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Sex-specific analysis in Behçet's disease reveals higher genetic risk in male patients

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Abstract

Objectives: Behçet's disease tends to be more severe in men than women. This study was undertaken to investigate sex-specific genetic effects in Behçet's disease.

Methods: A total of 1,762 male and 1,216 female patients with Behçet's disease from six diverse populations were studied, with the majority of patients of Turkish origin. Genotyping was performed using an Infinium ImmunoArray-24 BeadChip, or extracted from available genotyping data. Following imputation and extensive quality control measures, genome-wide association analysis was performed comparing male to female patients in the Turkish cohort, followed by a meta-analysis of significant results in all six populations. In addition, a weighted genetic risk score for Behçet's disease was calculated and compared between male and female patients.

Results: Genetic association analysis comparing male to female patients with Behçet's disease from Turkey revealed an association with male sex in *MICA/HLA-B* within the HLA region with a GWAS level of significance (rs2848712, OR=1.46, $P=1.22 \times 10^{-8}$). Meta-analysis of the effect in rs2848712 across six populations confirmed these results. Genetic risk score for Behçet's disease was significantly higher in male compared to female patients from Turkey. Higher genetic risk for Behçet's disease was observed in male patients in *HLA-B/MICA* (rs116799036, OR=1.45, $P=1.95 \times 10^{-8}$), *HLA-C* (rs12525170, OR=1.46, $P=5.66 \times 10^{-7}$), and *KLRC4* (rs2617170, OR=1.20, $P=0.019$). In contrast, *IFNGR1* (rs4896243, OR=0.86, $P=0.011$) was shown to confer higher genetic risk in female patients.

Conclusions: Male patients with Behçet's disease are characterized by higher genetic risk compared to female patients. This genetic difference, primarily derived from our Turkish cohort, is largely explained by risk within the HLA region. These data suggest that genetic factors might contribute to differences in disease presentation between men and women with Behçet's disease.

1. Introduction

Behçet's disease is a chronic inflammatory disease characterized by recurrent oral and genital ulcers. It is a multisystemic vasculitis affecting many organs, such as the eyes, skin, blood vessels, central nervous system, and gastrointestinal tract [1, 2]. Behçet's disease is also known as the "Silk Road disease". Although patients have been diagnosed with Behçet's disease worldwide, it is found to be most prevalent in populations originating from the region along the ancient trading route [3]. The etiology of Behçet's disease is not clearly understood, however, genetic factors along with environmental triggers are thought to play a role contributing to the susceptibility of the disease. It is suggested that infectious pathogens might contribute to the onset of the disease by triggering abnormal immune responses in genetically predisposed individuals [4]. The genetic studies performed to date in Behçet's disease have identified multiple robust genetic susceptibility loci for the disease [5]. However, until recent years, genome-wide association studies were limited to small sample sizes due to the low prevalence of Behçet's disease in select populations [6]. It is well established that the human leukocyte antigen (HLA) class I region is the most robust genetic susceptibility locus associated with Behçet's disease [7]. Several independent susceptibility loci located within the HLA region have been identified, such as *HLA-B/MICA*, *HLA-A*, and *HLA-C* [8-10]. In addition, susceptibility loci outside the HLA region with genome-wide level of significance have also been identified, such as *IL12RB2*, *IL10*, *IFNGR1*, *STAT4*, *LNCAROD/DKK1*, *IRF8*, *FUT2*, and many more, providing valuable insights into the pathogenesis of the disease [5, 11-18]. Behçet's disease is known to affect both sexes, however, the disease tends to be more severe in

men compared to women [19, 20]. The reasons for the difference in severity of the disease between male and female patients are unknown. To better understand sex-bias in Behçet's disease, we sought to investigate the sex-specific genetic effects in the disease by performing a case-case genetic association analysis in a large cohort of patients with Behçet's disease.

