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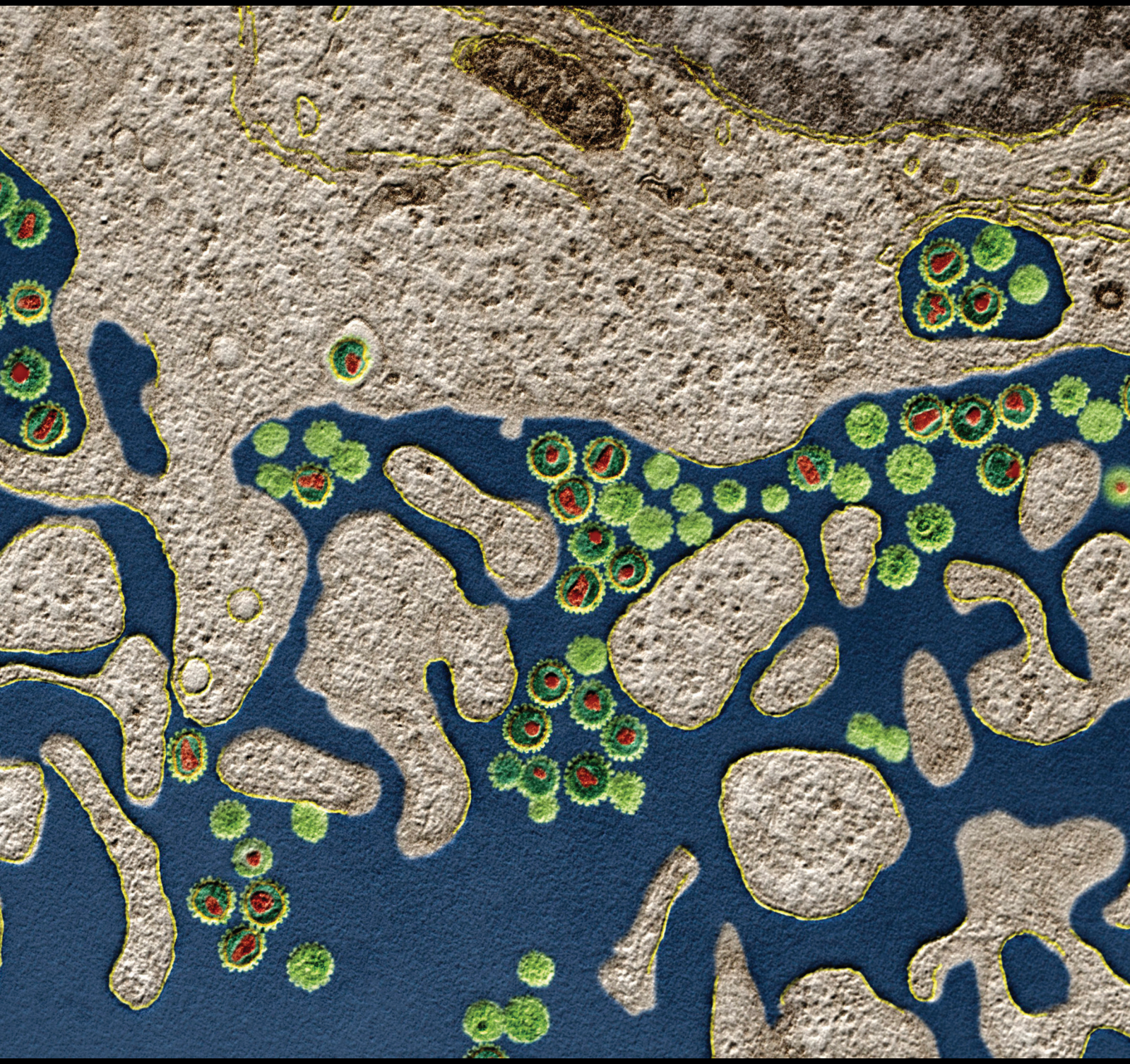
Autoimmune Disease Genetics 2013

Guest Editors: Timothy B. Niewold, George N. Goulielmos, and Shervin Assassi



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Editorial Board

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Editorial

Autoimmune Disease Genetics 2013

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The familial predilection toward autoimmunity is in part due to genetic risk factors, and recent studies have greatly expanded our understanding of the complex genetic architecture underlying autoimmune disease. Technical advances and the assembly of large patient cohorts have resulted in rapid progress in the field, which does not appear to be slowing. Studies which have included multiple ancestral backgrounds have demonstrated differences in risk factors between ancestral backgrounds, as well as some similarities [1–3]. Also, genetic studies examining subphenotypes in autoimmune diseases have illustrated the idea of biological diversity within a complex condition, that different individuals with a given condition have different genetic risk factors, and some of the clinical differences between patients are likely related to this fact [4–6]. In these ways, understanding the genetic basis of disease provides us with some tools that could eventually be useful in developing more individualized diagnostic and therapeutic strategies, enabling personalized medicine.

In the current issue, a series of papers highlight some exciting topics within autoimmune disease genetics. M. G. Zavala-Cerna et al. explore genetic associations between PAD4 and rheumatoid arthritis in a Mexican cohort. The PAD4 enzyme is implicated in posttranslational protein modification characteristically targeted by rheumatoid arthritis-associated autoantibodies. B. N. Frederiksen et al. examine genetic polymorphisms underlying type I diabetes and islet cell autoimmunity, finding both age- and disease-stage relevant differences in association. This study illustrates the complex ways in which genetic factors can influence disease, and it is likely that this complexity occurs in many different autoimmune diseases. Genetic risk alleles may depend on other factors such as age, disease stage, and environment to influence risk of disease. Discovering these relationships will greatly improve our understanding of autoimmune disease pathogenesis. C. E. Weckerle et al. report a familial aggregation study looking at circulating levels of tumor necrosis factor alpha, a cytokine which is elevated in lupus patients [7], in unaffected members of lupus families. Previous work had demonstrated familial correlation in type I interferon levels [8], and the current study documents a familial relationship in tumor necrosis factor alpha levels. Interestingly, while type I interferon was only correlated within genetically related family members and not correlated between patients and spouses, tumor necrosis factor alpha was correlated between lupus patients and their spouses, suggesting a potential environmental influence on tumor necrosis factor alpha levels.

S. A. Zavaleta-Muñiz et al. study polymorphisms in the IL6 gene with regard to susceptibility to rheumatoid arthritis. A. Zóka et al. provide a comprehensive review of the alterations in the immune system which are related to type I diabetes. H. C. Chai et al. examine polymorphisms in genes within the Toll-like receptor and type I interferon pathways in systemic lupus erythematosus patients from a South Asian population, extending our knowledge of these susceptibility genes to an additional world population. While one issue cannot be comprehensive, the studies included in this issue provide an overview of some of the current frontiers in the genetics of autoimmune disease.

Timothy B. Niewold
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Shervin Assassi

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Research Article

Insight into Gene Polymorphisms Involved in Toll-Like Receptor/Interferon Signalling Pathways for Systemic Lupus Erythematosus in South East Asia

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Polymorphisms in genes involved in toll-like receptor/interferon signalling pathways have been reported previously to be associated with SLE in many populations. This study aimed to investigate the role of seven single nucleotide polymorphisms within *TNFAIP3*, *STAT4,* and *IRF5*, which are involved in upstream and downstream pathways of type I interferon production, in SLE in the South East Asian populations. Genotyping of 360 Malaysian SLE patients and 430 normal healthy individuals revealed that minor alleles of *STAT4* rs7574865 and rs10168266 were associated with elevated risk of SLE in the Chinese and Malay patients, respectively ($P = 0.028$, odds ratio (OR) = 1.42; $P = 0.035$, OR = 1.80, respectively). Polymorphisms in *TNFAIP3* and *IRF5* did not show significant associations with SLE in any of the ethnicities. Combined analysis of the Malays, Chinese, and Indians for each SNP indicated that *STAT4* rs10168266 was significantly associated with the Malaysian SLE as a whole ($P = 0.014$; OR = 1.435). The meta-analysis of *STAT4* rs10168266, which combined the data of other studies and this study, further confirmed its importance as the risk factor for SLE by having pooled OR of 1.559 and P value of <0.001.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease affecting various parts of the body including skin, kidneys, lungs, joints, heart, nervous system, and hematopoietic organs. It is a disease whereby a diverse array of autoantibody production, complement activation, immune complex deposition, and inflammation cause damages in those organs. Although the exact aetiology of SLE still remains unclear, a combination of genetic risk factors and environmental events is believed to contribute to an irreversible break in immunological self-tolerance. With the introduction of genome-wide association studies, a huge breakthrough has been made in the discovery of SLE associated susceptibility genes that in turn advances our understanding of pathogenesis of SLE. Recently, several reviews have categorised the susceptible genes according to their immunological pathways and cell types. Three biological pathways involved in SLE have been forwarded by Harley et al. [1]: (i) innate immune response including toll-like receptor (TLR)/interferon (IFN) signalling pathways; (ii) adaptive immune response including B, T, and antigen-presenting cells immune signal transduction; and (iii) immune complex clearance mechanism.

Defects in TLR/IFN signalling pathways cause immune complexes containing self-nucleic acids to interact with TLR7 and TLR9 inside plasmacytoid dendritic cells and B cells endosomes, resulting in the secretion of type I IFN and interleukin (IL)-6. The combined triggering of both B cell receptors and TLR leads to autoreactive B-cell proliferation. Their further differentiation into plasmablasts and autoantibody-secreting plasma cells is induced by type I IFN and IL-6, respectively [2]. *TNFAIP3*, *STAT4*, *IRF5*, *TREX*, and *IRAK1* are the genes involved in upstream and downstream pathways of type I IFN production that have been recently identified. The *STAT4* gene consists of 24 exons that spread

Table 1: Distribution of samples according to ethnicity and gender. The percentage of SLE patients and healthy controls was ethnic- and gender-matched.

	SLE patients	Healthy controls
Total	360	430
Malay	93 (25.8%)	110 (25.6%)
Chinese	245 (68.1%)	294 (68.4%)
Indian	22 (6.1%)	26 (6.0%)
Female : Male	10.25 : 1	10.03 : 1

Table 2: SNPs that were investigated in this study on their association with SLE.

Genes	SNP	Chromosome	Position	Alleles
STAT4	rs7574865	2	191672878	G/T
	rs10168266	2	191644049	C/T
	rs7601754	2	191648696	A/G
TNFAIP3	rs2230936	6	138237759	T/G
	rs3757173	6	138231847	T/C
IRF5	rs4728142	7	128361203	G/A
	rs729302	7	128356196	A/C

over a 120 kb region on chromosome 2q32.3. It encodes a transcription factor that mediates signals induced by IL-12, IL-23, and type I IFN and activates the production of IFN-γ and IL-17. It also directs the differentiation of helper T cells toward the proinflammatory T-helper type 1 and T-helper type 17 lineages that have been shown to play a critical role in the pathogenesis of SLE. The *STAT4* null allele in lupus-prone mouse model confers reduced autoantibody production and glomerulonephritis, indicating that *STAT4* may be involved in multiple SLE-associated phenotypes [3]. There are a few studies involving *STAT4*-deficient lupus-prone mice which demonstrate the role of *STAT4* in autoantibody production only [4, 5]. Polymorphisms in the *STAT4* gene have been found to be strongly associated with SLE susceptibility, in particular rs7574865 [6, 7]. The simultaneous association of the risk allele T of *STAT4* rs7574865 with both lower serum IFN-α activity and increased IFN-α-induced gene expression has been reported, confirming that this polymorphism was associated with increased IFN-α sensitivity [8, 9].

TNFAIP3, or tumour necrosis factor alpha-induced protein 3 gene, encodes the A20 protein which is a negative regulator of the NF-κB signalling pathway, an essential pathway in the pathogenesis of SLE. A20 is an ubiquitin-editing enzyme required for effective termination of NF-κB-mediated proinflammatory responses induced by TLRs, TNF receptor, IL-1 receptor, and NOD2 [10]. A meta-analysis and imputation study identified a 109 kb risk haplotype spanning *TNFAIP3* region with lupus nephritis and hematologic manifestation [11]. A nonsynonymous mutation (c.380T > G), rs223092, in *TNFAIP3* gene which causes phenylalanine-to-cysteine change at position 127 of A20 protein has been consistently linked with SLE various ethnic groups.

The final candidate gene, *IRF5*, which is IFN regulatory factor 5, is a transcription factor that mediates inflammatory and immune responses [12]. This factor stimulates the production of the proinflammatory cytokines TNF-α, IL-12, and IL-6 following TLR signalling as well as transactivation of type I IFN and IFN-induced genes [13, 14]. Polymorphisms in *IRF5* cause functional changes in messenger RNA, which in turn alter *IFR5*-mediated transcription resulting in elevated SLE risk [15]. It was also suggested that SLE patients who carry *IRF5* risk haplotype and are positive for either anti-RBP or anti-dsDNA potentially have higher serum IFN-α activity [16]. In this study, we aimed to investigate the association between seven single nucleotide polymorphisms (SNPs) in *STAT4*, *TNFAIP3*, *IRF5* genes, and SLE in the South East Asian scenario, particularly in the Malaysian participants. We also attempted to compare and pool the ORs of SNPs which were significant in the Malaysian SLE with the other studies through meta-analysis.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction. A total of 790 Malaysians were included in this study, which is comprised of 360 SLE patients and 430 healthy controls. Blood samples were collected from patients diagnosed with SLE according to 4 out of ACR criteria and healthy volunteers recruited at the University of Malaya Medical Centre (UMMC), Kuala Lumpur, in compliance with requirements as stipulated by the UMMC Medical Ethics Committee (UMMC Ethics Approval Code: 733.19). The distribution of samples from Malays, Chinese, and Indians, as well as the ratio of females to males, is shown in Table 1. Genomic DNA was isolated from the peripheral blood samples by using the standard DNA extraction method as described previously [17]. The concentration and purity of the extracted DNA were further quantified by measuring the absorbance values at 260 nm and 280 nm via a spectrophotometer.

2.2. Genotyping with Tetraprimer ARMS-PCR. SNPs that were included in this study are listed in Table 2. Tetraprimer ARMS-PCR was performed in the genotyping of rs10168266 and rs7601754 in *STAT4* region, rs2230926 and rs3757173 in *TNFAIP3* region, and rs4728142 in *IRF5* region. Primers were designed using computer software accessible through the Internet at http://cedar.genetics.soton.ac.uk/public_html/primer1.html, developed by Ye and team [18]. *In silico* PCR as described previously was further carried out to ensure the self-designed primers were targeted to the gene regions of interest [19–21]. Each PCR reaction was carried out in a total of 10 μL, containing 50 ng of template DNA, appropriate concentration of inner and outer primers and $MgCl_2$ (Table 3), 200 μM dNTP, 20 mM Tris-HCl pH8.4, 50 mM KCl, and 0.15 U *Taq* polymerase (Fermentas, Vilnius, Lithuania.). The PCR mixture was then subjected to touchdown PCR, whereby it was incubated for 5 min at 95°C, followed by 30 cycles of 45 s denaturation at 95°C, 45 s of annealing (started at temperature 10°C higher than annealing temperature, decreasing by 1°C per cycle, maintained at annealing temperature for the remaining 20 cycles) and 45 s of extension at 72°C, and a final extension at 72°C for 10 min

Table 3: Touchdown PCR primers and conditions.

SNP	Primer sequence	T_m	Final concentration	Inner/outer primers ratio	Mg^{2+}	Annealing temperature
STAT4						
rs10168266	Forward inner primer (T allele) (29 bp) 5′-CAAAGTAGTAGCTATTGACTACATGAGAT	57°C	1.0 μM	4 : 1	2.5 mM	55°C
	Reverse inner primer (C allele) (27 bp) 5′-GTTATTACTACGGGTGGGTAGACATTG	62°C	1.0 μM			
	Forward outer primer (28 bp) 5′-AAAAGTATAGAATTTGGAGGAAGAGAGT	59°C	0.25 μM			
	Reverse outer primer (28 bp) 5′-TATTGGGGTATACTGAAAAGAAAGAGTA	59°C	0.25 μM			
rs7601754	Forward inner primer (A allele) (21 bp) 5′-GGGTGAAGAAAAGGAACTCCA	60°C	1.0 μM	5 : 1	1.25 mM	55°C
	Reverse inner primer (G allele) (23 bp) 5′-CAAGGTCTTAGTATCATCTTGGC	57°C	1.0 μM			
	Forward outer primer (28 bp) 5′-GGAGGTGATTACTATATTTCTAGGCTAA	58°C	0.2 μM			
	Reverse outer primer (27 bp) 5′-AAAAATTAAAAATTAGTTGGCTATGGT	58°C	0.2 μM			
TNFAIP3						
rs2230936	Forward inner primer (G allele) (28 bp) 5′-CAGACTTGGTACTGAGGAAGGCGCTATG	69°C	1.0 μM	4 : 1	2.5 mM	62°C
	Reverse inner primer (T allele) (23 bp) 5′-GTCTGTTTCCTTGAGCGTGCCGA	69°C	1.0 μM			
	Forward outer primer (28 bp) 5′-CTGAAAACCTTTGCTGGGTCTTACATGC	69°C	0.25 μM			
	Reverse outer primer (29 bp) 5′-GACCTAGTCCATCAGATGCTACCAGAGGG	69°C	0.25 μM			
rs3757173	Forward inner primer (T allele) (26 bp) 5′-GACCTTATTCCCTTCCCTGAAATGAT	64°C	1.0 μM	4 : 1	2.5 mM	53°C
	Reverse inner primer (C allele) (27 bp) 5′-CCTTAGCTGCAGACTAAGGTGGTATTG	64°C	1.0 μM			
	Forward outer primer (28 bp) 5′-TTAAACCATTCAGTCCCCTAGAATAGCA	64°C	0.25 μM			
	Reverse outer primer (28 bp) 5′-TAAAATCTTCCTACTGCCCATCTCTTTC	64°C	0.25 μM			
IRF5						
rs4728142	Forward inner primer (A allele) (26 bp) 5′-GTCACACCCCAAAAAGCTCTGAGACA	68°C	2.0 μM	5 : 1	1.25 mM	55°C
	Reverse inner primer (G allele) (26 bp) 5′-CCTTCCTCCCCATTTCTTACTAACCCC	68°C	2.0 μM			
	Forward outer primer (28 bp) 5′-GAAAGGTGGAGACTCCGAGTGTAGAGGT	68°C	0.2 μM			
	Reverse outer primer (28 bp) 5′-GACAGAGCGATACTCCGTCTCAAAAGAA	68°C	0.2 μM			

at the end of the cycles. The annealing temperatures for different PCRs are stated in Table 3. Five microlitres of PCR amplicons was electrophoresed on a 2% (w/v) agarose gel. The agarose gel was viewed under UV illumination and image was recorded using a gel documentation system. The results obtained were further verified by sequencing.

2.3. Genotyping with Real-Time PCR. Predesigned TaqMan SNP genotyping assays were used to genotype SNPs where tetraprimers could not be designed for ARMS-PCR (probe ID: rs7574865 in *STAT4* region, C_29882391_10; rs729302 in *IRF5* region, C_2691216_10; Applied Biosystems, NY, USA). Fifty nanograms of template DNA was mixed with 2X Taqman GTXpress master mix (Applied Biosystems) and 20X Taqman genotyping assay (Applied Biosystems) to make up to a total volume of 10 μL. Real-time PCR reaction was initiated with pre-PCR read step at 60°C for 1 min, followed by DNA polymerase activation at 95°C for 20 s, 40 cycles of

Table 4: Frequencies of alleles and genotypes for *STAT4* rs7574865 and rs10168266, and *TNFAIP3* rs2230926 in SLE patients and healthy control subjects of each ethnicity.

