European Review for Medical and Pharmacological Sciences 2024; 28: 2464-2482

Association of *SOD1* gene variants (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, rs17880135) with plasma protein levels in vitiligo patients and their analysis *in silico*

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Abstract. – OBJECTIVE: Vitiligo is a common systemic, idiopathic autoimmune disease. The aim of this study was to analyze the frequency of variants of the superoxide dismutase 1 (SOD1) gene (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, rs17880135) and circulating plasma protein levels through *in-silico* analysis.

PATIENTS AND METHODS: Blood samples were collected from adult patients of both sexes with a clinical diagnosis of vitiligo. ELISA tests for SOD and analysis of gene variants by qPCR were compared to a disease-free reference group.

RESULTS: The population analyzed was young people between 29 and 37 years old, with a higher percentage of women. The population was found in the Hardy-Weinberg equilibrium (HWE). The 50 bp Ins/Del, rs4817415, and rs2070424 variants showed no significant difference between groups ($p > 0.05$). Although, in the dominant model, the CT and CTTT genotypes of the rs1041740 and rs17880135 variants showed an association with susceptibility to vitiligo compared to the control. Plasma SOD levels showed significant differences between the groups, and when stratified according to the genotypes of each variant, there was a significant difference, except with the rs17880135 variant. The haplotypes InsC-GTC and InsAGCC are shown to be risk factors for susceptibility to vitiligo. The *in-silico* analysis demonstrated that the rs4817415, rs2070424, rs1041740, and rs17880135 variants of the *SOD1* gene participate in the modification of selected regulatory elements for differentiating the protein, transcription factors, and long non-coding RNA.

CONCLUSIONS: Information regarding the pathogenesis of vitiligo helps recognize risk factors and identify the relationship of diagnostic markers of cell damage inherent to the disease. This will help improve aspects of prevention and the choice of treatment alternatives appropriate to each case.

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Key Words:
Superoxide dismutase 1, Skin depigmentation, Hypopigmentation, Oxidative stress, Plasma SOD level, Vitiligo.

Introduction

Melanocytes synthesize a pigment known as melanin and distribute it among keratinocytes. The most important function of melanin is to protect the body from the deleterious effects of non-ionizing ultraviolet irradiation[1,2].

The disease vitiligo, also known as idiopathic acquired leukoderma, is a common, chronic, systemic, asymptomatic, and idiopathic disease that affects the skin, hair, and mucous membranes. It is characterized by macules and circumscribed hypochromic and achromic spots[1-3] and caused by the loss of function or destruction of melanocytes in the epidermis[4-6]. However, melanocyte loss occurs not only in the skin but also in the mucous membranes, eyes, and membranous labyrinth located in the middle ear, which causes the association between vitiligo and ocular manifestations, decreased hearing acuity, and autoimmune diseases[7,8].

This skin pigment disorder has an autoimmune component in which oxidative stress is proposed to be the initial triggering factor for subsequent immune dysregulation[9-12]. Evidence[9-17] reported that oxidative stress may also be present systemically in patients with vitiligo.

In addition, it is suggested that the deterioration of melanocytes could initially be related to an increase in oxidative stress; that is, the alteration of the redox balance in the skin could cause damage to melanocytes and cause hypopigmented macules. Consequently, there is an accumulation of hydrogen peroxide in the epidermis of patients with active disease[17] and increased activity of the enzyme superoxide dismutase 1 (*SOD1*; OMIM: 147450), the most abundant enzymatic antioxidant, which works by repairing cells[7,17-21].

The *SOD1* gene (21q22) has five exons and four introns. Some transcription factors have binding sites on the promoter region, and other polymorphisms have been studied for their association with different diseases, such as breast cancer[20]. The *SOD1* enzyme has antioxidant activity, binding to Cu and Zn ions and acting by deactivating superoxide radicals. Mutations in *SOD1* affect the activity of the protein, with gain or loss of activity. Deleterious mutations produce toxicity by the accumulation of hydroxyl radicals[22]. SOD deregulation can generate oxidative stress, which could cause DNA damage, activate transcription factors, and initiate multiple signal transductions that promote carcinogenesis and alterations in melanogenesis[1,20].

The rs1041740 variant of the *SOD1* gene has been linked[23-25] to heart conditions, hearing loss, and diabetic peripheral neuropathy, while the rs17880135 variant has been related to chronic kidney disease and diabetic nephropathy[26-28]. In addition, rs2070424 is associated with the risk of Alzheimer's disease and Parkinson's disease[29,30]. In addition, an association of amyotrophic lateral sclerosis with the rs4817415 variant has been described, with a founder effect reported[22,31] in *SOD1* A4V. The 50 bp Ins/Del variant, which is involved in reducing the activity of the gene promoter, has been related to the risk of breast cancer, osteoporosis, cardiovascular disease, bladder cancer, and preeclampsia, among other pathologies[18,20,31-34]. Vitiligo is not only a condition of cosmetic importance; it is a nosological entity involving genetic, neurological, autoimmune, and psychological factors[1]. In patients from Mexico, there is no information available regarding the status of *SOD1* gene variants in regulatory regions or the quantification of plasma protein levels. However, SOD supplementation is common as an adjuvant treatment, and therefore, having precise information on the genetic background of the Mexican population will allow us to have more precise elements for its treatment.

Patients and Methods

Blood samples (10 mL) were collected from 29 patients clinically diagnosed by a dermatologist as having vitiligo, classified as segmented and non-segmented, and 44 samples were collected from a control group of donors without vitiligo. The blood samples were divided into two tubes, one to obtain the serum after centrifugation and storage at -80°C until the determination of plasma SOD levels; while the other tube contained EDTA to extract the DNA.

The study groups have signed an informed consent letter approved by the Ethics Committee of the University of Guadalajara, ensuring compliance with all study procedures outlined in the Declaration of Helsinki. Clinical and demographic data were obtained through written questionnaires.

Plasma SOD levels were analyzed by the ELISA method according to the instructions for the SOD assay kit (Cayman Chemical, Ann Arbor,

MI, USA) expressed in U/mL. DNA extraction was performed using the Miller method[35], and the variants (rs4817415, rs2070424, rs1041740, and rs17880135) of the *SOD1* gene were extracted with real-time PCR using IDT probes (Integrated DNA Technologies, Inc., Coralville, IA, USA). Genotypes were identified using the CFX96 real-time PCR system C1000 Touch (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the conditions of 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 45 s, and 68°C for 28 s.

The 50 bp Ins/Del variant was carried out by endpoint PCR using the previously published primers[20,36] 5'-AATTCCTTACCCCTGTTCTA-3' and 5'-GGCAGATTTCAGTTCATTGT-3' at an annealing temperature of 58°C. After shifting electrophoresis in polyacrylamide gels at 6% (29:1) and silver nitrate staining, two bands were identified: one of 297 bp (Insertion) and one of 247 bp (Deletion).

In-Silico Analysis

In this study, *in-silico* analyses were performed to investigate how the rs4817415, rs2070424, rs1041740, and rs17880135 variants influence *SOD1* gene regulation. The tools HaploReg v.4.2 (available at: https://pubs.broadinstitute.org; accessed August 6, 2023), RegulomeDB, v.2.2 (available at: https://www.regulomedb.org; accessed August 7, 2023), and rSNPBASE V3.1 (available at: http://rsnp3.psych.ac.cn; accessed August 8, 2023) were used to analyze how the different variants on the *SOD1* gene participate or interfere in different *SOD1* gene regulation sites.

In addition, an eQTL (Expression Quantitative Trait Loci) analysis was performed in the GTEx Portal (available at: https://www.gtexportal.org/home/; accessed August 8, 2023) to observe how each variant is associated with the expression of the *SOD1* gene.