2. Methods

2.1 Study population and genotyping

A total of 2,213 patients (1,330 male and 883 female patients) and 1,533 healthy controls (897 male and 636 female controls) of Turkish origin were included in the initial phase of this study. To confirm the results, a multi-ancestral metanalysis was performed, including 5 additional populations. These consisted of 224 patients from Spain (118 female and 106 male patients), 194 patients from Korea (96 female and 98 male patients), 126 patients from Italy (59 female and 67 male patients), 117 patients from Japan (27 female and 90 male patients), and 104 patients from Tunisia (33 female and 71 male patients). All patients included in the study fulfilled the 1990 international study group classification criteria for Behçet's disease [21]. Genotyping was performed as previously described [6], using the Illumina ImmunoChip custom arrays (Infinium ImmunoArray-24 V.1.0 or V.2.0 BeadChip). Genotyping data from 654 male and 545 female Turkish patients, and 748 male and 530 female Turkish controls were obtained from dbGAP (accession number (phs000272.v1.p1) [12]. The study was approved by the institutional review boards and the ethics committees at the participating institutions, and all study participants signed a written informed consent.

2.2 Data quality assessment and imputation

Genotyping data quality assessment, imputation, and subsequent filtering of genetic variants and individuals was performed as previously described [6]. Briefly, samples were filtered out if they had a genotyping call <95%, and one individual from duplicates

or first-degree relatives ($P_i\text{-HAT}>0.4$) was excluded randomly. SNPs were removed if they had a genotyping call $<98\%$ or minor allele frequency (MAF) $<1\%$ or deviated from Hardy-Weinberg equilibrium (HWE, $P<0.001$). Sex chromosomes were excluded and not analyzed in this study. Principle component analysis was done using linkage disequilibrium (LD) pruned SNPs with r^2 threshold of <0.2 , with Eigensoft 6.1.4 software [22]. Samples that were more than 6 SD from the center of the cluster were considered outliers and removed [23]. Assembly GRCh37 hg19 was used for SNP annotation [24]. Imputation was performed using genotyping data post-quality control, using the Michigan Imputation Server using Minimac3 [25]. SHAPEIT haplotype phasing software was used for haplotype reconstruction with the Haplotype Reference Consortium r1.1 as the reference population [26]. Only SNPs with high imputation accuracy values ($r^2>0.9$) were used for association analyses, and imputed variants with $\text{MAF}<1\%$ or HWE $P<0.001$ were also excluded.

The total number of patients and controls evaluated in this study, as listed above, is the final number of samples following the exclusion of samples based on quality control measures, and following the exclusion of samples with missing sex information or with genotyping-based imputed sex that mismatched reported sex.

2.3 Data Analysis and genetic risk score calculation

Plink v. 1.9 [27] was used to conduct a case-case association analysis using a logistic regression model. Genome-wide association study (GWAS) threshold of $P<5 \times 10^{-8}$ was set for evidence of significant association (sex bias). The aggregate genetic risk was

measured by calculating cumulative genetic risk scores (GRS) of individuals with Behçet's disease. A total of 20 SNPs representing the previously reported susceptibility loci for Behçet's disease were used to calculate GRS. Each locus was represented by one SNP, apart from Interferon Regulatory Factor 8 (*IRF8*), displaying 2 distinct genetic effects in this locus [5]. Only individuals with 100% genotyping success rate for these markers were included. If a particular marker was not genotyped, imputed genotype data were used for the calculation. Odds ratios (OR) used to calculate the genetic risk scores at each risk locus were those obtained from case-control association analyses for accurate representation of the impact of each locus [5]. Risk scores were calculated by multiplying the natural logarithm of the OR at each locus by the number of effect alleles: $(\sum_{k=1}^i \ln(OR_i)n_i)$ [5, 28], then Welch's t-test was used to assess differences between male and female patients. We also implemented a non-parametric multifactor-dimensionality reduction (MDR) analysis to further validate our results [29, 30].

Confirmation followed by multi-ancestral meta-analysis was performed to evaluate and confirm the sex-bias detected in rs2848712 (*MICA/HLA-B*) in multiple populations. Meta-analysis was performed with programming language R [31-33], using fixed-effect inverse variance method measuring the odds ratio for rs2848712 (*MICA/HLA-B*) comparing male and female patients for all populations. Fixed effect model was chosen in this case because both heterogeneity index (I^2) and Cochran's Q test P value suggested no evidence of heterogeneity ($I^2 < 0.5$ and $Q > 0.1$) [6].

