An original Eurasian haplotype, HLA-DRB1*14:54-DQB1*05:03, influences the susceptibility to idiopathic achalasia

Janette Furuzawa-Carballeda1*, Joaquín Zuñiga2,3,*, Diana I. Hernández-Zaragoza4, Rodrigo Barquera5, Eduardo Marques-García2, Luis Jiménez-Alvarez2, Alfredo Cruz-Lagunas2, Gustavo Ramírez3, Nora E. Regino5, Ramón Espinosa-Soto3, Edmond J. Yunis6, Fernanda Romero-Hernández3, Daniel Azamar-Llamas1, Enrique Coss-Adame7, Miguel A. Valdivinos5, Samuel Torres-Landa6, Axel Palacios-Ramírez6, Blanca Breña6, Edgar Alejandro-Medrano6, Axel Hernández-Ávila6, Julián Granados6, Gonzalo Torres-Villalobos6,8*

1 Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico, 2 Laboratory of Immunobiology and Genetics, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico, 3 School of Medicine and Health Sciences, Instituto Tecnológico y de Estudios Superiores de Monterrey, Mexico City, Mexico, 4 Department of Archaeogenetics, Max Planck Institute for the Science of Human History, Jena, Germany, 5 Department of Cancer Immunology and AIDS, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, United States of America, 6 Department of Experimental Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico, 7 Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico, 8 Department of Transplants, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico, 9 Department of Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico.

☯ These authors contributed equally to this work.

* torresvgm@yahoo.com.mx (GTV); joazu@yahoo.com (JZ)

Abstract

Idiopathic achalasia is a relatively infrequent esophageal motor disorder for which major histocompatibility complex (MHC) genes are well-identified risk factors. However, no information about HLA-achalasia susceptibility in Mexicans has previously been reported. We studied a group of 91 patients diagnosed with achalasia and 234 healthy controls with Mexican admixed ancestry. HLA alleles and conserved extended haplotypes were analyzed using high-resolution HLA typing based on Sanger and next-generation sequencing technologies. Admixture estimates were determined using HLA-B and short tandem repeats. Results were analyzed by non-parametric statistical analysis and Bonferroni correction. P-values < 0.05 were considered significant. Patients with achalasia had 56.7% Native American genes, 24.7% European genes, 16.5% African genes and 2.0% Asian genes, which was comparable with the estimates in the controls. Significant increases in the frequencies of alleles DRB1*14:54 and DQB1*05:03 and the extended haplotypes DRB1*14:54-DQB1*05:03 and DRB1*11:01-DQB1*03:01, even after Bonferroni correction (pC<0.05), were found in the achalasia group compared to those in the controls. Concluding, the HLA class II alleles HLA-DRB1*14:54-DQB1*05:03 and DQB1*05:03:01 and the extended haplotype are risk factors for achalasia in mixed-ancestry Mexican individuals. These results also suggest that the HLA-DRB1*14:54-DQB1*05:03 haplotype was introduced by admixture with European and/or Asian populations.
data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** ANAs, antinuclear antibodies; CEH, conserved extended haplotype; CI, confidence interval; EH, expected heterozygosity; EM, expectation-maximization; GF, gene frequency; GSSP, group-specific sequencing primer; HF, haplotype frequency; HLA, human leukocyte antigen; HSV-1, herpes simplex virus 1; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; LES, lower esophageal sphincter; CI, confidence interval; EH, expected heterozygosity; OR, odds ratio; PBMC, peripheral blood mononuclear cells; PCr, p-corrected value; PCR, polymerase chain reaction; PD, power of discrimination; PIC, polymorphism information content; PV, pemphigus vulgaris; RA, rheumatoid arthritis; SBM, sequence-based method; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; STR, short tandem repeats; Δ, delta value; Δ', relative delta value.

**Introduction**

Idiopathic achalasia is a relatively uncommon major motility disorder of the esophagus that inflicts substantial morbidity upon affected individuals [1]. Clinically, idiopathic achalasia is characterized by dysphagia with aperistalsis and the absence of lower esophageal sphincter (LES) relaxation [2]. While the factors that influence achalasia development remain to be completely elucidated, viral, autoimmune and genetic host factor influences have been studied [3]. In this regard, familial aggregation in twin studies has suggested that genetic factors play an important role in the pathogenesis of this condition [4]. The contributions of major histocompatibility complex (MHC) class II loci in achalasia were first explored by Wong et al. in 1989 [5], showing an association of DQw1 with higher susceptibility to the disease. In 1998, De la Concha and colleagues found a significant association between the allele HLA-DQA1*01:01 and a protective role of HLA-DQB1*02 in a small group of Caucasian European patients [6]. Other studies have described a protective effect of the conserved extended haplotype (CEH) DRB1*15:01-DQA1*01:02-DQB1*06:02 [7]. Some association studies in large cohorts of achalasia patients with central European ancestry have demonstrated that haplotypes bearing HLA-DQB1*05:03 and DQB1*06:01 are linked with susceptibility to achalasia [8]. Additionally, differences in the distribution of these achalasia-risk HLA class II alleles among Central Europeans appear to influence the prevalence of the disease in Europe [9].

The frequency of CEHs and specific block combinations of HLA genes varies between major ethnic groups and/or different continental ancestries, and these variations can be used as measurements of MHC genetic diversity in autoimmune conditions [10,11]. Various population genetics studies have revealed that Mexican mixed-ancestry populations have complex genetic structures with contributions from Native American (50–60%), European (25–40%), African (4–12%), and more recently, Asian (1%) biological roots [11–14]. In this context, the role of HLA and ethnic backgrounds in the susceptibility of Mexicans to achalasia has not been explored, and we believe that the identification of specific HLA haplotypes associated with achalasia in Mexicans will be helpful for understanding the genetic background related to this condition. Thus, this study aimed to describe the distribution of HLA class I and class II alleles and CEHs and their most likely ancestral origins using high-resolution HLA typing in a group of Mexican mixed-ancestry patients with achalasia.

**Materials and methods**

**Patient samples**

Eligible patients included those born in Mexico whose parents and grandparents were also born in Mexico, with a diagnosis of idiopathic achalasia as described below. The diagnosis of achalasia was based on clinical evaluations as well as on esophagram, high-resolution manometry (classified based on Chicago v3.0) [15] and endoscopy results. All patients were recruited between 2014 and 2017 from the Outpatient Clinics of Gastroenterology and Surgery of the National Institute for Medical Sciences and Nutrition Salvador Zubirán in Mexico City, which is a referral center for this condition. We excluded patients from study participation according to diagnosis of secondary achalasia due to Chagas disease, esophageal stricture, gastric, esophageal cancer or esophageal scleroderma. A total of 182 HLA class I and class II haplotypes from 91 patients diagnosed with achalasia were analyzed in this study. All achalasia patients were of Mexican ancestry, and admixture estimations using HLA markers revealed a greater proportion of Native American genetic contributions, followed by an important component of European alleles.

As the control group, 234 unrelated Mexican admixed individuals were studied, including a group of 40 Mexican admixed families, providing a total of 468 haplotypes for this HLA-disease
association study. All participants had Mexican ancestry, and their parents and grandparents were born in Mexico. Admixture estimations using HLA-B and short tandem repeats (STRs) were performed in this group of controls to determine whether their genetic backgrounds was comparable to that of achalasia patients.

Ethics statement
The Institutional Review Board of the National Institute for Medical Sciences and Nutrition Salvador Zubirán (INCMNSZ) and the National Institute for Respiratory Diseases (INER) reviewed and approved the protocols for genetic studies. All subjects provided written informed consent for these studies, and they authorized the storage of their DNA samples at INER or INCMNSZ repositories for this and future studies. In this study, we collected samples only from adults older than 18 years.

Sanger sequencing-based HLA typing
Genomic DNA was obtained from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). High-resolution HLA class I and class II typing was performed using a sequence-based method (SBM) as described previously [11,16,17]. Briefly, we amplified exons 2 and 3 from HLA-A, HLA-B and HLA-C and exon 2 from HLA-DRB1 and HLA-DQB1. Polymerase chain reactions (PCRs) utilized 1.5 mm KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 200 mM dNTPs, 10 μM of each primer, 30 ng of DNA and 0.5 U of Taq DNA polymerase in a final volume of 25 μl. Amplifications were performed on a PE9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: 95˚C for 30 s, 65˚C for 30 s, 72˚C for 1 min, preceded by 5 min at 95˚C and followed by a final elongation step at 72˚C for 5 min. The amplified products were sequenced independently in both directions using BigDye Terminator™ chemistry on the ABI PRISM® 3730xl Genetic Analyzer (Applied Biosystems). Data were analyzed with match tools allele assignment software (Applied Biosystems) using the IMGT/HLA sequence database alignment tool (http://www.ebi.ac.uk/imgt/hla/align.html). Ambiguities were solved using group-specific sequencing primers (GSSPs) that had been previously reported and validated [16,17].