Ethnicity	Locus	Frequency		*P* value	*P* value (Bonferroni adjusted)	OR (95% CI)
		SLE patients	Healthy controls			
	STAT4 rs7574865					
Malay		$n = 93$	$n = 110$			
	Allele					
	G†	104 (55.9%)	148 (67.3%)	—	—	1.00
	T	82 (44.1%)	72 (32.7%)	0.019*	0.133	1.62 (1.08–2.43)
	Genotype					
	GG†	29 (31.2%)	51 (46.4%)	—	—	1.00
	GT	46 (49.5%)	46 (41.8%)	0.276	NA	1.76 (0.95–3.24)
	TT	18 (19.3%)	13 (11.8%)	0.137	0.959	2.44 (1.04–5.68)
Chinese		$n = 245$	$n = 294$			
	Allele					
	G†	263 (53.7%)	366 (62.2%)	—	—	1.00
	T	227 (46.3%)	222 (37.8%)	0.004*	0.028*	1.42 (1.12–1.82)
	Genotype					
	GG†	69 (28.2%)	114 (38.8%)	—	—	1.00
	GT	125 (51.0%)	138 (46.9%)	0.345	NA	1.50 (1.02–2.20)
	TT	51 (20.8%)	42 (14.3%)	0.046*	0.322	2.01 (1.21–3.33)
Indian		$n = 22$	$n = 26$			
	Allele					
	G†	30 (68.2%)	30 (57.7%)	—	—	1.00
	T	14 (31.8%)	22 (42.3%)	0.290	NA	0.64 (0.27–1.47)
	Genotype					
	GG†	9 (40.9%)	7 (26.9%)	—	—	1.00
	GT	12 (54.5%)	16 (61.5%)	0.624	NA	0.58 (0.17–2.01)
	TT	1 (4.6%)	3 (11.6%)	0.382	NA	0.26 (0.02–3.06)
	STAT4 rs10168266					
Malay		$n = 93$	$n = 110$			
	Allele					
	C†	104 (55.9%)	153 (69.5%)	—	—	1.00
	T	82 (44.1%)	67 (30.5%)	0.005*	0.035*	1.80 (1.20–2.71)
	Genotype					
	CC†	26 (28.0%)	53 (48.2%)	—	—	1.00
	CT	52 (55.9%)	47 (42.7%)	0.061	0.427	2.26 (1.22–4.16)
	TT	15 (16.1%)	10 (9.1%)	0.128	0.896	3.06 (1.21–7.73)
Chinese		$n = 245$	$n = 294$			
	Allele					
	C†	266 (54.3%)	363 (61.7%)	—	—	1.00
	T	224 (45.7%)	225 (38.3%)	0.014*	0.098	1.36 (1.07–1.73)
	Genotype					
	CC†	69 (28.2%)	108 (36.7%)	—	—	1.00
	CT	128 (52.2%)	147 (50.0%)	0.604	NA	1.36 (0.93–2.00)
	TT	48 (19.6%)	39 (13.3%)	0.047*	0.329	1.93 (1.15–3.24)

Table 4: Continued.

Ethnicity	Locus	Frequency		*P* value	*P* value (Bonferroni adjusted)	OR (95% CI)
		SLE patients	Healthy controls			
Indian		$n = 22$	$n = 26$			
	Allele					
	C†	30 (68.2%)	35 (67.3%)	—	—	1.00
	T	14 (31.8%)	17 (32.7%)	0.929	NA	0.96 (0.41–2.27)
	Genotype					
	CC†	9 (40.9%)	11 (42.3%)	—	—	1.00
	CT	12 (54.5%)	13 (50.0%)	0.753	NA	1.13 (0.35–3.67)
	TT	1 (4.6%)	2 (7.7%)	0.654	NA	0.61 (0.05–7.88)
	TNFAIP3 rs2230926					
Malay		$n = 93$	$n = 110$			
	Allele					
	T†	181 (97.3%)	203 (92.3%)	—	—	1.00
	G	5 (2.7%)	17 (7.7%)	0.025*	0.175	0.33 (0.12–0.91)
	Genotype					
	TT†	88 (94.6%)	93 (84.5%)	—	—	1.00
	TG	5 (5.4%)	17 (15.5%)	0.021*	0.147	0.31 (0.11–0.88)
	GG	0 (0%)	0 (0%)	NA	NA	NA
Chinese		$n = 245$	$n = 294$			
	Allele					
	T†	476 (97.1%)	563 (95.7%)	—	—	1.00
	G	14 (2.9%)	25 (4.3%)	0.222	NA	0.66 (0.34–1.29)
	Genotype					
	TT†	231 (94.3%)	270 (91.8%)	—	—	1.00
	TG	14 (5.7%)	23 (7.8%)	0.335	NA	0.71 (0.36–1.42)
	GG	0 (0%)	1 (0.4%)	0.361	NA	NA
Indian		$n = 22$	$n = 26$			
	Allele					
	T†	44 (100%)	52 (100%)	—	—	1.00
	G	0 (0%)	0 (0%)	0	NA	NA
	Genotype					
	TT†	22 (100%)	26 (100%)	—	—	1.00
	TG	0 (0%)	0 (0%)	NA	NA	NA
	GG	0 (0%)	0 (0%)	NA	NA	NA

†Reference category; *$P < 0.05$.

denaturation (95°C for 3 s) and annealing/extension (60°C for 30 s), and ended with a final extension step at 60°C for 1 min. Fluorescence was detected using an Applied Biosystems 7500 Fast Real-Time PCR System. The results were verified by sequencing.

2.4. Association Test. Allele and genotype frequencies were calculated, followed by performing a χ^2 goodness-of-fit test to evaluate whether or not the observed genotype frequencies of each polymorphisms were departures from Hardy-Weinberg equilibrium (HWE) in control subjects (P values > 0.05). An open access HWE calculator developed by Rodriguez et al. [22] was used. Fisher's exact test was conducted on 2×2 contingency table using SPSS software to assess the association of each SNP with SLE susceptibility in Malays, Chinese, and Indians. P values were adjusted according to Bonferroni correction and $P < 0.05$ was regarded as significant. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Adjusted ORs were computed using logistic regression, whereby major allele and major homozygous

Table 5: Association of each SNP with the Malaysian SLE, resulting from combined analysis of the three ethnicities.

Gene	SNP	Minor allele	OR (95% CI)	*P*-value (Bonferroni adjusted)
STAT4	rs7574865	T	1.337 (0.948–1.885)	0.686
	rs10168266	T	1.435 (1.143–1.802)	0.014*
	rs7601754	G	0.800 (0.589–1.085)	NA
TNFAIP3	rs2230926	G	0.522 (0.273–0.999)	0.350
	rs3757173	C	1.660 (1.088–2.531)	0.133
IRF5	rs4728142	A	1.272 (0.934–1.733)	0.889
	rs729302	C	0.947 (0.768–1.168)	NA

*$P < 0.05$.

genotype of each SNP were set as reference group and their ORs were adjusted to 1.

The ORs of the three ethnicities were combined using Mantel-Haenszel test to evaluate the overall association of each SNP in the Malaysian population. The Mantel-Haenszel ORs were calculated using Comprehensive Meta-Analysis Version 2.0 software (Biostat, NJ, USA). The between-subgroup heterogeneity was tested using Cochran's Q statistic. Random effect model was used when heterogeneity was significant ($P < 0.10$); otherwise, the fixed effect model was used.

2.5. Meta-Analysis. Meta-analysis was conducted using Comprehensive Meta-Analysis Version 2.0 software (Biostat) for the SNP(s) which was/were significantly associated with SLE in the Malaysian population by including data from other studies as well as the current study. Due to insufficient number of Indian subjects, analysis was not carried out for this ethnic group.

We examined the association between *STAT4* polymorphisms and SLE fully and rigorously, with the use of the key words "*STAT4*," "polymorphisms," "systemic lupus erythematosus," and "SLE." Electronic databases including Pubmed, Embase, and Web of Science were thoroughly searched until December 2013. Only fully published articles were included and the eligible studies were identified based on the following criteria: (a) the study was original, (b) the patients were sporadic cases, (c) having available allele and genotype frequency data, and (d) having sufficient published data to determine OR with 95% CI.

Data extraction was performed by collecting the following information from each study: the first author's name, year of publication, ethnicity, the number of cases and controls, and the frequency of minor allele (MAF) of each polymorphism in both cases and controls. For studies including several independent case-control populations, each case-control population was extracted separately. Malay and Chinese populations from this study were also included in the meta-analysis.

The heterogeneity across studies was evaluated by using Cochran's Q statistic. Random effect model was used for meta-analysis when heterogeneity was significant ($P < 0.10$); otherwise, fixed effect model was used. By inputting the study name, total number of cases and controls, and MAF of cases and controls, Comprehensive Meta-Analysis Version 2.0 software calculated the ORs, 95% CI, and *P* values for each study, as well as the pooled OR, 95% CI, and *P* values for the meta-analysis.

3. Results

3.1. Polymorphisms and SLE Risk. The χ^2 goodness-of-fit test demonstrated that all polymorphisms investigated in this study fulfilled HWE in the control group. The Malay SLE patients were significantly associated with minor alleles of *STAT4* rs7574865, rs10168266, and *TNFAIP3* rs2230926, and heterozygous genotype TG of *TNFAIP3* rs2230926 (Table 4). Increased SLE susceptibility in Chinese population was significantly conferred by minor alleles and minor homozygous genotypes TT of *STAT4* rs7574865 and rs10168266. However, after Bonferroni adjustment, significant associations were only observed between minor allele T of *STAT4* rs7574865 and Chinese SLE patients ($P = 0.028$, OR = 1.42, 95% CI: 1.12–1.82) and between minor allele T of *STAT4* rs10168266 and Malay SLE patients ($P = 0.035$, OR = 1.80, 95% CI: 1.20–2.71). *STAT4* rs7601754, *TNFAIP3* rs3757173, and *IRF5* rs4728142 and rs729302 did not show significant association with SLE in any of the ethnicities.

Combined analysis of the three ethnicities was carried out to represent the association of each SNP with SLE in the Malaysian population as a whole. The analysis revealed that only minor allele T of *STAT4* rs10168266 was significantly associated with the Malaysian SLE ($P = 0.014$, OR = 1.435, 95% CI: 1.143–1.802) (Table 5).

3.2. Meta-Analysis of STAT4 rs10168166. Since *STAT4* rs10168166 showed significant association with the Malaysian SLE, meta-analysis was carried out to combine the data from other studies with the current one [6, 23–25]. Four relevant articles were identified eligible and a total of 5 subgroups were included for comparison. Four subgroups were from Asian population, while one was from European population. Data extracted from these articles is shown in Table 6.

In the overall analysis, significant association of *STAT4* rs10168166 with SLE was observed. The fixed effect model was used as the heterogeneity test did not appear significant ($P > 0.10$). The pooled OR for the minor allele T was 1.559, with 95% CI of 1.459–1.665 and *P* value of <0.001 (Figure 1).

Table 6: Main data extracted from the studies included in the meta-analysis of *STAT4* rs10168266.

Study	Year	Ethnicity	Minor allele	SLE patients		Controls		OR (95% CI)
				Total	MAF	Total	MAF	
Kawasaki et al. [6]	2008	Japanese	T	308	0.378	306	0.258	1.75 (1.39–2.63)
Namjou et al. [23]	2009	European	A	2583	0.251	3099	0.184	1.49 (1.35–1.65)
		Korean	A	661	0.400	781	0.289	1.64 (1.40–1.91)
Yang et al. [24]	2010	Hong Kong Chinese	T	1484	0.4543	1484	0.3369	1.64
Kim et al. [25]	2013	Korean	A	553	0.395	663	0.297	1.55 (1.31–1.83)
Current study	2013	Malay	T	93	0.441	110	0.305	1.80 (1.20–2.71)
		Malaysian Chinese	T	245	0.457	294	0.383	1.36 (1.07–1.73)

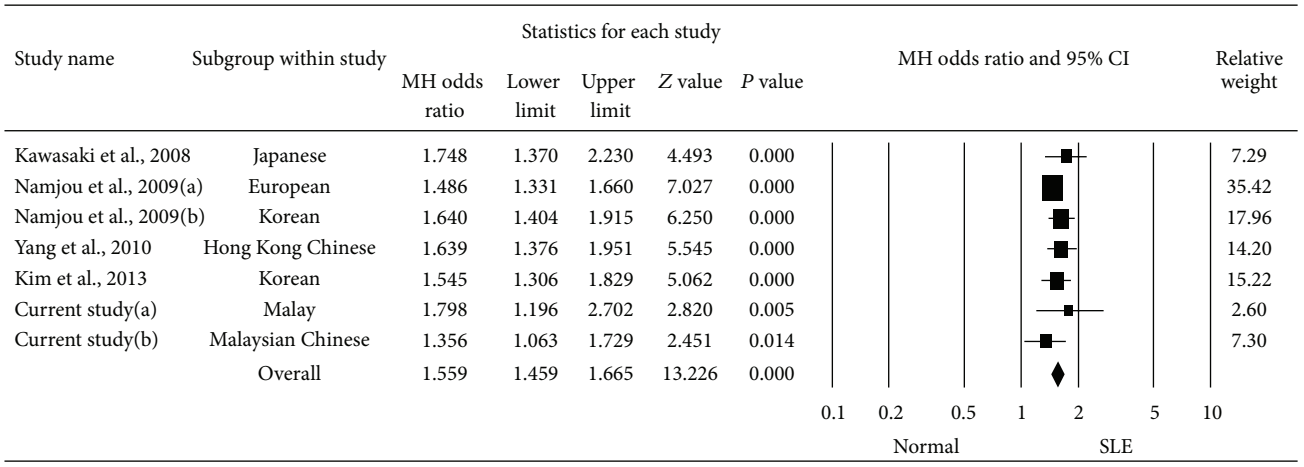

Figure 1: Forest plot of individual and pooled ORs with 95% CI for *STAT4* rs10168266 with SLE risk.

4. Discussion

Understanding the full molecular pathology of SLE remains a great challenge, although many insights have been revealed. Recognition of self-nucleic acids by toll-like receptors TLR7 and TLR9 on plasmacytoid dendritic cells and B cells is believed to be an important step in the pathogenesis of this disease [26]. Increased antinuclear antibodies and production of type I IFN are both correlated with the severity of disease. *STAT4*, *TNFAIP3*, and *IRF5* are genes involved in regulating TLR/IFN signalling pathways. SNPs investigated in this study have consistently shown associations with SLE susceptibility in many populations, especially in Asians [7, 24, 27–29]. When the various ethnic groups were considered, rs7574865 and rs10168266 of *STAT4* gene were significant in Chinese and Malays, respectively. However, only rs10168266 of *STAT4* was observed to have correlations with SLE in the Malaysians generally. None of the SNPs seemed to influence SLE in Indians. Due to population demographics and lower SLE risk predisposition, the fewer Indians recruited in this study may have impacted the results. The SNPs of *IRF5* were not significant. This suggests that the *IRF5* genetic variants tested for in this study are not linked to SLE in our cohort and that there may be other variants that are more important in the Indian ethnic group.

Therefore, it may be concluded from the present study that *STAT4* gene polymorphisms feature more prominently as the genetic risk factors in the Malaysian SLE rather than those polymorphisms in *TNFAIP3* and *IRF5*. Rs10168266 which is located in intron 5 of *STAT4* gene has been frequently related to SLE susceptibility in the Asian population, particularly in Korean population, and also in the European population [6, 23–25, 30]. This was also reflected in the findings of this study. Nevertheless, not many studies were done on this SNP and thus only four studies were included in the meta-analysis of this study. After the analysis, the pooled OR and *P* value once again showed that this SNP was overall an important risk factor for SLE and more attention should be taken.

Rs7574865, which was significantly associated with SLE in Chinese in this study, is located in the third intron of the *STAT4* gene. The minor/risk allele T has reported associations with other immune-mediated diseases such as rheumatoid arthritis, primary Sjögren's syndrome, type-1 diabetes, Crohn's disease, and ulcerative colitis [31–34]. The association of this particular SNP with SLE susceptibility was observed in many populations, including both European and Asian populations [7]. SNP haplotype in the third intron of *STAT4* marked by rs7574865 was found to be associated with SLE susceptibility and it could be responsible for splice variation or regulatory effects of *STAT4* [31, 35].

The next SNP that may be important in the Malaysian SLE was *TNFAIP3* rs2230936. This coding SNP is a nonsynonymous variant causing a phenylalanine-to-cysteine change at residue 127 of the A20 protein. It has been already proven that minor Cys127 is relatively stable compared to the Phe127 protein, causing it to be less effective at inhibiting TNF-induced NF-κB activity [36]. This reduced autoinflammatory activity of A20 could result in excessive cellular response to TNF. Interestingly, as opposed to other findings suggesting that minor allele G was the risk factor of SLE, the results of this study demonstrated that it conferred protection against SLE in our cohort [11, 24, 27, 36–40]. This study speculates that apart from rs223093, other factors such as adjacent SNPs may possibly alter the structure of A20 protein. This may play a role in SLE susceptibility in the Malays. We suggest that multiple (at least four) genes may collectively play critical roles in the development of this disease [41–43]. This speculation has yet to be validated.

Finally, both SNPs in *IRF5* gene investigated in our study were not significant in the Malaysian patients although both are fairly established SLE risk factors for Europeans and some Asians [44–47]. Presumably, other SNPs of this gene would have to be considered.

5. Conclusion

The present study was relatively small in contrast to larger studies of SLE by other researchers. Nevertheless, we present evidence to suggest that the genes involved in TLR/IFN signalling pathways, especially *STAT4* rs10168266 polymorphisms, contribute to the development of SLE in Malays and Chinese.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

PADI4 Haplotypes in Association with RA Mexican Patients, a New Prospect for Antigen Modulation

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Peptidyl arginine deiminase IV (PAD 4) is the responsible enzyme for a posttranslational modification called citrullination, originating the antigenic determinant recognized by anti-cyclic citrullinated peptide antibodies (ACPA). Four SNPs (single nucleotide polymorphisms) have been described in *PADI4* gene to form a susceptibility haplotype for rheumatoid arthritis (RA); nevertheless, results in association studies appear contradictory in different populations. The aim of the study was to analyze if the presence of three SNPs in *PADI4* gene susceptibility haplotype (GTG) is associated with ACPA positivity in patients with RA. This was a cross-sectional study that included 86 RA patients and 98 healthy controls. Polymorphisms PADI4_89, PADI4_90, and PADI4_92 in the *PADI4* gene were genotyped. The susceptibility haplotype (GTG) was more frequent in RA patients; interestingly, we found a new haplotype associated with RA with a higher frequency (GTC). There were no associations between polymorphisms and high scores in Spanish HAQ-DI and DAS-28, but we did find an association between RARBIS index and PADI4_89, PADI4_90 polymorphisms. We could not confirm an association between susceptibility haplotype presence and ACPA positivity. Further evidence about proteomic expression of this gene will determine its participation in antigenic generation and autoimmunity.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease, characterized by articular inflammation which can lead to joint destruction. RA prevalence is 1% worldwide with considerable variation between ethnic groups, with a higher prevalence in Caucasians compared with Asiatic populations [1, 2]. This disease is more frequent in females (3 : 1) around the fourth decade [3]; some studies suggest that sexual hormones, specifically estrogens, can cause hyperactivity in B and T cell functions [4]. RA represents a disease with risk of function disability due to articular damage as a result of ongoing inflammation, which is irretrievable. In order to limit illness incapability, it is necessary to establish the diagnostic as soon as possible and treat the condition.

Genetic predisposition for this disease is supported by the following findings: (1) first degree relatives of patients

with RA have a four to six times greater risk to develop the disease [5]; (2) presence of some HLA-DR molecules (HLA-DRB1*0401 and HLA-DRB1*0404) are genetic factors commonly found in RA, and its presence is associated with a more severe disease [6, 7]. The epidemiological genetic information suggests that the heritability for this disease ranges between 53 and 60%. Linkage disequilibrium studies revealed susceptibility *loci* for RA located within several chromosomes, one consistently implicated is the HLA-DRB1 gene [8]. Since this *locus* represents approximately one third of the total genetic effect, other *loci* should be considered to be part of RA development.

The peptidyl arginine deiminase IV gene denominated *PADI4*, located in 1p36.13, was recently acknowledged as one in association with RA, mainly in Japanese populations [9]. Suzuki and cols. described 17 single nucleotide polymorphisms (SNPs), four of them located in gene coding regions (exons 2–4). They found five haplotypes differing in four polymorphic sites; one denominated the susceptibility haplotype and was associated with RA. The SNPs involved are named PADI4_89, PADI4_90, PADI4_92, and PADI4_104; the first three determine an amino acid change, and the last one is a silent polymorphism [9–11]. In this same study, Suzuki and cols. described that this functional haplotype affected transcript stability, decreasing its degradation four times, and also demonstrated an association between haplotype homozygous individuals and ACPA positivity in patients with RA. In another study, this increase in *PADI4* mRNA stability was confirmed when mononuclear cells of peripheral blood from patients with RA were analyzed [12].

The protein peptidylarginine deiminase (PAD 4) consists of 663 amino acid residues with a 74 kDa molecular weight [13] and is the only isotype out of five described to be expressed in cell nucleus [14]. PAD enzymes have diverse physiologic functions including aggregation of keratin during terminal differentiation in the epidermis [15], involvement in brain development [16], and gene expression regulation by chromatin modeling [14, 17]. PAD 4 enzyme is responsible for a posttranslational modification called citrullination, originating the antigenic determinant recognized by anti-cyclic citrullinated peptide antibodies (ACPA). PAD 4 is a calcium dependant enzyme, an increase in cytosolic Ca^{+2} concentration (2 μM) is needed for citrullinated antigens to appear [13]. Since calcium ions induce conformational changes that create the active site in the α/β catalytic domain of the enzyme. Intracellular calcium concentrations range from ~ 200 nM (resting cells) to ~1 μM (activated cells) [18], and calcium concentrations in the cytosol can be increased during apoptosis or necrosis, leading to PAD activation and protein citrullination [19, 20]. Consequences of protein citrullination include protein charge neutralization, change in isoelectric point, ionic interaction breakage, partial protein unfolding, decrease in photolytic degradation, increase in antigenicity, and affinity changes with HLA.