Statistical Analysis

We determined the frequency of the genotypes and alleles of each *SOD1* gene variant by direct counting. The Hardy-Weinberg equilibrium (HWE) was calculated by means of the Chi-squared test, and the frequencies of the observed genotypes were compared with what was expected. Non-parametric tests were used to compare the means and standard deviations of protein levels because the study populations were not homogeneous. The Kolmogorov-Smirnov test was used because the sample size was less than 50, and the Mann-Whitney U-test and the receiver operating characteristic (ROC) curve were used to determine the cut-off point for plasma SOD levels. Odds ratios were also calculated in SPSS Statistic Base 24 software (IBM Corp., Armonk, NY, USA). The pairwise linkage disequilibrium (D′) and haplotype frequency were analyzed using SHEsis Online (available at: http://shesisplus.bio-x.cn/SHEsis.html; accessed August 5, 2023). In this study, p-values < 0.05 were considered indicative of statistical significance.

Results

The general characteristics of the study groups are described in Table I. The average age was 36.5 years in patients with vitiligo and 29 in controls, with participants being mostly female in patients (n = 21, 72%) and in controls (n = 24, 54.5%). Tobacco consumption was 55% (n = 16) in patients and 20% (n = 9) in controls. Alcohol consumption was 27% in both study groups, while marijuana type drug consumption was 21% (n = 6) in patients and 14% (n = 6) in controls.

The clinical phenotype of patients with vitiligo was distributed from 1 to 4 in 64% (n = 18), while it was unknown in the remaining 36%.

The frequencies of genotypes and alleles of the 50 bp Ins/Del, rs4817415, rs2070424, rs1041740, and rs17880135 variants of the *SOD1* gene in patients with vitiligo and the control group are described in Table II. The genotypes and alleles of the 50 bp Ins/Del, rs4817415, and rs2070424 variants did not show statistically significant differences when comparing the study groups ($p > 0.05$). However, the CT [OR 3.7 (95% CI 1.36-9.8), $p = 0.01$] and CTTT [dominant model; OR 3.1 (95% CI 1.15-8.6), $p = 0.04$] genotypes of the rs1041740 variant showed an association of susceptibility to the risk of vitiligo when compared to the control group. The CT [OR 6.6 (95% CI 1.27-34.9), $p = 0.02$] and CTTT [dominant model; OR 6.6 (95% CI 1.27-34.9), $p = 0.02$] genotypes and the T allele [OR 5.9 (95% CI 1.18-29.5), $p = 0.02$] of the rs17880135 variant showed an association of vitiligo risk susceptibility when compared with the control group.

Serum levels of SOD in the patient group with vitiligo (n = 29, 3.5341 ± 0.87837 U/mL) and the control group (n = 44, 3.2480 ± 1.35566 U/mL) showed statistically significant differences when compared ($p < 0.05$) (Table III and Figure 1). These serum levels of SOD were stratified by the genotypes of each of the variants (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, and rs17880135) of the *SOD1* gene analyzed in this study, showing

Table I. General characteristics of the study groups.

	Vitiligo patients (n = 29)	Controls (n = 44)	*p*-value
Age (years)			
Mean ± SD	36.58 ± 11.89	29.02 ± 12.14	0.0001*
Gender			
Male [(n), %]	(8) 27.6	(20) 45.5	0.19
Female [(n), %]	(21) 72.4	(24) 54.5	
Tobacco consumption			
Yes [(n), %]	(16) 55	(9) 20	0.005*
No [(n), %]	(13) 45	(35) 80	
Alcohol consumption			
Yes [(n), %]	(8) 27.5	(12) 27.2	1.0
No [(n), %]	(21) 72.5	(32) 72.8	
Drugs consumption (marijuana)			
Yes [(n), %]	(6) 21	(6) 14.0	0.63
No [(n), %]	(23) 79	(38) 86.0	
Vitiligo phenotype			
1	1	4	
2	5	17	
3	12	41	
Unknown	11	38	

*Mann-Whitney U test. 1 incomplete depigmentation, 2 complete depigmentation (hair whitening in a minority of hairs < 30%), 3 complete depigmentation plus significant hair whitening (> 30%).

Table II. Genotype and allelic distribution of the variants of *SOD1* in vitiligo patients and controls.

Variant		Vitiligo		Controls*		OR	95% (CI)	*p*-value
50 bp Ins/Del	Genotype	(n = 29)	%	(n = 44)	%			
	Ins/Ins	(21)	72	(35)	80	1.0		
	Ins/Del	(6)	21	(8)	18	1.1	(0.36-3.82)	1.0
	Del/Del	(2)	7	(1)	2	3.1	(0.27-36.8)	0.55
Dominant	Ins/Ins	(21)	72	(35)	80			
	Ins/Del+Del/Del	(8)	28	(9)	20	1.48	(0.49-4.42)	0.50
Recessive	Del/Del	(2)	13	(1)	7	3.1	(0.27-36.8)	0.55
	Ins+Ins/Del	(27)	87	(43)	93			
	Allele (2n = 58)			(2n = 88)				
	Ins	(48)	0.827	(78)	0.748	0.61	(0.23-1.58)	0.44
	Del	(10)	0.173	(10)	0.252	1.62	(0.53-4.19)	0.44
rs4817415	Genotype	(n = 29)	%	(n = 44)	%			
	AA	(2)	7	(2)	5	1.0		
	AC	(10)	34	(15)	34	1.0	(0.37-2.73)	1.0
	CC	(17)	59	(27)	61	0.9	(0.34-2.32)	1.0
Dominant	AA	(2)	7	(2)	5			
	AC+CC	(27)	93	(42)	95	0.6	(0.08-4.81)	1.0
Recessive	CC	(17)	8	(27)	4	0.9	(0.34-2.32)	1.0
	AA+AC	(12)	92	(17)	96			
	Allele (2n = 58)			(2n = 88)				
	A	(14)	0.241	(19)	0.215	1.1	(0.52-2.53)	0.87
	C	(44)	0.759	(69)	0.785	0.8	(0.39-1.90)	0.87
rs2070424	Genotype	(n = 29)	%	(n = 44)	%			
	AA	(11)	38	(20)	45	1.0		
	AG	(13)	45	(20)	45	1.0	(0.38-2.50)	1.0
	GG	(5)	17	(4)	10	2.0	(0.50-8.52)	0.5
Dominant	AA	(2)	7	(2)	5			
	AG+GG	(27)	93	(42)	95	0.6	(0.08-4.84)	1.0
Recessive	GG	(17)	8	(27)	4	2.0	(0.50-8.52)	0.5
	AA+AG	(12)	92	(17)	96			
	Allele (2n = 58)			(2n = 88)				

(Table continued)

Table II. ***(Continued).*** Genotype and allelic distribution of the variants of *SOD1* in vitiligo patients and controls.

Variant		Vitiligo		Controls*		OR	95% (CI)	*p*-value
	A	(35)	0.603	(60)	0.681	0.7	(0.35-1.41)	0.42
	C	(23)	0.397	(28)	0.319	1.4	(0.70-2.81)	0.42
rs1041740	Genotype	(n = 29)	%	(n = 44)	%			
	CC	(8)	27	(24)	54	1.0		
	CT	(19)	66	(15)	34	3.7	(1.36-9.8)	0.01
	TT	(2)	7	(5)	12	0.6	(0.10-3.1)	0.81
Dominant	CC	(8)	28	(24)	55			
	CT+TT	(21)	72	(20)	45	3.1	(1.15-8.6)	0.04
Recessive	TT	(17)	8	(27)	4	0.6	(0.10-3.1)	0.81
	CC+CT	(12)	92	(17)	96			
	Allele (2n = 58)			(2n = 88)				
	C	(35)	0.603	(63)	0.715	0.6	(0.29-1.21)	0.21
	T	(23)	0.397	(25)	0.285	1.6	(0.82-3.33)	0.21
rs17880135	Genotype	(n = 29)	%	(n = 44)	%			
	CC	(22)	76	(42)	95	1.0		
	CT	(7)	24	(2)	5	6.6	(1.27-34.9)	0.02
	TT	(0)	0	(0)	0			
Dominant	CC	(22)	28	(42)	55			
	CT+TT	(7)	72	(2)	45	6.6	(1.27-34.9)	0.02
Recessive	TT	(0)	0	(0)	0	1.4	(0.09-24.6)	0.78
	CC+CT	(29)	100	(44)	100			
	Allele (2n = 58)			(2n = 88)				
	C	(51)	0.880	(86)	0.980	0.1	(0.03-0.84)	0.02
	T	(7)	0.120	(2)	0.020	5.9	(1.18-29.5)	0.02

OR (odds ratio), CI (confidence intervals), *p*-value (significant < 0.05). * Hardy-Weinberg equilibrium in controls for 50 bp Ins/Del (Chi-square test = 0.4177, $p = 0.5180$), rs4817415 (Chi-square test = 0.002; $p = 0.963$), rs2070424 (Chi-square test = 0.099; $p = 0.752$), rs1041740 (Chi-square test = 1.15; $p = 0.282$), rs17880135 (Chi-square test = 0.02; $p = 0.877$).