3. Results

A Case-Case genetic association analysis was performed in the Turkish population to compare male and female patients with Behçet's disease using a logistic regression model (**Figure 1A**). Our results revealed that the most significant differences between the male and female patients were in the *HLA* region with 6 genetic variants reaching the GWAS level of significance ($P < 5 \times 10^{-8}$). Differences were observed in the HLA class I region (*MICA/HLA-B*), and the most significant sex-associated SNPs in Behçet's disease were rs2848712, rs2596527, rs2848713 (OR=1.46 (95% CI 1.28 to 1.66), $P=1.22 \times 10^{-8}$), followed by rs77563643 (OR=1.45 (95% CI 1.27 to 1.65), $P=2.47 \times 10^{-8}$), rs3819272 (OR=1.44 (95% CI 1.27 to 1.64), $P=3.15 \times 10^{-8}$), and rs79953803 (OR=1.43 (95% CI 1.26 to 1.63), $P=4.30 \times 10^{-8}$) (**Table 1, Figure 1B**). It is important to note that a similar control-control genetic association analysis including 897 male and 636 female Turkish healthy individuals revealed no significant differences across the genome (**Supplementary Figure 1**).

To confirm the sex-bias effect detected within the HLA region in patients with Behçet's disease, we evaluated the genetic association with male sex in a multiethnic cohort of patients with Behçet's disease, representing 5 additional populations. A meta-analysis between all 6 cohorts confirmed that the frequency of the minor allele in the lead SNP within the HLA region, rs2848712 (*MICA/HLA-B*), was significantly higher in male compared to female patients with Behçet's disease ($OR_{meta}=1.39$ (95% CI 1.23 to 1.56), $P_{meta}=1.83 \times 10^{-8}$) (**Figure 2**). Indeed, the direction trend of this effect was consistent in almost all populations studied (Turkish OR=1.46 (95% CI 1.28 to 1.66) $P=1.22 \times 10^{-8}$; Korean OR=1.14 (95% CI 0.73 to 1.76), $P=0.57$; Italian OR=1.75 (95% CI

1.00 to 3.06), $P=0.048$; Japanese OR=1.15 (95% CI 0.60 to 2.17), $P=0.68$; Tunisian OR 2.42 (95% CI 0.94 to 5.71), $P=0.068$), with an exception being the Spanish population with OR=0.76 (95% CI 0.47 to 1.23), $P=0.26$. The Turkish cohort had the majority of the weight (76.3%) in the analysis due to the number of individuals in the study resulting in greater power.

Next, we investigated sex-specific differences in overall genetic risk between men and women with Behçet's disease in the Turkish cohort by calculating and comparing a cumulative genetic risk score (GRS). Scores were based on the OR obtained from case-control association analyses [5]. Our results indicate that on average, men with Behçet's disease have higher genetic risk for the disease than women ($P=3.18 \times 10^{-8}$; **Figure 3A**). This difference was largely driven by the genetic variants within the HLA region, and disappeared after the HLA loci were excluded ($P=0.34$; **Figure 3B**). No difference in GRS was detected between male and female Turkish healthy controls ($P=0.61$; **Figure 3C**).

Of the previously reported disease susceptibility loci for Behçet's disease, the locus within *HLA-B/MICA* (rs116799036, OR=1.45, $P=1.95 \times 10^{-8}$) had the largest impact on the cumulative genetic risk score difference between male and female patients, followed by the locus in *HLA-C* (rs12525170, OR=1.46, $P=5.66 \times 10^{-7}$) (**Table 2**). Of note, rs116799036 was not included in the case-case GWAS analysis, as this variant was filtered out due to failing to pass the HWE test filter (HWE, $P<0.001$). The susceptibility locus in *KLRC4* (rs2617170, OR=1.20 $P=0.019$) also showed higher risk in male patients, though the imputation accuracy ($r^2=0.51$) was low and therefore data on this locus should be investigated further for confirmation. Interestingly, the risk locus in

IFNGR1 (rs4896243, OR=0.86, P=0.011) was associated with higher disease risk in female compared to male patients.