High-resolution HLA typing by next-generation sequencing
We also used the next-generation sequencing Trusight Illumina (Illumina, San Diego, CA, USA) HLA system to confirm HLA allele-level typing. Briefly, genomic DNA samples from achalasia patients were adjusted to a working concentration of 10 ng/μl using the real-time PCR assay Qubit BR and Qubit equipment (Thermo Fisher Scientific, Waltham, MA, USA). Long-range PCR templates of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci were prepared using specific primers included in the Trusight HLA Pre 24 sample kit (Illumina) and MasterAmp™ Extra-Long DNA Polymerase (Lucien Corporation, Middleton, WI, USA). PCR reactions were performed in a 96-well plate on the 9700 PE thermal cycler (Applied Biosystems/Thermo Fisher Scientific) under the following PCR conditions: 25 μl of HPM (HLA-PCR Mix), 2 μl of MasterAmp™ Extra-Long DNA Polymerase, 13 μl of water and 5 μl of gDNA (10 ng/μl). For HLA-DQB1, the locus conditions were 94˚C for 3 min; followed by 10 cycles at 94˚C for 30 sec, 55˚C for min, 72˚C for 15 min; 20 cycles of 94˚C for 30 sec, 60˚C for 2 min, 72˚C for 15 min, 72˚C for 10 min; and a final hold at 10˚C. Simultaneously, the PCR reactions for HLA-A, HLA-B, HLA-C and DRB1 loci were performed under the following conditions: initial denaturation at 94˚C for 3 min, 30 cycles at 94˚C for 30 sec, 60˚C for 2 min, 68˚C for 15 min, 68˚C for 10 min and a final hold at 10˚C. PCR products were confirmed by 1% agarose gel electrophoresis. Using magnetic beads (LNA1, LNB1, Trusight HLA, Illumina), we proceeded to normalize the concentrations of the
PCR products of all loci for multiplex library preparation and next-generation sequencing. After normalization, 40 μl of each PCR product was used for fragmentation (800 and 1200 pb), and fragmentation buffers HTM and HTB (Trusight HLA Pre-PCR 24, Illumina) were added to the reaction (10 μl each) and incubated at 58°C for 12 min in the presence of sequencing primers. The purified fragmented PCR products were pooled, and adaptor addition was performed using the Nextera XT DNA sample preparation kit (Illumina). Amplification was performed under the following conditions: denaturation at 72°C for 3 min and 98°C for 30 sec, followed by 10 cycles at 98°C for 10 sec, 60°C for 30 sec, 72°C for 5 min, and a final hold at 10°C. Seven microliters of the PCR sequencing products were denatured with 10 μl of 0.1N NaOH and sequenced on a MiSeq instrument using the paired-end 300 cycle (2 x 150 bp paired-end) MiSeq Reagent Kit (Illumina).

**Next-generation sequencing data analysis**

After the sequencing, MiSeq Reporter analysis software generated FASTQ sequence files, BAM alignment files and allele calling were generated using Trusigh HLA ASSIGN 2.1 software (v2.1.0.943RUO.msi, Illumina). The software used reference sequences from the IMGT/HLA database (release 3.28.0).

**Assignation of HLA class I and class II conserved extended haplotypes (CEHs)**

Allele, haplotype and CEH HLA class I and class II frequencies at allelic resolution from both achalasia patients and controls were obtained by family segregation analysis. Maximum likelihood haplotype frequencies for two-point, three-point, four-point and five-point associations were estimated using an expectation-maximization (EM) algorithm provided by the computer program Arlequin ver. 3.1 [18]. Hardy-Weinberg equilibrium (HWE) at a locus-by-locus level was also calculated using this software. The polymorphism information content (PIC), observed heterozygosity (OH), and expected heterozygosity (EH) for each HLA class I and class II locus were also calculated. CEHs of known African, Asian and Caucasian origin were assigned based on previous reported frequencies in different ethnic groups including Mexican admixed and Native American populations [11]. Delta (Δ) and relative delta (Δ') values were calculated using previously described standardized methods to measure linkage disequilibrium (LD) [19], defined as the non-random association of alleles at two or more loci and their statistical significance <0.05.

**Admixture estimations using HLA genes**

Admixture estimations in achalasia patients and healthy controls were obtained by the maximum likelihood method using the population genetics Leadmix software [20] with k = 4 parental populations (Europe, Africa, Asia, and America) and HLA-B as the genetic estimator. European components were estimated based on HLA data from southern Portugal and USA inhabitants [21,22]. African components were calculated using parental populations from Nandi from Kenya [23], and Native American Mexican components were calculated using previously reported HLA data from Oaxaca Mixtecs, a population from southeastern Mexico [24], and Tarahumaras from Chihuahua in northern Mexico [25]. Finally, Southern Han Chinese data (N = 281) were used to mate the Asian contributions in both samples [26].

**Admixture estimations using STRs**

To estimate the genetic backgrounds of the healthy controls, we used the distribution of autosomal STR markers (CSF1PO, FGA, THO1, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179,
D13S317, D16S539, D18S51, D21S11, D19S433, and D2S1338) along with amelogenin using the Applied Biosystems AmpF/STR Identifier Kit (Applied Biosystems). PCR amplification and capillary electrophoresis were carried out as previously described [14]. An analysis of admixture estimation using STR data was performed using a model-based clustering method with Structure software v. 2.3.4 [27]. For this analysis, we assumed that \( k = 3 \) for the parental populations and performed 10,000 dememorization steps using STR data previously published in Spaniards [28], Fang Africans [29], and Native American Huastecos [30] and Tepehuas [31] populations from the central region of Mexico.

**Antinuclear antibodies (ANAs) testing**

Only patients in the idiopathic achalasia group newly diagnosed and without previous treatment donated blood samples, which were used for ANAs assessment by the indirect immunofluorescence of HEp-2 cells with the IgG isotype (Inova Diagnostics Inc., San Diego, CA, USA). Positivity was assigned according to our local cut-off values (i.e., speckled: > 1:160; nucleolar: > 1:40; cytoplasmic: > 1:40; mitochondrial: > 1:160; others: > 1:40) [32].

**Statistical analysis**

Differences in the frequencies of HLA class I and class II alleles as well as in HLA conserved haplotypes were analyzed using \( \chi^2 \) or Fisher’s exact test, and \( p \)-values less than 0.05 were considered statistically significant. If appropriate, the \( p \)-values were also corrected using the Bonferroni (for allele frequencies, multiplying the original \( p \)-value by the number of alleles) or Yates methods (for block and haplotype frequencies); odds ratios and 95% confidence intervals (CI) were calculated to measure association strength (EPIINFO v7 software).

Numerical variables were analyzed using Student’s \( t \)-test, and Pearson correlations were also calculated using SPSS software, version 15 (IBM Corp., Armonk, NY, USA).

The minimal data set necessary to replicate the study findings is in the Supporting Information files (S1 Data).

**Results**

**Clinical and demographic characteristics**

The clinical and demographic characteristics of the Mexican patients with achalasia are summarized in Table 1. The patients (66% female) had a mean age of 42.3 ± 15.8 years, while the mean age of the control patients (51% female) was 38.0 ± 15.0 years. As expected, 100% of the patients diagnosed with achalasia had dysphagia, 91% had regurgitation, 69% heartburn and 88% exhibited an important weight loss. The mean time of disease onset was 24.3 months, and the percentage of autoimmune disease among the achalasia patients was 13%. The most frequent associated disease was thyroid autoimmune disease (6.3%). Two patients (2.2%) had more than one concomitant autoimmune diseases (1 with both hyperthyroidism and vitiligo and 1 with hypothyroidism, scleroderma and rheumatoid arthritis), 1 patient (1.1%) had Sjögren’s syndrome, 1 patient (1.1%) had Guillain-Barré syndrome, and 1 patient (1.1%) had ankylosing spondylitis, as previously described by Romero-Hernández et al. [33] More than 25% of the patients had been exposed to wood smoke, and 33% had been exposed to tobacco smoke.

ANAs testing was performed on all patients with achalasia, showing positivity in 62 (68%). The most prevalent pattern observed was speckled, followed by nucleolar and homogenous.
HLA genetic diversity and admixture estimations revealed no differences in the proportions of Native American or European components between Mexican admixed patients with achalasia and healthy controls

Maximum likelihood analysis revealed that the HLA-DQB1 locus in patients with achalasia and the HLA-DRB1 locus in controls had marginal significant deviations from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($p < 0.05$). The degrees of HLA class I and class II locus polymorphisms in both the Mexican mixed-ancestry patients with achalasia and the controls were analyzed using PIC values $>0.5$. As expected, the HLA-B and HLA-DRB1 loci were the most polymorphic in both groups, whereas HLA-DQB1 was the least polymorphic locus. The power of discrimination (PD) values of each locus ranged from 0.9538 to 0.9856 in achalasia patients and from 0.9447 to 0.9907 in the controls (Table 2).

Admixture estimations using HLA-B as the genetic estimator revealed that among the patients with achalasia, 56.7% of their genes were of Native American descent, 24.7% were of European descent, 16.5% were of African descent and 2.0% were of Asian descent. In the healthy controls group, 59.8% Native American, 25.7% European, 14.1% African and 1.8% Asian contributions were found. These results were comparable with the estimates in the controls obtained using other polymorphic markers, such as STRs (Native American contribution: 60.5%, European: 25.9%, and African: 13.6%). These findings demonstrate that the achalasia patients and the controls had comparable genetic backgrounds in the context of their parental populations.

Table 1. Clinical and demographic information of Mexican patients with achalasia.

| Variable                  | Achalasia (n = 91) | Type I Achalasia (n = 15) | Type II Achalasia (n = 71) | Type III Achalasia (n = 5) |
|---------------------------|--------------------|---------------------------|---------------------------|---------------------------|
| Age, years, mean±SD       | 42.3±15.8          | 45.3±17.6                 | 41.1±14.9                 | 48.7±17.8                 |
| Female, n (%)             | 66 (73)            | 9 (60)                    | 53 (75)                   | 4 (80)                    |
| Male, n (%)               | 25 (27)            | 6 (40)                    | 18 (25)                   | 1 (20)                    |
| Dysphagia, n (%)          | 91 (100)           | 15 (100)                  | 71 (100)                  | 5 (100)                   |
| Regurgitation, n (%)      | 83 (91)            | 13 (87)                   | 66 (93)                   | 4 (80)                    |
| Heartburn, n (%)          | 63 (69)            | 5 (33)                    | 56 (79)                   | 2 (40)                    |
| Weight loss, n (%)        | 80 (88)            | 11 (73)                   | 64 (90)                   | 5 (100)                   |
| Time of disease diagnosis, months mean±SD | 24.3±35.1          | 38.0±37.5                 | 21.8±26.9                 | 11.6±7.8                  |
| Autoimmune disease, n (%) | 12 (13)            | 2 (13)                    | 8 (11)                    | 2 (40)                    |
| ANA autoantibodies, n (%) | 62 (68)            | 9 (60)                    | 49 (69)                   | 4 (80)                    |
| Wood smoke, n (%)         | 24 (25)            | 4 (27)                    | 18 (25)                   | 2 (40)                    |
| Tobacco use, n (%)        | 30 (33)            | 5 (33)                    | 19 (27)                   | 3 (60)                    |

https://doi.org/10.1371/journal.pone.0201676.t001

Table 2. Estimations of genetic diversity of HLA class I and class II loci in Mexican admixed individuals with achalasia and controls.