Our interest in the present study comes after the discovery of ACPA, since not only have they shown high sensitivity (80%) and specificity (98%) [21], but they have also demonstrated a positive predictive value [22] in healthy blood donors who developed RA over the years [23] and in patients with undifferentiated arthritis [24]. Several authors have suggested that protein citrullination and autoantibody production are two processes implicated in RA development [24, 25], and nevertheless, the exact mechanism has not been elucidated.

One theory involves the possibility that susceptibility haplotype presence may induce an increase in transcript stability, which would lead to an elevated PAD 4 level and as a consequence citrullination of more epitopes that would break tolerance and induce production of autoantibodies against citrullinated peptides, thus initiating an autoimmune response.

Although evidence exists supporting the presence of *PADI4* susceptibility haplotype in RA Japanese patients [9] and Taiwan patients [26], it could not be extrapolated to other populations [27–29], and it is important to repeat association studies in populations with different ethnic background, in order to find and replicate previous findings related to *PADI4* susceptibility haplotype. The purpose of the present study was to analyze if the presence of three SNPs in *PADI4* gene susceptibility haplotype (GTG) is associated with ACPA positivity in Mexican patients with RA.

2. Material and Methods

2.1. Patients and Samples. We carried out a cross-sectional study that included 86 patients and 98 healthy subjects from northwestern Mexico who attended to the rheumatology outpatient clinical facilities at "Instituto Mexicano del Seguro Social" in Guadalajara, JAL, Mexico. All patients were classified as RA according to the 1987 ACR classification criteria [30] and fulfilled other inclusion criteria: voluntary acceptance to participate in the study and being able to answer questionnaires. We only included patients with Mestizo ethnicity since two previous generations; patients were not related to each other. Clinical data was obtained from direct interrogatory and physical examination, as well as a chart review in order to identify clinical variables such as disease duration, characteristics of the disease, and therapeutics. Two rheumatologists systematically evaluated the following indexes: DAS-28 [31] to establish severity of disease activity and Spanish HAQ-DI [32] to determine patient disability. We also obtained information from clinical charts in order to evaluate the RARBIS [33] that constitutes a medical records-based index to evaluate RA severity. Patients were included in any functional class according to Steinbrocker Functional Classification, and all of them were receiving treatment; these data was recorded.

Exclusion criteria included patients who had a diagnosis of other rheumatic disease, inability to access patient clinical chart, insufficient amount of sample, or bad quality DNA after extraction.

Healthy controls were blood donors who attended to "Instituto Mexicano del Seguro Social" blood bank and denied having any chronic disease.

2.2. Genotyping. DNA from 86 patients with rheumatoid arthritis and 98 healthy subjects was extracted from blood

Table 1: Genotyping strategies for *PADI4* polymorphism variants detection.

SNP	Primers	T_m (°C)	Band size (bp)	Restriction enzyme	Recognized sequence	Band size after digestion (bp)
PADI4_89	5′-TCTGCTTTCCCATGTGTCTTG-3′ 5′-AGGACAGAGTGTGTGTGGCTG-3′	61	278	*HaeIII*	GGCC	**G** 100, 95, 43, and 40 **A** 140, 95, and 43
PADI4_90	5′-AAATCCACAGGTTCCTCCACA-3′ 5′-CATCACGAGCTCTTCCACAGG-3′	62	221	*MscI*	TGGCA	**T** 154 and 67 **C** 221
PADI4_92	5′-CCCAACTTTGTCTCCCCAGT-3′ 5′-TTGTGGTTCACTGACTAAGGAT-3′	61	363	*MspI*	CCGG	**G** 195, 134, and 34 **C** 329 and 34

SNP: single nucleotide polymorphism, T_m: fusion mean temperature, and bp: base pair.

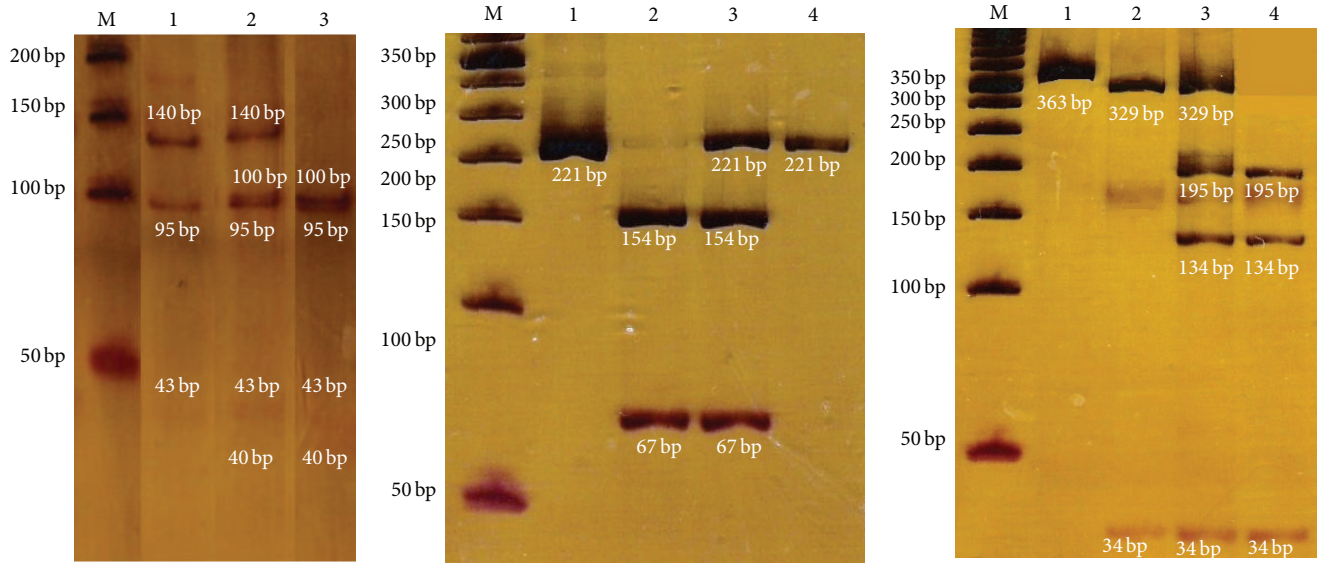

Figure 1: *PADI4* SNPs enzyme digestion. The figure shows digestion of three SNPs in the *PADI4* gene. (a) Shows digestion of PADI4_89, with *HaeIII* enzyme; lane 1 represents the A/A genotype, lane 2 A/G and 3 G/G. (b) Demonstrates PADI4_90 amplification (221 bp) in lane 1 and digested products with *MscI* enzyme in lanes 2 (C/C genotype), 3 (C/T genotype), and 4 (T/T genotype). (c) Shows amplification product of PADI4_92 in lane 1 (363 bp) and restriction products obtained with the enzyme *MspI;* lane 2 corresponds to the G/G genotype, lane 3 G/C, and lane 4 C/C. Visualized in 8% (29 : 1) polyacrylamide gel with silver staining. M: molecular weight marker (50 bp).

samples using conventional methods [34] and stored frozen at −80°C.

Genotyping for polymorphisms PADI4_89, PADI4_90, and PADI4_92 in the *PADI4* gene was determined by three polymerase chain reaction-restriction fragment length polymorphisms (PCR_RFLPs) protocols designed using Oligo 0.4 software, BLAST, and NEBcutter V.2.0. Table 1 shows primer sequence and obtained products. Each PCR reaction was carried out in 10 μL final volume containing (final concentrations): 1X buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, and 4 mM $MgCl_2$); 5 pmol/mL each of the pair primers according to polymorphism (Table 1); 10 mM each of the four deoxyribonucleoside triphosphates; 1 U of taq DNA polymerase (Invitrogen, Carslbad, CA, USA); and 200 to 300 ng DNA template. The PCR products were visualized by electrophoresis in 8% (29 : 1) polyacrylamide gels at 150 V for 1 h, followed by silver staining. The PADI4_89, PADI4_90, and PADI4_92 genotypes were identified after restriction enzyme digestion with *HaeIII*, *MscI,* and *MspI,* respectively (New England Biolabs, MS, USA), shown in Figure 1.

2.3. Haplotypes. Haplotype analysis was done using the software PHASE v 1.0 for haplotype reconstruction, and recombination rate estimation was done using the genotypic data [35].

2.4. Anti-Cyclic Citrullinated Peptide (ACPA) Antibody Assay. ACPA (IgG) was measured using a commercially available second generation enzyme-linked immunosorbent assay (ELISA-II), according to the manufacturer instructions (EUROIMMUN, UK). Briefly, test samples, calibrator, and controls are incubated in the respective wells, containing the citrullinated peptide. Antibodies will bind, and nonbound material is removed by washing. Next, peroxidase conjugated anti-human IgG is added to each well. After incubation with substrate solution, the reaction is stopped, and density values were obtained with a spectrophotometer at a wave length of 405/620 nm. Results were expressed in relative units per milliliter (RU/mL), considering positive when the result was >5 RU/mL.

Table 2: Demographic and clinical characteristics of RA patients.

Characteristic	RA ($n = 86$)
Age ($x \pm$ SD)	50 ± 12
Smoking	17%
Years with RA ($x \pm$ SD)	11 ± 7
Functional class	
I	8%
II	79%
III	11%
IV	2%
HAQ-DI ($x \pm$ SD)	1.22 ± 1.00
DAS 28 ($x \pm$ SD)	4.4 ± 1.3
RARBIS ($x \pm$ SD)	6.26 ± 2.70
RF (IgG) +	66%
ACPA (IgM) +	74%
Treatments	*n* (%)
Chloroquine	33 (38)
Sulfasalazine	47 (55)
Methotrexate	65 (76)
Azathioprine	25 (29)
Cyclosporine	1 (1)
Infliximab	2 (2)
Etanercept	10 (11)
Cyclophosphamide	1 (1)
Corticosteroids	72 (80)

HAQ-DI: health assessment questionnaire disability index, DAS: disease activity score, RARBIS: RA medical records-based index of severity, and RF: rheumatoid factor.

2.5. Statistical Analysis. Allelic and genotypic frequencies were determined by gene count. Comparisons between groups for nominal and categorical variables were performed by applying *chi* square test or Fisher exact test as indicated. A P value of ≤0.05 was considered significant. Hardy-Weinberg equilibrium was tested.

3. Results

3.1. Demographics and Clinical. We included 89 patients; all of them attended to rheumatology clinic and were classified as RA according to ACR classification criteria [30]. Three of them were excluded because of poor DNA quality. Mean age was 50 ± 12 years, 100% of the studied subjects were women, mean duration disease was 11 ± 7, years and all of them were receiving treatment, some of them with monotherapy and others combined therapy, and the number and percentages of patients taking a specific drug are listed in Table 2. Results from HAQ-DI, DAS-28, and RARBIS are also shown in Table 2.

3.2. Molecular Analysis. In this comparative study of 86 patients and 98 controls, we found a significant association between three exonic SNPs of *PADI4* gene and RA ($P < 0.05$). Genotypic and allelic frequencies are listed on Table 3 confirming previous association studies.

Carriage of PADI4_89 G allele (OR 2.51, 95% CI 1.19–5.32) and PADI4_90 T allele (OR 2.64 95% CI 1.21–5.75) was associated with susceptibility to RA. The PADI4_92 G allele could not be associated with RA (OR 2.08, 95% CI 0.81–5.36).

The three nonsynonymous polymorphisms PADI4_89 (163G/A), PADI4_90 (245T/C), and PADI4_92 (335G/C) constituted mainly eight haplotypes; their sequence and frequencies are represented in Table 4. We found significant association ($P < 0.0005$) of the susceptibility haplotype (Haplotype 1) and RA condition with an OR (95% CI) of 19 (2.4 to 147), and this haplotype was present in 14 patients with RA and only one control. Interestingly, another haplotype was associated significantly with RA (Haplotype 3); this haplotype was present in a higher frequency: 42 patients and 28 controls ($P = 0.006$) OR (95% CI) = 2.4(1.3–4.4). The nonsusceptibility haplotype (Haplotype 2) did not display significant differences.

3.3. Antibodies against Cyclic Citrullinated Peptide (ACPA) (IgG). From 86 patients with RA, 74% had ACPA positivity, and mean ± SD was 5.98 ± 4.15 RU/mL. We did not find an association between ACPA positivity and the presence of genetic variants in PADI4_89, PADI4_90, and PADI4_92 polymorphisms of *PADI4* gene; results are shown in Figure 2. We neither found an association between high titers of ACPA and risk allele (data not shown in graph). Fifteen patients had positive smoking, and we searched antibody positivity among them and found eight positive cases with mean ± SD titers of 8.41 ± 2.98 RU/mL.

3.4. Clinical Data and PADI4 Gene Polymorphisms. We searched for possible associations between clinical data, high punctuation score of HAQ-DI DAS-28 and RARBIS indexes with susceptibility haplotype, or the presence of polymorphisms PADI4_89, PADI4_90, and PADI4_92 of *PADI4* gene in RA patients. The only clinical variable significantly associated was the presence of high score in RARBIS index with G allele (susceptibility) of PADI4_89 SNP ($P = 0.007$) and with T/T genotype (homozygous susceptible) of PADI4_90 ($P = 0.04$). For PADI4_92, we did not find any significant association ($P = 0.84$) (data not shown in tables).

4. Discussion and Conclusion

During the last decade, RA pathogenesis research has been centered in the idea that multiple cells and molecular mechanisms of the immune system are involved; nevertheless, from this complicated view, a common point emerges: escape from immunological control leading to loss of tolerance with lymphocyte T activation and B cells production of autoantibodies [36]. The self/nonself theory of the immune system fails to explain how autoimmune responses are being generated, since our immune system should be tolerant to self molecules. Matzinger [37] proposed an alternative model that suggested, in addition to antigen presentation, the presence of danger signals that are released after tissue injury and can trigger immune responses [38, 39]. First observations of the relationship between cellular lyses and inflammation go back to inflammasome description; produced spontaneously after

Table 3: Genotypic and allelic frequencies of PADI4_89, PADI4_90, and PADI4_92 SNPs of *PADI4* gene in controls (HC $n = 98$) and rheumatoid arthritis (RA $n = 86$) patients.

SNPs	Genotypes	RA	HC	Allele	RA	HC	P	OR (95% CI)
PADI4_89	A/A	0.148	0.305	A	0.488	0.581	0.040	2.51 (1.19–5.32)
	A/G	0.679	0.551	G	0.512	0.419		
	G/G	0.173	0.144					
PADI4_90	C/C	0.136	0.278	C	0.420	0.562	0.004	2.64 (1.21–5.75)
	C/T	0.568	0.567	T	0.580	0.438		
	T/T	0.296	0.155					
PADI4_92	C/C	0.086	0.165	C	0.438	0.454	0.732	2.08 (0.81–5.36)
	C/G	0.704	0.578	G	0.562	0.546		
	G/G	0.210	0.257					

SNP: single nucleotide polymorphism, HC: healthy controls, RA: rheumatoid arthritis, OR: odds ratio, and 95% CI: 95% confidence interval.
In order to compute OR (95% CI), the following alleles were used as reference: PADI4_89 allele A, PADI4_90 allele C, and PADI4_92 allele C in correspondence with the nonsusceptibility haplotype (ACC).

Table 4: Haplotype sequence and frequency of *PADI4* gene SNPs (PADI4_89, PADI4_90, and PADI4_92) in healthy controls and RA patients.

Haplotypes	Condition	Frequencies		P	OR (95% CI)
		RA ($n = 86$)	HC ($n = 98$)		
Haplotype 1 susceptibility GTG	Present	14	1	0.0002	18.9 (2.4–146.8)
	Absent	72	97		
Haplotype 2 nonsusceptibility ACC	Present	5	6	1	0.95 (0.3–3.2)
	Absent	81	92		
Haplotype 3 new susceptibility GTC	Present	42	28	0.006	2.4 (1.3–4.4)
	Absent	44	70		
Haplotype 4 ACG	Present	43	39	0.18	1.5 (0.8–2.7)
	Absent	43	59		
Haplotype 5 GCG	Present	1	10	0.011	0.1 (0.01–0.8)
	Absent	85	88		
Haplotype 6 ATC	Present	3	8	0.22	0.4 (0.1–1.6)
	Absent	83	90		
Haplotype 7 ATG	Present	6	6	1	1.1 (0.4–3.7)
	Absent	81	92		
Haplotype 8 GCC	Present	1	4	0.4	0.3 (0.03–2.5)
	Absent	85	94		

HC: healthy controls, RA: rheumatoid arthritis, and SNPs: single nucleotide polymorphisms.

cellular membrane disruption [40], inflammasome activation causes the release of IL-1β, IL-18, and IL-33 cytokines, which can activate B and T lymphocytes and contribute to the development of inflammatory and autoimmune diseases [41].

Over the years, several paradigms have been broken with respect to RA pathogenesis. In 1997, Weyand and Goronzy [42] proposed a new hypothetical model; this model integrates genetic risk factors to immune inflammatory responses [42]. *PADI4* susceptibility haplotype could be a genetic risk factor, but before *PADI4* gene polymorphisms are considered genetic markers for RA, it was mandatory to replicate the association findings first reported in Asiatic populations; nevertheless, such findings could not be replicated in other populations (Table 5) [27, 28]. In the last years, two meta-analyses have been published pretending to clarify the existence of a true association in different populations [43, 44], since such studies can increase sample size and precision, thus reducing the probability of incising in false positive or false negative results. In Lee and colleagues. study, three Asiatic and six European populations were included [44]; a significant association between RA and *PADI4* polymorphisms was found (PADI4_94, PADI4_104, and PADI4_90).

The present study represents the first one in Mexican population which is the result of genetic admixture of Spanish, Indian, and Black populations, and it should be emphasized that the genetic background introduced from Spaniards includes genes from Romans, Greeks, Visigods, Arabs, and Jews [45]. We should point out that three new PCR-RFLP protocols were designed for the conduction of the present study; we pretend that this approach of association can be replicated in laboratories where equipment for DNA sequencing or RT-PCR is not available, since these have been methodologies described in previous reports [9, 46].

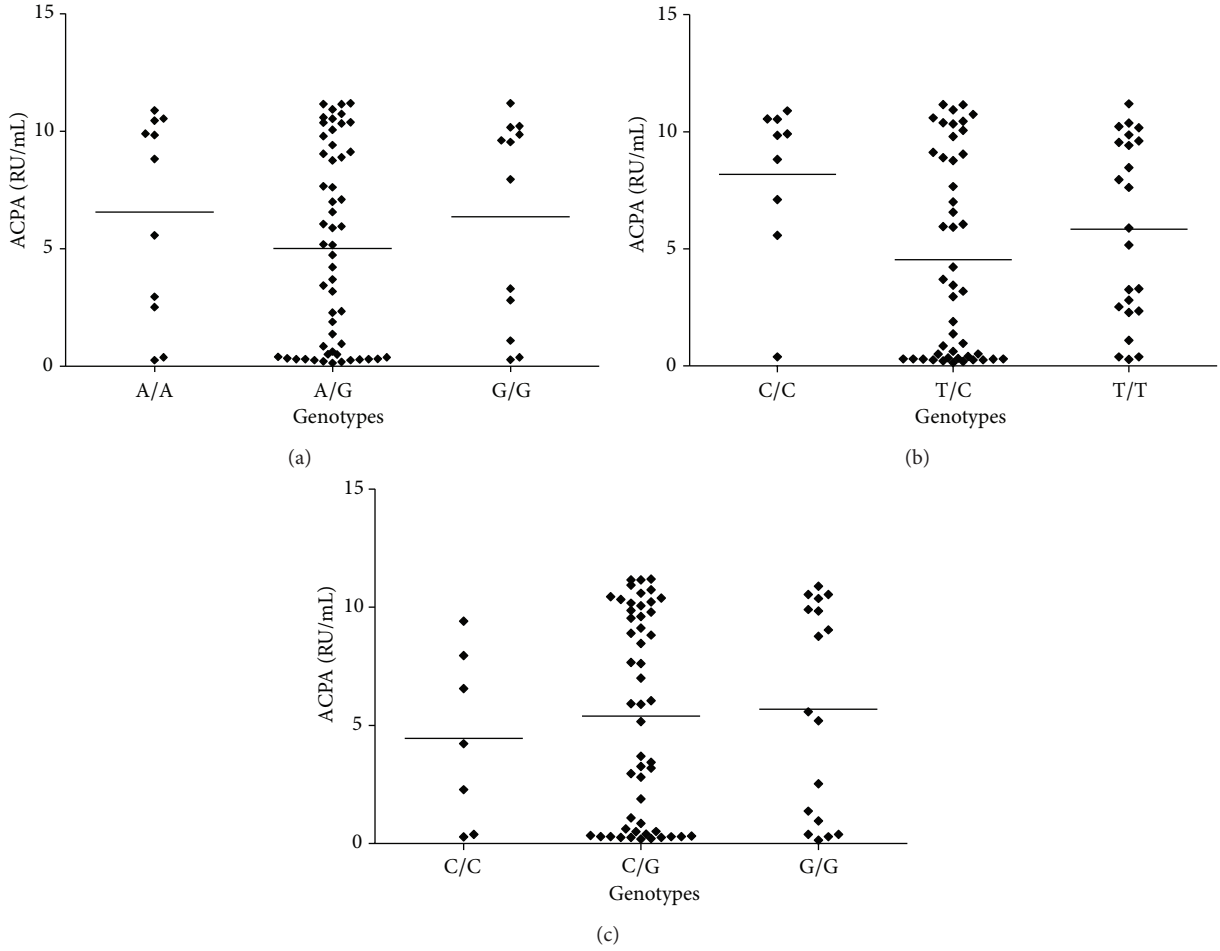

Figure 2: Anti-cyclic citrullinated peptide antibodies (ACPA) titers in the presence of genetic variants in PADI4_89, PADI4_90, and PADI4_92 polymorphisms of *PADI4* gene.