We then, performed a non-parametric analysis to confirm sex-gene interactions in the specific loci that had significant allele frequency differences between male and female patients. This was applied to test sex-gene interactions in *HLA-B/MICA*, *HLA-C*, *KLRC4* and *IFNGR1*, and confirmed our results obtained from parametric tests (**Table 3**).

4. Discussion

We explored for the first time the genetic differences between male and female patients with Behçet's disease, using a large set of patients from 6 independent populations and a genome-wide approach. The most significant sex-specific genetic differences in Behçet's disease were observed in the HLA class I region, specifically in the *HLA-B/MICA* locus (tagged by rs2848712). Indeed, the difference in GRS for Behçet's disease between male and female patients was abrogated when genetic variants within the HLA region were excluded from the analysis.

Genetic susceptibility loci in the HLA region are among the most robust contributing factors to many immune-mediated diseases, including Behçet's disease. Several independent susceptibility loci within the HLA region have been reported in Behçet's disease, with the most robust association localized to the *HLA-B/MICA* locus [8, 9]. The genetic variant rs2848712, which showed the most significant difference between male and female patients with Behçet's disease, resides in the *HLA-B/MICA* region. This variant is in LD with the previously reported *HLA-B/MICA* intergenic SNP rs116799036, which is associated with genetic risk for Behçet's disease [8], ($r^2=0.79$ in our Turkish population).

Although sexual dimorphism in Behçet's disease is poorly understood, our data demonstrated significant genetic differences in the HLA region between male and female patients. Whether these differences in the HLA region also affect disease manifestations needs further investigation. A previous meta-analysis demonstrated that *HLA-B*51/HLA-B5* was slightly but significantly present at a higher frequency in male patients (OR=1.14), and that *HLA-B*51/HLA-B5* moderately increases the risk of ocular,

skin, and genital involvement [34]. In an earlier study, male-sex has been found to be the strongest determinant of disease severity in Behçet's disease, although *HLA-B*51* was not associated with a more severe disease course [35]. Several *HLA-A* alleles were associated with various manifestations of Behçet's disease in Korean and Japanese populations. *HLA-A*26:01* and *HLA-A*30:04* were shown to have higher prevalence in patients with uveitis and vascular lesions, respectively [36].

Some studies have reported other possible explanations for sexual dimorphism in Behçet's disease. Bang et al. reported that 18 out of 27 Korean patients with Behçet's disease experienced worsening of disease during pregnancy, mostly in the first trimester, suggesting possible influence from hormones in the disease [37]. However, conflicting results have been reported, and in larger studies it appears that the majority of patients showed no exacerbation of disease during pregnancy [38]. Testosterone activates neutrophils, and male patients with Behçet's disease manifest increased neutrophil oxidative burst responses compared to female patients [39]. Nonetheless, the effect of hormonal influences on the presentation and sex-bias in Behçet's disease remains incompletely evaluated. Measurements of serum vitamin D3 levels failed to demonstrate significant differences between male and female patients with Behçet's disease [40].

Our data suggest that the risk variant in rs7749390 (*IFNGR1*) is more common in female compared to male patients with Behçet's disease. Interestingly, rs7749390 has been also identified as a possible genetic factor associated with the development of recurrent mouth ulcers (OR=1.08 (95% CI 1.07 to 1.08)) [41]. It has been reported that in Behçet's disease patients, oral ulcers are more prevalent in female compared to male

patients [20]. *IFNGR1* polymorphisms are also observed to be associated with systemic lupus erythematosus [42]. Though autoimmune clinical features and autoantibody production are not classical features of Behçet's disease, immune responses to some autoantigens and evidence for the activation of intracellular signaling pathways such as JAK/STAT pathway have been reported [43].

KLRC4 is a member of killer cell lectin-like receptor subfamily C, and encodes a calcium dependent (C type) lectin receptor. The exact function of *KLRC4* remains unknown, although it was closely associated with cytotoxic activity in natural killer cells and $\gamma\delta$ T cells [44]. It is important to note that while the Behçet's disease risk allele in *KLRC4* (rs2617170) was more prevalent in male patients, our imputation accuracy was low at this locus ($r^2=0.51$).