| HLA loci | Achalasia | Controls |
|----------|-----------|----------|
|          | O.H.      | E.H.     | pCorr | PIC | PD | O.H. | E.H. | pCorr | PIC | PD |
| HLA-A    | 0.8557    | 0.8918   | ns    | 0.8794 | 0.9740 | 0.8718 | 0.8919 | ns | 0.8776 | 0.9761 |
| HLA-B    | 0.9588    | 0.9698   | ns    | 0.9623 | 0.9856 | 0.9487 | 0.9668 | ns | 0.9544 | 0.9907 |
| HLA-C    | 0.8454    | 0.9012   | ns    | 0.8891 | 0.9723 | 0.9009 | 0.8947 | ns | 0.8845 | 0.9767 |
| HLA-DRB1 | 0.8969    | 0.9265   | ns    | 0.9171 | 0.9793 | 0.9013 | 0.9193 | 0.03 | 0.9123 | 0.9835 |
| HLA-DQB1 | 0.7835    | 0.8555   | 0.0100 | 0.8354 | 0.9538 | 0.8205 | 0.8256 | ns | 0.8020 | 0.9447 |

pCorr: p corrected value after Bonferroni correction, O.H.: Observed heterozygosity, E.H.: Expected heterozygosity, PIC: polymorphism information contents. PD: Power of discrimination

https://doi.org/10.1371/journal.pone.0201676.t002

An original Eurasian haplotype, HLA-DRB1*14:54-DQB1*05:03, influences the susceptibility to achalasia
Achalasia is associated with HLA class II alleles in Mexicans

The frequencies of HLA class I (HLA-A, HLA-B, HLA-C) alleles are summarized in S1, S2 and S3 Tables, respectively. The most frequent HLA-A alleles in both the achalasia and control groups were A*02:01, A*24:02 and A*02:06, with frequencies greater than 10%. For the HLA-B locus, the most common alleles in achalasia patients were B*39:05, B*35:01 and B*44:03, with gene frequencies of 0.0989, 0.0549 and 0.0495, respectively. Regarding the HLA-C locus, the most common alleles in the achalasia patients were C*07:02, C*04:01 and C*12:03, whereas in the controls, the most common alleles were C*07:02, C*04:01 and C*01:02. No significant differences in the distribution of HLA class I alleles among patients with achalasia and the controls were observed.

We found a significant increase in the frequency of the DRB1*14:54 allele in the achalasia group (G.F. = 0.0604) compared to that in the control group (G.F. = 0.0171, pC = 0.0200, OR = 3.7, 95% CI = 1.35–10.26). We also found a significant increase in the frequency of the HLA-DRB1*0407 allele (pC = 0.01, OR = 1.85; 95% CI = 1.14–3.01) in the achalasia group. In contrast, a decreased frequency of the DRB1*08:02 allele was observed (Table 3).

Analysis of HLA-DQBI alleles revealed a significant association between the DQBI*05:03 allele and achalasia (pC value = 0.0036, OR = 4.06; 95% CI = 1.52–11.07, Table 4).

Distribution of the HLA-C/B and DRB1/DQBI blocks confirm the relevance of the HLA class II region in the susceptibility of admixed Mexicans to achalasia

In this study, we classified HLA class I (HLA-C/-B) and class II (HLA-DRB1/-DQBI) blocks according to their most probable ancestry (MPA) in both the achalasia group and the control group. No significant differences in the distributions of the HLA-C/-B blocks from Native American, European, Asian and African MPAs were detected between the patients and controls (S4 Table). In the achalasia patients, 7 Native American-specific HLA-DRB1/-DQBI haplotypes, 21 European haplotypes, two Asian MPA haplotypes and five haplotypes of unknown origin were detected. Analysis of the HLA-DRB1/-DQBI blocks revealed an association between Eurasian origin haplotypes and achalasia (Table 5). The haplotype HLA-DRB1*11:01/-DQBI*03:01 was markedly more common in the achalasia group (pC value = 0.008, OR = 7.94; 95% CI = 1.65–57.29). We also found a significant increase in the frequency of the haplotype HLA-DRB1*14:54/-DQBI*05:03 in patients with achalasia compared to that in the controls (pC = 0.009, OR = 4.06; 95% CI = 1.57–11.09, Table 5).

We also found a significant correlation between the presence of HLA-DRB1*14:54-carrying haplotypes in patients with achalasia that use tobacco (p = 0.02) and patients with BMIs higher than 25 (p = 0.03). Other significant correlations with other clinical variables and the HLA-DQBI*06:03 allele was not detected. Next-generation HLA sequencing allowed to us to determine that the DRB1*14:54-DQBI*05:03-carrying haplotypes in achalasia patients were DRB1*14:54:01-DQA1*01:04:01-DQBI*05:03:01-DPA1*01:03:01.

Analysis of HLA class I/class II CEHs in achalasia patients and controls

The distributions of HLA class I/class II CEHs and their MPAs in achalasia patients and controls are summarized in Table 6, and this analysis was extended to HLA-A in Table 7. Interestingly, unlike in the HLA class II region analysis, no significant differences in CEHs carrying HLA-DRB1*14:54 and DQBI*05:03 were observed between the two groups. We detected only a slight increase in the frequency of the Caucasian European haplotype
Interestingly, CEHs carrying the susceptibility alleles HLA-DRB1*14:54 and DQB1*05:03, presumably of Eurasian MPA origin, were detected in only achalasia patients and not in the controls (Table 7). No significant associations between these susceptibility alleles and early onset of the disease were found.

**Discussion**

Numerous HLA disease case-control studies have demonstrated that an achalasia susceptibility region is associated with the MHC class II genomic transect in the short arm of human six chromosome [5–9,34]. Nevertheless, most studies did not consider the genetic admixtures or probable ancestral origins of the studied populations or the HLA class II alleles and haplotypes.

**Table 3. Gene frequencies of HLA-DRB1 in achalasia patients and healthy controls.**

| Allele  | Achalasia (N = 182) | Controls (N = 468) | pCorr | OR (95%CI) |
|---------|---------------------|--------------------|-------|------------|
|         | n | G.F. | n | G.F. | pCorr |      |
| DRB1*01:01 | 5  | 0.0274 | 9  | 0.0192 | ns   |      |
| DRB1*01:02 | 5  | 0.0274 | 11 | 0.0235 | ns   |      |
| DRB1*01:03 | 1  | 0.0054 | 3  | 0.0064 | ns   |      |
| DRB1*03:01 | 7  | 0.0385 | 15 | 0.0321 | ns   |      |
| DRB1*04:01 | 3  | 0.0164 | 3  | 0.0064 | ns   |      |
| DRB1*04:02 | 2  | 0.0109 | 10 | 0.0214 | ns   |      |
| DRB1*04:03 | 1  | 0.0054 | 10 | 0.0214 | ns   |      |
| DRB1*04:04 | 8  | 0.0439 | 31 | 0.0662 | ns   |      |
| DRB1*04:05 | 1  | 0.0054 | 1  | 0.0021 | ns   |      |
| DRB1*04:07 | 36 | 0.1978 | 55 | 0.1175 | 0.0114 | 1.85 (1.14–3.01) |
| DRB1*04:08 | 1  | 0.0054 | 1  | 0.0021 | ns   |      |
| DRB1*04:11 | 3  | 0.0164 | 9  | 0.0192 | ns   |      |
| DRB1*07:01 | 12 | 0.0659 | 33 | 0.0705 | ns   |      |
| DRB1*08:01 | 1  | 0.0054 | 1  | 0.0021 | ns   |      |
| DRB1*08:02 | 22 | 0.1208 | 91 | 0.1944 | 0.0284 | 0.57 (0.33–0.96) |
| DRB1*10:01 | 3  | 0.0164 | 6  | 0.0128 | ns   |      |
| DRB1*11:01 | 6  | 0.0329 | 6  | 0.0128 | ns   |      |
| DRB1*11:04 | 1  | 0.0054 | 8  | 0.0171 | ns   |      |
| DRB1*12:01 | 2  | 0.0109 | 2  | 0.0043 | ns   |      |
| DRB1*13:01 | 5  | 0.0274 | 12 | 0.0256 | ns   |      |
| DRB1*13:02 | 9  | 0.0495 | 10 | 0.0214 | ns   |      |
| DRB1*13:03 | 1  | 0.0054 | 3  | 0.0064 | ns   |      |
| DRB1*13:05 | 1  | 0.0054 | 1  | 0.0021 | ns   |      |
| DRB1*14:02 | 3  | 0.0164 | 11 | 0.0235 | ns   |      |
| DRB1*14:06 | 12 | 0.0659 | 47 | 0.1004 | ns   |      |
| DRB1*14:54 | 11 | 0.0604 | 8  | 0.0171 | 0.0200 | 3.7 (1.35–10.26) |
| DRB1*15:01 | 5  | 0.0274 | 17 | 0.0363 | ns   |      |
| DRB1*15:02 | 3  | 0.0164 | 5  | 0.0107 | ns   |      |
| DRB1*15:03 | 2  | 0.0109 | 1  | 0.0021 | ns   |      |
| DRB1*16:02 | 10 | 0.0549 | 30 | 0.0641 | ns   |      |

G.F.: Gene Frequency; ns: not significant; pCorr: p Corrected value using Bonferroni method; OR: Odds ratio; 95%CI: 95% Confidence Interval.

https://doi.org/10.1371/journal.pone.0201676.t003

HLA-A*29:02-B*44:03-C*16:01-DRB1*07:01-DQB1*02:02 in achalasia patients (pC = 0.02, OR = 9.32; 95% CI = 1.76–65.52).

