.05) (Fig 3A). In the analysis of mild influenza A against the general population 35 SNPs (Fig 3B) met the same conditions, and in the analysis of all influenza cases against the general population 14 SNPs had significant differences (Fig 3C). Out of these SNPs, 31 were specific of severe influenza A cases and 23 of mild influenza A cases (Fig 3D). The 34 SNPs detected in the comparison between severe influenza cases and the general population comprised 16 intergenic SNPs, 14 intronic SNPs, two synonymous SNPs, and two nonsynonymous SNPs: rs11551002 in gene APLP1 (amyloid precursor-like protein 1, involved in neural function), and rs11216131 in BUD13, which participates in the regulation of serum lipid levels.

Next, we turned to the 49 genes that contained or were adjacent to the 34 SNPs with significant differences between severe cases and the general population (S2 Table). We screened their definitions in the Refseq database and their biological functions according to GO categories for involvement in immunity and inflammation, and the Genetic Association Database and CDC HuGe Published Literature for associations with autoimmune and inflammatory diseases. SNP rs1996377 was 3.09 Mb downstream from DPP10, which is associated with asthma [18]; and rs28447319 was 51.8 Kb upstream of B4GALT1, which is upregulated by proinflammatory TNFα [19].

**Discussion**

In a GWAS for genetic contributors to the severity of influenza A infection, we did not find any SNP with p<5×10^{-8}; the SNP with the lowest p-value (and one order of magnitude smaller than the next most associated SNP) was rs28454025, which lies in an intron of the SGK223 gene. This gene encodes an enzyme that belongs to the tyrosine protein kinase family. A similar protein in rat binds to Rho family GTPase 2 (Rnd2) and regulates neurite outgrowth via activation of Ras homolog gene family, member A (RhoA) [20]. SNPs in SGK223 have only been putatively associated with carotid artery disease [21], an association that has not been subsequently replicated. The association between rs28454025 and influenza A severity seems, then, implausible.
Previous studies had produced a number of SNPs that were associated with influenza infection or severity. None of those were contained in the array we genotyped, but, by using as proxies SNPs in the genomic vicinity, we indirectly replicated the association with CD55, while IFITM3 was close to statistical significance.

When we compared the severe influenza A cases with a much larger sample of the general population, 34 SNPs had significant allele frequency differences (p < 5×10^{-8}). However, the biological plausibility of most of these associations was tenuous (e.g. SNPs with distances > 100 Kb from the closest gene, or in or near genes with biological functions unlikely to be related to inflammation or immunity).

Sample sizes in our study were low: 41 severe and 90 mild influenza A cases. Still, it has sufficient statistical power to rule out common genetic variants as highly penetrant contributors to poor prognosis in influenza A infection. The prevalence of severe influenza A among influenza A cases without obvious risk factors was estimated as 0.26% in a Spanish population[22]. We used this figure and Genetic Power Calculator [23] to estimate that our study had 0.415...
power to detect a recessive variant with an allele frequency of 0.2 and an odds ratio of 25 with \( \alpha = 5 \times 10^{-8} \) and a dominant model; for a recessive model, power increased to 0.805. Admittedly, these parameters imply a penetrance sufficiently high so as that familial aggregation of non-cohabiting relatives may have been observed. In particular, in these conditions the relative risk for a sibling would be 1.562 in the dominant model and 2.404 in the recessive model.

In conclusion, our study did not detect what was powered to find, namely one or a few host genes with a major impact in poor influenza course. However, this does not rule out the presence of genes with a more limited contribution.

**Supporting Information**

**S1 Fig.** Population stratification analysis from \( K = 2 \) to \( K = 5 \). Top left, coefficient of variation for each \( K \) value.

(PPTX)

**S1 Table.** SNPs with association p-values < \( 10^{-5} \) when comparing mild and severe influenza cases. Only rs16954376 remained significant after Bonferroni correction.

(XLSX)

**S2 Table.** SNPs with significant association p-values (\( p < 5 \times 10^{-8} \)) when comparing severe influenza cases against a sample of the general population.

(XLSX)

**S3 Table.** SNPs with significant association p-values (\( p < 5 \times 10^{-8} \)) when comparing mild influenza cases against a sample of the general population.

(XLSX)

**S4 Table.** SNPs with significant association p-values (\( p < 5 \times 10^{-8} \)) when comparing all influenza cases against a sample of the general population.