We found that *PADI4* polymorphisms are associated with RA susceptibility, regardless of ACPA titers. This is consistent with results published by Hoppe and Kang et al. in German [11] and Korean [47] populations, respectively. Our findings also show partial concordance with results in Japanese population [9], since PADI4_89 and PADI4_90 were associated with RA in our study. A recent meta-analysis [44] concluded that *PADI4* gene polymorphisms could have a higher susceptibility role in Asiatic populations compared to Caucasians, but soon after that, an association between *PADI4* gene and RA was confirmed in a French population [48]. The lack of replication in different studies could be attributed to (1) false positive results in the primary report due to sample bias, (2) false negative in the replication study due to lack of statistical power, or (3) true genetic heterogeneity exists.

When we analyzed haplotype frequencies, we found that the susceptibility haplotype (GTG) was significantly associated with RA ($P = 0.0002$), but more interestingly, we found a new haplotype (GTC) which was both significantly associated ($P = 0.006$) and more frequent than previous susceptibility haplotype. We believe that since this second haplotype was present in a higher frequency among RA patients, it should be looked forward.

In the present study, our patients with RA had an active disease according to DAS 28 and a significant limitation in functioning according to HAQ-DI. Nevertheless, an exploratory subanalysis did not observe a significant association between the presence of polymorphisms PADI4_89, PADI4_90, and PADI4_92 of *PADI4* gene with high scores in DAS 28 or HAQ-DI. Instead, a G allele of PADI4_89 SNP and T/T genotype of PADI4_90 were significantly associated with higher score in RARBIS. The RARBIS index is based on patient records and could represent a confident tool to measure disease severity, but it is not yet validated in Spanish. It would be desirable that other association studies could further characterize their population with the use of these clinical scores, since most of the association studies do not present clinical data. Furthermore, a deficient clinical characterization of the group of study could contribute to inconsistencies in results.

In our RA patient group, ACPA (IgG) positivity was present in 74%, similar to previous reports [44, 49, 50]. We did not find an association between polymorphisms or haplotype susceptibility of the *PADI4* gene and positivity or elevated titers of ACPA. It should be noted that previous association between ACPA positivity and susceptibility haplotype was described in homozygous subjects to the susceptibility

Table 5: Association studies for *PADI4* SNPs and rheumatoid arthritis.

SNPs	Country RA/HC	Results	Reference
PADI4_89-105	Japan (830/736)	PADI4_92, 94, 95, 97, 99, 100, 101 and 104 ($P = 0.0000084$–0.00051)	Suzuki et al. 2003 [9]
PADI4_89, 90, 92, 104	UK (839/481)	Susceptibility haplotype more frequent in RA patients (32.3 versus 29.6) without significance ($P = 0.79$)	Barton et al. 2004 [27]
PADI4_92, 96 and 102	France (100 families)	No significant associations	Caponi et al. 2005 [28]
PADI4_92, 94, 97, 99, 100, 103 and 104	England (111/111)	PADI4_100 and 103 ($P = 0.03$). Increase in mRNA expression of *PADI4* in PBM from RA versus C	Harney et al. 2005 [12]
PADI4_94, 102 and 104	Japan (1170/926)	PADI4_94, 102, and 104 ($P = 0.0008$–0.010)	Ikari et al. 2005 [26]
PADI4_94 and 104	Spain (354/498)	No significant associations	Martinez et al. 2005 [29]
PADI4_94	Sweden and North America	Association with RA ($P = 0.02$)	Plenge et al. 2005 [55]
PADI4_89, 90, 92 and 104	Korea (545/392)	Strong association of RA with susceptibility haplotype ($P = 1.0 \times 10^{-4}$)	Kang et al. 2006 [47]
PADI4_89, 90, 92, 94, 95, 96 and 104	Germany (102/102)	PADI4_89, 90, and 94 ($P = 0.04$)	Hoppe et al. 2006 [11]
PADI4_89 and 90	France (405/275)	Association with RA ($P = 0.03$ and 0.003)	Gandjbakhch et al. 2009 [48]

RA: rheumatoid arthritis, HC: healthy controls, and PBM: peripheral blood mononuclear.

haplotype according to Suzuki et al. [9] and Gandjbakhch et al. [48]. We did not find individuals who were homozygous for the susceptibility haplotype. Additionally, besides IgG isotype ACPA positivity, more recently, the presence of the IgM isotype was demonstrated at different times during the course of RA, indicating that ACPA production is a constant phenomenon during RA evolution [51] and seems to be an ongoing phenomena along disease duration [52]. For this reason, a lack of association between ACPA positivity and susceptibility haplotype presence in our study does not confirm that they are unrelated. Another important finding in ACPA research is the fact that citrullinated proteins can be found in synovium with inflammation caused by several pathologies [53], but the presence of ACPA remains specific for RA patients [10]. As a consequence, it has been suggested that ACPA could participate in RA generation, and there are two possible explanations for their development: the first one considers a high expression of citrullinated antigens which can originate loss of tolerance and as a result ACPA production contributing to inflammation and specific immune responses toward citrullinated antigens. The second theory postulates that RA patients have an abnormal humoral immune response towards citrullinated proteins and they start producing elevated amounts of antibodies against them.

Supporting the first theory is the fact that susceptibility haplotype presence can affect *PADI4* translation, as the increase in mRNA stability has been demonstrated, generating an increase in protein levels and as a consequence a higher occurrence of citrullinated proteins [54]. Citrullination can contribute to the generation of antigens since it originates a change in amino acid charge (amine group is positively charged and citrulline is neutral). This may affect the tertiary or quaternary structure of the protein, allowing protein domains that where otherwise maintained in the internal protein structure to be exposed.

Finally, we did not find associations between *PADI4* gene polymorphisms and clinical variables, except for high punctuation in RARBIS index and presence of susceptibility allele (G) in PADI4_89 or susceptibility homozygous (T/T) in PADI4_90 of *PADI4* gene.

Limitations in this study include sample size since only one gender is represented in the present investigation; it is well known that RA is a condition that affects mainly women, but it would be desirable to increase sample size in order to be able to include male patients and to verify if association with the susceptibility haplotype remains constant, since in our study we were able to find only one HC with the susceptibility haplotype.

In conclusion, we confirm the association between the susceptibility haplotype (GTG) with RA patients, observing also that a new haplotype (GTC) could be associated with RA in Mexican mestizo patients. Further studies including other regions in Mexico and increasing the sample size are required in order to confirm our findings; furthermore, the biggest challenge for association studies is the identification of genetic variants that, combined in haplotypes, can be described as causal effects to RA.

Ethical Considerations

This research was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants, and the study protocol was approved by the Local Committees for Ethical and Health Research (CLIEIS 1301), and all data were managed anonymously.

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Clinical Study

Evidence of Stage- and Age-Related Heterogeneity of Non-HLA SNPs and Risk of Islet Autoimmunity and Type 1 Diabetes: The Diabetes Autoimmunity Study in the Young

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Previously, we examined 20 non-HLA SNPs for association with islet autoimmunity (IA) and/or progression to type 1 diabetes (T1D). Our objective was to investigate fourteen additional non-HLA T1D candidate SNPs for stage- and age-related heterogeneity in the etiology of T1D. Of 1634 non-Hispanic white DAISY children genotyped, 132 developed IA (positive for GAD, insulin, or IA-2 autoantibodies at two or more consecutive visits); 50 IA positive children progressed to T1D. Cox regression was used to analyze risk of IA and progression to T1D in IA positive children. Restricted cubic splines were used to model SNPs when there was evidence that risk was not constant with age. *C1QTNF6* (rs229541) predicted increased IA risk (HR: 1.57, CI: 1.20–2.05) but not progression to T1D (HR: 1.13, CI: 0.75–1.71). SNP (rs10517086) appears to exhibit an age-related effect on risk of IA, with increased risk before age 2 years (age 2 HR: 1.67, CI: 1.08–2.56) but not older ages (age 4 HR: 0.84, CI: 0.43–1.62). *C1QTNF6* (rs229541), SNP (rs10517086), and *UBASH3A* (rs3788013) were associated with development of T1D. This prospective investigation of non-HLA T1D candidate loci shows that some SNPs may exhibit stage- and age-related heterogeneity in the etiology of T1D.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which the insulin-producing beta cells of the pancreas are destroyed. There is typically a preclinical phase of circulating autoantibodies, called islet autoimmunity (IA), which precedes the clinical diagnosis of T1D. T1D is widely believed to be caused by an environmental factor on a susceptible genetic background. The major susceptibility locus for T1D maps to the HLA class II genes at chromosome 6p21. These HLA class II alleles account for 30–50% of the familial clustering of T1D [1].

More than 50 non-HLA T1D susceptibility gene markers have been confirmed. The major non-HLA loci include *INS* [2], *CTLA4* [3], *PTPN22* [4], *IL2RA* [5], and *IFIH1* [6]. The DAISY study has previously investigated 20 non-HLA SNPs and found SNPs in *PTPN22*, *UBASH3A*, *INS*, and *IFIH1* associated with IA and/or progression to T1D [7–11].

Prospective birth cohorts have the unique ability to study two stages in the natural history of T1D: development of IA and progression to T1D in IA positive children. Different exposures have been associated with one or both stages. For instance, DAISY recently identified an association between a gene-gene interaction involving the vitamin D receptor gene (*VDR*) and protein tyrosine phosphatase, nonreceptor type 2 gene (*PTPN2*) with progression to T1D in IA positive children, but not with development of IA [12]. This would be an example of stage-related heterogeneity in the natural history of T1D. There is also evidence of age-related heterogeneity in the etiology of T1D when a gene or exposure is associated with the disease at certain ages, but not others. One example is a recent study that found

differences in metabolite profiles relative to age, in which there was an association between lower methionine levels and presence of diabetes autoantibodies in younger onset (≤2 years), but not older onset (≥8 years) autoimmunity [13]. The purpose of this analysis was to investigate stage- and age-related heterogeneity of fourteen non-HLA T1D candidate SNPs for their association with development of IA and progression to T1D in a prospective birth cohort of non-Hispanic white (NHW) children at increased genetic risk of T1D. Additionally, we investigated whether the fourteen T1D candidate SNPs that were originally detected by GWAS using a case-control study design would be detected in time-to-event analyses of T1D risk in a prospective birth cohort.

2. Materials and Methods

2.1. Subjects. The Diabetes Autoimmunity Study in the Young (DAISY) is a prospective study composed of two groups of children at increased risk for T1D who were recruited between 1993 and 2004 and are being followed prospectively for the development of IA and T1D. One group is made up of first degree relatives of patients with T1D, identified and recruited between birth and eight years of age, mainly through the Barbara Davis Center for Childhood Diabetes ($n = 815$). The second group consists of infants born at St. Joseph's Hospital in Denver, CO, whose umbilical cord blood was screened for diabetes-susceptibility HLA-DR, DQ genotypes ($n = 819$). Details of the newborn screening, sibling and offspring recruitment, and followup of both cohorts have been published previously [14, 15]. Cord blood or the first available blood sample (depending on enrollment group) was sent to Roche Molecular Systems, Inc., Alameda, CA, for PCR-based HLA-DR, DQ typing. All study protocols were approved by the Colorado Multiple Institutional Review Board, and informed consent was given by parents of all participating children.

2.2. Measurement of Autoantibodies. Autoantibodies were tested at 9, 15, and 24 months, and annually thereafter, or at their first visit and annually thereafter if the child enrolled after birth. Radioimmunoassays were used to measure serum autoantibodies to insulin, GAD-65, and IA-2 (BDC512), as previously described [16–19], with rigorous confirmation of all positive and a subset of negative results. The cut-off for positivity was established as the 99th percentile of healthy controls. Children who tested autoantibody positive were put on an accelerated testing schedule of every 3–6 months.

Cases of IA were defined as those children positive for at least one islet autoantibody (IAA, GAD-65, IA-2) on two or more consecutive visits. T1D was diagnosed by a physician and defined as random blood glucose >200 mg/dL and/or HbA1c (A1C) > 6.5% with clinical symptoms of T1D.

2.3. Non-HLA SNP Genotyping. DAISY children were genotyped for fourteen non-HLA T1D candidate SNPs: *C1QTNF6* (rs229541), *C6orf173* (rs9388489), *C14orf181* (rs1465788), *IL2* (rs2069762), *IL2* (rs4505848), *IL2RA* (rs12722563), *IL2RA* (rs2104286), *IL7R* (rs6897932), *PRKCQ* (rs947474), *SKAP2* (rs7804356), *SMARCE1* (rs7221109), *TLR8* (rs5979785), *UBASH3A* (rs3788013), and SNP (rs10517086). Thirteen of the fourteen SNPs were chosen from three GWAS meta-analyses [20–22]. *UBASH3A* (rs3788013) was chosen based on its strong LD with *UBASH3A* (rs876498), which was discovered for its association with T1D by Concannon et al. [23].

The SNPs were genotyped utilizing the Taqman SNP genotype based OpenArray platform (Applied Biosystems, CA, USA). Custom designed 48-sample arrays and normalized genomic DNA were loaded using the OpenArray AccuFill system and cycling was performed on a GeneAmp 9700 PCR system (Applied Biosystems, CA, USA), all according to manufacturer protocol. Alleles were analyzed using the OpenArray SNP genotyping analysis software v.1.0.3 and Taqman Genotyper Software 2.0 (Applied Biosystems, CA, USA). All fourteen SNPs had a 95% call rate or higher.

Each SNP was tested for consistency with Hardy-Weinberg proportions using a 1-degree of freedom χ^2 goodness-of-fit test with a P value of 0.05 considered as evidence of a departure from Hardy-Weinberg equilibrium; all fourteen SNPs were in Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) was tested in our population using Haploview version 4.2 as measured by r^2 and D', with $r^2 = 0.257$ and $D' = 0.862$ for the two *IL2RA* SNPs and $r^2 = 0.222$ and $D' = 1.0$ for the two *IL2* SNPs.

2.4. Analysis Population. We obtained genetic data on at least one of the fourteen non-HLA T1D candidate SNPs for 1634 non-Hispanic white children in the DAISY cohort. This included 132 children who developed IA, of whom 50 went on to develop T1D. Fifteen IA cases were positive for autoantibodies on their first clinic visits; these left-censored cases were removed from the development of IA analysis cohort but retained in the progression from IA to T1D cohort. The same 50 IA positive children who went on to develop T1D are the same 50 T1D cases in the development of T1D analyses. However, not all IA positive children went on to develop T1D. All statistical analyses were limited to non-Hispanic whites in the DAISY cohort.

2.5. Statistical Analyses. SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) statistical software package was used for all statistical analyses. SNPs were tested for violation of the proportional hazards assumption using a supremum test, with a P value < 0.20 indicating possible departure from proportional hazards [24]. If a SNP appeared not to meet this assumption, restricted cubic splines were used to evaluate the nature and extent of the violation. SNPs were analyzed for their association both with development of IA and with progression from IA to T1D. For each model, hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox regression analyses. There were 302 sibling pairs in the analysis cohort, so a clustered time to event analysis was performed treating siblings from the same family as a cluster, and robust sandwich variance estimates were used for statistical inference [25]. Analyses of time to development of IA were adjusted for the HLA-DR genotype (HLA-DR3/4,

Table 1: Demographic characteristics of DAISY non-Hispanic white population.

Characteristic	Development of islet autoimmunity (IA) (n = 1619)				Progression from IA to type 1 diabetes (T1D) (n = 132)			
	Children positive for IA (n = 117)	Children negative for IA (n = 1502)	Univariate HR and 95% CI	P value	IA positive children who progressed to T1D (n = 50)	IA positive children who have not progressed to T1D (n = 82)	Univariate HR and 95% CI	P value
Mean age (years)	6.2 ± 4.2[a]	9.9 ± 5.7[b]	N/A	N/A	8.7 ± 3.9[c]	14.1 ± 4.2[b]	N/A	N/A
Mean age at first IA positive visit (years)	N/A	N/A	N/A	N/A	3.9 ± 2.9	7.2 ± 4.2	0.86 (0.77, 0.95)	0.003
HLA-DR3/4, DQB1*0302	43 (36.8%)	248 (16.5%)	2.97 (2.01, 4.39)	<0.0001	28 (56.0%)	19 (23.2%)	2.79 (1.65, 4.72)	0.0001
First degree relative with T1D	75 (64.1%)	737 (49.1%)	1.41 (0.96, 2.07)	0.08	36 (72.0%)	54 (65.9%)	1.02 (0.56, 1.85)	0.96
Sex (female)	62 (53.0%)	712 (47.4%)	1.22 (0.85, 1.74)	0.28	24 (48.0%)	44 (53.7%)	1.03 (0.59, 1.83)	0.91

CI: confidence interval; DAISY: Diabetes Autoimmunity Study in the Young; HLA: human leukocyte antigen; HR: hazard ratio; IA: islet autoimmunity; T1D: type 1 diabetes.
[a]Age at first IA positive visit.
[b]Age at last followup.
[c]Age at T1D diagnosis.

DQB1*0302 versus other genotypes) and presence of a first degree relative with T1D. Analyses of time to progression to T1D were adjusted, in addition, for age at first positive autoantibody visit. *TLR8* (rs5979785) was additionally adjusted for sex because it is on the X chromosome. The significance threshold was defined as $\alpha = 0.05$. Because our analyses were based on *a priori* hypotheses with SNPs previously found to be associated with T1D, P values were not corrected for multiple testing. We analyzed each non-HLA SNP in separate, covariate adjusted models. In the analyses examining development of IA, all of the SNPs were treated additively, except *IL2RA* (rs12722563) and *PRKCQ* (rs947474), which were dichotomized on the minor allele due to small sample sizes. Additionally, in the progression to T1D analyses, SNPs were treated additively, except *C14orf181* (rs1465788), *IL2RA* (rs12722563), *IL2RA* (rs2104286), *IL7R* (rs6897932), *PRKCQ* (rs947474), *SKAP2* (rs7804356), and SNP (rs10517086), which were dichotomized on the minor allele due to small sample sizes.

3. Results

3.1. Development of IA. We first examined whether non-HLA variants were associated with development of IA. The mean age at first IA positive visit was 6.2 years, and the mean age at last follow-up visit in children who did not develop IA was 9.9 years (Table 1). IA positive children were more likely to have the HLA-DR3/4, DQB1*0302 genotype compared to DAISY children who did not develop IA (HR: 2.97, 95% CI: 2.01, 4.39).

Unadjusted SNP association analyses are presented in (see Supplemental Table 1 in Supplemetary Material available online at http://dx.doi.org/10.1155/2013/417657). Adjusting for HLA-DR3/4,DQB1*0302 and first degree relative with T1D,

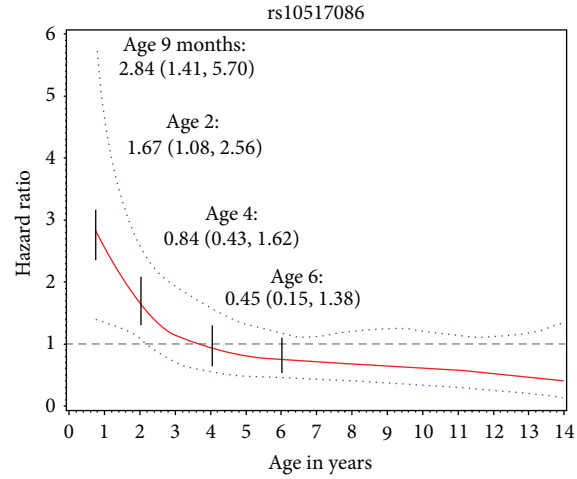

Figure 1: Association between SNP rs10517086 and development of IA modeled using a restricted cubic spline. The hazard ratio at different ages is represented by the solid line and the 95% confidence intervals are represented by the dotted lines. Hazard ratios and 95% confidence intervals are shown at 9 months, 2 years, 4 years, and 6 years (denoted with short vertical lines) illustrating the increased risk of developing IA in the younger ages, but not in older ages.