Table III. Differences in serum levels of SOD in the study groups and their distribution by genotypes of *SOD1* gene variants.

	Vitiligo		Controls		
	SOD serum value (U/mL)				
	n	Mean ± SD	n	Mean ± SD	*p*-value*
	(29)	3.534 ± 0.878	(44)	3.248 ± 1.355	0.038
Variant					
Ins	(21)	3.32 ± 0.819	(35)	3.33 ± 1.474	0.351
Ins/Del	(6)	3.78 ± 0.229	(8)	2.92 ± 0.757	0.029
Del	(2)	5.01 ± 0.016	(1)	3.11	0.000
rs4817415					
AA	(2)	5.00 ± 2.660	(0)		
AC	(10)	3.66 ± 1.070	(16)	3.64 ± 1.690	0.531
CC	(17)	3.41 ± 0.716	(28)	2.89 ± 0.873	0.015
rs2070424					
AA	(11)	3.90 ± 0.554	(20)	3.73 ± 1.800	0.338
AG	(13)	2.99 ± 0.837	(20)	2.89 ± 0.660	0.758
GG	(5)	4.11 ± 0.888	(4)	2.62 ± 0.356	0.032
rs1041740					
CC	(8)	4.13 ± 0.618	(24)	3.22 ± 1.590	0.002
CT	(19)	3.33 ± 0.885	(15)	3.26 ± 0.943	0.864
TT	(2)	3.10 ± 0.848	(5)	3.18 ± 1.520	0.857
rs17880135					
CC	(22)	3.34 ± 0.737	(42)	3.26 ± 1.380	0.195
CT	(7)	4.12 ± 1.070	(2)	2.80 ± 0.107	0.222
TT	(0)		(0)		

*Mann-Whitney U test.

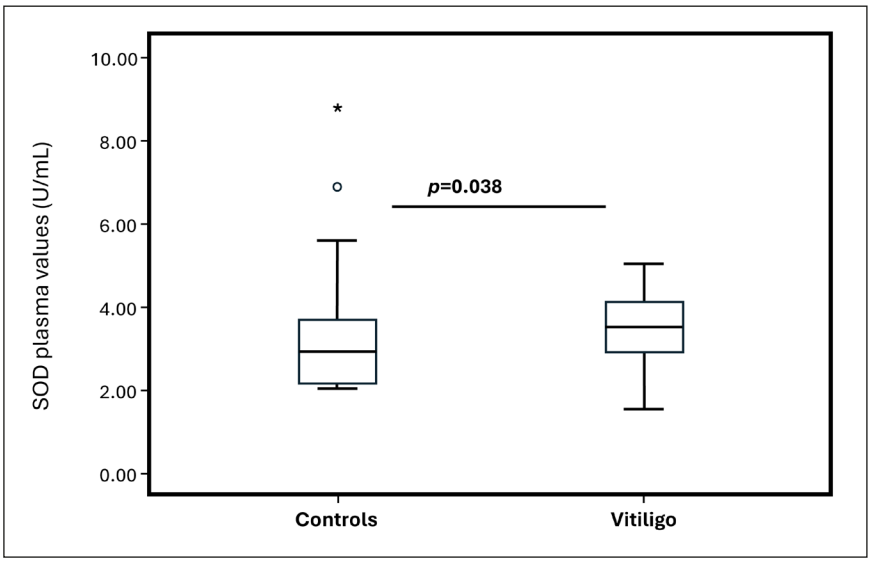


Figure 1. Plasma SOD values in the study groups. ROC curve sensitivity of 83% and specificity of 66%. The cut-off point for SOD values was 2.6480 U/mL. *=atypical value.

statistically significant differences when comparing the study groups ($p < 0.05$), except with the rs17880135 variant of the *SOD1* gene (Table III).

Haplotypes of the variants (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, and rs17880135) of the *SOD1* gene were analyzed in the patients with vitiligo and the control group (Table IV). It was observed that the InsCGCC haplotype showed a protection susceptibility factor [0.28 (0.09-0.90), $p=0.03$], while the InsCGTC [10 (0.91-85.3), $p=0.01$] and InsAGCC [8.1 (0.92-71.6), $p=0.03$] haplotypes showed risk susceptibility factors for vitiligo, although the number of cases analyzed was low, which would be considered a limitation of the study. However, characteristic haplotypes, which represented the Mexican population, were observed in the analyzed sample. Unfortunately, there are no studies in other populations that compare the analyzed haplotypes. The linkage disequilibrium was strong between the rs1041748 and rs17880135 variants (96%) and the rs17880135 and rs4817415 variants (98%) and moderate between the 50 bp Ins/Del and rs1041748 variant (74%) (Figure 2).

In-Silico Analysis

Prediction of the impact of the variants

Through the HaploReg tool, we observed that the rs4817415, rs2070424, rs1041740, and rs17880135 variants of the *SOD1* gene participated in the modification of selected regulatory elements. The rs4817415 variant is bound to the binding sites of the Arid5b and RREB1 proteins. The rs2070424 variant altered the anchor points of the Barx2, Dlx3, Dlx5, HNF1, Hlxb9, Hoxa3, Hoxa5, Hoxa7, Hoxb4, Hoxb7, Hoxb8, Hoxc6, Hoxc9, Hoxd8, Lhx3, Msx-1, Nkx6-1, and Phox2a proteins and Vax2. The rs1041740 variant affected the interaction sites of the EBPB, CEBPB, and p300 proteins, and the rs17880135 variant interfered with the GR and PLZF binding of the proteins.

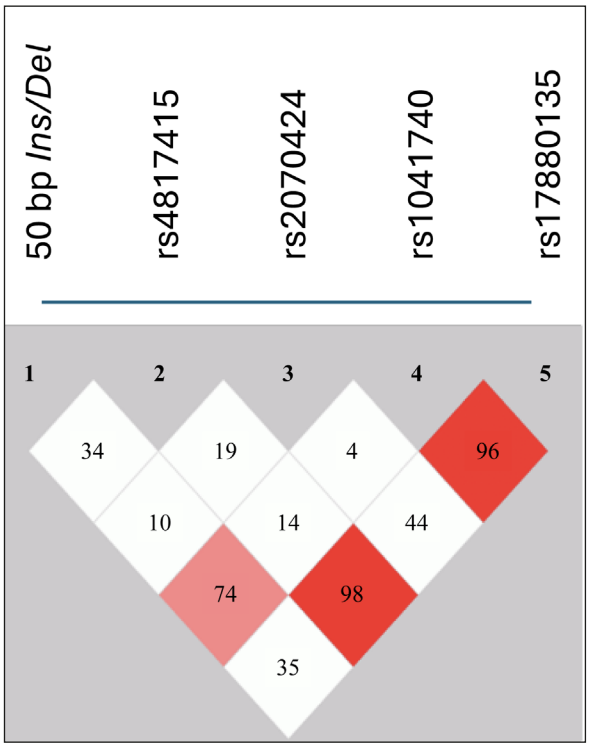


Figure 2. Linkage disequilibrium of 50 bp Ins/Del, rs4817415, rs2070424, rs1041740, and rs17880135 variants in the *SOD1* gene.