Interestingly, CEHs carrying the susceptibility alleles HLA-DRB1*14:54 and DQB1*05:03, presumably of Eurasian MPA origin, were detected in only achalasia patients and not in the controls (Table 7). No significant associations between these susceptibility alleles and early onset of the disease were found.
associated with achalasia in different populations. In this study, we determined that 1) HLA class I and class II alleles are associated with the susceptibility of Mexican admixed individuals to achalasia using high-resolution sanger sequencing and next-generation HLA typing and 2) the ancestral origin (Native American, European, African and Asian) of these HLA-achalasia-associated alleles and haplotypes. We found a significant association between the HLA class II haplotype HLA-DRB1*14:54-DQB1*05:03 and achalasia, but no significant associations between HLA class I alleles or haplotypes were found. These findings are helpful for understanding that the susceptibility gene(s) are mapped within the HLA class II region and that genetic admixture with Eurasian populations contributes to the presence of these susceptibility loci together with potential environmental triggering factors, such as infections, which results in the development of achalasia.

Specific DNA blocks (with important and predictable patterns of LE) within specific HLA haplotypes are critical for mapping susceptibility or protection alleles in different autoimmune inflammatory conditions [10–11].

Protective effects of the allele HLA-DQB1*02 [6] and the haplotype DRB1*15:01-DQA1*01:02-DQB1*06:02 were reported in Spaniards, whereas DQA1*01:03, DQA1*01:01 and DQB1*06:03 were associated with achalasia in Spaniards [34]. HLA-DQB1*05:02 and DQB1*06:01 have been associated with achalasia susceptibility in Italians [35]. In addition, in 1999, Verne and colleagues described a significant contribution of the DQB1*06:02 allele in the susceptibility of a small group of European American descendants to achalasia [36]. More recent studies in large multinational cohorts of Central European origin support that alleles HLA-DQA1*01:01, HLA-DQB1*05:03 and DQB1*06:01 and their associated haplotypes are strong susceptibility factors to achalasia [8], and their distributions among these populations influence the prevalence of achalasia in Europe. In these studies, the presence of an eight-residue insertion at position 227–234 of the cytoplasmic region of the HLA-DQB1 chain (specifically encoded in DQB1*05:03 and DQB1*06:01 alleles) determines the susceptibility to achalasia; however, the mechanisms underlying this result are still poorly understood [9].
Table 5. Frequencies of HLA-DRB1/-DQB1 block in achalasia patients and healthy controls.

| HLA-DRB1/-DQB1 haplotypes | Achalasia (N = 182) | Controls (N = 468) | H.F. | Δ’ | n | H.F. | Δ’ | pCorr | OR (95% CI) |
|---------------------------|---------------------|--------------------|------|----|----|------|----|-------|-------------|
| **Amerindian**            |                     |                    |      |    |    |      |    |       |             |
| DRB1*04:07-DQB1*03:02     | 32                  | 0.1758             | 0.9628 | 53 | 0.1133 | 0.9518 | ns |
| DRB1*08:02-DQB1*04:02     | 23                  | 0.1263             | 1.0000 | 89 | 0.1902 | 0.9723 | ns |
| DRB1*14:06-DQB1*03:01     | 15                  | 0.0824             | 1.0000 | 46 | 0.0983 | 0.9717 | ns |
| DRB1*16:02-DQB1*03:01     | 10                  | 0.0549             | 1.0000 | 30 | 0.0641 | 1.0000 | ns |
| DRB1*14:02-DQB1*03:01     | 2                   | 0.0109             | 0.5801 | 11 | 0.0235 | 1.0000 | ns |
| DRB1*04:11-DQB1*03:02     | 1                   | 0.0054             | 0.1081 | 8  | 0.0171 | 0.8526 | ns |
| DRB1*04:11-DQB1*04:02     | 1                   | 0.0054             | 0.2161 | 1  | 0.0022 | -0.4595 | ns |
| **European**              |                     |                    |      |    |    |      |    |       |             |
| DRB1*03:01-DQB1*02:01     | 7                   | 0.0384             | 1.0000 | 15 | 0.0320 | 1.0000 | ns |
| DRB1*11:01-DQB1*03:01     | 6                   | 0.0329             | 1.0000 | 2  | 0.0043 | 0.1130 | ns |
| DRB1*13:01-DQB1*06:03     | 5                   | 0.0274             | 1.0000 | 6  | 0.0128 | 1.0000 | ns |
| DRB1*15:01-DQB1*06:02     | 5                   | 0.0274             | 1.0000 | 15 | 0.0320 | 0.8779 | ns |
| DRB1*04:02-DQB1*03:02     | 2                   | 0.0109             | 1.0000 | 10 | 0.0214 | 1.0000 | ns |
| DRB1*04:01-DQB1*03:02     | 2                   | 0.0109             | 0.5540 | 3  | 0.0064 | 1.0000 | ns |
| DRB1*11:04-DQB1*03:01     | 1                   | 0.0054             | 1.0000 | 8  | 0.0171 | 1.0000 | ns |
| DRB1*07:01-DQB1*03:03     | 1                   | 0.0054             | 1.0000 | 5  | 0.0107 | 0.462  | ns |
| **European shared with other populations** |                     |                    |      |    |    |      |    |       |             |
| DRB1*14:54-DQB1*05:03     | 12                  | 0.0659             | 1.0000 | 8  | 0.0171 | 1.0000 | ns |
| DRB1*07:01-DQB1*02:02     | 11                  | 0.0604             | 1.0000 | 28 | 0.0598 | 1.0000 | ns |
| DRB1*13:02-DQB1*06:04     | 7                   | 0.0384             | 1.0000 | 9  | 0.0192 | 0.8978 | ns |
| DRB1*04:04-DQB1*03:02     | 6                   | 0.0329             | 0.6655 | 29 | 0.0620 | 0.9144 | ns |
| DRB1*01:01-DQB1*05:01     | 5                   | 0.0274             | 1.0000 | 9  | 0.0192 | 1.0000 | ns |
| DRB1*01:02-DQB1*05:01     | 5                   | 0.0274             | 1.0000 | 11 | 0.0235 | 1.0000 | ns |
| DRB1*15:02-DQB1*06:01     | 3                   | 0.0164             | 1.0000 | 5  | 0.0107 | 1.0000 | ns |
| DRB1*01:03-DQB1*05:01     | 1                   | 0.0054             | 1.0000 | 3  | 0.0064 | 1.0000 | ns |
| DRB1*04:03-DQB1*03:02     | 1                   | 0.0054             | 1.0000 | 10 | 0.0214 | 1.0000 | ns |
| DRB1*04:05-DQB1*03:02     | 1                   | 0.0054             | 1.0000 | 1  | 0.0022 | 1.0000 | ns |
| DRB1*08:01-DQB1*04:02     | 1                   | 0.0054             | 1.0000 | 1  | 0.0022 | 1.0000 | ns |
| DRB1*13:03-DQB1*03:01     | 1                   | 0.0054             | 1.0000 | 3  | 0.0064 | 1.0000 | ns |
| DRB1*13:05-DQB1*03:01     | 1                   | 0.0054             | 1.0000 | 1  | 0.0022 | 1.0000 | ns |
| **African**               |                     |                    |      |    |    |      |    |       |             |
| DRB1*10:01-DQB1*05:01     | 3                   | 0.0164             | 1.0000 | 5  | 0.0107 | 0.8211 | ns |
| DRB1*15:03-DQB1*06:02     | 2                   | 0.0109             | 1.0000 | 1  | 0.0022 | 1.0000 | ns |
| **Asian**                 |                     |                    |      |    |    |      |    |       |             |
| DRB1*04:04-DQB1*04:02     | 2                   | 0.0109             | 0.1182 | 2  | 0.0043 | -0.6862 | ns |
| DRB1*12:01-DQB1*03:01     | 2                   | 0.0109             | 1.0000 | 1  | 0.0022 | 0.3348 | ns |
| **Unknown**               |                     |                    |      |    |    |      |    |       |             |
| DRB1*03:01-DQB1*03:01     | 1                   | 0.0054             | 0.1601 | ND |        |        |     |
| DRB1*04:07-DQB1*05:03     | 1                   | 0.0054             | -0.6151 | ND |        |        |     |
| DRB1*04:08-DQB1*03:04     | 1                   | 0.0054             | 1.0000 | ND |        |        |     |
| DRB1*04:11-DQB1*05:01     | 1                   | 0.0054             | 0.2774 | ND |        |        |     |
| DRB1*14:02-DQB1*03:04     | 1                   | 0.0054             | 0.4921 | ND |        |        |     |

*Uncorrected p value: 0.001; * Uncorrected p value: 0.0008; H.F.: Haplotype Frequency; ns: not significant; ND: Not detected; Δ: Delta max; pCorr: Corrected value using Bonferroni method; OR: Odds ratio; 95%CI: 95% Confidence Interval.

https://doi.org/10.1371/journal.pone.0201676.t005
Table 6. HLA-B/-C/-DRB1/-DQB1 conserved extended haplotypes in achalasia patients and healthy controls.