Our data suggest that genetic factors might contribute to differences in disease presentation between men and women with Behçet's disease. However, a sub-phenotype analysis to examine the relationship between genetic risk and Behçet's disease severity, or the frequency of organ threatening involvement, in men compared to women with the disease are necessary to support this conclusion. In addition, as in any genetic study, independent replication of our findings is needed to confirm our results. Our data might also be interpreted to suggest that men require a higher genetic load to develop Behçet's disease than women. This logic would then suggest that Behçet's disease should be more common in women than men, as the genetic threshold to develop the disease is lower in women. However, this is not consistent with epidemiologic data for Behçet's disease [45]. Therefore, an alternative explanation would be that non-genetic factors, such as environmental or hormonal factors, play a

more dominant role in the etiopathogenesis of Behçet's disease in women. A different explanation might be that there are other genetic factors that play a more dominant role in women, and which could not be assessed by our genetic approach, such as genetic regions not assessed in our study, gene-gene interactions, rare genetic variants, or epigenetic differences between men and women with Behçet's disease. Indeed, epigenetic differences between patients with Behçet's disease and healthy controls have been reported [46]. However, a comparative epigenetic analysis between men and women with Behçet's disease would be insightful.

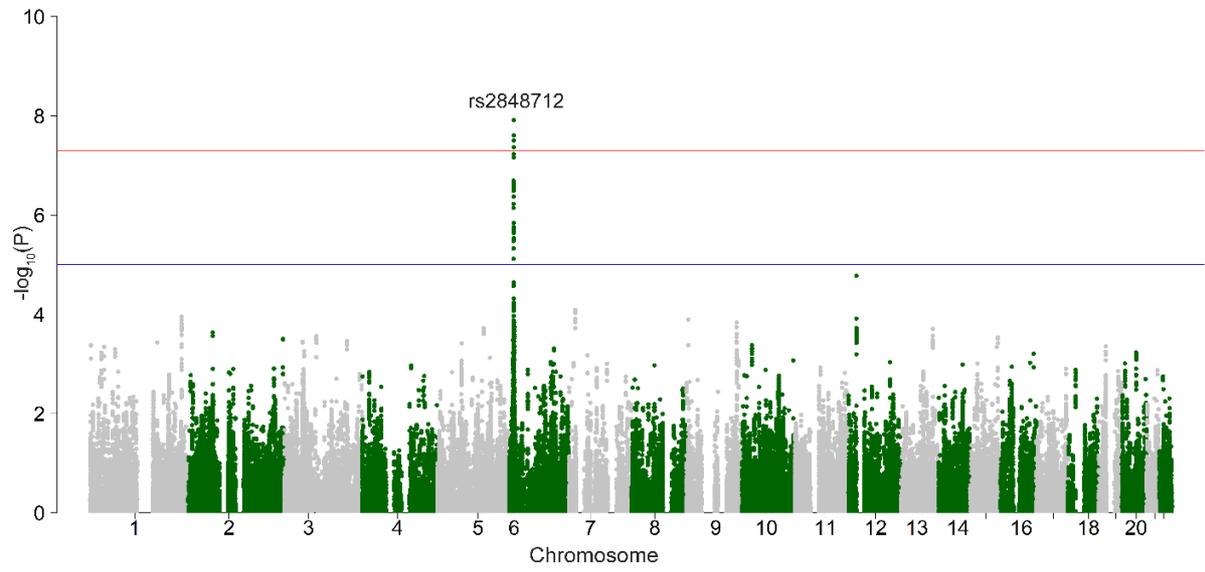
5. Conclusion

Our study comprehensively evaluated genetic contribution to sex-bias in Behçet's disease for the first time. We demonstrated that the largest genetic difference between men and women with Behçet's disease is in the HLA region, resulting in a higher overall genetic risk for the disease in male compared to female patients across multiple populations. Further studies to evaluate epigenetic differences between male and female patients, and how genetic and epigenetic factors affect specific disease manifestations in Behçet's disease are warranted.