C1QTNF6 (rs229541) was associated with development of IA (HR: 1.57, 95% CI: 1.20, 2.05 (for each additional minor allele)) (Table 2). SNP (rs10517086) did not appear to meet the assumptions of proportional hazards in the development of IA analysis and therefore was modeled using a restricted cubic spline. The restricted cubic spline shows that SNP (rs10517086) is associated with an increased risk of developing IA before the age of two or in younger ages but is not associated with developing IA in older ages (Figure 1). Children

Table 2: Association between non-HLA T1D candidate SNPs and development of IA, progression from IA to T1D, and development of T1D adjusted for HLA-DR3/4, DQB1*0302 genotype and first degree relative with T1D.

SNP	Minor allele	Development of IA (n = 1619)			Progression from IA to T1D (n = 132)		Development of T1D (n = 1619)	
		MAF[a]	Adjusted HR[b] and 95% CI	P value	Adjusted HR[c] and 95% CI	P value	Adjusted HR[b] and 95% CI	P value
C1QTNF6 (rs229541)	A	0.44	**1.57 (1.20, 2.05)[d]**	**0.001**	1.13 (0.75, 1.71)[d]	0.56	**1.95 (1.33, 2.87)[d]**	**0.001**
C6orf173 (rs9388489)	G	0.46	1.15 (0.88, 1.51)[d]	0.31	0.87 (0.61, 1.24)[d]	0.44	1.03 (0.69, 1.54)[d]	0.87
C14orf181 (rs1465788)	T	0.28	0.97 (0.73, 1.27)[d]	0.80	1.09 (0.62, 1.92)[e]	0.77	0.70 (0.40, 1.23)[e]	0.21
IL2 (rs2069762)	C	0.29	1.08 (0.82, 1.43)[d]	0.58	1.22 (0.79, 1.87)[d]	0.38	1.20 (0.79, 1.83)[d]	0.40
IL2 (rs4505848)	G	0.35	1.04 (0.80, 1.34)[d]	0.78	0.93 (0.67, 1.28)[d]	0.65	1.18 (0.82, 1.70)[d]	0.37
IL2RA (rs12722563)	A	0.12	0.90 (0.58, 1.41)[e]	0.65	0.52 (0.17, 1.57)[e]	0.25	0.43 (0.17, 1.08)[e]	0.07
IL2RA (rs2104286)	C	0.27	0.85 (0.63, 1.15)[d]	0.28	1.26 (0.70, 2.27)[e]	0.44	0.77 (0.43, 1.38)[e]	0.38
IL7R (rs6897932)	T	0.28	0.93 (0.68, 1.28)[d]	0.66	0.86 (0.49, 1.53)[e]	0.61	0.88 (0.50, 1.55)[e]	0.65
PRKCQ (rs947474)	G	0.17	1.19 (0.80, 1.77)[e]	0.40	0.76 (0.41, 1.42)[e]	0.39	0.87 (0.47, 1.64)[e]	0.67
SKAP2 (rs7804356)	C	0.25	0.90 (0.66, 1.22)[d]	0.49	1.54 (0.83, 2.85)[e]	0.17	1.01 (0.57, 1.78)[e]	0.99
SMARCE1 (rs7221109)	T	0.35	0.94 (0.72, 1.22)[d]	0.63	0.94 (0.57, 1.57)[d]	0.82	0.73 (0.45, 1.20)[d]	0.21
TLR8 (rs5979785)	C	0.20	0.82 (0.64, 1.05)[d]	0.11	0.84 (0.61, 1.16)[d]	0.29	0.86 (0.60, 1.24)[d]	0.43
UBASH3A (rs3788013)	A	0.44	1.19 (0.89, 1.59)[d]	0.25	1.03 (0.72, 1.48)[d]	0.86	**1.63 (1.04, 2.54)[d]**	**0.03**
rs10517086	A	0.30	*	*	1.36 (0.77, 2.41)[e]	0.30	**2.03 (1.35, 3.03)[d]**	**0.001**

CI: confidence interval; DAISY: Diabetes Autoimmunity Study in the Young; HLA: human leukocyte antigen; HR: hazard ratio; IA: islet autoimmunity; MAF: minor allele frequency; T1D: type 1 diabetes.

[a]Minor allele frequency (MAF) calculated for children negative for IA.

[b]Adjusted for HLA-DR3/4, DQB1*0302 genotype and first degree relative with T1D. *TLR8* (rs5979785) is additionally adjusted for sex because it is on the X chromosome.

[c]Adjusted for HLA-DR3/4, DQB1*0302 genotype, first degree relative with T1D, and age at first antibody positive visit. *TLR8* (rs5979785) is additionally adjusted for sex because it is on the X chromosome.

[d]SNP analyzed additively with HR representing increase in risk for each additional minor allele.

[e]SNP analyzed dichotomously with HR representing increase in risk for at least one minor allele.

*SNP rs10517086 did not meet the assumptions of proportional hazards in the development of IA analysis and therefore was modeled using a restricted cubic spline (Figure 1).

with a minor allele developed IA significantly earlier than children with no minor alleles (mean age at onset of IA: 7.2, 5.2, and 3.2 for 0, 1, and 2 minor alleles, resp., $P = 0.003$).

3.2. Progression to T1D in Children with IA. We then examined whether non-HLA variants were associated with progression to T1D in IA positive children. Of the 132 IA positive children in DAISY, 50 developed T1D; the mean age at T1D diagnosis was 8.7 years (Table 1). The mean age at last followup visit in nondiabetic children with IA was 14.1 years. Children who developed T1D were younger when they first tested positive for an autoantibody than IA positive children who have not progressed to T1D, 3.9 and 7.2 years, respectively ($P = 0.003$). Children with IA who developed T1D were more likely to have the HLA-DR3/4, DQB1*0302 genotype compared to children with IA who did not progress to T1D (HR: 2.79, 95% CI: 1.65, 4.72). Adjusting for HLA-DR3/4, DQB1*0302, first degree relative with T1D, and age at first IA positive visit, none of the fourteen non-HLA T1D candidate SNPs was associated with progression to T1D in IA positive children (Table 2). SNP association analyses adjusted only for age at first IA positive visit are presented in Supplemental Table 1.

3.3. Development of T1D. In order to evaluate the same outcome as previous GWAS to see if similar associations could be seen using a time-to-event analysis in a prospective birth cohort and to better understand the role these SNPs play in the natural history of T1D, we examined whether these non-HLA variants were associated with T1D in our population of 1619 children. All 50 children who developed T1D had developed IA previously, so the same 50 T1D cases

were included in both the progression from IA to T1D (presented in Section 3.2) and these development of T1D analyses. However, not all IA positive children went on to develop T1D during followup. Unadjusted SNP association analyses are presented in Supplemental Table 1. Adjusting for HLA-DR3/4, DQB1*0302 and first degree relative with T1D, three of the fourteen non-HLA T1D candidate SNPs were associated with development of T1D (Table 2). Two of the SNPs associated with development of T1D, *C1QTNF6* (rs229541) and SNP (rs10517086), were also associated with development of IA, but not with progression to T1D in IA positive children. The other SNP associated with development of T1D, *UBASH3A* (rs3788013), was not associated with development of IA nor progression to T1D in IA positive children.

4. Discussion

In exploring associations between fourteen previously discovered non-HLA T1D candidate SNPs and the development of IA and progression to T1D in the prospective DAISY cohort, we found that *C1QTNF6* (rs229541) predicts IA but not progression to T1D, demonstrating stage-related heterogeneity. Moreover, SNP (rs10517086) demonstrates age-related heterogeneity with predicting IA only in the youngest ages. These two SNPs were also associated with development of T1D in our cohort, as well as *UBASH3A* (rs3788013). It is possible that the observed associations between both *C1QTNF6* (rs229541) and SNP (rs10517086) and development of T1D were driven by their association with development of IA. Given that all of our T1D cases developed IA prior to clinical diagnosis, it was not possible to determine whether a gene was associated with T1D via a pathway other than IA.

C1QTNF6 (rs229541), which was first identified through a meta-analysis of data from three genome-wide association studies (GWAS) (combined P value $= 1.98 \times 10^{-8}$) [20], is an intronic SNP located on chromosome 22q13 between two genes, *C1QTNF6* (C1q and tumor necrosis factor related protein 6) and *SSTR3* (somatostatin receptor 3). We found that *C1QTNF6* (rs229541) is associated with development of IA but not associated with progression to T1D in IA positive children, suggesting that this gene may play a role early in the development of T1D related to the initial appearance of autoimmunity.

SNP (rs10517086) exhibits an age-related effect with development of IA, with an increased risk of developing IA before the age of two or in younger ages, and a null effect in older ages. Children carrying a risk allele for SNP (rs10517086) developed IA significantly earlier than children without a risk allele. Based on these epidemiologic analyses, future studies should investigate mechanisms as to how this SNP influences risk of early autoimmunity. SNP (rs10517086), which was first discovered through another GWAS and meta-analysis of T1D, is located within a gene desert on chromosome 4. Loci in or near genes without a known function or in regions not containing annotated genes may indicate involvement of long-range gene expression regulatory elements and/or nonprotein-coding RNA genes [26].

UBASH3A (rs3788013) was associated with development of T1D, but not with either of the stages preceding this (development of IA and progression from IA to T1D). *UBASH3A* (rs3788013) is an intronic SNP located on chromosome 21q22 in the *UBASH3A* (ubiquitin associated and SH3 domain containing A) gene. *UBASH3A* is expressed predominantly in T cells suppressing T-cell receptor signaling [27]. *UBASH3A* has also been associated with other autoimmune diseases, such as celiac disease and rheumatoid arthritis [28]. Another *UBASH3A* SNP, *UBASH3A* (rs11203203), was found to be associated with both development of IA and development of T1D in a previous DAISY analysis [11]. The LD between *UBASH3A* (rs3788013) and *UBASH3A* (rs11203203) is $r^2 = 0.491$ and $D' = 0.801$. DAISY uses two definitions of IA, one that defines IA as the presence of at least one islet autoantibody on two consecutive visits (which is the definition used in the present analysis) and the other that further requires that the children still be autoantibody positive or diabetic on their most recent visit (which is the definition used in the previous analysis). The definition used in the previous study is closer to T1D, which makes our *UBASH3A* (rs3788013) association with T1D consistent with what was previously found with *UBASH3A* (rs11203203).

In combination with those presented in this paper, DAISY has now investigated 34 non-HLA T1D candidate SNPs for association with development of IA, progression from IA to T1D, and/or development of T1D with multiple examples of stage-related heterogeneity, which are presented in Table 3. Investigating 20 non-HLA SNPs for development of IA, progression from IA to T1D, and/or development of T1D, Steck et al. found *PTPN22* (rs2476601) associated with development of IA, but not progression from IA to T1D, and *CTLA4* (rs231775) associated with progression from IA to T1D, but not development of IA [9]. *PTPN2* (rs1893217) was only associated with development of IA, while *UBASH3A* (rs11203203) was associated with development of IA and development of T1D [11]. *INS* (rs689) was not associated with development of IA nor progression from IA to T1D but was associated with development of T1D [9, 11]. *PTPN22* (rs2476601) was also associated with development of T1D [11]. Here we investigated fourteen additional non-HLA T1D candidate SNPs and found *C1QTNF6* (rs229541) associated with development of IA and development of T1D, but not progression from IA to T1D. *UBASH3A* (rs3788013) was associated with development of T1D, but not with either of the stages preceding this (development of IA and progression from IA to T1D) and SNP (rs10517086) was associated with development of T1D, while exhibiting an age-related effect with IA risk but was not associated with progression from IA to T1D. The SNPs investigated in these three studies are summarized in Table 3. The distinction between the risk factors for islet autoimmunity versus progression to type 1 diabetes in IA positive children is important because it may allow us to explore potentially different mechanisms of *triggering* islet autoimmunity versus *epitope spreading and progressive loss of beta-cell mass* leading to overt diabetes.

GWAS are important for identifying new candidate regions associated with a clinical outcome, such as T1D,

Table 3: Non-HLA T1D candidate SNPs associated with development of IA, progression from IA to T1D, and/or development of T1D in DAISY.

SNP	Development of IA		Progression from IA to T1D		Development of T1D	
	Adjusted HR and 95% CI	*P* value	Adjusted HR and 95% CI	*P* value	Adjusted HR and 95% CI	*P* value
C1QTNF6 (rs229541)	**1.57 (1.20, 2.05)**[abc]	**0.001**	1.13 (0.75, 1.71)[abd]	0.56	**1.95 (1.33, 2.87)**[abc]	**0.001**
CTLA4 (rs231775)	1.12 (0.86, 1.46)[aef]	0.42	**0.54 (0.33, 0.88)**[aeg]	**0.01**	1.00 (0.70, 1.43)[ahc]	1.00
INS (rs689)	1.39 (0.99, 1.95)[aef]	0.05	1.34 (0.72, 2.52)[aeg]	0.35	**1.75 (1.08, 2.83)**[ahc]	**0.02**
PTPN2 (rs1893217)	**1.42 (1.02, 1.99)**[ach]	**0.04**	0.65 (0.27, 1.60)[ijk]	0.35	0.99 (0.60, 1.66)[ach]	0.98
PTPN22 (rs2476601)	**1.83 (1.27, 2.63)**[aef]	**0.001**	0.98 (0.50, 1.93)[aeg]	0.96	**1.74 (1.04, 2.90)**[ahc]	**0.03**
UBASH3A (rs11203203)	**1.46 (1.11, 1.91)**[ach]	**0.01**	**	**	**1.83 (1.28, 2.64)**[ach]	**0.001**
UBASH3A (rs3788013)	1.19 (0.89, 1.59)[abc]	0.25	1.03 (0.72, 1.48)[abd]	0.86	**1.63 (1.04, 2.54)**[abc]	**0.03**
rs10517086	*	*	1.36 (0.77, 2.41)[bdj]	0.30	**2.03 (1.35, 3.03)**[abc]	**0.001**

DAISY: Diabetes Autoimmunity Study in the Young; HLA: human leukocyte antigen; HR: hazard ratio; CI: confidence interval; IA: islet autoimmunity; T1D: type 1 diabetes.

[a]SNP analyzed additively with HR representing increase in risk for each additional minor allele.

[b]Listed in Table 2 of the current paper.

[c]Adjusted for HLA-DR3/4, DQB1*0302 genotype and first degree relative with T1D.

[d]Adjusted for HLA-DR3/4, DQB1*0302 genotype, first degree relative with T1D, and age at first antibody positive visit.

[e]From Steck et al. (2009) [9].

[f]Adjusted for HLA-DR3/4, DQB1*0302 genotype, ethnicity, sex, and first degree relative with type 1 diabetes.

[g]Adjusted for HLA-DR3/4, DQB1*0302 genotype, ethnicity, sex, first degree relative with type 1 diabetes, and age at first antibody positive visit.

[h]From Steck et al. (2012) [11].

[i]From Frederiksen et al. (2013) [12]. Frederiksen et al. (2013) [12] did not find an association between *PTPN2* (rs1893217) and development of IA, which can be attributed to the use of different IA case definitions used in the manuscripts by Steck et al. (2009) [9] and Frederiksen et al. (2013) [12].

[j]SNP analyzed dichotomously with HR representing increase in risk for at least one minor allele.

[k]Adjusted for *PTPN2* (rs478582), HLA-DR3/4, DQB1*0302 genotype, first degree relative with type 1 diabetes, ethnicity, and age at first IA positive visit.

**Analysis not conducted.

*SNP rs10517086 did not meet the assumptions of proportional hazards in the development of IA analysis and therefore was modeled using a restricted cubic spline (Figure 1 of the current paper).

in a large number of cases and controls. Prospective birth cohort studies, like DAISY, are then able to take these newly identified candidate regions and look for associations with different stages in the disease process and at different ages. As a prospective birth cohort study following children at increased risk for developing T1D from birth, we are able to capture the preclinical phase of T1D, islet autoimmunity. This allows us to study two separate stages in the natural history of T1D: development of IA and progression to T1D in IA positive children. We are also able to study whether certain exposures are important at one age, but not another.

Due to the cost associated with following a large group of children from birth into adulthood, our sample sizes are much smaller than those obtained for GWAS. Our lack of association for many of these SNPs is not evidence against their association with T1D but may result from limited power, especially in the progression from IA to T1D stage. We also have a very unique population comprised of children with high risk HLA genotypes and it is possible that the effect of these SNPs differs based on one's HLA risk status. The risk for non-HLA loci appears to be lower in individuals carrying high-risk HLA genotypes, as has been seen with *PTPN22* (rs2476601) [21, 29] and *TCF7* (rs5742913) [30, 31].

We believe that taking these GWAS identified candidate regions and studying them in the context of the natural history of T1D are central to better understanding the disease process and where in the disease process genetic loci may be important. This will allow us to create more accurate risk prediction models for both stages in the natural history of the disease, as well as inform the design of targeted interventions to prevent or slow the progression of IA and subsequent development of T1D.

5. Conclusions

The effect of a SNP may act nonlinearly, with an effect at early ages but not later ages or vice versa. Our results provide evidence that SNP (rs10517086) is acting on early risk of IA, with the age at onset of IA occurring significantly earlier in children with a minor allele compared to children with no minor alleles. By ignoring heterogeneity in the etiology of

disease, valuable associations may be missed that could aid in better understanding complex diseases, such as T1D.

Abbreviations

CI:	Confidence interval
C1QTNF6:	C1q and tumor necrosis factor related protein 6
C6orf173:	Chromosome 6 open reading frame 173
C14orf181:	Chromosome 14 open reading frame 181
CTLA4:	Cytotoxic T-lymphocyte-associated protein 4
HLA:	Human leukocyte antigen
HR:	Hazard ratio
IA:	Islet autoimmunity
IFIH1:	Interferon induced with helicase C domain 1
IL2:	Interleukin 2
IL2RA:	Interleukin 2 receptor, alpha
IL7R:	Interleukin 7 receptor
INS:	Insulin
PRKCQ:	Protein kinase C, theta
PTPN22:	Protein tyrosine phosphatase, nonreceptor type 22
SKAP2:	Src kinase associated phosphoprotein
SMARCE1:	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily E, member 1
SNP:	Single nucleotide polymorphism
T1D:	Type 1 diabetes
TLR8:	Toll-like receptor 8
UBASH3A:	Ubiquitin associated and SH3 domain containing A.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Familial Aggregation of High Tumor Necrosis Factor Alpha Levels in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) patients frequently have high circulating tumor necrosis factor alpha (TNF-α) levels. We explored circulating TNF-α levels in SLE families to determine whether high levels of TNF-α were clustered in a heritable pattern. We measured TNF-α in 242 SLE patients, 361 unaffected family members, 23 unaffected spouses of SLE patients, and 62 unrelated healthy controls. Familial correlations and relative recurrence risk rates for the high TNF-α trait were assessed. SLE-affected individuals had the highest TNF-α levels, and TNF-α was significantly higher in unaffected first degree relatives than healthy unrelated subjects ($P = 0.0025$). No Mendelian patterns were observed, but 28.4% of unaffected first degree relatives of SLE patients had high TNF-α levels, resulting in a first degree relative recurrence risk of 4.48 ($P = 2.9 \times 10^{-5}$). Interestingly, the median TNF-α value in spouses was similar to that of the first degree relatives. Concordance of the TNF-α trait (high versus low) in SLE patients and their spouses was strikingly high at 78.2%. These data support a role for TNF-α in SLE pathogenesis, and TNF-α levels may relate with heritable factors. The high degree of concordance in SLE patients and their spouses suggests that environmental factors may also play a role in the observed familial aggregation.

1. Introduction

Systemic lupus erythematosus (SLE) is a severe multisystem autoimmune disease which is caused by a combination of genetic and environmental factors [1]. Many lines of evidence underscore the importance of cytokines in SLE susceptibility. Circulating interferon alpha (IFN-α) levels are high in many SLE patients [2, 3]. One of the most direct lines of evidence suggesting that high IFN-α is a primary pathogenic factor is that some individuals treated with recombinant interferon alpha (IFN-α) for viral hepatitis develop de novo SLE, which typically resolves when IFN-α treatment is discontinued [4, 5]. Additionally, many of the genetic risk loci for SLE are in or near genes which play roles in cytokine pathways [6, 7]. A number of SLE-associated genes impact serum cytokine levels in SLE patients, providing further support for this idea [8, 9]. In the case of IFN-α, we have previously shown that high serum IFN-α is aggregated within SLE families, supporting the idea that high IFN-α is a heritable risk factor for SLE [10]. We have also demonstrated that high IFN-α is more common in family members of SLE patients who have a different non-SLE autoimmune disease, suggesting that high

IFN-α may be a heritable factor predisposing to a number of autoimmune diseases [11]. Subsequent studies directed at defining the genetic architecture of the high IFN-α trait have implicated both established SLE and autoimmune disease risk genes [12–19], as well as novel genes which impact circulating IFN-α levels in SLE patients [20–24].