| HLA-B/-C/-DRB1/-DQB1 haplotypes | Achalasia (N = 182) | Controls (N = 468) | Controls (N = 468) | Δ | pCorr | OR (95%CI) |
|----------------------------------|---------------------|---------------------|---------------------|---|-------|-----------|
|                                  | n                   | H.F.                | n                   | H.F. | Δ       |           |
| Amerindian                       |                     |                     |                     |     |        |           |
| B*39:05-C*07:02-DRB1*04:07-DQB1*03:02 | 13                  | 0.0714              | 19                  | 0.0406 | 0.5025  | ns        |
| B*35:12-C*04:01-DRB1*08:02-DQB1*04:02 | 3                   | 0.0164              | 7                   | 0.0150 | 0.3054  | ns        |
| B*35:17-C*04:01-DRB1*08:02-DQB1*04:02 | 3                   | 0.0164              | 14                  | 0.0299 | 0.7256  | ns        |
| B*39:06-C*07:02-DRB1*14:06-DQB1*03:01 | 3                   | 0.0164              | 16                  | 0.0342 | 0.5482  | ns        |
| B*40:02-C*03:04-DRB1*16:02-DQB1*03:01 | 3                   | 0.0164              | 4                   | 0.0086 | 0.3200  | ns        |
| B*48:01-C*08:01-DRB1*08:02-DQB1*04:02 | 3                   | 0.0164              | 8                   | 0.0171 | 1.0000  | ns        |
| B*39:05-C*07:02-DRB1*08:02-DQB1*04:02 | 2                   | 0.0109              | -0.0871             | 5   | 0.0107 | -0.2267  | ns        |
| B*39:05-C*07:02-DRB1*16:02-DQB1*03:01 | 2                   | 0.0109              | 0.0866              | 3   | 0.0064 | 0.0295   | ns        |
| B*15:15-C*01:02-DRB1*08:02-DQB1*04:02 | 2                   | 0.0109              | 1.0000              | 8   | 0.0171 | 0.5251   | ns        |
| B*39:02-C*07:02-DRB1*16:02-DQB1*03:01 | 2                   | 0.0109              | 0.3604              | 2   | 0.0043 | 0.4658   | ns        |
| Admixed                          |                     |                     |                     |     |        |           |
| B*35:12-C*04:01-DRB1*04:07-DQB1*03:02 | 5                   | 0.0274              | 2                   | 0.0043 | 0.0133  | ns        |
| B*35:01-C*04:01-DRB1*04:07-DQB1*03:02 | 3                   | 0.0164              | 14.159              | ND  |        |           |
| B*40:02-C*03:04-DRB1*04:07-DQB1*03:02 | 3                   | 0.0164              | 0.3899              | ND  |        |           |
| B*35:17-C*04:01-DRB1*04:07-DQB1*03:02 | 2                   | 0.0109              | -1.0000             | ND  |        |           |
| B*39:03-C*07:02-DRB1*14:06-DQB1*03:01 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| B*48:01-C*08:01-DRB1*04:04-DQB1*03:02 | 2                   | 0.0109              | 0.3809              | 3   | 0.0064 | 0.1472   | ns        |
| B*35:01-C*07:02-DRB1*04:07-DQB1*03:02 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| Caucasian                        |                     |                     |                     |     |        |           |
| B*44:03-C*16:01-DRB1*07:01-DQB1*02:02 | 7                   | 0.0384              | 0.8668              | 6   | 0.0128 | 0.7341   | ns        |
| B*07:02-C*07:02-DRB1*15:01-DQB1*06:02 | 3                   | 0.0164              | 0.5894              | 7   | 0.0150 | 0.4478   | ns        |
| B*14:02-C*08:02-DRB1*01:02-DQB1*05:01 | 3                   | 0.0164              | 0.5850              | 5   | 0.0107 | 0.4414   | ns        |
| B*14:02-C*08:02-DRB1*11:01-DQB1*03:01 | 2                   | 0.0109              | 0.2590              | ND  |        |           |
| B*18:01-C*05:01-DRB1*03:01-DQB1*02:01 | 2                   | 0.0109              | 1.0000              | 3   | 0.0064 | 0.5868   | ns        |
| B*18:01-C*12:03-DRB1*14:54-DQB1*05:03 | 2                   | 0.0109              | 0.6447              | ND  |        |           |
| B*38:01-C*12:03-DRB1*14:54-DQB1*05:03 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| Caucasian Shared with other populations |                     |                     |                     |     |        |           |
| B*44:02-C*05:01-DRB1*13:01-DQB1*06:03 | 2                   | 0.0109              | 0.4868              | 1   | 0.0021 | 0.2403   | ns        |
| B*41:01-C*17:01-DRB1*13:02-DQB1*06:04 | 2                   | 0.0109              | 0.6542              | ND  |        | N.D.     |
| B*35:01-C*04:01-DRB1*14:54-DQB1*05:03 | 2                   | 0.0109              | 0.1473              | 2   | 0.0043 | 0.2252   | ns        |
| B*52:01-C*12:02-DRB1*15:02-DQB1*06:01 | 2                   | 0.0109              | 0.6596              | 1   | 0.0021 | 0.4946   | ns        |
| African                          |                     |                     |                     |     |        |           |
| B*45:01-C*06:02-DRB1*10:01-DQB1*05:01 | 2                   | 0.0109              | 0.6614              | ND  |        |           |
| Unknown                          |                     |                     |                     |     |        |           |
| B*35:03-C*12:03-DRB1*14:54-DQB1*05:03 | 3                   | 0.0164              | 0.7335              | ND  |        |           |
| B*15:01-C*03:04-DRB1*04:01-DQB1*03:02 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| B*15:10-C*03:04-DRB1*12:01-DQB1*03:01 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| B*27:05-C*01:02-DRB1*01:01-DQB1*05:01 | 2                   | 0.0109              | 1.0000              | ND  |        |           |
| B*51:01-C*14:02-DRB1*04:04-DQB1*03:02 | 2                   | 0.0109              | 0.6560              | ND  |        |           |

H.F.: Haplotype Frequency; ns: not significant; ND: Not detected; Δ: Delta max; pCorr: p Corrected value using Bonferroni method; OR: Odds ratio; 95%CI: 95% Confidence Interval.

https://doi.org/10.1371/journal.pone.0201676.1006

To our knowledge, this is the first study reporting the distribution of HLA alleles in Mexican mixed-ancestry patients with achalasia using high-resolution typing at the allelic level. We performed admixture estimations that revealed a greater contribution of Native American and European genes and lower contributions of African and Asian genes in patients with achalasia compared to those in healthy controls. Here, we found a significant association between HLA-DQB1*05:03 and HLA-DRB1*14:54 alleles and achalasia, and strong LD results associated the entire HLA-DRB1*14:54-DQB1*0503 haplotype with this clinical condition.
Table 7. Most frequent HLA-A/-B/-C/-DRB1/-DQB1 conserved extended haplotypes in Achalasia patients and healthy controls.

| HLA-?A/-B/-C/-DRB1/-DQB1 haplotype | Achalasia (N = 182) | Controls (N = 468) | pCorr | OR (95%CI) |
|-------------------------------------|---------------------|-------------------|--------|------------|
|                                     | n        | H.F.  | Δ'   | n        | H.F.  | Δ'   |        |           |
| 1 A*29:02 B*44:03 C*16:01 DRB1*07:01 DQB1*02:02 | 7        | 0.0384 | 1.0000 | 2        | 0.0043 | 1.0000 | 0.02* | 9.32 (1.76–65.52) |
| 3 A*02:06 B*39:05 C*07:02 DRB1*04:07 DQB1*03:02 | 5        | 0.0274 | 0.4641 | 5        | 0.0107 | 0.2834 | ns    |            |
| 3 A*02:06 B*39:05 C*07:02 DRB1*04:07 DQB1*03:02 | 3        | 0.0164 | 0.1324 | 5        | 0.0107 | 0.1848 | ns    |            |
| 1 A*02:01 B*35:03 C*12:03 DRB1*14:54 DQB1*05:03* | 3        | 0.0164 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 4 A*02:01 B*15:01 C*03:04 DRB1*04:54 DQB1*03:02 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 5 A*02:01 B*35:12 C*04:01 DRB1*04:07 DQB1*03:02 | 2        | 0.0109 | 0.1917 | 1        | 0.0021 | 0.3518 | ns    |            |
| 3 A*02:01 B*39:02 C*07:02 DRB1*16:02 DQB1*03:01 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 5 A*02:01 B*39:05 C*07:02 DRB1*04:07 DQB1*03:02 | 2        | 0.0109 | -0.4031 | 6        | 0.0128 | 0.1130 | ns    |            |
| 1 A*02:01 B*44:02 C*05:01 DRB1*13:01 DQB1*06:03 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 3 A*02:06 B*35:17 C*04:01 DRB1*08:02 DQB1*04:02 | 2        | 0.0109 | 0.6240 | 2        | 0.0043 | 0.0517 | ns    |            |
| 5 A*02:06 B*40:02 C*03:04 DRB1*04:07 DQB1*03:02 | 2        | 0.0109 | 0.6240 | 0        | 0.0000 | N.D   |       |            |
| 2 A*11:01 B*35:01 C*04:01 DRB1*14:54 DQB1*05:03* | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 3 A*24:02 B*39:06 C*07:02 DRB1*14:06 DQB1*03:01 | 2        | 0.0109 | 0.6174 | 12       | 0.0256 | 0.6992 | ns    |            |
| 2 A*24:02 B*41:01 C*17:01 DRB1*13:02 DQB1*06:04 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 1 A*25:01 B*18:01 C*12:03 DRB1*14:54 DQB1*05:03* | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 6 A*29:02 B*45:01 C*06:02 DRB1*10:01 DQB1*05:01 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 1 A*33:01 B*14:02 C*08:02 DRB1*01:02 DQB1*05:01 | 2        | 0.0109 | 0.6596 | 2        | 0.0043 | 0.3922 | ns    |            |
| 4 A*33:03 B*51:01 C*14:02 DRB1*04:04 DQB1*03:02 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 3 A*68:01 B*35:17 C*04:01 DRB1*04:04 DQB1*03:02 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 5 A*68:01 B*48:01 C*08:01 DRB1*04:04 DQB1*03:02 | 2        | 0.0109 | 1.0000 | 1        | 0.0021 | 0.2761 | ns    |            |
| 4 A*68:02 B*15:10 C*03:04 DRB1*12:01 DQB1*03:01 | 2        | 0.0109 | 1.0000 | 0        | 0.0000 | N.D   |       |            |
| 3 A*68:05 B*39:05 C*07:02 DRB1*04:07 DQB1*03:02 | 2        | 0.0109 | 0.3569 | 0        | 0.0000 | N.D   |       |            |

*Uncorrected p value: 0.001;

HLA-DRB1*14:54-DQB1*05:03 carrying haplotypes;

F: Haplotype Frequency; ns: not significant; N.D: Not detected; NotDelta max; pCorr: p Corrected value using Bonferroni method; OR: Odds ratio; 95%CI: 95% Confidence Interval; 1: European; 2: European shared; 3: Amerindian; 4: Unknown; 5: Admixed; 6: African.

https://doi.org/10.1371/journal.pone.0201676.t007

Interestingly, these alleles have been previously associated with pemphigus vulgaris (PV), a rare and severe autoimmune condition, in different populations. Among Europeans, including Slovaksians and Serbians, the HLA-DRB1*14:54 and DQB1*05:03 alleles have been strongly associated with PV [37–39]; remarkably, DQB1*05:03 is a strong genetic determinant for PV in non-Jewish groups and Asians [40,41].