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Figure 1

A



B

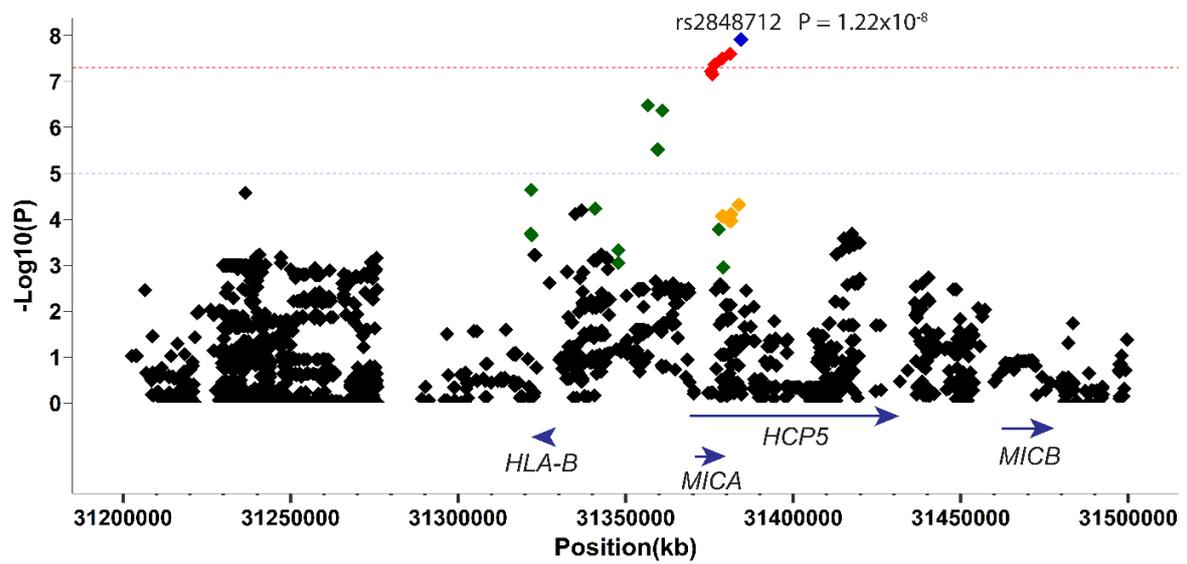


Figure 2

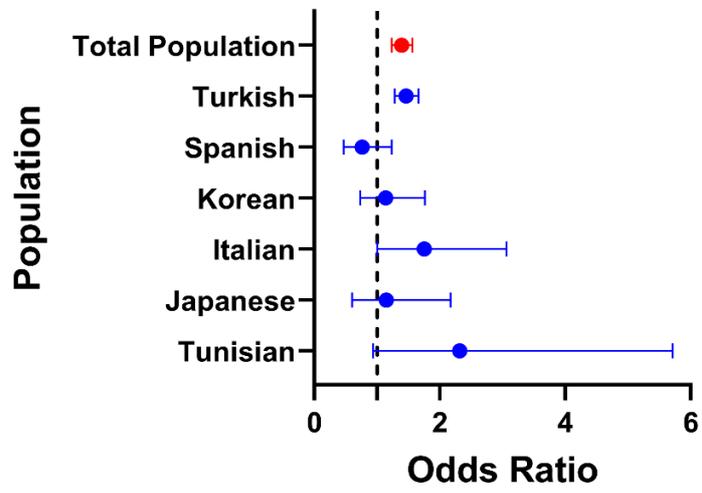


Figure 3

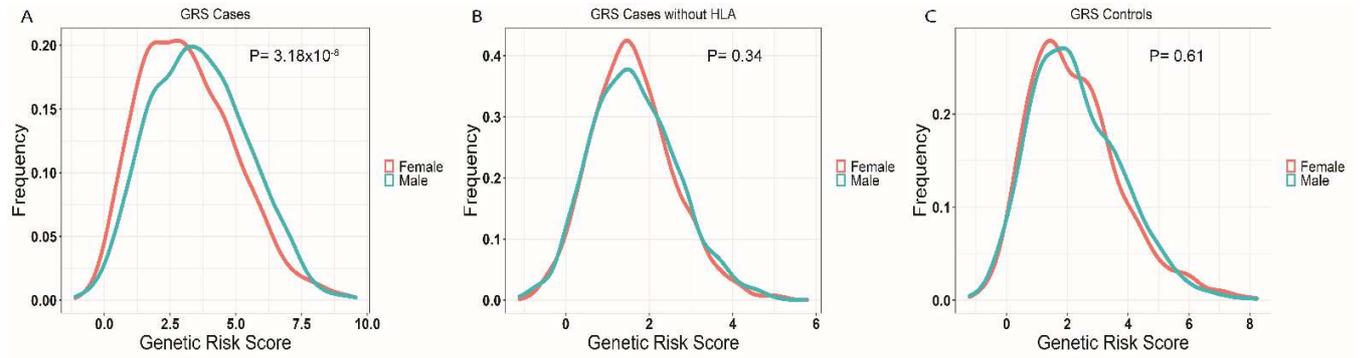


Figure Legends

Figure 1. (A) A Manhattan plot showing the results of the case-case genetic association analysis of male and female patients with Behçet's disease of Turkish origin. The $-\log_{10}(P)$ value for each variant is plotted against its chromosomal position. The red line represents the genome-wide level of significance ($P < 5.0 \times 10^{-8}$) and the blue line represents the suggestive level of significance ($P < 1 \times 10^{-5}$). **(B)** Regional plot displaying SNPs in LD with rs2848712 in the HLA region in the Turkish population (LD=1 is blue, LD=0.99-0.80 is red, LD=0.79-0.60 is orange, LD=0.59-0.40 is green, and LD=0.39-0.00 is black). The $-\log_{10}(P)$ value for each variant is plotted against its physical position in chromosome 6. The red line represents GWAS level of significance ($P < 5.0 \times 10^{-8}$) and the blue line represents suggestive level of significance ($P < 1.0 \times 10^{-5}$). (Assembly_GRCh37/hg19 by Ensembl was used).

Figure 2. Meta-analysis forest plot of rs2848712 (*HLA-B/MICA*) depicting the sex-specific differences in genetic association from different populations at this locus. Individual populations are shown in blue and total populations in red.