While much attention has been focused on IFN-α in SLE in recent years, many other cytokines will also play important roles in SLE pathogenesis. Serum tumor necrosis factor alpha (TNF-α) levels are elevated in many patients with SLE [13, 25, 26]. High levels of TNF-α have been correlated with increased clinical disease activity and the presence of anti-dsDNA antibodies [27]. High levels of TNF-α have been demonstrated in patients with lupus nephritis, and TNF-α is overexpressed in renal tissue in lupus nephritis [26, 28]. The role of TNF-α in murine models of SLE has been controversial. In some models TNF-α improved disease features [29], while in others TNF-α blockade has been beneficial [26]. Small scale clinical trials in human SLE suggest that short-term TNF-α blockade may have benefit in lupus nephritis, as well as transient benefit in SLE arthritis [26]. Significant side effects have been reported in a small group of SLE patients who have received long-term anti-TNF-α therapy [30], and there are no large-scale trials of TNF blockade in human SLE to date.

It is not clear whether high TNF-α predisposes to SLE or if the levels rise after the disease is established. Genetic studies have implicated a promoter polymorphism in the TNF-α gene in SLE susceptibility [31], although the TNF-α gene is within the HLA locus which is characterized by multiple association signals that are difficult to resolve due to high linkage disequilibrium in the region. It is also not clear that the TNF-α promoter polymorphism functionally confers a propensity for excess TNF-α mRNA or protein production [32]. In support of the idea that background genetic factors influence TNF-α levels, some non-HLA polymorphisms have been associated with differences in TNF-α in SLE patients [13, 24].

To explore this question further, we studied TNF-alpha levels in SLE families to determine whether high levels of TNF-alpha were heritable and aggregated in SLE families. We also examine the spouses of SLE patients to detect potential environmental contributions to familial tendencies. We also examine family TNF-α data in the context of IFN-α data in the same subjects from the same blood sample to detect potential relationships between these two cytokines in SLE patients and their families.

2. Methods

2.1. Patients and Samples. Serum and plasma samples were obtained from the Lupus Family Registry and Repository (LFRR) at the Oklahoma Medical Research Foundation and the Hospital for Special Surgery (HSS) Lupus Family Registry. There were no significant differences between the two cohorts in familial TNF-α or in the measures of familial clustering, and data from the two cohorts are presented in aggregate. A total of 206 samples from the HSS Lupus Family Registry were studied, including 106 SLE patients and 100 healthy family members. Clinical data are available for all samples in the registry, and serologic data are available for all of the SLE-affected individuals. A total of 397 samples from the LFRR were studied, including 136 SLE patients and 261 unaffected family members. Samples from 23 unaffected spouses were also available. Samples from 62 healthy unrelated controls were obtained from healthy blood donors. Demographic and clinical information for the SLE patients and healthy controls are shown in Table 1.

Table 1

	SLE patients	Unrelated controls
Age (yrs.)	40.8	45.6
Female gender	87.9	90.3
African-American	31.8	39.2
European-American	41.7	43.6
Hispanic-American	26.5	15.4
Malar rash	57.7	—
Discoid rash	8.3	—
Photosensitivity	43.5	—
Oral ulcer	31.0	—
Arthritis	75.0	—
Serositis	33.3	—
Renal d/o	42.9	—
Neuro d/o	13.7	—
Heme d/o	60.1	—
Immuno d/o	73.8	—
ANA	100.0	—
Anti-Ro	28.7	—
Anti-La	8.9	—
Anti-Sm	12.7	—
Anti-RNP	24.1	—
Anti-dsDNA	46.1	—

2.2. Measurement of TNF-α in Serum. TNF-α is measured using the Pierce Human Monoclonal TNF-α ELISA per manufacturer instructions. This ELISA has performed well in our hands to date with SLE samples [13, 25]. For categorical analyses, we used a cutoff for high TNF-α of two standard deviations above the mean of our nonautoimmune control population. Samples from families were not run together on the same plates or on the same days to prevent spurious correlations potentially related to a batch effect.

2.3. Reporter Cell Assay for IFN-α. The reporter cell assay for IFN-α has been described in detail elsewhere [10, 33]. Reporter cells were used to measure the ability of patient sera to cause IFN-α-induced gene expression. The reporter cells (WISH cells, ATCC #CCL-25) were cultured with 50% patient sera for 6 hours and then lysed. mRNA was purified from cell lysates, and cDNA was made from total cellular mRNA. cDNA was then quantified using real-time PCR using an Applied Biosystems 7900HT PCR machine with the SYBR

Green fluorophore system. Forward and reverse primers for the genes *MX1*, *PKR*, and *IFIT1*, which are known to be highly and specifically induced by IFN-α, were used in the reaction [10]. *GAPDH* was amplified in the same samples to control for background gene expression.

The amount of PCR product of the IFN-α-induced gene was normalized to the amount of product used for the housekeeping gene *GAPDH* in the same sample. The relative expression of each of the three tested IFN-induced genes was calculated as a fold increase compared to its expression in WISH cells cultured with media alone. Results from the IFN-α assay were standardized to a healthy multiancestral reference population as previously described, and a serum IFN-α activity score was calculated based upon the mean and SD of the reference population [10]. This assay has been highly informative when applied to SLE as well as other autoimmune disease populations [10, 34–37]. For categorical analyses, we used a cutoff for high IFN-α as of 2 standard deviations above the mean of the control group.

2.4. Statistical Analysis and Methods for Determining Familial Clustering and Heritability. Data from both TNF-α and IFN-α were nonnormally distributed. Median and interquartile range are used for graphical representation of quantitative data, and Mann-Whitney U test is used for comparison of groups. Correlation analyses were performed using the Spearman's rho rank order correlation. For categorical data analyses, we used the cutoffs noted above for each cytokine measurement to categorize subjects as high versus low. In the pedigree analyses, family members are first classified as SLE affected or unaffected. The unaffected individuals are then categorized by their closest relationship to an SLE-affected individual in the family. Each person in each registry is represented only once. Unaffected family members were classified by their most direct relationship to an SLE patient (for example, in an SLE family with multiple affected generations, sometimes a person could be both an SLE mother and an SLE grandmother—in this case the person is categorized as an SLE mother). Familial clustering is detected using the Fisher's exact test with categorical data, as outlined in [10]. Odds ratio (OR) for concordance in categorical IFN-α activity between SLE patients and their nuclear family members was calculated using a standard procedure, with input variables being the number of families with each of the following IFN-α activity patterns: patient high/family high, patient high/family low, patient low/family high, and patient low/family low. Relative recurrence risk ratios (λ) were calculated using a standard approach [38].

3. Results

3.1. Unaffected First Degree Relatives of SLE Patients had Higher TNF-α Than Unrelated Controls. Serum TNF-α was highest in the SLE patients, and TNF-α levels resembled those of previously published SLE cohorts [25]. Similar to previous work, we did not find any significant differences in serum TNF-α in the SLE patients by ancestral background or sex [25]. Patients had significantly higher levels of TNF-α than unaffected first degree relatives or unrelated controls (Figure 1, $P = 0.0024$ and $P = 2.3 \times 10^{-7}$, resp.). Interestingly, unaffected first degree relatives had significantly higher median serum TNF-α than unrelated controls ($P = 0.0025$).

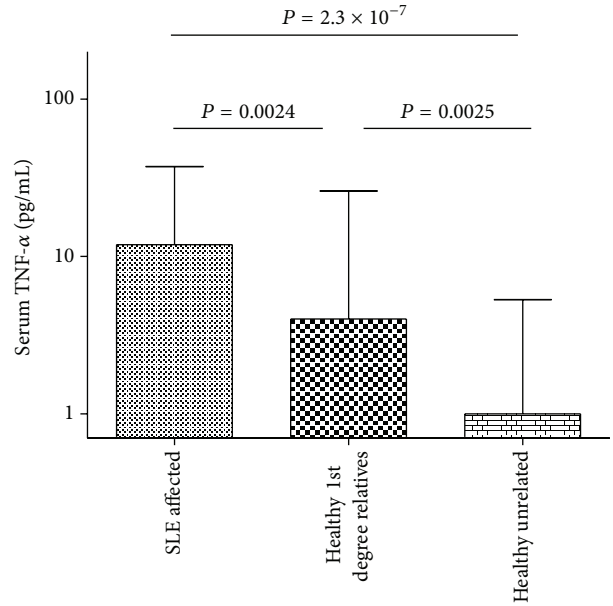

Figure 1: Serum TNF-α levels in SLE patients, first degree relatives, and unrelated controls. Bars show the median; error bars show the interquartile range, P values by Mann-Whitney U test.

3.2. High TNF-α Was Strongly Aggregated in Nuclear SLE Families. No Mendelian patterns were observed in categorical analysis of TNF-α within the pedigrees. In this analysis, patients and relatives were categorized as having high versus low circulating TNF-α (see Section 2). 28.4% of unaffected first degree relatives of SLE patients had high TNF-α levels. We did observe strong familial aggregation of high TNF-α within SLE patients and their first degree relatives ($\lambda_{1st} = 4.48$, 2.9×10^{-5}; see Table 2). Families with a high TNF-α SLE patient were likely to have a first degree relative with high TNF-α, and low TNF-α SLE patients were more likely to have low TNF-α relatives. As might be expected, the least common scenario was a patient with low TNF-α who had a first degree family member with high TNF-α. These analyses cannot be easily adjusted for factors such as disease activity or treatment in the SLE patient group because of course these factors are not applicable to the unaffected relatives. It is easy to think that an SLE patient might have had high TNF-α level which was decreased by aggressive immunosuppressive therapy, and this may result in a decreased correlation between the patient and their relatives. This of course would introduce a conservative bias into our study, biasing toward the null hypothesis. It is striking that we observe correlations between patients and family members despite some of these uncontrollable factors which may reduce the ability to detect familial correlations. We did not observe any significant

Table 2

TNF-α designation	No. of Instances	P value for familial aggregation	1st degree relative recurrence risk
High patient/high relative	63	2.9×10^{-5}	4.48
High patient/low relative	86		
Low patient/high relative	46		
Low patient/low relative	166		

Table 3

TNF-α high versus low concordance	No. of couples	Odds ratio	P value
Concordant	18	3.60	0.03
Discordant	5		

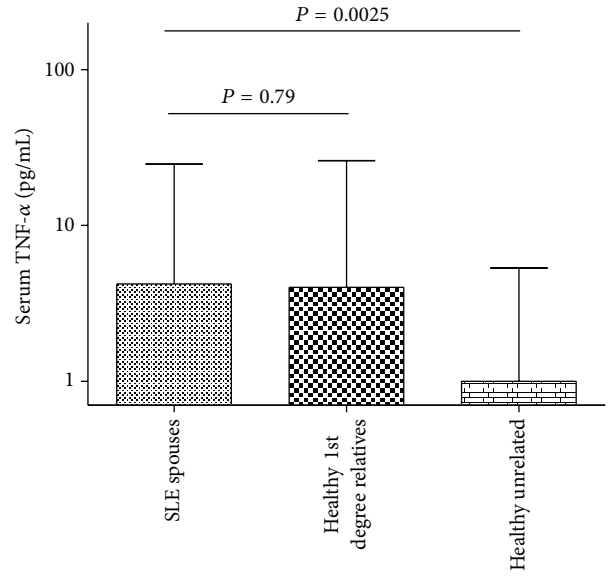

Figure 2: Serum TNF-α levels in first degree relatives of SLE patients, spouses of SLE patients, and unrelated controls. Bars show the median; error bars show the interquartile range, P values by Mann-Whitney U test.

increase in relative recurrence risk in second degree relatives ($P = 0.24$, data not shown).

3.3. Unaffected Spouses of SLE Patients Had TNF-α Levels, Which Were Highly Concordant with Their SLE Affected Spouse. Finding a correlation between closely related individuals in circulating TNF-α levels would suggest an inherited predisposition to high TNF-α within SLE families. Controlling environmental factors is difficult in human studies, but spouses provide an opportunity to study the unrelated individuals that share many environmental factors. We looked at circulating TNF-α levels in spouses of SLE patients and found that many spouses had high TNF-α levels, and as a group the median level resembled that of first degree relatives of SLE patients (Figure 2). Concordance of the TNF-α trait (high versus low) in SLE patients and their spouses was strikingly high at 78.2% in categorical analyses of TNF-α (OR = 3.60, $P = 0.03$, Table 3).

3.4. IFN-α and TNF-α Were Correlated in SLE Patients, but Not in Unaffected Family Members. We observed evidence for a correlation between serum IFN-α and serum TNF-α levels in the SLE patients when comparing the measurements of these two cytokines from the same serum sample (Spearman's rho = 0.18, $P = 0.0066$). This is concordant with previous studies of concomitant TNF-α and IFN-α levels in SLE patients [25]. Interestingly, we did not observe the same correlation between these cytokines in first degree relatives of the SLE patients (Spearman's rho = 0.07, $P = 0.25$). This suggests that the factors underlying high IFN-α and high TNF-α within the SLE families are distinct, but converge upon the affected members of the families.

4. Discussion

In this study, we have demonstrated familial clustering of high TNF-α levels in SLE families. We have previously shown that high IFN-α is aggregated within SLE families [10] and that TNF-α and IFN-α are correlated to some degree in SLE patients [25], so perhaps it does not seem surprising that TNF-α is also clustered within SLE families. Important differences between the patterns of familial aggregation of these two cytokines that support the idea of the findings related to TNF-α reported in this study are not simply secondary to familial clustering of IFN-α. First, TNF-α levels in first degree relatives are not correlated with IFN-α levels, suggesting that different family members are contributing to the observed clustering of each of these cytokines in SLE families. In the SLE patients, a correlation is observed between these two cytokines, as has been observed in previous studies. This supports the idea that the factors predisposing to high levels of TNF-α and IFN-α within the SLE families are distinct and are generally present in different unaffected family members. These factors then presumably coalesce in the SLE affected members of the family.

Secondly, the fact that spouses of SLE patients had high TNF-α levels which were frequently concordant between the patients and their spouses suggests an environmental factor leading to high TNF-α levels. This was not the case with IFN-α [10], as spouses did not have any significant increase in IFN-α above that of unrelated controls. Previous studies have documented a similar familial aggregation of IL-10 production from peripheral blood cells in SLE families, and in this study the familial correlation of IL-10 also extended to spouses of SLE patients [39]. Our study findings may be related to the findings in this IL-10 study, as high levels of TNF-α may induce a compensatory increase in IL-10, and

both studies may be observing a similar phenomenon. We can only speculate about what environmental factor might induce increased TNF-α in SLE spouses and family members. Viral triggers of SLE have been proposed, and Epstein-Barr virus is a leading candidate in this regard [40]. Nearly everyone is infected with Epstein-Barr virus at some point in their lives, but there could be differences in quantitative exposures to Epstein-Barr virus within SLE families that could differ from healthy controls. These could relate with an increased propensity for subclinical reactivation in SLE patients, either related to the altered immunity produced by the disease itself or the immunosuppression given to treat the disease.

In previous studies of circulating IFN-α levels in SLE families, we have followed up familial clustering of the cytokine trait with evidence that a number of candidate genes are associated with high levels of IFN-α [41]. This has provided further support for heritability of IFN-α. With TNF-α, it is quite possible that we will identify candidate genes that are associated with this cytokine phenotype as well. An environmental effect on TNF-α levels within SLE families does not rule out genetic influences, and if genetic polymorphisms are associated with TNF-α levels, then the case for heritability would be strengthened. To date, we have observed one coding-change polymorphism in the ILT3 receptor which is associated with TNF-α levels in SLE patients [24]. Future work may establish additional genetic associations with the high TNF-α phenotype, and this would assist in defining differences in the molecular pathogenesis of SLE in different individuals affected by this heterogeneous condition.

Conflict of Interests

The authors report no financial conflict of interest.

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Clinical Study

The −174G/C and −572G/C Interleukin 6 Promoter Gene Polymorphisms in Mexican Patients with Rheumatoid Arthritis: A Case-Control Study

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Objective. There is a lack of information about the genotype frequencies of IL-6 −174G/C and −572G/C polymorphisms in Mexicans with rheumatoid arthritis (RA). Therefore, the aim of this study was to evaluate the association of the IL-6 −174G/C and −572G/C polymorphisms in Mexican mestizo with RA. *Methods*. We included 137 patients with RA and 102 healthy controls. Patients were assessed for clinical characteristics. IL-6 −174G/C and −572G/C polymorphisms were genotyped using PCR-RFLP analysis. Allele and genotype frequencies and the Hardy-Weinberg equilibrium were computed. Odds ratios (ORs) were computed to identify the risk for RA associated with the presence of GG genotype in comparison with the GC or CC genotypes. *Results*. The genotype −174GG occurred at a higher frequency in cases and controls (77.4% versus 78.4%, $P = 0.845$). We found similar results for the genotype −572GG (54% in patients versus 60.8% in controls, $P = 0.295$). *Conclusions*. This is the first study to evaluate the association of −174G/C and −572G/C polymorphisms of the IL-6 gene with RA in Mexican mestizo patients. These two polymorphisms were not associated with RA in the studied sample. Additional studies are required to evaluate if these IL-6 polymorphisms have relevance to the development of more severe disease.

1. Introduction

Rheumatoid arthritis (RA) is a multisystemic autoimmune disease that leads to destruction of the joints and affects 1.6% of the Mexican population [1, 2]. Although the etiology of RA is multifactorial, some genetic factors contribute to individuals' susceptibility to this disease [3, 4]. HLA-DR loci constitute the genetic factor most associated with predisposition to RA [5], although only approximately one-third of the genetic predisposition to RA is explained by HLA. Other gene candidates for susceptibility to RA are cytokine genes. Numerous proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), are mediators that participate in the inflammatory response and play a role in the pathogenesis of RA [6, 7]. Of these cytokines, IL-6 is considered a key mediator of systemic and localized inflammation in RA, and high levels of IL-6 have been detected in synovial fluid of inflamed joints [8, 9]. Changes in IL-6 level are regulated by multiple factors, including the modulation of variations by polymorphisms within the promoter regions of the IL-6 gene [10, 11].

The human IL-6 gene is located on chromosome 7p21. Among the polymorphic sites described in the IL-6 gene promoter, there are two biallelic polymorphisms that may be associated with differences in cytokine production: −572G/C and −174G/C. These polymorphisms consist of a single nucleotide change from guanine (G) to cytosine (C) at positions −572 and −174 in the promoter region, respectively [12, 13].

Some previous reports have investigated a possible association between IL-6 gene polymorphisms and RA or juvenile chronic arthritis (JCA). One study performed in the Caucasian population associated the −174G/C IL-6 gene polymorphism with systemic JCA [14]. However, this association has not been reproduced in adults with RA. In two independent studies performed in Caucasians from Western Europe in Spain [15] and patients from United Kingdom [16], no association was observed between −174G/C polymorphism and RA. Meanwhile, the unique report assessing the relationship between the −572G/C polymorphism and RA was performed in Taiwan by Lo et al. without observing association with this polymorphism [17].

The genotype frequencies of polymorphisms are known to vary according to race or ethnicity. To date, no studies have been performed in Latin America to evaluate whether the −174G/C and −572G/C IL-6 polymorphisms are associated with RA. Therefore, we decided to investigate genotype and allele frequencies of these two polymorphisms in Mexican mestizo patients with RA and to compare these frequencies with those observed in controls of similar ethnic origin.

2. Materials and Methods

2.1. Design. This is a case-control study.

2.2. Clinical Setting. The study was performed from January 2009 to January 2011 in an outpatient rheumatology clinic of a secondary-care hospital in Guadalajara, Mexico (Hospital General Regional 110, del Instituto Mexicano del Seguro Social, IMSS). The RA group was comprised of patients who met the 1987 revised American College of Rheumatology (ACR) criteria. Healthy subjects in a similar age range were selected as controls. Study participants (both patients with RA and healthy controls) were included if they were Mexican mestizo (defined as having at least two generations of ancestors born in Western Mexico); only one person per family (18 years of age or older) was recruited. Individuals who were adopted or of unknown ancestral origin were excluded from both study groups. Healthy control participants were also excluded if they had a family history of rheumatic inflammatory disease; patients with overlapping syndrome in RA were also excluded.

2.3. Clinical Assessment. All patients and controls were interviewed to assess sociodemographic characteristics. Patients with RA were evaluated regarding functionality using the Health Assessment Questionnaire Disability Index (HAQ-DI) and regarding disease severity using the 28-item Disease Activity Score (DAS-28) and history of RA medication.