Table IV. Distribution of haplotypes of *SOD1* gene variants in patients with vitiligo and the control group.

Haplotype					Vitiligo patients		Control group			
50 bp Ins/Del	rs4817415	rs2070424	rs1041740	rs17880135	n	(%)	n	(%)	OR (95% CI)	*p*-value
Del	A	A	C	C	3	(0.05)	1	(0.01)	4.7 (0.48-46.7)	0.30
Del	A	G	C	C	3	(0.05)	2	(0.02)	2.3 (0.37-11.4)	0.38
Del	C	A	C	C	2	(0.03)	6	(0.07)	0.4 (0.09-2.5)	0.47
Del	C	A	C	T	0	(0.00)	1	(0.01)	0.7 (0.06-8.3)	1.0
Ins	A	A	C	C	1	(0.02)	8	(0.09)	0.1 (0.02-1.4)	0.08
Ins	A	G	T	C	2	(0.03)	6	(0.07)	0.4 (0.07-2.0)	0.30
Ins	C	A	C	C	14	(0.24)	28	(0.31)	0.6 (0.31-1.4)	0.37
Ins	C	A	T	C	15	(0.26)	17	(0.19)	1.4 (0.66-3.2)	0.46
Ins	C	G	C	C	4	(0.07)	18	(0.21)	0.28 (0.09-0.90)	0.03
Ins	C	G	C	T	4	(0.075)	1	(0.01)	6.3 (0.68-58.1)	0.16
Ins	C	G	T	C	6	(0.10)	1	(0.01)	10 (0.91-85.3)	0.01
Ins	A	G	C	C	4	(0.075)	0	(0.0)	8.1 (0.92-71.6)	0.03

The RegulomeDB tool predicted the regulatory scores for each of the variants: rs4817415 (0.55436), rs2070424 (0.5985), rs1041740 (0.55436), and rs17880135 (0.58955). The analysis of the transcription factor (TF) binding sites using the ChIP-Seq tool (avaialbale at: https://epd.expasy.org/chipseq/) revealed that the rs4817415 variant was located in the TF binding sites of MAX (score 36.81) and CEBPA (scores 3.78 and 5.39). The rs2070424 variant was found in the sequence of the TF binding sites POLR2A (scores 21.70, 16.60, and 46.92), ATF3 (score 71.63), and GM12878 (score 41.75). The rs1041740 variant showed an association with the TF OSR2, ZNF652, PRDM6, and GLI2 (scores 73.97, 37.53, 117.48, and 12.42, respectively). Finally, the rs17880135 variant showed interference in the binding of PML, FOXM1, AGO2, POLRG2, and SUPT5H (scores 33.34, 14.04, 24.05, 33.99, and 101.90, respectively) (Figure 3).

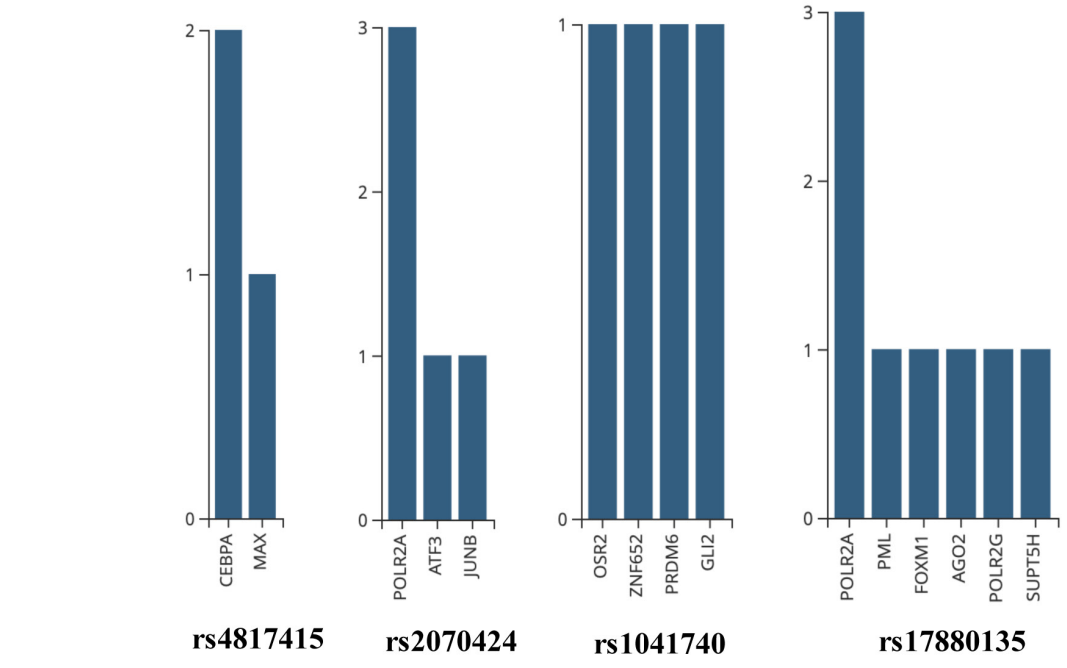
Using the rSNPBASE tool, it was observed that each variant analyzed was linked to different regulatory elements of the gene. In the case of the rs4817415 variant, its location in a topologically associated domain (TAD) was evidenced in the following regions: chr21:32960001-33000000___ chr21:34880001-34920000. Furthermore, the tool showed that the rs2070424 variant resided in the junction points of the transcription factors POLR2A, CEBPB, NFIC, and CUX1, as well as in the coding region of the long non-coding RNA (lncRNA) lnc-SCAF4-2, and in the regions of the circular RNAs (cirRNA) hsa-circ-*SOD1*-overlap.3 and hsa-circ-*SOD1*-overlap.4. Furthermore, it was confirmed that the rs1041740 variant was localized in the binding sites of the transcription factors POLR2A, PRDM1, and STAT3. Notably, this variant is present in six different topologically associated domains and junction points of the hsa-circ-*SOD1*-overlap.4 cirRNA (Figure 4). Unfortunately, the tool did not provide any relevant information about the rs17880135 variant.

Analysis with eQTL

When it was demonstrated that the previously mentioned variants of the *SOD1* gene were related to regula-tory elements of the gene, an eQTL analysis was carried out with the GTEx portal, and the following data were obtained. The genotypes of the rs4817415 variant were not identified as eQTL in the expression of *SOD1* in skin not exposed to the sun (PNES) (p=0.70), as well as in skin exposed to the sun (PES) (p=0.63), obtaining the following mean expression by PNES genotypes: AA, 0.01936; AC, -0.007260; and CC, -0.007260. The following means of expression by genotype in PES were also obtained: AA, -0.004136; AC, 0.02896; and CC, -0.04552.

However, the expression of the rs2070424 variant genotypes was statistically significant in PNES (p=0.0014) and PES (p= 7.7e-10). The following means of expression by genotypes in PNES were obtained: AA, 0.04357; AG, 0.1802; and GG, undefined. In PES, the means of expression were AA, -0.07038; AG, 0.2253; and GG, undefined.