Figure 3. (A) Density plot of genetic risk scores (GRS) for Behçet's disease in Turkish patients. The frequencies of individuals are plotted against their respective GRS (men in blue and women in red), showing higher genetic risk in men than women ($P = 3.18 \times 10^{-8}$). **(B)** Density plot of GRS in Turkish patients with Behçet's disease without the SNPs in the HLA region (men in blue and women in red). The difference between men and women disappears ($P = 0.34$). **(C)** Density plot of GRS in healthy Turkish controls (men in blue and women in red) displaying no significant difference between men and women ($P = 0.61$).

Table 1. Results showing SNPs with GWAS level of significance ($P < 5.0 \times 10^{-8}$) from the genetic association analysis comparing men and women with Behçet's disease in the Turkish cohort.

Locus	Chr	SNP	MAF Frequency		OR	95% CI LL	95% CI UL	P-value
			Male	Female				
<i>HLA-B/MICA</i>	6	rs2848712	0.41	0.33	1.46	1.28	1.66	1.22×10^{-8}
<i>HLA-B/MICA</i>	6	rs2596527	0.41	0.33	1.46	1.28	1.66	1.22×10^{-8}
<i>HLA-B/MICA</i>	6	rs2848713	0.41	0.33	1.46	1.28	1.66	1.22×10^{-8}
<i>HLA-B/MICA</i>	6	rs77563643	0.41	0.33	1.45	1.27	1.65	2.47×10^{-8}
<i>HLA-B/MICA</i>	6	rs3819272	0.41	0.33	1.44	1.27	1.64	3.15×10^{-8}
<i>HLA-B/MICA</i>	6	rs79953803	0.41	0.33	1.43	1.26	1.63	4.30×10^{-8}

SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio of male compared to female patients (case-case analysis); CI LL, confidence interval lower limit; CI UL, confidence interval upper limit; P-value, P-values from the logistic regression analysis.