2.4. Determination of Polymorphisms. Genomic DNA was isolated from white blood cell using the standard protocol. To identify the −174G/C polymorphism, a 198 bp fragment was amplified using the forward primer 5′ TGACTTCAGCTTTACTCTTTGT 3′and the reverse primer 5′ CTGATTGGAAACCTTATTAAG 3′; the conditions of the reaction were as follows: denaturalization at 94°C for 60 seconds, followed by 35 cycles of annealing at 53°C for 1 minute and 20 seconds, extension at 72°C for 1 minute and 20 seconds, and a final elongation step extension at 72°C for 5 minutes. To identify the −572G/C polymorphism, we amplified a 163 bp using the forward primer 5′ GGAGACGCCTTGAAGTAACTGC 3′ and the reverse primer 5′ GAGTTTCCTCTGACTCCATCGCAG 3′; the conditions of the reaction were as follows: denaturalization at 94°C for 60 seconds, followed by 35 cycles of annealing at 55°C for 60 seconds, extension at 72°C for 60 seconds, and a final extension step at 72°C for 60 seconds. Polymerase chain reactions (PCRs) were performed using a PCR thermal cycler (ESCO). PCR products were digested with SfaNI enzyme and BsrBI enzyme to identify the −174/G/C and −572G/C polymorphisms, respectively, according to the manufacturer's instructions.

Each PCR product was electrophoresed on a 6% polyacrylamide gel stained with silver nitrate. The resultant genotypes for both polymorphisms were classified as the nonexcisable homozygote allele (CC), the excisable homozygote allele (GG), and the heterozygote allele (CG). After enzymatic digestion of the amplified fragment for the −174G/C polymorphism, we were able to identify the different GG (148 pb and 50 bp), GC (198 bp, 148 bp, and 50 bp), and CC (198 bp) genotypes. For the −572G/C polymorphism, we identified the different genotypes as 102 bp and 61 bp fragments for the GG genotype; 163 bp, 102 bp, and 61 bp fragments for the GC genotype; a 163 bp fragment for the CC genotype.

2.5. IL-6 Measurement. IL-6 in serum was measured with an enzyme-linked immunosorbent assay using commercial kits (R&D Systems, Minneapolis, MN, USA). The detection range

Table 1: Comparison in selected characteristics between patients with rheumatoid arthritis and healthy controls.

Characteristics	Cases with RA $n = 137$	Controls $n = 102$	P
Age (years), mean ± SD	50 ± 9	48 ± 10	0.191
Females (%)	135 (98.5)	96 (94.1)	0.076
Serum IL-6 (pg/mL)	10.9 ± 17.9	1.14 ± 3.4	<0.001

Quantitative variables are expressed as means ± standard deviations (SD) and qualitative variables as frequencies and percentages (%) as noted. Abbreviations: RA: rheumatoid arthritis, IL-6: interleukin 6.
Comparisons between means were performed with unpaired Student's t-test. Comparisons between proportions were performed with Chi-squared test.

Table 2: Clinical and laboratory characteristics of patients with RA.

Characteristic	RA $n = 137$
Disease duration (years), mean ± SD	10 ± 7.9
Females (%)	135 (98.5)
DAS-28, mean ± SD	4.1 ± 1.5
HAQ-DI, mean ± SD	0.67 ± 0.6
Laboratory findings	
Serum Rheumatoid factor (UI/L), mean ± SD	200.5 ± 384.9
C-reactive protein (mg/L), mean ± SD	17.0 ± 25.9
Erythrocyte sedimentation rate (mm/h), mean ± SD	30.7 ± 12.6

Quantitative variables are expressed as means ± standard deviations (SD) and qualitative variables as frequencies and percentages (%) as noted. Abbreviations: RA: rheumatoid arthritis, DAS-28: disease activity score of 28 joints, HAQ-DI: Health Assessment Questionnaire Disability Index.

provided is 3.12 to 300 pg/mL, and the minimum detectable dose (MDD) of IL-6 is typically less than 0.70 pg/mL.

2.6. Statistical Analysis. The allele and genotype frequencies of both polymorphisms were obtained by direct counting. Hardy-Weinberg equilibrium was evaluated for the control group using the Chi-squared test. Genotype and allele frequencies between RA and controls were compared using the Chi-squared test or Fisher's exact test if required.

Odds ratios (ORs) and their 95% confidence intervals (95% CI) were computed to identify the RA risk associated with the presence of GG genotype in comparison with the GC or CC genotypes (used as a referent). A similar strategy was used to identify the risk associated with the G allele. The P value was set at 0.05. All statistical analysis was performed using SPSS version 8.0 or EPI INFO version 6.04.

2.7. Ethics. This study was approved by a research committee from the participant center R-2009-1301-78. All study participants voluntarily provided written informed consent. All procedures in the protocol were performed according to the guidelines of the Declaration of Helsinki.

3. Results

We included 137 patients with RA and 102 healthy controls. Comparison in selected characteristics between patients with rheumatoid arthritis and healthy controls is shown in Table 1. No significant differences were observed between the study groups with regard to age ($P = 0.191$) or sex ($P = 0.076$). Serum levels of IL-6 were higher in patients with RA than in the control group (mean of levels 10.9 versus 1.14 pg/mL, resp., $P < 0.001$).

Table 2 describes the clinical and laboratory characteristics in patients with RA. They had a mean disease duration of 10 years, a mean DAS-28 of 4.1, and a mean HAQ-Di of 0.67. Mean titles for rheumatoid factor were 200.5 ± 384.2 UI/mL, for C-reactive protein 17.0 ± 25.9 mg/L, and for ESR 30.7 ± 12.6 mm/hr. At the time of the study, 80.3% of patients were taking methotrexate, 21.9% chloroquine, 18.9% leflunomide, and only 13.8% received biologic agents.

For the control group, both −174G/C and −572G/C IL-6 polymorphisms were in Hardy-Weinberg equilibrium ($P = 0.52$ and $P = 0.31$, resp.).

Table 3 shows the genotype and allele frequencies as well as the comparison between patients and controls of both polymorphisms. There were no statistical differences between the study groups.

4. Discussion

The results of the present study identified that, for both −174G/C and −572G/C polymorphisms, the GG genotype is the most frequently observed in the Mexican mestizo population among patients with RA and healthy controls. We observed no differences in allele or genotype frequencies of these polymorphisms between RA and controls.

Our findings regarding GG being the most frequently encountered genotype for the −174G/C polymorphism in patients with RA are similar to those observed in Spain by Pascual et al. [15]. This group did not find an association between this polymorphism and RA; however, it must be noted that they obtained a genotype frequency different from ours (GG genotype 46% versus 77.4%, GC 44.2% versus 21.9%, and CC 9.8% versus 0.7%, resp.). In another contrasting study by Marinou et al. in the United Kingdom [16], the GC genotype was the most frequent, although not associated with RA (GC genotype 51.8% versus 21.9% in ours study). This finding underlines the relevance of racial mixing to the polymorphism frequency in Mexican mestizos.

For the polymorphism −572G/C, only Lo et al. in Taiwan [17] have reported a comparison between genotype frequencies in patients with RA and controls. They found a genotype distribution with wide differences in frequency compared

Table 3: Comparison in genotype and allele frequencies of −174G/C and −572G/C polymorphisms between the groups with rheumatoid arthritis and controls.

Genotype	Polymorphism −174G/C				Polymorphism −572G/C			
	RA n (%) $n = 137$	Controls n (%) $n = 102$	OR (95% CI)	P	RA n (%) $n = 137$	Controls n (%) $n = 102$	OR (95% CI)	P
GG	106 (77.4)	80 (78.4)	0.94	0.845	74 (54)	62 (60.8)	0.76	0.295
GC	30 (21.9)	20 (19.6)	(0.48–1.82)		58 (42.3)	37 (36.3)	(0.44–1.32)	
CC	1 (0.7)	2 (2)			5 (3.6)	3 (2.9)		
Allele								
G	242 (88.3)	180 (88.2)	1.01	0.976	206 (75.2)	161 (78.9)	0.81	0.338
C	32 (11.7)	24(11.8)	(0.57–1.8)		68 (24.8)	43 (21.1)	(0.1–1.28)	

RA: Rheumatoid arthritis; OR: odds ratio; 95% CI: confidence interval. For genotype, OR was computed using GG as a risk factor and GC + CC as the referent.

with our study and observed no association between RA and any particular genotype. In our study population, the GG genotype was the most frequently observed, contrasting with the results described by Lo et al., (54% versus 4.5%, resp.).

Our results show the variability in genotype frequencies for both polymorphisms observed in Mexican patients and controls. It has been observed that polymorphisms in the promoter region of the IL-6 gene may be responsible for changes in the expression of IL-6, which could in turn lead to greater inflammation and thus affect the clinical status of RA patients [12]. Additional studies should include the evaluation of whether changes in serum levels of IL-6 are associated with these genotypes; however, this aim was beyond the scope of the present study.

One strength of our study is the use of a carefully defined population in which patients and controls were Mexican mestizos with a family history of living for at least three generations in the western region of Mexico, and healthy controls were selected to closely match our RA patients regarding age and sex, minimizing these confounding factors present in other studies. Nevertheless, because the Mexican population features great ethnic diversity, therefore one limitation of the present study is that we limited the inclusion to subjects born in Western region of the country. We do not know whether our results are generalizable to other regions.

In conclusion, this is the first study to evaluate the association of −174G/C and −572G/C polymorphisms of the IL-6 gene with RA in Mexican mestizo patients. These results are relevant to improving the understanding of the genetic factors associated with RA in patients of this ethnicity. Although these two polymorphisms were not associated with RA, additional studies are required to evaluate the relevance of these IL-6 polymorphisms to the development of more severe disease.

Ethical Approval

This study protocol was approved by the Research and Ethics Board of the Mexican Institute for Social Security (Instituto Mexicano del Seguro Social, Mexico). Number of approval: R-2009-1301-78.

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Review Article

Altered Immune Regulation in Type 1 Diabetes

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Research in genetics and immunology was going on separate strands for a long time. Type 1 diabetes mellitus might not be characterized with a single pathogenetic factor. It develops when a susceptible individual is exposed to potential triggers in a given sequence and timeframe that eventually disarranges the fine-tuned immune mechanisms that keep autoimmunity under control in health. Genomewide association studies have helped to understand the congenital susceptibility, and hand-in-hand with the immunological research novel paths of immune dysregulation were described in central tolerance, apoptotic pathways, or peripheral tolerance mediated by regulatory T-cells. Epigenetic factors are contributing to the immune dysregulation. The interplay between genetic susceptibility and potential triggers is likely to play a role at a very early age and gradually results in the loss of balanced autotolerance and subsequently in the development of the clinical disease. Genetic susceptibility, the impaired elimination of apoptotic β-cell remnants, altered immune regulatory functions, and environmental factors such as viral infections determine the outcome. Autoreactivity might exist under physiologic conditions and when the integrity of the complex regulatory process is damaged the disease might develop. We summarized the immune regulatory mechanisms that might have a crucial role in disease pathology and development.

1. Introduction

Type 1 diabetes (T1DM) is known to be the result of the selective damage of pancreatic β-cells. Other cell types of the Langerhans islets are preserved. However, the lack of insulin causes a secondary disinhibition of the glucagon-secreting α-cells [1]. The destruction of the β-cells is the consequence of a cell-mediated immune response mediated by islet-infiltrating lymphocytes and macrophages (insulitis). Cytokines secreted by macrophages are toxic for β-cells [2]; CD8+ cytotoxic lymphocytes are able to damage them by pore formation [3]. Autoantibodies that give the basis of clinical diagnosis are known to be secondary factors. However, promising experimental data has been published based on autoantibody neutralisation in nonobese diabetic (NOD) mice, one of the best known models of the human disease [4]. The knowledge about genetic predisposition has increased significantly in the last few decades. We know more genetic variants besides the HLA (human leukocyte antigen) alleles which are responsible for the most significant susceptibility. Although the role of genetic factors in the development of the disease is well established, by itself it seems to be insufficient to explain all the pathophysiological features of the disease. The role of numerous environmental factors is justified by experimental and epidemiological data. Nevertheless, none has been proven to be a main or generally accepted cause of the disease. More and more publications are raising the question whether autoimmunity under all circumstances would possess a definitely pathologic nature [5]. In this paper, we summarized the immunoregulatory processes including those recently put into the focus of T1DM development.

2. Immune Tolerance and Genetic Factors

According to twin studies, the cumulative significance of genetic features is estimated to be as high as 50 to 65% [6, 7]. Approximately 70% of type 1 diabetic carry a HLA risk allele

as the strongest genetic factor. However, only 3–7% of those carrying such a HLA haplotype will ever become a patient with manifest type 1 diabetes [8]. Although NOD mice are the most commonly used models of the human disease and have high susceptibility, not all develop diabetes. However, insulitis occurs in each animal [9]. Two questions might be raised: what is necessary for the development of the insulitis? and what is necessary for its progression to diabetes? The discovery of genetic features and immunological events went on separate threads for a long time. The correlation between susceptibility and some major histocompatibility complex II (MHC-II) alleles (DR3-DQ2, DR4-DQ8) has been known to be linked to the highest (with an odds ratio—OR—of nearly seven) increase in risk [10]. Recently, some MHC-I loci were also identified among the genetic risk factors [11]. Besides the obvious role of the MHC class II and I in antigen presentation, there is an increasing number of non-HLA alleles associated with the development of type 1 diabetes. More than seventy non-HLA genes have been reported to date in the GWAStudies (genomewide association study) Catalogue based on ten large GWAScans that have variants contributing to the genetic susceptibility for T1DM with odds ratios typically below 2 (also see Table 1) [10, 12–20].

The first selection to filter autoreactive B- and T-lymphocyte clones occurs in the primary immune organs (bone marrow and thymus, resp.) where clones strongly bound to own (auto)antigens are eliminated. During the formation of the central tolerance, the autoreactive clones can be deleted or their receptor might be corrected through editing [21, 22]. Autoreactive T-cells which reach the periphery may become anergic due to being continuously flooded with self-antigens without costimulatory signals. The selection of T- and B-lymphocytes offers a mutual protection against autoimmune tendencies of each other. This is partly because B-lymphocytes are professional antigen-presenting cells (APCs), and partly because an autoreactive B-lymphocyte needs Th2 (helper T-cell, subset 2) help to expand. However, it is still possible that in some infections the pathogen-specific T-cells might be able to provide Th2 help for the autoreactive B-lymphocytes through bystander activation (as discussed later) [23]. Furthermore, in pathological conditions previously hidden self-antigens may become recognizable, leading to antigen spreading [24]. While the central tolerance is overwhelmingly genetically determined, peripheral tolerance is multifactorial.

In the selection of autoreactive T-cell clones, the antigen presentation in central lymphoid organs is crucial. Genetic background influencing the presentation of insulin in the thymus is able to weaken central immune tolerance. Those having shorter variable number tandem repeat sequences in the IDDM2 (insulin-dependent diabetes mellitus 2) locus located upstream from the insulin gene are more susceptible to autoimmune diabetes [10, 25]. In the presence of longer VNTRs, higher levels of insulin mRNA expression could be detected in the thymus. This is likely to contribute to antigen presentation with higher efficacy eventually leading to better immune tolerance [25]. When insulin expression was abrogated in the thymus using a knockout of Ins2 gene specifically in the AIRE (autoimmune regulator) expressing medullary thymic epithelial cells of mice, without affecting its expression in the beta-cells, diabetes developed in three weeks of age independent of sex [26].

In the central lymphoid organs, not all human antigens are present. Furthermore, after the central selection, the escape of autoreactive clones is minimal but not excluded in healthy individuals. The generation of autoreactive B-lymphocyte clones is possible even in the germinal centers through somatic hypermutation [23]. As the immune system contacts a large number of antigens during life, the optimal balance should be set in the periphery. In this process, the role of T_{reg} cells is critical. However, they do not form a homogenous population. Autoreactivity is not a binary property, even in the thymus the affinity of a T-cell to a peptide might vary on a wide range. Some of the T-cells that bind self-antigens become natural T_{reg} cells (nT_{reg}) and support active immune tolerance [27]. At the same time, induced T_{reg} cells (iT_{reg}) develop in the periphery from naive CD4+ T-cells. These iT_{reg} cells may have a function either in maintaining immune tolerance under tolerogenic circumstances (such as the antigens sensed in the gut mucosa under physiologic conditions) and in limiting the process in inflammation. Besides the T-cell receptor (TCR), signal transforming growth factor β (TGF-β) and interleukin 2 (IL-2) seem to be minimal requirements for iT_{reg} cell induction and also the role of APCs seems to be crucial in iT_{reg} development [28]. In this perspective, we should mention that T1DM-associated SNPs (single-nucleotide polymorphisms) were reported directly in the IL-2 [29, 30]—IL2-RA (IL-2 receptor α-chain, also known as CD25) [12–14] axis (see Table 1). Expression of high levels of CD25 had been the most important marker of T_{reg} cells before Foxp3 (forkhead box P3) became known. The antigen presentation will be tolerogenic and favorable for T_{reg} induction provided that the MHC-II expression on the APC is low and also the amount of costimulatory signals from APC remains limited. This can happen when there are no induction signals (as described later) for the maturation of dendritic cells (DCs). Furthermore, certain DCs are able to produce retinoic acid which is able to abrupt the effect of cytokines (such as IL-4, IFN-γ) that would otherwise suppress the T_{reg} inductive effect of TGF-B and IL-2, and retinoic acid eventually enhances iT_{reg} development [28, 31, 32]. Macrophages in the intestinal mucosa exhibited lower Toll-like receptor (TLR) sensitivity compared to splenic macrophages, and by IL-10 and retinoic acid production they contribute to T_{reg} induction and oral tolerance [33]. The T_{reg} cells inhibit inflammation and contribute to further T_{reg} induction via their own IL-10 and TGF-β secretion. Besides humoral stimuli, low cell surface CD28 and high cytotoxic T lymphocyte antigen 4 (CTLA-4) signaling promote iT_{reg} induction [34]. The binding of the T-cell surface CD28 to the B7 complex of the APC is the best known and most important costimulus in T-cell activation. CTLA-4 also binds to the B7 complex and this way abrogates this signaling. Its expression in high levels is typical of T_{reg} cells [23]. Lühder et al. could achieve prompt manifestation of T1DM in BDC2.5 TCR transgenic mice backcrossed onto the NOD genetic background by CTLA-4 inhibition which

Table 1: T1DM-associated non-MHC risk polymorphisms (SNPs) with an odds ratio above 1.4 and the reported/mapped candidate genes in GWAStudies ranked by the highest corresponding odds ratio (OR) values in addition to the candidate genes (CTLA4, IFIH1, IL2, and PTPN2) that due to their immunological functions have been discussed in detail in the text.

Region	SNPs	Reported gene(s)	Mapped gene	Odds ratio	Risk allele frequency	*P* value	Gene product function [42]	Context	Initial sample size	Replication sample size	Platform (SNPs passing QC)	References
1p13.2	rs6679677	PHTF1, PTPN22	PHTF1-RSBN1	1,89	0,1	$1E-40$	PHTF1: transcription factor; RSBN1: not fully characterized	Intergenic	1,963 cases, 2,938 cntrl	2997 trios, 4,000 cases, 5,000 cntrl	See [15]	[10]
				NR	NR	$9E-85$		Missense	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
	rs2476601		PTPN22	1,8	0,09	$1E-07$	Lymphoid-specific intracellular phosphatase, a negative regulator of many signal transduction pathways	Missense	467 trios, 561 cases, 1,143 cntrl	2,350 individuals in 549 families; 390 trios	Illumina (543,071)	[16]
		PTPN22		1,98	0,09	$2E-80$		Missense	1,963 cases, 2,938 cntrl	2997 trios, 4,000 cases, 5,000 cntrl	See [15]	[10]
	rs6679677		PHTF1-RSBN1	NR	NR	$1E-40$		Intergenic	3,561 cases, 4,646 cntrl	6,225 cases, 6,946 cntrl, 3,064 trios	Affymetrix (up to 335,565)	[14]
				1,82	0,1	$5E-26$		Intergenic	1,963 cases, 2,938 cntrl	See [10]	Affymetrix (469,557)	[15]
11p15.5	rs1004446	INS	IGF2; IGF2-AS; INS-IGF2	1,61	0,65	$4E-09$	Insulin hormone, IGF-2 growth factor	Intron	467 trios, 561 cases, 1,143 cntrl	2,350 individuals in 549 families; 390 trios	Illumina (543,071)	[16]
	rs3741208		IGF2; IGF2-AS; INS-IGF2	1,25	0,38	$2E-07$		Intron; ncRNA	1,963 cases, 2,938 cntrl	2997 trios, 4,000 cases, 5,000 cntrl	See [15]	[10]
	rs7111341		MIR4686-ASCL2	NR	NR	$4E-48$	MIR4686: micro-RNA; ASCL2: a helix-loop-helix transcription factor involved in the determination of the neuronal precursors in the central and peripheral nervous system	Intergenic	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
10p15.1	rs61839660	IL2RA	IL2RA	1,6	NR	$5E-09$	IL2RA: as homodimer, a low-affinity receptor of IL-2; RPL32P23: pseudogene; RBM17: an RNA-binding protein, part of the spliceosome complex	Intron	16,179 Eu individuals	NR	NR (6,233,112) (imputed)	[12]
	rs12251307		RPL32P23-RBM17	NR	NR	$1E-13$		Intergenic	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
				NR	NR	$2E-06$		Intergenic	3,561 cases, 4,646 cntrl	6,225 cases,6,946 cntrl, 3,064 trios	Affymetrix (up to 335,565)	[14]
12p13.31	rs3764021	NR	CLEC2D	1,57	0,47	$5E-08$	C-type lectin, a member of the natural killer cell receptor family, inhibits osteoclast formation	Cds-synon	1,963 cases, 2,938 cntrl	See [10]	Affymetrix (469,557)	[15]

Table 1: Continued.