Likewise, for the rs1041740 variant, the expression means of the genotypes were different, with p=0.000027 in PNES and p=0.0034 in PES. The means of expression by genotype in PNES were CC, 0.1286; CT, -0.1310; and TT, -0.2146. In PES, the means of expression by genotype were CC, 0.1233; CT, -0.07452; and TT, -0.1767.



rs4817415

ChIP data

Method	Peak location	Biosample	Targets	Organ	Dataset	File	Value
ChIP-seq	chr21:31619173..31619569	K562	MAX	bodily fluid, blood	ENCSR000BLP	ENCFF822FKQ	36.81932
ChIP-seq	chr21:31618700..31619442	BLaER1	CEBPA	bone element, bone marrow	ENCSR490DXW	ENCFF438BMZ	5.39907
ChIP-seq	chr21:31619069..31619351	BLaER1	CEBPA	bone element, bone marrow	ENCSR195PUH	ENCFF295MNC	3.78232

rs207042

ChIP data

Method	Peak location	Biosample	Targets	Organ	Dataset	File	Value
ChIP-seq	chr21:31666841..31667311	K562	POLR2A	bodily fluid, blood	ENCSR000FAW	ENCFF215BYD	21.70330
ChIP-seq	chr21:31666939..31667423	K562	POLR2A	bodily fluid, blood	ENCSR000FAX	ENCFF552DZV	16.60223
ChIP-seq	chr21:31666962..31667441	K562	POLR2A	bodily fluid, blood	ENCSR388QZF	ENCFF355MNE	46.92355
ChIP-seq	chr21:31667002..31667666	K562	ATF3	bodily fluid, blood	ENCSR632DCH	ENCFF107DBQ	71.63857
ChIP-seq	chr21:31666598..31667028	GM12878	JUNB	bodily fluid, blood	ENCSR897MMC	ENCFF912OPT	41.75384

rs1041740

ChIP data

Method	Peak location	Biosample	Targets	Organ	Dataset	File	Value
ChIP-seq	chr21:31667666..31668086	HEK293	OSR2	kidney, epithelium	ENCSR324LTM	ENCFF632WJK	73.97261
ChIP-seq	chr21:31667717..31668097	HepG2	ZNF652	endocrine gland, epithelium, liver, exocrine gland	ENCSR502GAX	ENCFF170GIQ	37.53656
ChIP-seq	chr21:31667714..31668182	HEK293	PRDM6	kidney, epithelium	ENCSR892QHR	ENCFF540USW	117.48736
ChIP-seq	chr21:31667595..31667899	HEK293	GLI2	kidney, epithelium	ENCSR978EQY	ENCFF576SDC	12.42015

rs17880135

ChIP data

Method	Peak location	Biosample	Targets	Organ	Dataset	File	Value
ChIP-seq	chr21:31669409..31669965	PFSK-1	POLR2A	connective tissue, brain	ENCSR000BPC	ENCFF881IAR	32.18376
ChIP-seq	chr21:31669017..31669697	GM12878	PML	bodily fluid, blood	ENCSR000BQM	ENCFF242KVU	33.34596
ChIP-seq	chr21:31669193..31669709	GM12878	FOXM1	bodily fluid, blood	ENCSR000BRU	ENCFF549GKZ	14.04497
ChIP-seq	chr21:31669670..31669924	K562	POLR2A	bodily fluid, blood	ENCSR000EGF	ENCFF951KHS	24.05397
ChIP-seq	chr21:31669613..31670277	HepG2	AGO2	endocrine gland, epithelium, liver, exocrine gland	ENCSR151NQL	ENCFF348MDS	33.99610
ChIP-seq	chr21:31669663..31670307	K562	POLR2G	bodily fluid, blood	ENCSR283ZRI	ENCFF413LLO	9.27673
ChIP-seq	chr21:31668887..31670121	K562	POLR2A	bodily fluid, blood	ENCSR388QZF	ENCFF355MNE	94.59458
ChIP-seq	chr21:31669008..31669723	K562	SUPT5H	bodily fluid, blood	ENCSR894CGX	ENCFF738XMN	101.90111

Figure 3. Analysis of the transcription factor of rs4817415, rs2070424, rs1041740, and rs17880135 variants by ChIP-Seq tool. *The maximum numbers on the Y-axis in each bar represent the number of samples in which a specific TF exhibits a maximum signal (value) at a given variant location.

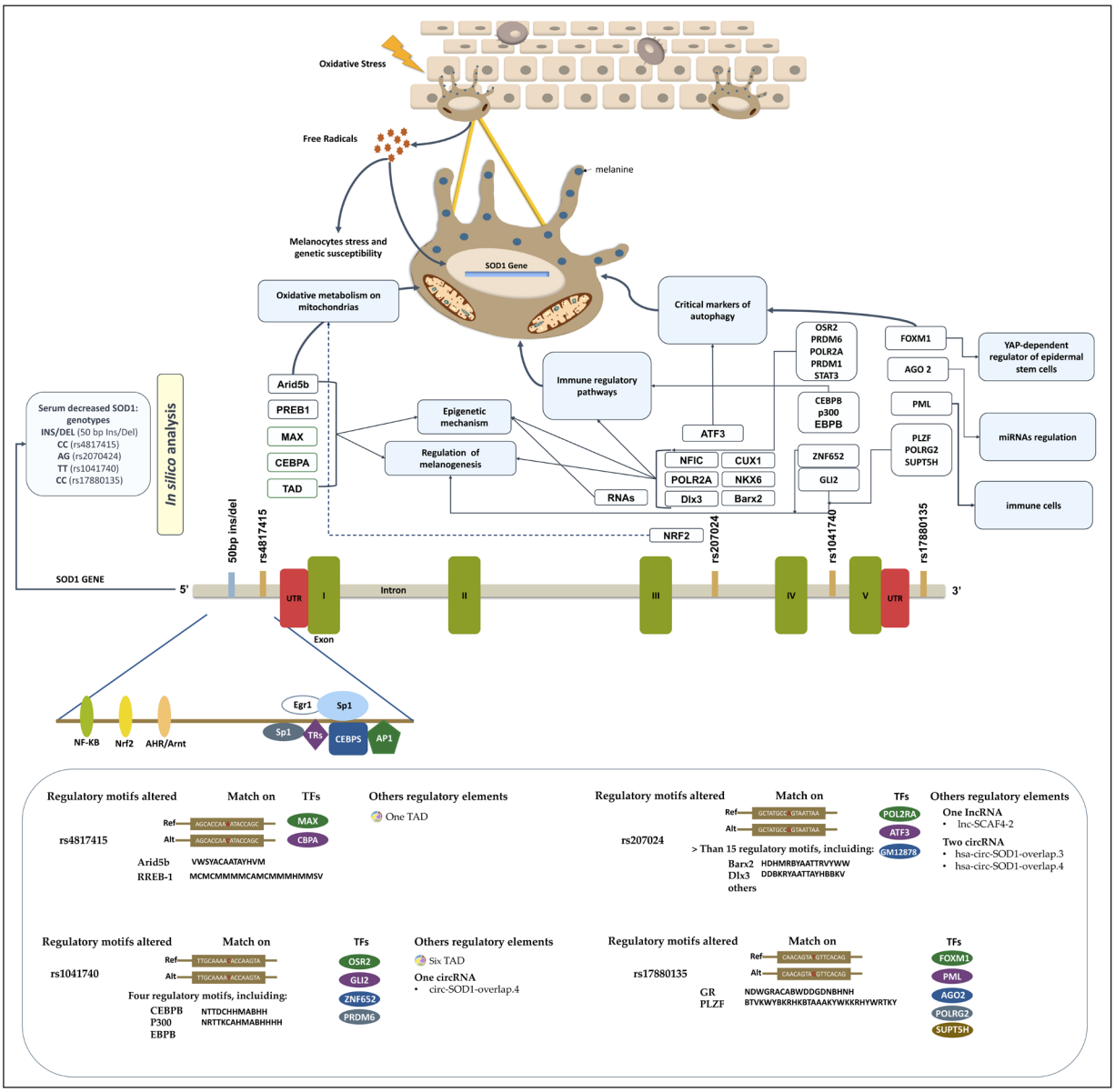


Figure 4. Union sites of regulation factor and circRNA at rs4817415 and rs1041740 variants of the *SOD1* gene. One of the characteristics of the SOD enzyme family is that they are the first line of cellular defense for the elimination of reactive oxygen species (ROS) produced by oxidative stress, chemicals, and ultraviolet radiation, during the melanogenesis process. The imbalance between ROS generation and their elimination can alter gene expression levels and, therefore, alter the level of ROS detoxification, consequently damaging proteins and nucleic acids. The variants analyzed in this study are located in regulatory regions of the *SOD1* gene, which can contribute to epigenetic modulation or be target sites for other regulatory regions of transcription, signaling pathways, micro (mi) RNA, and contribute to the modulation of adipogenesis, fibrogenesis, melanogenesis, retinal pigment epithelium, adaptive immune activation, oxidative stress and mitophagy, processes that participate in the modulation of oxidative metabolism of the mitochondrial membrane potential, that are important mechanisms in vitiligo.