Historical reconstructions and HLA association studies with PV support that the DRB1*04:02 and DQB1*03:02 susceptibility genes possibly originated from central Asian populations, including ancient northwest Iranians, and then affected Europe with the migration of Ashkenazi Jews after the 9th century A.D. [42,43].

From this perspective, the presence of the HLA-DRB1*14:54 and DQB1*05:03 alleles in Mexicans could be the result of genetic admixture between Mexicans and Spaniards, who arrived in Mexico in the 16th century. Spaniards came from the western coast of Spain, mainly from the regions of Andalucía, Leon, Extremadura, and the Castillas, as well as from Portugal and Genoa. However, by the end of the colonial period, almost half a million Europeans, mainly peninsula Spaniards but also French, German, and English colonizers, found a place within Mexican society [44]. The HLA-DRB1 allele has been found at frequencies ranging from 0.0078 to 0.0330 in Irish [45] and Italians [46]. However, HLA-DRB1*14:54 could also reflect admixture events with Asian groups. Asian migrants from South East Asia who arrived throughout
the colonial period [47] would have contributed to the presence of this allele in present-day Mexican populations. HLA-DRB1*14:54 has been reported in several East Asian populations, such as Chinese from Hong Kong [48] and Maori and Polynesians from New Zealand [49], in frequencies ranging from 0.0273 to 0.0950. HLA-DQB1*05:03 is present throughout the entire Eurasian region [50], with the highest allelic frequencies being found in Romani from both Spain (0.2672) [51] and the Czech Republic (0.2331) [52]. The Romani people are a traditionally nomadic ethnic group originating in northern India nearly 1500 years ago [53,54].

Moreover, the HLA genes that confer susceptibility to autoimmunity might also be preserved in specific populations because they play a key role in protection against pathogens [55,56]. In Mexican admixed populations, different studies have reported high frequencies of HLA class II alleles, including HLA-DRB1*04:04, DRB1*14:02, and DRB1*01:02 in rheumatoid arthritis (RA); DRB1*03:01 in systemic lupus erythematosus (SLE); and DRB1*11:04 in systemic sclerosis (SSc), that predispose the recipients to different autoimmune disorders [57–59]. Autoimmunity risk HLA class II genes may exhibit increased frequencies due to past selective processes or infectious diseases that developed in different environments, thus partially explaining the susceptibility to autoimmune diseases in our populations [60]. While the pathogenesis of idiopathic achalasia is still unknown, viral, bacterial and neurodegenerative mechanisms have been proposed [3,61–64]. However, the fact that alleles and associations reported elsewhere [5–9] could not be replicated in this study demonstrates the heterogeneity of this disease and that genetic factors in the patient may be linked to environmental factors and the patient’s immune response to both cellular and humoral factors. For instance, all patients with achalasia in previous reports [3,64] were positive for Herpes simplex virus 1 (HSV-1) infection. Since promiscuous binding of the viral sequence to HLA-DR molecules could suggest a potential for HSV-1 to manipulate antigen processing and presentation [62,63], an association among susceptible HLA class II alleles, HSV-1 and a number of triggering factors could detonate an aberrant response that may be linked to an autoimmune origin for achalasia [33, 64]. Furthermore, changes in the binding affinities of HSV-1 viral glycoprotein B and human protein invariant chain (Ii; involved in preventing the premature binding of peptides to clefts in MHC class II molecules) via stabilization of the proline-rich segment of both proteins may indicate the mechanism by which such aberrant responses are achieved, furthering the development of autoimmune conditions (such as achalasia, potentially). Thus, a number of alleles sharing a common sequence of amino acid motifs could potentially be associated with the development of achalasia. Such alleles may have differential distributions in human populations, and ancestry and population genetics studies may aid in their identification.

Idiopathic achalasia may occur at any age and it has been suggested that susceptibility genes inherited to the siblings may influence the age of disease presentation [64]. In our study the age of onset of the disease was heterogeneous and the analysis of distribution of HLA-DRB1/DQB1 susceptibility haplotypes did not display significant associations with early onset of the disease.

It has been also suggested that achalasia may be the result of a self-sustained inflammatory process secondary to acute gastrointestinal infections and that individual susceptibility of developing achalasia following such an initial trigger may be genetically determined. In this complex scenario, neuronal nitric oxide (NO) represents a unique molecule since, depending on its concentration, it is involved in either inhibitory neurotransmission, lower esophageal sphincter relaxation, or defense against infections [65]. A single nucleotide polymorphism has been found within the 3’-untranslated region (UTR) of exon 29. Additionally, an exome analysis of two siblings with infant-onset achalasia revealed homozygosity for a premature stop codon in the gene encoding NOS1 (at residue Tyr1202, instead of at residue 1435). Kinetic analyses and molecular modeling indicated that the truncated protein product has defective
folding capacity, as well as defective capabilities for NO production and binding of co-factors. Other genetic polymorphisms of NOS gene isoforms that have been discovered involve the endothelial NOS4a4a, inducible NOS2GA and neuronal NOS29TT [65]. Recently, Sarnelli et al., have provided evidence that genetic variations in the promoter region of the iNOS gene are associated with susceptibility to achalasia. Analysis of the allele frequencies revealed that individuals carrying 10 and 13 CCTTT repeats were respectively less and more frequent in achalasia (OR 0.5, 95% CI 0.3–0.5 and OR 1.6, 95% CI 1–2.4, all p < 0.05). Long repeats were also significantly associated with an earlier onset of the disease (OR 1.69, 95% CI 1.13–2.53, p = 0.01) [66].

Finally, the high prevalence of ANAs, as well as autoimmune comorbidity in achalasia patients have all favored the hypothesis that achalasia could be an autoimmune disorder [3, 33, 65].

This work has some potential limitations, such as the relatively small sample size, which was limited by the low prevalence of achalasia, and the criterion to recruit only patients born in Mexico whose parents and grandparents were also born in Mexico. With such a modest sample size, we were able to find statistically significant associations and confirm previously reported associations with HLA class II alleles in other populations. Another limitation was the lack of a validation cohort because there are no referral centers in Mexico, and to our knowledge, there is no other cohort of patients with achalasia in Mexico that could potentially validate, or at least replicate, our results.

**Conclusion**

MHC class II genes (HLA-DRB1*15:54 and DQB1*05:03) and the conserved haplotype DRB1*14:54-DQB1*05:03 confer risk for the development of achalasia in mixed-ancestry Mexicans. These achalasia-associated MHC class II genes are possibly of Eurasian origin, and they could be important in the development of aberrant immune responses against triggering factors, such as viral, bacterial or parasitic infections, that may lead to the development of this autoimmune condition.

**Supporting information**

S1 Table. Gene frequencies of HLA-A in achalasia patients and healthy controls.
(DOCX)

S2 Table. Gene frequencies of HLA-B in achalasia patients and healthy controls.
(DOCX)

S3 Table. Gene frequencies of HLA-C in achalasia patients and healthy controls.
(DOCX)

S4 Table. Frequencies of HLA-B/-C block in achalasia patients and healthy controls.
(DOCX)

S1 Data. Data_Raw data HLA, arlequin analysis, haplotypes, LD, Mexican healthy controls.
(XLSX)

**Author Contributions**

Conceptualization: Janette Furuzawa-Carballeda, Joaquín Zuñiga, Diana I. Hernández-Zaragoza, Rodrigo Barquera, Gonzalo Torres-Villalobos.
Data curation: Janette Furuzawa-Carballeda, Joaquin Zuñiga, Diana I. Hernández-Zaragoza, Rodrigo Barquera, Eduardo Marques-García, Luis Jiménez-Alvarez, Alfredo Cruz-Lagunas, Gustavo Ramírez, Nora E. Regino, Ramón Espinosa-Soto, Edmond J. Yunis, Fernanda Romero-Hernández, Daniel Azamar-Llamas, Enrique Coss-Adame, Miguel A. Valdovinos, Samuel Torres-Landa, Axel Palacios-Ramírez, Blanca Breña, Edgar Alejandro-Medrano, Axel Hernández-Ávila, Julio Granados, Gonzalo Torres-Villalobos.

Formal analysis: Janette Furuzawa-Carballeda, Joaquin Zuñiga, Diana I. Hernández-Zaragoza, Rodrigo Barquera, Eduardo Marques-García, Luis Jiménez-Alvarez, Alfredo Cruz-Lagunas, Gustavo Ramírez, Nora E. Regino, Ramón Espinosa-Soto, Edmond J. Yunis, Fernanda Romero-Hernández, Daniel Azamar-Llamas, Enrique Coss-Adame, Miguel A. Valdovinos, Samuel Torres-Landa, Axel Palacios-Ramírez, Blanca Breña, Edgar Alejandro-Medrano, Axel Hernández-Ávila, Julio Granados, Gonzalo Torres-Villalobos.

Funding acquisition: Janette Furuzawa-Carballeda.

Investigation: Janette Furuzawa-Carballeda, Joaquin Zuñiga.

Methodology: Janette Furuzawa-Carballeda, Joaquin Zuñiga, Diana I. Hernández-Zaragoza, Fernanda Romero-Hernández, Daniel Azamar-Llamas, Enrique Coss-Adame, Miguel A. Valdovinos, Samuel Torres-Landa, Axel Palacios-Ramírez, Blanca Breña, Edgar Alejandro-Medrano, Axel Hernández-Ávila, Julio Granados.

Supervision: Gonzalo Torres-Villalobos.

Writing – original draft: Janette Furuzawa-Carballeda, Joaquin Zuñiga, Diana I. Hernández-Zaragoza, Gonzalo Torres-Villalobos.

Writing – review & editing: Janette Furuzawa-Carballeda, Joaquin Zuñiga, Diana I. Hernández-Zaragoza, Rodrigo Barquera, Eduardo Marques-García, Luis Jiménez-Alvarez, Alfredo Cruz-Lagunas, Gustavo Ramírez, Nora E. Regino, Ramón Espinosa-Soto, Edmond J. Yunis, Fernanda Romero-Hernández, Daniel Azamar-Llamas, Enrique Coss-Adame, Miguel A. Valdovinos, Samuel Torres-Landa, Axel Palacios-Ramírez, Blanca Breña, Edgar Alejandro-Medrano, Axel Hernández-Ávila, Julio Granados, Gonzalo Torres-Villalobos.