(XLSX)

**Acknowledgments**

Genotyping services were provided by Centro Nacional de Genotipado—Plataforma de Recursos Biomoleculares y Bioinformáticos—Instituto de Salud Carlos III (CeGen-PRB2-ISCIII), Santiago de Compostela, Spain. We want to express our gratitude to all donors who contributed their samples as well as to their general practitioners. The members of the CIBERESP Cases and Controls in Pandemic Influenza Working Group are: **Andalusia:** E. Azor, J. Carrillo, R. Moyano, J.A. Navarro, M. Vázquez, F. Zafra (Red de Médicos Centinela, Andalucía), M.A. Bueno, M.L. Gómez, M. Mariscal, B. Métez, J.P. Quesada, M. Sillero (Complejo Hospitalario de Jaén), M. Carnero, J. Fernández-Crehuet, J. del Diego Salas (Hospital Virgen de la Victoria), M. Delgado Rodríguez (CIBERESP, Universidad de Jaén), V. Fuentes (Hospital Costa del Sol), V. Gallardo, E. Pérez (Servicio de Epidemiología, Junta de Andalucía), R. López (Hospital Infanta Elena, Huelva), J.R. Maldonado (Hospital de Torrecárdenas), J.M. Mayoral (Servicio de Vigilancia de Andalucía), Á. Morillo (Hospital Virgen del Rocío), J.M. Navarro, M. Pérez (Laboratorio de Referencia de Gripe), S. Oña (Hospital Carlos Haya), M.J. Pérez (Hospital Virgen de Valme), M.C. Ubagó (Hospital Virgen de las Nieves), M. Zarruela (Hospital Puerta del Mar). **Valencian Community:** J. Blanquer (Hospital Clínico Universitario, Valencia), F. González Candelas (CIBERESP, CSISP-Universitat de València), M. Morales (CIBERESP, CSISP-Universitat de València, Hospital Doctor Peset). **Castile and Leon:** D. Carriedo, F. Díez, I. Fernández, S. Fernández, M.P. Sanz (Complejo Asistencial Universitario de León), J.J. Castrodeza, A. Pérez, S. Tamames (Dirección General de Salud Pública, Desarrollo e Innovación, Junta de
Castilla y León), V. Martín (CIBERESP, Universidad de León), A. Molina (Instituto de Biomedicina, Universidad de León), J. Ortiz de Saracho (Hospital del Bierzo), R. Ortiz de Lejarazu (Centro Nacional de Gripe, Valladolid), A. Pueyo, J.L. Viejo (Complejo Asistencial, Burgos), P. Redondo (Servicio Territorial de Sanidad y Bienestar Social, León). Catalonia: A. Agustí, A. Torres, A. Trilla, A. Vilella (Hospital Clinic de Barcelona), J. Alonso, O. Garín (CIBERESP, Universitat Pompeu Fabra, IMIM-Instituto Recerca Hospital del Mar), F. Barbé (Hospital Arnau de Vilanova), M. Baricot, N. Soldevila (CIBERESP), L. Blanch, G. Navarro (Hospital de Sabadell), X. Bonfill, J. López-Contreras, V. Pomar, M.T. Puig (Hospital de Sant Pau), E. Borras, A. Martínez, Núria Torner (Dirección General de Salud Pública, Generalitat de Catalunya), C. Bravo, F. Moraga (Hospital Vall d’Hebrón), F. Calafell (Universitat Pompeu Fabra), J. Caylà, C. Tortajada (Agencia de Salud Pública de Barcelona), A. Domínguez (CIBERESP, Universitat de Barcelona), I. García, J. Ruiz (Hospital Germans Trias i Pujol), J.J. García (Hospital Sant Joan de Deu), (Universitat Pompeu Fabra), J. Gea, J.P. Horcajada (Hospital del Mar), P. Godoy (CIBERESP, Departament de Salut Generalitat de Catalunya), N. Hayes (Hospital Clinic-CRESIB), T. Pumarola (REIPI, Universitat de Barcelona), A. Rosell (Hospital de Bellvitge), M. Sáez (CIBERESP, Universitat de Girona).

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Author Contributions
Conceived and designed the experiments: KGE FGC FC. Performed the experiments: MAB IQ NB MGG. Analyzed the data: KGE FC. Contributed reagents/materials/analysis tools: JCG TP JC ROdL MRD AD FGC. Wrote the paper: KGE FGC FC.

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