Finally, the rs17880135 variant did not show statistically significant differences in the expression means, with $p=0.13$ in PNES and $p=0.44$ in PES. The expression means observed in PNES were TT, 0.000; TG, 0.07750; and GG, undefined. The means of expression in PES were TT, 0.002068; TG, -0.07452; and GG, undefined (Figure 5).

Discussion

General Characteristics of the Study Groups

Vitiligo is a disorder that affects between 0.5% and 1% of the world's population[37]. In Mexico, the exact frequency is unknown. It has been observed[38]

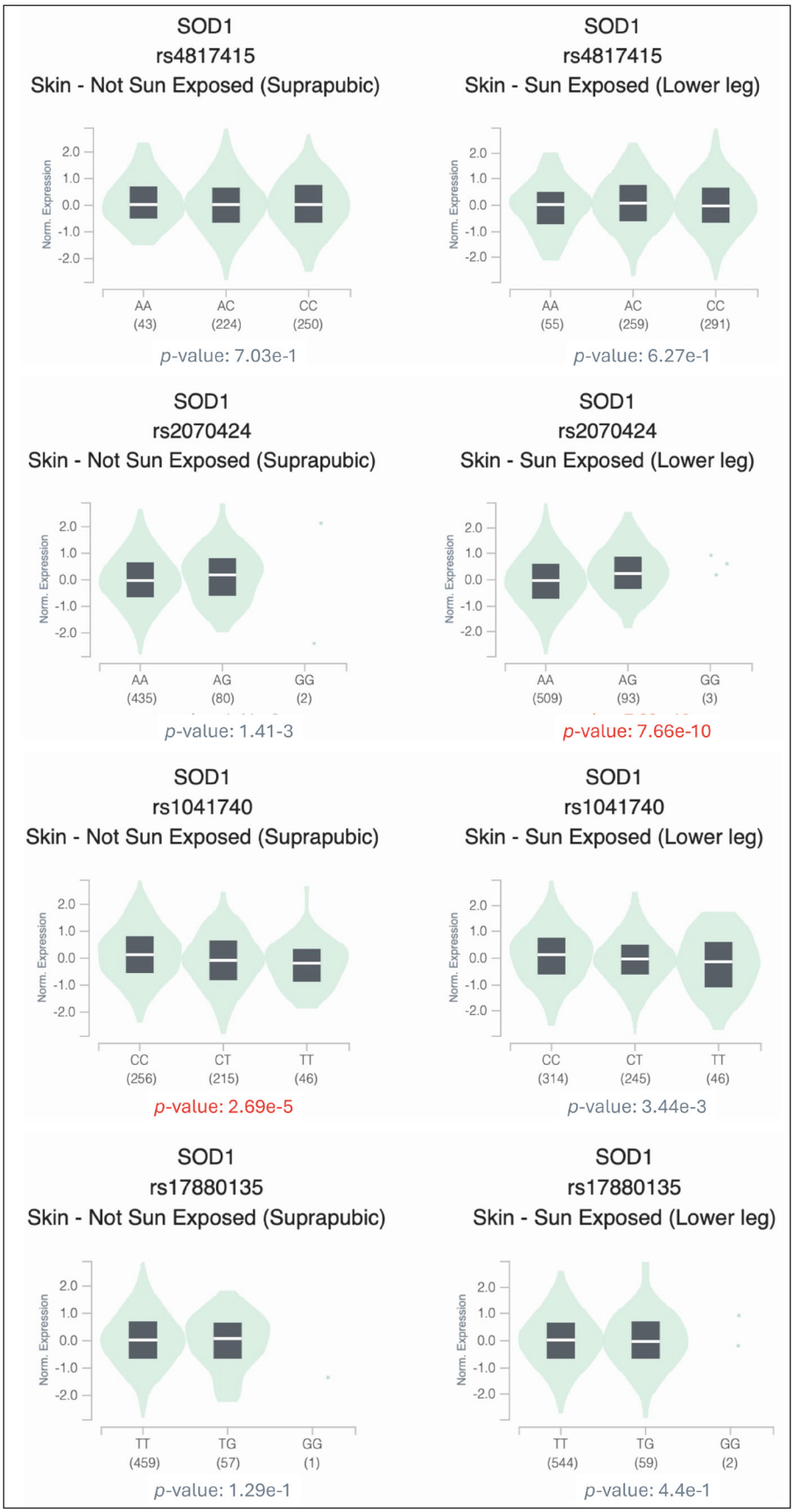


Figure 5. Expression analysis of the *SOD1* gene in Sun Exposed and Unexposed Skin Tissue from healthy individuals by the genotype of variants rs4817415, rs2070424, rs1041740, and rs17880135 variants.

in Mexico that 50% of individuals with vitiligo start to develop symptoms around the age of 20, and its incidence decreases with increasing age. This is consistent with our study, in which we identified that the mean age was 36.58 years old (range 26.69-48.49). However, different risk factors related to the presence of vitiligo were noted. We observed that it occurs more frequently in women with phenotype 3. These data agree with what was observed in studies[39,40] carried out on the Mexican population, in which 63% of patients with vitiligo were women.

In Mexico, it is necessary to carry out campaigns for the early detection of this progressive dermatological disease that is distinguished by depigmentation in certain areas of the skin. The cause of the disease is not determined; therefore, it is necessary to carry out more studies to understand its cause and the genomics of the Mexican population and to gain a better understanding of the biological mechanisms of the disease[41,42]. Although vitiligo is often considered a cosmetic disorder, its effects on the physical and psychosocial health of sufferers cannot be ignored[41-43]. Various areas of the body, including the skin, hair, and mucous mem-branes, develop discolored white markings. The lack of melanin production by melanocytes and the development of white spots on the skin have been associated with different mechanisms, including genetic, immunological, neural, and oxidative stress mechanisms[1,37-41]. Previous studies[8,14,15,20,22,23,26,31,42] have associated the regulation process of oxidative stress as an important factor in the development of the disease.

Molecular Mechanisms of SOD

SOD, one of the important antioxidant enzymes, has previously been studied[1,12,18,19,37,42-44] in relation to vitiligo, but to date, the exact mechanism by which oxidative stress produces vitiligo is unknown. It is known[2,15] that melanocytes stop producing melanin necessary for skin pigmentation due to the accumulation of reactive oxygen species (ROS), such as hydrogen peroxide, which causes alterations in the mitochondria when there is an imbalance in its concentration, leading to apoptosis and death of melanocytes. It has been observed[1,37,38,42-44] that patients with vitiligo have significantly greater amounts of SOD, decreased erythrocyte glutathione peroxidase (GPx) activity, low levels of the CAT (Catalase) enzyme, and low levels of the vitamins C and E in both their epidermis and serum. Oxidative stress has been indicated as the main triggering event in the pathogenesis of vitiligo, and various studies[18,43-46] have observed circulating levels of SOD in its three isoforms (SOD1, SOD2, and SOD3) in high concentrations in patients with vitiligo. However, there are few studies[47,48] regarding the association of variants in the genes that code for the different isoforms of SOD with vitiligo.

Association Variant Studies with Vitiligo Disease

In this sense, this is the first study to analyze the association between different variants (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, rs17880135) of the *SOD1* gene and vitiligo. We observed an association of susceptibility to the risk of heterozygous CT genotype in both the rs1041740 and rs17880135 variants of the *SOD1* gene. Unfortunately, no other studies have analyzed this association.