Table 2. Sex-specific differences in Behçet's disease susceptibility loci between men and women with Behçet's disease in the Turkish cohort.

Susceptibility Loci	Gene/Locus	Chr	SNP	Effect Allele	Effect Allele Frequency		OR	P-value	Imputation Accuracy (r^2)
					Male	Female			
	<i>IL12RB2</i>	1	rs924080	A	0.66	0.67	0.95	0.44	0.99
	<i>IL10</i>	1	rs1518111	A	0.38	0.36	1.09	0.30	0.93
	<i>IL1A/IL1B</i>	2	rs3783550	G	0.35	0.36	1.04	0.55	0.95
	<i>STAT4</i>	2	rs7574070	A	0.46	0.46	1.03	0.61	0.63
	<i>CCR1/CCR3</i>	3	rs7616215	T	0.73	0.73	1.01	0.88	0.94
	<i>IL12A</i>	3	rs17753641	G	0.073	0.072	1.01	0.97	0.95
	<i>ERAP1</i>	5	rs17482078	T	0.18	0.16	1.15	0.11	0.97
	<i>IFNGR1</i>	6	rs4896243	C	0.45	0.48	0.86	0.011	0.99
	<i>HLA-A</i>	6	rs114854070	A	0.27	0.25	1.11	0.12	0.94
	<i>HLA-C</i>	6	rs12525170	A	0.26	0.19	1.46	5.66×10^{-7}	0.90
	<i>HLA-B/MICA</i>	6	rs116799036	A	0.38	0.30	1.45	1.95×10^{-8}	0.94
	<i>LNCAROD/DKK1</i>	10	rs1660760	T	0.48	0.50	0.91	0.13	0.93
	<i>ADO/EGR2</i>	10	rs224127	A	0.46	0.48	0.96	0.56	0.96
	<i>JRKL/CNTN5</i>	11	rs2848479	A	0.45	0.48	1.014	0.83	0.98
	<i>KLRC4</i>	12	rs2617170	C	0.80	0.77	1.20	0.019	0.51
	<i>LACC1</i>	13	rs2121033	G	0.24	0.25	0.98	0.71	0.96
	<i>IRF8</i>	16	rs7203487	C	0.16	0.15	1.08	0.54	0.84
	<i>IRF8</i>	16	rs11117433	C	0.073	0.089	0.82	0.081	0.82
	<i>FUT2</i>	19	rs681343	T	0.53	0.53	0.99	0.92	0.78
	<i>CEBPB/PTPN1</i>	20	rs913678	C	0.32	0.34	0.91	0.17	0.62

SNP, single nucleotide polymorphism; OR, odds ratio of male compared to female patients (case-case analysis); P-value, p-value from comparing allele frequency differences between male and female patients.

Table 3. Multifactor dimensionality reduction (MDR) analysis in the Turkish cohort providing confirming evidence for interaction between sex and susceptibility loci in Behçet's disease.

Locus	SNP	MDR Analysis			
		Cross-validation consistency	Balanced Accuracy	χ^2	P-value
<i>HLA-B/MICA</i>	rs116799036	10/10	0.56	30.76	2.92x10 ⁻⁸
<i>HLA-C</i>	rs12525170	10/10	0.56	31.81	1.7x10 ⁻⁸
<i>KLRC4</i>	rs4896243	10/10	0.52	4.26	0.039
<i>IFNGR1</i>	rs4896243	10/10	0.52	5.23	0.019

Cross-validation consistency is a measure of the number of times the same MDR model is identified in each possible 90% of the subjects [31]; Balanced Accuracy is defined as (sensitivity+specificity/2). Sensitivity represents true positive rate (true positives/(true positives + false negatives)), and specificity represents true negative rate (true negatives/(false positives + true negatives)). Balanced accuracy is used as an estimator of true accuracy to mitigate imbalanced sampling [34].

Degree of freedom (df) of 1 was used to calculate the P Values from χ^2 .

SNP, single nucleotide polymorphism.

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