References
1. Vaezi MF, Felix VN, Penagini R, Mauro A, de Moura EF, Pu LZ, et al. Achalasia: from diagnosis to management. Ann NY Acad Sci. 2016; 1381(1): 34–44. https://doi.org/10.1111/nyas.13176 PMID: 27571581
2. Vaezi MF, Pandolfino JE, Vela MF. ACG clinical guideline: diagnosis and management of achalasia. Am J Gastroenterol. 2013; 108(8): 1238–1249. https://doi.org/10.1038/ajg.2013.196 PMID: 23877351
3. Furuzawa-Carballeda J, Aguilar-León D, Gamboa-Domínguez A, Valdovinos MA, Nuñez-Álvarez C, Martín-del-Campo LA, et al. Achalasia—An Autoimmune Inflammatory Disease: A Cross-Sectional Study. J Immunol Res. 2015; 2015: 729217. https://doi.org/10.1155/2015/729217 PMID: 26078981
4. Stein DT, Knauer CM. Achalasia in monozygotic twins. Dig Dis Sci. 1982; 27(7): 636–640. PMID: 7200858
5. Wong RK, Maydonovitch CL, Metz SJ, Baker JR Jr. Significant DQw1 association in achalasia. Dig Dis Sci. 1989; 34(3): 349–352. PMID: 2920639
6. de la Concha EG, Fernandez-Arquero M, Mendoza JL, Conejero L, Figueredo MA, Perez de la Serna J, et al. Contribution of HLA class II genes to susceptibility in achalasia. Tissue Antigens. 1998; 52(4): 381–384. PMID: 9820602
7. de la Concha EG, Fernandez-Arquero M, Conejero L, Lazaro F, Mendoza JL, Sevilla MC, et al. Presence of a protective allele for achalasia on the central region of the major histocompatibility complex. Tissue Antigens. 2000; 56(2): 149–153. PMID: 11019915
8. Gockel I, Becker J, Wouters MM, Niebish S, Gockel HR, Hess T, et al. Common variants in the HLA-DQ region confer susceptibility to idiopathic achalasia. Nat Genet. 2014; 46(8): 901–904. https://doi.org/10.1038/ng.3029 PMID: 24997987
9. Becker J, Haas SL, Mokrowiecka A, Wasielica-Berger J, Ateeb Z, Bister J, et al. The HLA-DQB1 insertion is a strong achalasia risk factor and displays a geospatial north-south gradient among Europeans. Eur J Hum Genet. 2016; 24(8): 1228–1231. https://doi.org/10.1038/ejhg.2015.262 PMID: 26733285

10. Yunis EJ, Larsen CE, Fernandez-Viña M, Awdeh ZL, Romero T, Hansen JA, et al. Inheritable variable sizes of DNA stretches in the human MHC: conserved extended haplotypes and their fragments or blocks. Tissue Antigens. 2003; 62(1): 1–20. PMID: 12859592

11. Zuñiga J, Yu N, Barquera R, Alocos S, Ohashi M, Lebedeva T, et al. HLA class I and class II conserved extended haplotypes and their fragments or blocks in Mexicans: implications for the study of genetic diversity in admixed populations. PLoS One. 2013; 8(9): e74442. https://doi.org/10.1371/journal.pone.0074442 PMID: 24086347

12. Lisker R, Ramírez E, Briceño RP, Granados J, Babinsky V. Gene frequencies and admixture estimates in four Mexican urban centers. Hum Biol. 1990; 62(6): 791–801. PMID: 2262003

13. Barquera R, Zuñiga J, Hernández-Díaz R, Acuña-Alonso V, Montoya-Gama K, Moscoso J, et al. HLA class I and class II haplotypes in admixed families from several regions of Mexico. Mol Immunol. 2008; 45(4): 1171–1178. https://doi.org/10.1016/j.molimm.2007.07.042 PMID: 17904223

14. Juárez-Cedillo T, Zuñiga J, Acuña-Alonso V, Pérez-Hernández N, Rodríguez-Pérez JM, Barquera R, et al. Genetic admixture and diversity estimations in the Mexican Mestizo population from Mexico City using 15 STR polymorphic markers. Forensic Sci Int Genet 2008; 2(3): e37–e39. https://doi.org/10.1016/j.fsigen.2007.08.017 PMID: 19083813

15. Kahrilas PJ, Bredenoord AJ, Fox M, Gyawali CP, Roman S, Smout AJ, et al. The Chicago classification of esophageal motility disorders, v3.0. Neurogastroenterol Motil. 2015; 27(2): 160–174. https://doi.org/10.1111/ngeo.12477 PMID: 25465969

16. Lebedeva TV, Mastromarino SA, Lee E, Ohashi M, Alosco SM, Yu N. Resolution of HLA class I sequence-based typing ambiguities by group-specific sequencing primers. Tissue Antigens. 2011; 77(3): 247–250. https://doi.org/10.1111/j.1399-0039.2010.01616.x PMID: 21299532

17. Robinson J, Waller MJ, Parham P, Bodmer JG, Marsh SG. IMGT/HLA Database—a sequence database for the human major histocompatibility complex. Nucleic Acids Res. 2001; 29(1): 210–213. PMID: 11120504

18. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online. 2007; 1: 47–50. PMID: 19325852

19. Haseman JK, Elston RC. The investigation of linkage between a quantitative trait and a marker locus. Behav Genet. 1972; 2(1): 3–19. PMID: 4157472

20. Wang J. Maximum Likelihood estimation of admixture proportions from genetic data. Genetics. 2003; 164(2): 747–765. PMID: 12807794

21. Spinola H, Brehm A, Williams F, Jesus J, Middleton D. Distribution of HLA alleles in Portugal and Cabo Verde. Relationships with the slave trade route. Ann Hum Genet. 2002; 66(Pt4): 285–296.

22. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernández-Viña MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum Immunol. 2001; 62(9): 1009–1030. PMID: 11543903

23. Cao K, Moormann AM, Lyke KE, Masaberg C, Sumba OP, Doumbo OK, et al. Differentiation between African populations is evidenced by the diversity of alleles and haplotypes of HLA class I loci. Tissue Antigens. 2004; 63(4): 293–325. PMID: 15009803

24. Hollenbach JA, Thomson G, Cao K, Fernández-Vina M, Erlich H, Bugawan TL, et al. HLA diversity, differentiation, and haplotype evolution in Mesoamerican Natives. Hum Immunol. 2001; 62(4): 378–390. PMID: 11295471

25. García-Ortíz JE, Sandoval-Ramírez L, Rangel-Villalobos H, Maldonado-Torres H, Cox S, García-Sepúlveda CA, et al. High-resolution molecular characterization of the HLA class I and class II in the Tarahumara Amerindian population. Tissue Antigens. 2006; 68(2): 135–146. https://doi.org/10.1111/j.1399-0039.2006.00636.x PMID: 16866883

26. Trachtenberg E, Vinson M, Hayes E, Hsu YM, Houtchens K, Erlich H, et al. Southern Han Chinese from People's Republic of China. Anthropology/human genetic diversity population reports. In: Hansen JA (ed.). Immunobiology of the Human MHC; Proceedings of the 13th International Histocompatibility Workshop and Conference. Seattle: IHWG Press. 2006; pp. 616–617.

27. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics. 2003; 164(4): 1567–1587. PMID: 12930761

28. Sanz P, Prieto V, Flores I, Torres Y, López-Soto M, Fárrán MJ. Population data of 13 STRs in Southern Spain (Andalusia). Forensic Sci Int. 2001; 119(1): 113–115. PMID: 11348802
29. Calzada P, Suárez I, García S, Barrot C, Sánchez C, Ortega M, et al. The Fang population of Equatorial Guinea characterized by 15 STR-PCR polymorphisms. Int J Legal Med. 2005; 119: 107–110. https://doi.org/10.1007/s00414-005-0505-9 PMID: 15565295

30. Barrot C, Sánchez C, Ortega M, González-Martín A, Brand-Casadevall C, Gorostiza A, et al. Characterisation of three Amerindian populations from Hidalgo State (Mexico) by 15 STR-PCR polymorphisms. Int J Legal Med. 2005; 119(2): 111–115. https://doi.org/10.1007/s00414-004-0488-6 PMID: 15378309

31. González-Martín A, Gorostiza A, Rangel-Villalobos H, Acunha V, Barrot C, Sánchez C, et al. Analyzing the genetic structure of the Tepehua in relation to other neighbouring Mesoamerican populations. A study based on allele frequencies of STR markers. Am J Hum Biol. 2008; 20(5): 605–613. https://doi.org/10.1002/ajhb.20787 PMID: 18464267

32. Barahona-Garrido J, Camacho-Escobedo J, García-Martínez Cl, Tocay H, Cabiedes J, Yamamoto-Furusho JK. Antinuclear antibodies: a marker associated with steroid dependence in patients with ulcerative colitis. Inflamm Bowel Dis. 2009; 15(7): 1039–1043. https://doi.org/10.1002/ibd.20852 PMID: 19107779

33. Romero-Hernández F, Furuzawa-Carbeddela J, Hernández-Molina G, Alejandro-Medrano E, Núñez-Alvarez CA, Hernández-Ramírez DF, et al. Autoimmune comorbidity in achalasia patients. J Gastroenterol Hepatol. 2018; 33(1): 203–208. https://doi.org/10.1111/jgh.13839 PMID: 28568312

34. Ruiz-de-León A, Mendoza J, Sevilla-Mantilla C, Fernández AM, Pérez-de-la-Serna J, González VA, et al. Myenteric antiplexus antibodies and class II HLA in achalasia. Dig Dis Sci. 2002; 47(1): 15–19. PMID: 11837716