A comprehensive understanding of molecular interactions in pathways is important for integrating multiomics data, can help understand the pathogenesis of vitiligo, and can help propose new therapies to mitigate the effects of the disease[44,49].

Regarding the analysis of the variants of *SOD1*, we observed a strong linkage disequilibrium between the rs1041748 and rs17880135 variants that showed an individual risk association. The rs17880135 and rs4817415 variants also showed an association with individual risk, and a moderate association was shown with the 50 bp Ins/Del and rs1041748 variants. Both protection and risk haplotypes were also distinguished. The combination InsCGCC is the protection haplotype, while the InsCGTC and InsAGCC haplotypes were shown as risk susceptibility factors for vitiligo. However, the number of cases analyzed was low, which is a limitation of the study. However, characteristic haplotypes in the analyzed sample represented the Mexican population. Unfortunately, there are no studies in other populations that compare the analyzed haplotypes.

One of the characteristics of the 50 bp Ins/Del, rs17880135, rs1041748, and rs4817415 variants is that they are located in regulatory regions of the gene, which may contribute to gene modulation or be target sites for other regulatory regions that can alter the levels of expression and thus may alter the ROS level detoxification[20]. It is known[2,15] that the production of melanin by melanocytes is a mechanism of protection against UV in the skin. During this process, called melanogenesis, ROS and intracellular stress are generated due to UV absorption. The SOD family of enzymes is the main line of defense against ROS[46-49]. The mitochondrial SOD2

isoform has shown high activity in patients with vitiligo. Mitochondrial metabolism, cell proliferation, cell apoptosis, cell differentiation, and immune reactions are known to generate ROS. Therefore, the variants in the SOD genes can participate in the mechanisms of regulation of melanogenesis, such as signaling pathways, miRNA, and immunological mechanisms[43-49].

SOD Circulating Levels in Vitiligo Disease

Regarding the circulating levels of SOD, although the sample size analyzed was small, statistically significant differences were observed in patients and controls, where levels were slightly higher in the group of patients with vitiligo. These differences will likely be more evident by increasing the size of the sample. In this regard, there is evidence[50-52] that high levels of SOD2 have been observed in patients with vitiligo.

Conversely, we observed significant differences in the variants analyzed (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, and rs17880135) and the circulating levels of SOD by genotype, except with the rs17880135 variant of the *SOD1* gene. This is the first study where this association was analyzed.

In-Silico Analysis

Recognition of target sites of the variants (50 bp Ins/Del, rs4817415, rs2070424, rs1041740, rs17880135) analyzed in vitiligo disease

The accessibility of open-access databases (HaploReg, RegulomeDB, and rSNPBASE) contributed to the recognition of target sites of the variants analyzed in this study. We identified two regulatory motifs in transcription factors of the rs4817415 variant (Arid5b and RREB1 proteins). Arid5b, a member of the AT-rich (Adenine and Thymine rich) interactive domain protein family, participates in the regulation of transcription through histone. It is associated with the regulation of adipogenesis, leptin, and melanogenesis and is retinoic acid-induced. Moreover, it participates in the modulation of oxidative metabolism of mitochondrial membrane potential and has been associated with acute lymphoblastic leukemia[53,54]. The Ras-responsive element binding protein 1 (RREB1) participates in the RAS/RAF (Rat sarcoma/Rapidly Accelerated Fibro-sarcoma) signal transduction cascade, resulting in cellular effects. The differentiation process, which is an increase in the expression of the calcitonin gene, is an important factor in the regulation of fibrogenesis and melanogenesis[55]. However, there are still no studies on vitiligo regarding the association of these proteins.

The *MAX* protein belongs to the family of leucine helix-loop-helix-zipper (bHLHZ) transcription factors. MAX forms homodimers and heterodimers with other members of the same family of proteins, such as Mad, Mxi1, and Myc, and participates in the transcriptional regulation[56,57]. In this regard, some studies[56,57] described that some patients with sporadic pheochromocytomas had mutations in the *MAX* gene, and the presence of depigmented areas on the skin produced by the depigmentation of melanin has also been observed in them. Although the relationship of the *MAX* gene with vitiligo has not yet been described, the results of a previous study[58] and those described in this analysis are probably important evidence of its participation in the melanocyte regulation pathways in patients with vitiligo. On the other hand, the participation of the MAX protein in the development of cells of the pigment epithelium of the retina of the eye has also been observed. A strong link between vitiligo and ocular pathology seems to lie in dry eye disease and pigmentary abnormalities of various ocular structures, especially the retinal pigment epithelium[59].

Regarding the participation of the CEBPA protein, whose gene encodes the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα), its participation has been observed[60] as an epigenetic mechanism due to its interaction with other regulatory pathways in leukemia. Likewise, in studies[61] with keratinocyte cell cultures, it participates as a DNA damage response mechanism, where this C/EBPα protein is induced after DNA damage by UVB, through the inhibition of G1 in the cell cycle. Another study[62] has investigated the potential relationship between HLA-A (Human Leukocyte Antigen-A) and vitiligo and ob-served its association with the HLA-A*02:01 haplotype and the *C/EBPα* gene and proposed that HLA functions as a transcriptional regulator that contains multiple bidirectional promoters.

Regarding the POLR2A protein, different studies[63-65] have analyzed the complete genome in patients with vitiligo and identified more than 50 susceptibility loci and 7 loci suggestive of the disease, proposing them as evidence of polygenic transmission. Among these genes, POLR2A was found to participate in adaptive immune activation in the etiology of multiple cases of vitiligo. Additionally, it has been proposed that most of these loci participate in immune regulatory pathways, apoptotic, melanocytes, oxidative stress, and mitophagy.

The TAD protein is a component of the TAD domain (topologically associated domains). Its role is to regulate promoter enhancer interactions, thereby preserving the spatiotemporal pattern of genetic activity. Notably, it plays a crucial role in the expression of tyrosine kinase receptors such as KIT, KDR, and PDGFRA. A study[66] carried out in melanocyte cells in mice showed that TAD acts epigenetically and that mice with deletion of TAD (*KIT* and *KDR* genes) had a lighter coloring phenotype.

Although there are no studies on the function of the aforementioned proteins with vitiligo, the findings of the present study may be evidence of their possible involvement in vitiligo.

We also observed that the rs2070424 variant alters the anchor points of the Barx2, Dlx3, Dlx5, HNF1, Hlxb9, Hoxa3, Hoxa5, Hoxa7, Hoxb4, Hoxb7, Hoxb8, Hoxc6, Hoxc9, Hoxd8, Lhx3, Msx-1, Nkx6-1, and Phox2a proteins and Vax2[67-72]. These proteins are important target sites for the regulation of cell transcription, proliferation, and differentiation of melanocytes[67], as well as the POLR2A protein explained above[60-65]. NLRP1 and FOXD3 have been identified as regulators of melanoblast differentiation, and Nrf2 and Vax2 are transcription factors that regulate the expression of detoxifying and antioxidant genes[73,74]. The Lhx3 protein participates in pituitary regulation[75]. The protein NKX6.1 is an insulin transcription factor that acts as a marker of β-cell maturity, although not related to vitiligo patients, it has been observed[76] to be associated with diabetes, and this is probably evidence of its involvement with vitiligo.

Regarding the CUX1 protein, there are studies[77] in relation to its participation in the inhibition of the proliferation of dermal papilla cells regulated by miR-143. Regarding Nuclear factor 1 C-type (NFIC), it has been observed[78] that NFI proteins have affinity with regulatory regions of genes with high expression and are enriched with active chromatin modifications such as H3K4me3 and H3K36me3. On the other hand, it is known[78,79] that nuclear factor erythroid 2-related 2 (Nrf2) regulates the expression of antioxidant proteins. Oxidative stress is now one of the accepted theories on the development of vitiligo. In a study[79] carried out with the Nrf2 variant (-617 T/G) and (-653 T/C), it was found to be associated with a risk susceptibility factor in patients with vitiligo.