35. Latiano A, De Giorgio R, Volta U, Palmieri O, Zagaria C, Stanghellini V, et al. HLA and enteric antineuro antibodies in patients with achalasia. Neurogastroenterol Motil. 2006; 18(7): 520–525. https://doi.org/10.1111/j.1365-2982.2006.00772.x PMID: 16771767

36. Verne GN, Hahn AB, Pineau BC, Hoffman BJ, Wojciechowski BW, Wu WC. Association of HLA-DR and -DQ alleles with idiopathic achalasia. Gastroenterology. 1999; 117(1): 26–31. PMID: 10381906

37. Saha M, Harman K, Mortimer NJ, Binda V, Black MM, Kondeatis E, et al. Pemphigus vulgaris in Whites is linked with HLA Class II allele HLA DRB1*1454 but not DRB1*1401. J Invest Dermatol. 2010; 130(1): 311–314. https://doi.org/10.1038/jid.2009.241 PMID: 19847191

38. Zivanovic D, Bojc S, Medenica L, Andric Z, Popadic D. Human leukocyte antigen class II (DRB1 and DQB1) alleles and haplotypes frequencies in patients with pemphigus vulgaris among the Serbian population. HLA. 2016; 87(5): 367–374. https://doi.org/10.1111/tan.12796 PMID: 23551624

39. Pámková Z, Švecová D, Javor J, Shawkatová I, Buc M. High susceptibility to pemphigus vulgaris due to HLA-DRB1*14:54 in the Slovak population. Int J Immunogenet. 2013; 40(6): 471–475. https://doi.org/10.1111/iji.12052 PMID: 23702514

40. Ahmed AR, Wagner R, Khati K, Notani G, Awdeh Z, Alper CA, et al. Major histocompatibility complex haplotypes and class II genes in non-Jewish patients with pemphigus vulgaris. Proc Natl Acad Sci USA. 1991; 88(11): 5056–5060. PMID: 1675792

41. Nižeki H, Inoko H, Mizuki N, Inamoto N, Watababe K, Hashimoto T, et al. HLA-DQA1, -DQB1 and -DRB1 genotyping in Japanese pemphigus vulgaris patients by the PCR-RFLP method. Tissue Antigens. 1994; 44(4): 248–251. PMID: 7871526

42. Das R, Wexler P, Pirooznia M, Elhaik E. Localizing Ashkenazi Jews to Primeval Villages in the Ancient Iranian Lands of Ashkenaz. Genome Biol Evol. 2016; 8(4): 1132–1149. https://doi.org/10.1093/gbe/evw046 PMID: 26941229

43. Mobini N, Yunis EJ, Alper CA, Yunis JJ, Delgado JC, Yunis DE, et al. Identical MHC markers in non-Jewish Iranian and Ashkenazi Jewish patients with pemphigus vulgaris: possible common central Asian ancestral origin. Hum Immunol. 1997; 57(1): 62–67. PMID: 9438197

44. Buchenau J. Small numbers, great impact: Mexico and its migrants, 1821–1973. J Am Ethn His. 2001; 20(3): 23–49.

45. Dunne C, Crowley J, Hagan R, Rooney G, Lawlor E. HLA-A, B, Cw, DRB1, DQB1 and DPB1 alleles and haplotypes in the genetically homogenous Irish population. Int J Immunogenet. 2008; 35(4–5): 295–302. https://doi.org/10.1111/j.1744-313X.2008.00779.x PMID: 18976432

46. Rendine S, Ferrero NM, Sacchi N, Costa C, Pollichiere S, Amoroso A. Estimation of human leukocyte antigen class I and class II high-resolution allele and haplotype frequencies in the Italian population and comparison with other European populations. Hum Immunol. 2012; 73(4): 399–404. https://doi.org/10.1016/j.humimm.2012.01.005 PMID: 22342872

47. Hu-Dehart E. The Chinese of Peru, Cuba, and Mexico. In: Cohen R (ed.). The Cambridge survey of world migration. Cambridge: Cambridge University Press; 1995: 220–391.

48. Kwok J, Guo M, Yang W, Lee CK, Ho J, Tang WH, et al. HLA-A, -B, -C, and -DRB1 genotyping and haplotype frequencies for a Hong Kong Chinese population of 7595 individuals. Hum Immunol. 2016; 77(12): 1111–1112. https://doi.org/10.1016/j.humimm.2016.10.005 PMID: 27769748
49. Edinur HA, Dunn PP, Hammond L, Selwyn C, Brescia P, Askar M, et al. HLA and MICA polymorphism in Polynesians and New Zealand Maori: implications for ancestry and health. Hum Immunol. 2013; 74 (9): 1119–29. https://doi.org/10.1016/j.humin.2013.06.011 PMID: 23792058

50. Mack SJ, Bugawan TL, Moonsamy PV, Erlich JA, Trachtenberg EA, Paik YK, et al. Evolution of Pacific/Asian populations inferred from HLA class II allele frequency distributions. Tissue Antigens. 2000; 55 (5): 383–400. PMID: 10885559

51. Fernández O, Fernández V, Martinez-Cabrerona V, Mayorga C, Alonso A, León A, et al. Multiple sclerosis in Gypsies from southern Spain: prevalence, mitochondrial DNA haplogroups and HLA class II association. Tissue Antigens. 2008; 71 (5): 426–433. https://doi.org/10.1111/j.1399-0039.2008.01016.x PMID: 18312478

52. Cerna M, Fernandez Viña M, Ivásková E, Stastny P. Comparison of HLA class II alleles in Gypsy and Czech populations by DNA typing with oligonucleotide probes. Tissue Antigens. 1992; 39(3): 111–116. PMID: 1598683

53. Hancock IF. Aam sam e romane džen [We are the Romani People]. New York: The Open Society Institute, 2001.

54. Mendizabal I, Lao O, Marigorta UM, Wollstein A, Gusmão L, Ferak V, et al. Reconstructing the population history of European Romani from genome-wide data. Curr Biol. 2012; 22(24): 2342–2349. https://doi.org/10.1016/j.cub.2012.10.039 PMID: 23219723

55. Sanchez-Mazas A, Lemaitre JF, Currat M. Distinct evolutionary strategies of human leucocyte antigen loci in pathogen-rich environments. Philos Trans R Soc Lond B Biol Sci. 2012; 367(1590): 830–839. https://doi.org/10.1098/rstb.2011.0312 PMID: 22312050

56. Penn DJ, Damjanovich K, Potts WK. MHC heterozygosity confers a selective advantage against multiple-strain infections. Proc Natl Acad Sci USA. 2002; 99(17): 11260–11264. https://doi.org/10.1073/pnas.162006499 PMID: 12177415

57. Rodríguez-Carreón AA, Zúñiga J, Hernández-Pacheco G, Rodríguez-Pérez JM, Pérez-Hernández N, Montes de Oca JV et al. Tumor necrosis factor-α -308 promoter polymorphism contributes independently to HLA alleles in the severity of rheumatoid arthritis in Mexicans. J Autoimmun. 2005; 24(1): 63–68. https://doi.org/10.1016/j.jaut.2004.11.002 PMID: 15725578

58. Granados J, Vargas-Alarcón G, Andrade F, Melíñ-Aldana H, Alcocer-Varela J, Alarcón-Segovia D. The role of HLA-DR alleles and complotypes through the ethnic barrier in systemic lupus erythematosus in Mexicans. Lupus. 1996; 5(3): 184–189. https://doi.org/10.1177/096120339600500304 PMID: 8803888

59. Rodríguez-Reyna TS, Mercado-Velázquez P, Yu N, Alosco S, Ohashi M, Lebedeva T, et al. HLA Class I and II Blocks Are Associated to Susceptibility, Clinical Subtypes and Autoantibodies in Mexican Systemic Sclerosis (SSc) Patients. PLoS One. 2015; 10(5): e0126727. https://doi.org/10.1371/journal. pone.0126727 PMID: 25993664

60. Booy JD, Takata J, Tomlinson G, Urbach DR. The prevalence of autoimmune disease in patients with esophageal achalasia. Dis Esophagus. 2012; 25(3): 209–213. https://doi.org/10.1111/j.1442-2050.2011.01249.x PMID: 21899655

61. Boeckxstaens GE. Achalasia: virus-induced euthanasia of neurons? Am J Gastroenterol. 2008; 103 (7): 1610–1612. https://doi.org/10.1111/j.1572-0241.2008.01967.x PMID: 18557706

62. Facco M, Brun P, Baesso I, Costantini M, Rizzetto C, Berto A, et al. T cells in the myenteric plexus of achalasia patients show a skewed TCR repertoire and react to HSV-1 antigens. Am J Gastroenterol. 2008; 103(7): 1598–1609. https://doi.org/10.1111/j.1572-0241.2008.01956.x PMID: 18557707

63. Sievers E, Neumann J, Raftery M, Schönrich G, Eis-Hüibinger AM, Koch N. Glycoprotein B from strain 17 of herpes simplex virus type I contains an invariant chain homologous sequence that binds to MHC class II molecules. Tissue Antigens. 2002; 59(5): 383–400. PMID: 10885559

64. Paladini F, Cocco E, Cascino I, Belfiore F, Badiali D, Piretta L, et al. Age-dependent association of idiopathic achalasia with vasoactive intestinal peptide receptor 1 gene. Neurogastroenterol Motil. 2009; 21 (6): 597–602. https://doi.org/10.1111/j.1365-2982.2009.01284.x PMID: 19309439

65. Furuzawa-Carballeda J, Torres-Landa S, Valdivinos MA, Coss-Adame E, Martín Del Campo LA, Torres-Villalobos G. New insights into the pathophysiology of achalasia and implications for future treatment. World J Gastroenterol. 2016; 22(35): 7892–7907. https://doi.org/10.3748/wjg.v22.i35.7892 PMID: 27672286

66. Sarnelli G, Grosso M, Palumbo I, Pesce M, D’Alessandro A, Zaninotto G, et al. Allele-specific transcriptional activity of the variable number of tandem repeats of the inducible nitric oxide synthase gene is associated with idiopathic achalasia. United European Gastroenterol J. 2017; 5(2): 200–207. https://doi.org/10.1177/2050640616648870 PMID: 28344787