Although there is no evidence of the PRDM1 protein being associated with vitiligo, studies[80,81] have shown its involvement in the development of dermal and hair mustache color in mice. On the other hand, in the present study we evidenced binding sites of the rs2070424 variant in the coding region of the lncRNA lnc-SCAF4-2 and in the regions of the circRNA hsa-circ-*SOD1*-overlap.3 and hsa-circ-*SOD1*-overlap.4.

miRNAs are important regulators of gene expression, participating in various biological and pathological processes. A study[82] carried out in patients with vitiligo has shown that miRNAs are differentially expressed in skin lesions and peripheral blood mononuclear cells. miRNAs are significantly correlated with the development and progression of vitiligo. The abundance of some miRNAs in serum was also correlated with the severity of vitiligo lesions, indicating that miRNAs could serve as prognostic biomarkers. One of the functions of miR-25 is to promote the dysfunction and destruction of melanocytes induced by increased oxidative stress and the increase in its expression is related to the development of vitiligo.

The rs1041740 variant affects the interaction sites of the EBPB, CEBPB, and p300 proteins, and the rs17880135 variant interferes with the GR and PLZF binding of the proteins. These factors are known to participate in many cellular processes, such as cell growth, immune responses, cell differentiation, apoptosis, chromatin remodeling, autophagy, response to DNA damage, and melanogenesis pathway[83-85].

In relation to the ATF3 protein and FOXM1, a bioinformatics study[69] revealed that these proteins are one of the critical markers of autophagy, and it is thought that the deregulation of autophagy may be an important mechanism in the pathogenesis of vitiligo. Another identified protein is Odd-skipped-related 2 (OSR2) (Drosophila), which has been related to the regulation of pigmentation. In patients with melasma, a greater amount of melanin and proteins associated with melanogenesis were observed[86] in the epidermis of injured skin. A bioinformatics study[87] revealed that the Zinc Finger Protein 652 protein (ZNF 652) is regulated by miR-155 and acts in the differentiation of melanocytes in patients with psoriasis. On the other hand, regard-ing the Protein Putative Histone-Lysine N-Methyltransferase (PRDM6), there are no studies associated with vitiligo. However, it has been proposed that it is regulated by the signal transducer and activator of transcription 3 (STAT3), which has been implicated in vitiligo[88]. Regarding the GLI2 protein, it has been observed[60-65] that it participates in the control of cell proliferation and tumorigenesis of epidermal cells. In human keratinocytes, it has been shown[89,90] to activate the transcription of

several genes involved in cell cycle progression, such as *E2F1, CCND1, CDC2* and *CDC45L*, while repressing genes associated with epidermal differentiation. The identification of the recognition site with POLR2A was previously mentioned.

Concerning the binding sites present in various regulatory regions, it has been noted that the rs17880135 variant shares these sites with the promyelocytic leukemia zinc finger protein (PLZF). PLZF has been characterized as a transcriptional repressor of homeobox (HOX)-containing genes during embryogenesis. It has been shown[91] that the overexpression of HOXB7 is due to the lack of PLZF. Expression of PLZF has been observed in melanocytes but not in melanoma cells, a pattern inversely related to that of HOXB7.

Regarding the PML protein, an *in-silico* study[92] identified different genes that participate in the infiltration of immune cells in vitiligo. Regarding the FOXM1 protein, a recent genome wide association study[93] of generalized vitiligo identified the involvement of the rs17008723 variant of the FOXP1 gene in the development of vitiligo, as well as the participation of *FOXM1* as a key YAP (Yes-associated protein)-dependent regulator of epidermal stem cells[94].

In relation to the participation of the Argonaute 2 (Ago2) protein, although there are no studies on the relationship of this protein with vitiligo, it has been proposed[95] that in patients with skin cancer, the presence of miRNAs contributes to tumorigenesis and tumor progression. Furthermore, the deregulation of miR-NA-processing enzymes, such as the miRNA-binding protein Ago2, significantly affects miRNA function. Expression of Ago2-ex1/3 provides a survival advantage for melanoma cells, while deletion results in significantly reduced proliferation and increased apoptosis. Considering the aforementioned information, we can infer the important function of this gene in the regulation of the melanocyte that is involved in vitiligo.

There is no evidence of the POLRG2 protein's involvement with vitiligo or participation in the regulation of pigmentation in melanocytes. Similarly, the association of SUPT5H protein with vitiligo has not been demonstrated.

SOD1 Expression Levels of Variants Analyzed

Through the eQTL analysis, we observed differences in the *SOD1* expression levels of the rs2070424 and rs1041740 variants in skin exposed to the sun and skin not exposed to the sun. There are no studies on this association. However, it is known[1,12,18,19,37,42-44] that SOD functions as a potent antioxidant, and the increased activity of SOD isoforms under the influence of genetic factors may lead to the accumulation of hydrogen peroxide in cytoplasmic, mitochondrial, and extracellular compartments, resulting in oxidative damage to melanocytes[45,96].

These variants have been proposed[8,14,15,20,22,23,26,31,42] as biomarkers of therapeutic response, participating in lipid metabolism and oxidative stress in different pathologies, such as Alzheimer's and cancer. However, there are no studies on vitiligo. Interestingly, in this *in-silico* analysis showed that these variants may be part of regulatory regions of the gene itself and of other genes that regulate other proteins, such as Arid5b, RREB, MAX, CBPA, p300, OSR2, GLI2, ZNF6S2, PRDM6, and circ-*SOD1*-overlap-4. New studies are recom-mended to validate this information.

Vitiligo is a multifactorial disease characterized by the accumulation of environmental and epigenetic genetic changes that increase the risk of the disease, making it even more difficult to understand the molecular mechanisms that trigger its development[44-55,96]. In addition, the high activity of *SOD1*, depending on genetic variants, may contribute to the differentiation of melanocytes[46,48,49]. These variants are found within regulatory regions of the *SOD1* gene itself, as well as in regulatory target sites of other genes that can influence its expression, for example, transcription factors[44,48,49].

Conclusions

Our results showed that the CT genotype and CTTT dominant model of the rs1041740 and rs17880135 variants were associated with a risk for vitiligo. There were significant differences in plasma circulating SOD levels between the groups, and when stratified according to the genotypes of each variant, there was a significant difference between the groups, except with the rs17880135 variant. Linkage disequilibrium was evident between the variants rs1041748 and rs17880135 and between rs17880135 and rs 4817415 in the study groups. Two haplotypes in both groups showed that InsCGCC is related to protection, while InsCGTC and InsAGCC are shown to be risk factors for susceptibility to vitiligo. The *in-silico* analysis demonstrated that the rs4817415, rs2070424, rs1041740, and rs17880135 variants of the *SOD1* gene participated in the modification of selected regulatory elements in different proteins, transcription factors, lncRNA, immune modulation,

melanogenesis, oxidative stress, epigenetics, and mitophagy. In addition, differences were observed in the expression levels of the genotypes of the rs2070424 and rs1041740 variants in healthy skin tissue, both exposed and not exposed to the sun. Because of the low number of cases analyzed, a limitation of the study, more studies are needed to confirm the observed findings.

Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this article.

Ethics Approval
The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee of Universidad de Guadalajara (Protocol code CI-01620, October 20, 2020). The integrity of the patients was respected in accordance with good ethical practices and the Declaration of Helsinki.

Informed Consent
Written informed consent was obtained from all subjects involved in the study.

Authors' Contributions
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Funding
This research received no external funding.

Data Availability
The datasets generated or analyzed during the current study are available from the corresponding author upon reasonable request.

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Acknowledgments
We thank Sharon Elizabeth Valencia González, Braulio Diaz Villaseñor, Alejandro Salvador Gómez Cabrera, Saulo Oswaldo Sánchez Rivera, and Andrea Miranda for their invaluable support in the realization of this study.

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