Region	SNPs	Reported gene(s)	Mapped gene	Odds ratio	Risk allele frequency	P value	Gene product function [42]	Context	Initial sample size	Replication sample size	Platform (SNPs passing QC)	References
16p13.13	rs2903692	KIAA0350	CLEC16A	1,54	0,62	$7E-11$	Member of the C-type lectin domain containing family	Intron	467 trios, 561 cases, 1,143 cntrl	2,350 individuals in 549 families; 390 trios	Illumina (543,071)	[16]
	rs12708716			1,19	0,65	$5E-07$		Intron	1,963 cases, 2,938 cntrl	See [10]	Affymetrix (469,557)	[15]
				1,23	0,68	$3E-18$		Intron	1,963 cases, 2,938 cntrl	2997 trios, 4,000 cases, 5,000 cntrl	See [15]	[10]
12p13.31	rs11052552	NR	NPM1P7-CLECL1	1,49	0,49	$7E-07$	NPM1P7: pseudogene CLECL1: transmembrane, C-type lectin-like protein highly expressed on dendritic and B cells, may act as a T-cell costimulatory molecule	Intergenic	1,963 cases, 2,938 cntrl	See [10]	Affymetrix (469,557)	[15]
12q24.12	rs1265564	CUX2	CUX2	1,45	NR	$1E-16$	A protein that contains three CUT domains and a homeodomain; both domains are DNA-binding motifs	Intron	16,179 eu individuals	NR	NR (6,233,112) (imputed)	[12]
13q22.2	rs539514	LMO7	LMO7	1,43	0,5	$6E-11$	May be involved in protein-protein interactions	Intron	9,934 eu cases, 16,956 eu cntrl	1,120 eu affected trios	Affymetrix and illumina (~2.54 million) (imputed)	[17]
2q33.2	rs3087243	CTLA4	CTLA4	NR	NR	$1E-15$	Transmits an inhibitory signal to T-cells	NearGene-3	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
				NR	NR	$8E-11$		NearGene-3	3,561 cases, 4,646 cntrl	6,225 cases, 6,946 cntrl, 3,064 trios	Affymetrix (up to 335,565)	[14]
2q24.2	rs1990760	IFIH1	IFIH1	NR	NR	$7E-11$	Acts as a cytoplasmic sensor of viral nucleic acids	Missense	7,514 cases, 9,045 controls	4,267 cases, 4,670 controls, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
				1,18	0,6	$2E-11$		Missense	1,963 cases, 2,938 controls	2997 trios, 4,000 cases, 5,000 controls	See [15]	[10]
4q27	rs4505848	IL2	KIAA1109	NR	NR	$5E-13$	KIAA1109: thought to function in the regulation of epithelial growth and differentiation and in tumor development	Intron	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
	rs2069762	IL2	IL2	0,889	NR	NR	Cytokine, key activator of T-cells	Intron	8506 T1DM samples		Affymetrix and illumina (841,622) (imputed)	[29]

Table 1: Continued.

Region	SNPs	Reported gene(s)	Mapped gene	Odds ratio	Risk allele frequency	P value	Gene product function [42]	Context	Initial sample size	Replication sample size	Platform (SNPs passing QC)	References
18p11.21	rs1893217	PTPN2	PTPN2	NR	NR	$4E-15$	Member of the protein tyrosine phosphatase (PTP) family. Reported to have inhibitory role in beta-cell apoptosis	Intron	7,514 cases, 9,045 cntrl	4,267 cases, 4,670 cntrl, 4,342 trios	Affymetrix and illumina (841,622) (imputed)	[13]
	rs2542151		PSMG2-PTPN2	NR	NR	$9E-08$	PSMG2: proteasome assembly chaperone	Intergenic	3,561 cases, 4,646 cntrl	6,225 cases, 6,946 cntrl, 3,064 trios	Affymetrix (up to 335,565)	[14]
				1,3	0,16	$1E-14$		Intergenic	1,963 cases, 2,938 cntrl	2997 trios, 4,000 cases, 5,000 cntrl	See [15]	[10]

was not the result of a global T-cell activation but was caused by a more aggressive T-cell infiltration of the islets [35]. CTLA-4-deficient mice die at 2-3 weeks of age due to uncontrolled lymphoproliferation [36]. Risk polymorphisms of the CTLA-4 gene are linked to T1DM in GWAStudies [10]. CTLA-4 expression was found to be significantly lower on the mRNA level in T-lymphocytes of children with newly diagnosed T1DM [37]. Transgenic expression of an agonistic, membrane-bound single-chain anti-CTLA-4 on pancreatic β-cells in NOD mice could inhibit the autoimmune processes by selectively targeting CTLA-4 on pathogenic T-cells [38, 39]. The T-cell CTLA-4 expression of NOD mice was found to be diminished when examined in relation to in vitro anti-CD3 stimulation. However, the addition of exogenous IL-2 could restore the CTLA-4 expression of NOD CD8 cells to the level of healthy controls [40]. There is a clinical study in progress using low-dose IL-2 for therapeutic T_{reg} induction in T1DM [41]. The study has been completed, but not yet published.

3. The Janus-Character of Autoimmunity?

Although the role of B-lymphocytes is known to be secondary in T1DM, which can even develop in cases of severe congenital B-lymphocyte immunodeficiency [43], Xiu et al. could significantly delay disease onset with B-lymphocyte depletion by anti-CD20 antibody in NOD mice. They indicated that this was neither the result of T-cell reduction nor of T_{reg} induction but it was likely to be the consequence of the reduced induction of autoreactive T-cells [44]. The B-lymphocyte activating factor (BAFF, also known as tumor necrosis factor superfamily member 13b—TNFSF13B—or B-lymphocyte stimulator—BLyS) secreted by lymphoid stromal cells is necessary for B-lymphocyte survival and in cases of normal peripheric lymphocyte count autoreactive B-lymphocytes escaping central deletion might lose in the competition for BAFF. B-lymphocyte depletion using an anti-BAFF therapy in prediabetic NOD mice resulted in that the NOD mice remained diabetes free for at least 50 weeks [45]. On the other hand, transgenic overexpression of BAFF in mice resulted in the survival of autoreactive B-lymphocytes in the periphery [46]. Nevertheless, B-lymphocyte depletion might be a controversial therapy as it might delay the onset of diabetes in NOD mice, but theoretically it might also be able to enhance autoimmunity. Although B-lymphocytes as professional APCs are able to launch immune response, in vitro results suggest that they might play a regulatory role as well. In dextran sulfate sodium-induced experimental model of ulcerative colitis, Yanaba et al. described the presence of a unique B-lymphocyte population characterized by CD1d and CD5 marker positivity, which plays a regulatory role via IL-10 secretion [47]. In the coculture of primary B- and allogenic T-lymphocytes (B-lymphocytes as APCs) without additional cytokines, the expansion of T_{reg} cells characterized by Foxp3 expression was described. The partial inhibition of the MHC-II-TCR interaction enhanced this process, while CD28 stimulation by antibodies led to the generation of effector T-cells [48].

A theory first postulated by Jerne in 1974 might also be of notable significance. He indicated that the variable regions of immunoglobulins (idiotypes) may serve as antigens, and anti-idiotypic immunoglobulins can be produced against them [49]. Such a system might have a significant role in determining the intensity of immunological responses and mediating tolerance. As B-lymphocyte receptors are analogous to the immunoglobulins secreted by the cells and the B-lymphocytes present the bound antigens through MHC-II, blockade of B-lymphocyte receptors is supposed to have a profound influence on T-cell responses [50]. Furthermore, variable regions of immunoglobulins share common patterns with TCRs [51], and effective humoral immune response will not develop when there is lack of Th2 cells. Wang et al. were able to delay or, in some cases, even inhibit the onset of T1DM in NOD mice by anti-idiotypic antibodies [4]. A dysfunction of the anti-idiotypic system in autoimmunity was recently summarized by Hampe, who also cited that autoantibodies against glutamate acid decarboxylase in healthy individuals could be detected, but anti-idiotypic antibodies prevented them from binding to their target antigen [50].

It is described in rodents [52, 53] and also humans [54] that a shub of β-cell death occurs in the perinatal period. Trudeau et al. mention that in NOD mice insulitis appears approximately during the fifth week of life and never before the 15th day, subsequently following the peak of the apoptotic death wave of β-cells on the 13th day [53]. Theoretically, this timeframe might exist due to the immaturity of immune cells, although Höglund et al.'s finding suggests that T-cells and APCs in (their) mice are functional on the 10th day of life [55]. Trudeau et al. compared the apoptotic rate of β-cells in diabetes prone and resistant mice and rat strains and reported no significant difference. However, in NOD mice a higher amount of apoptotic β-cell remnants could be shown using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method [53]. The dysfunction of phagocytes (predominantly macrophages) as the first islet infiltrating cells and their diminished capacity to clean apoptotic remnants might contribute to this phenomenon [56, 57]. Although apoptotic cell remnants are generally thought to have a weak potential to induce immune response, due to the impaired disposal they can become victims of secondary necrosis and encounter a stronger immunogenic potential. APCs have a crucial role in determining the nature of the antigen presentation. The inhibition of the macrophage function turned out to be protective against diabetes in NOD mice—both by the inhibition of antigen presentation and also by the diminished induction of Th1 type response (due to weaker IL-12 stimuli) [58]. In contrast, either the enhancement or the inhibition of the apoptotic rate of β-cells does not influence the initiation of the disease significantly [59]. On the analogy that in SLE, macrophages have an impaired apoptotic cell clearance [60], it could be hypothesized that such macrophage dysfunction may be relevant in T1DM also. As early as the 80s, it had been demonstrated that human macrophages had decreased phagocytic capacity in patients with T1DM [61]. Recently—in addition to the alternative activation of macrophages in T1DM [62]—hypopresponsivity of macrophages to IFN-γ has been demonstrated in the NOD

mouse animal model [63], and it is possible that apoptotic cell clearance itself inhibited the macrophage responses to a major macrophage activator providing priming signal IFN-γ [64] and its downstream signaling (JAK-STAT1—Janus kinase and signal transducer and activator of transcription 1-path).

Turley et al. found that of the NOD and B6.H2g7 mice strains that carry the same MHC-I and MHC-II haplotype only the NOD mice develop diabetes, although in both strains potentially diabetogenic T-cells are primed [65]. Danke et al. described the presence of autoreactive T-cells in healthy individuals, but in vitro their expansion was inhibited by adding CD4+ CD25+ T-cells (T_{reg} cells) of the same individual to the culture [66]. Among others, Orban et al. have carried out a human study based on T_{reg} induction by insulin β-chain immunotherapy with no obvious benefit [67].

Hugues et al. could prevent NOD mice from developing diabetes by a single low dose of streptozotocin provided that β-cell apoptosis occurred but not in the RIP-CrmA transgenic NOD mice in which β cells expressed the caspase inhibitor CrmA [68]. Rayat et al. achieved similar prevention by intraperitoneal injection of in vitro streptozotocin-treated islet cells into prediabetic female NOD mice [69]. According to Hauben's review, autoimmunity is not under all circumstances a pathologic process and provided that the activation of T_{reg} cells might keep the process limited and the nature of the early immune response adequate; it may participate in the elimination of damaged tissues [5]. In BDC2.5 RAG-1-(recombination activating gene-1-) deficient mice, the generation of both B- and T-lymphocytes is impaired, and, in addition, they develop severe T1DM. The development of diabetes in the mouse model could be abrogated by the transfer of splenocytes on the 10th day of life from NOD mice, irrespective of whether the CD25 (IL-2 receptor α-chain) has or has not been present on the cell population [70]. Surprisingly, Balb/c mice expressing IFN-γ in their β-cells turned out to be resistant to streptozotocin-induced diabetes, while the controversial role of IL-10 (in conjunction with NOD MHC homozygosity) in autoimmune diabetes development has been suggested in experiments with transgenic mice expressing pancreatic IL-10 [71]. These data suggest that limited autoimmunity is likely to have a role in active tolerance [5], and the successful preventive attempts by supporting insulitis lead us to considering the sequence of other, for example, environmental factors.

4. Factors Influencing the Development of Immune Response

The existence of synergic immunological stimuli affecting separate pathways has been known for decades. However, their relevance in autoimmune diabetes is the product of recent investigation. In case of vaccination, it has been known that one-peptide antigen by itself is not always sufficient for triggering immune response. Furthermore, it is likely to induce tolerance. In general, the antigens, which are bigger and easily form aggregates, are more likely to induce immune response, while smaller soluble antigens primarily induce tolerance [72, 73]. Dresser proved in the 1960s that immunization with highly purified antigens induce immunity only in a case when an adjuvant is provided [74]. Dresser used aspecific bacterial adjuvant, but similar results could be achieved with endotoxin [75]. These observations led to the classical theory that two concurrent triggers are necessary for the appropriate induction of an immune response [73]. This basic theory by Bretscher and Cohen for B-lymphocytes [76] was later also proven in T-cell-mediated responses [77, 78]. As later clarified, during antigen presentation the APC provides numerous contact costimuli (e.g., the APC surface B7 complex binding to the surface CD28 of the T-cell) besides the MHC-II bound antigen which—together with cytokines—are necessary for T-cell activation. Janeway postulated that antigen presentation is the decision making step of the immune response, and the outcome (immunization or tolerance induction) depends on whether the APC could bind any pathogen-associated molecular pattern (PAMP) by its specific receptors (stranger hypothesis). In Janeway's theory, infections were the evolutionary drive for the immune system which was justified by the discovery of the TLRs by which the APCs detect highly conserved nonhuman (primarily bacterial and fungal) patterns. However, this theory seemed to be insufficient for the interpretation of the immune response to tumors, grafts, most viruses, and autoimmunity [73, 79]. Some of these exceptions become questionable as some viral components are ligands of intracellular TLRs through which they induce the secretion of antiviral interferons (IFN-α and β): double-stranded RNA of rota viruses is the ligand of TLR3, the single-stranded RNA of the coxsackie-B virus, probably the most often associated to T1DM, is the ligand of TLR7, while cytomegalovirus (CMV) can be sensed by the recognition of CpG DNA motif by TLR9 [80]. TLR4 functions as the receptor of bacterial lipopolysaccharide (LPS), while TLR2 recognizes cell wall components mainly from gram positive bacteria, mycobacteria, and yeast [81]. TLR2 and TLR4 are located on the cell surface but they are also involved in sensing some viruses. They primarily activate the nuclear factor kappa B (NF-κB) pathway and partly alternative pathways, which results in the secretion of inflammatory cytokines [80]. β-cell apoptosis might participate in the priming of diabetogenic T-cells via a TLR2-dependent APC stimulation [57].

The danger hypothesis by Matzinger states that the critical signals for APCs besides the antigen are cell components released from damaged cells (danger-associated molecular patterns—DAMP) [73]. Shi et al. found that subcutaneous injection of syngenic necrotic or apoptotic cells exerts significant adjuvant effect on cytotoxic T-cell responses to ovalbumine, while the cells alone were not immunogenic [82]. Some authors describe that particles of apoptotic cells can be engulfed by macrophages and DCs, and after degradation some components are presented both on MHC-I and MHC-II complexes, hence their ability to trigger cytotoxic response [83, 84]. The properties of the antigen presentation are determined by the circumstances of the antigen-uptake: if it occurs in a "peaceful" environment, the presentation will be tolerogenic such as when apoptotic remnants are eliminated.

If the appropriate factors potentiate the maturation of the DCs, it will result in T-cell activation [85].

Heat shock proteins (HSPs) are highly conserved immunogenic proteins that are involved in the pathogenesis of a variety of immune-mediated disorders, including autoimmune diseases. HSPs might serve as autoantigens, and antibodies against two epitope regions on HSP60 (AA394–413 and AA435–454) were detected in high titres in sera of children with T1DM [86]. Recently autoantibody against HSP 10 was found in sera from the majority of patients with fulminant type 1 diabetes (FT1DM) and also with autoimmune pancreatitis (AIP), and authors even suggested that an autoantibody against HSP 10 is a new diagnostic marker for both AIP and FT1DM [87]. The lymphocyte proliferative response to *Mycobacterium leprae* HSP65 of NOD mice was higher compared to their counterparts from I-E^{d}_{α} transgenic mice that show no insulitis. In addition, splenocytes from NOD mice were able to transfer insulitis to the previously resistant transgenic strain [88]. Many cytoplasmatic components are suspected to exert adjuvant effect as DAMPs, including HSPs [73], for example, HSP70, HSP90, and HSP100 [89], which are able to stimulate the maturation of DCs [90, 91]. Certain HSPs (e.g., HSP60 and 70) generate a signal via the LPS receptor (TLR4-CD14 complex) [92], and other authors suggested that LPS-free HSP preparates were ineffective [93]. HSPs, also as carriers of peptides, are taken up by APCs and the peptide they carried can be presented on MHC-I [94]. Although a specific HSP receptor is known (CD91) [95], the TLR2/4 cluster is also involved in the uptake of HSP-peptide complexes [94]. Miyagawa et al. even indicated the role of TLR4 in binding the chemokine CXCL10, which is known to have an important role in the development of insulitis [96]. Uric acid is also able to promote the maturation of dendritic cells via binding to membrane lipids. After phagocytosis, it potentiates inflammation through enhancing IL-1β secretion. Uric acid is able to exert this effect only in such high concentration when crystal forming appears [97].

5. Environmental Factors and Apoptosis

According to the DIPP (diabetes prediction and prevention) study, autoantibody positivity is detectable by the age of 2 in the overwhelming majority of those who develop diabetes until the age of 10 years [98]. Therefore, the interplay between genetic susceptibility and potential triggers is likely to play a role at a very early age that gradually results in the loss of balanced autotolerance in the upcoming years and subsequently to the development of the clinical disease. Seasonal accumulation of new cases led Adams in 1926 to hypothesize the pathogenic role of viral infections. Rubella, CMV, enteroviruses, mumps, and coxsackie virus are among the most frequently suspected pathogens [99]. The potential role of coxsackie-B virus was based on the serological results of newly diagnosed T1DM patients in 1969 [100]. Later, the viral RNA was detected from the sera of such patients [101]. It is not yet clear how these viruses might be able to exert a diabetogenic effect. There is evidence that the rubella, coxsackie, and mumps viruses are able to infect β-cells [24], and there are experimental models for other viral infections that might lead to fulminant diabetes [102]. The similarity between human and viral proteins might offer an additional explanation, and the coxsackie-B virus indeed does contain a protein similar to the human glutamic acid decarboxylase-65 (GAD65) [24]. Experimentally, it has been proven that molecular mimicry is only able to trigger autoimmune diabetes provided that there is a full identity between the amino acid sequences as modeled in the RIP-gp (rat insulin promoter) mice which expresses a glycoprotein (gp) of the lymphocytic choriomeningitis virus (LCMV) in their β-cells and after an infection rapidly develop diabetes [24, 103, 104]. On the other hand, an infection with a virus containing an analogous but not identical epitope is able to promote the ongoing autoimmunity [105]. The intensity of the ongoing immune response might determine the effect of an infection [24]. When older NOD mice—in which insulitis was already present—were infected with certain coxsackie virus strains, it resulted in the speed-up of the development of diabetes [106]. However, inoculating coxsackie-B virus (CBV) into young NOD mice devoid of insulitis diminished the incidence of diabetes until the fixed endpoint. Interestingly, the more pancreatovirulent the CBV strain was, the greater the protection from T1DM onset was seen in coxsackie-B3-virus-induced pancreatitis. The immunopathology of the protection in this genetically susceptible mouse strain is not fully clarified. Authors considered that virus induced pancreatitis reveals specific host pancreatic antigens to the immune system that suppress the autoimmune insulitis in the NOD mice [106]. Recently, acceleration of murin T1DM by rotavirus was also described and associated with virus spread in regional lymph-nodes and induction of Th1-dependent antibody and cytokine response [107].

Other environmental factors such as bovine insulin containing homologous epitopes (molecular mimicry) might also boost an ongoing autoimmune process. The suspected environmental trigger bovine insulin differs only in three amino acids from human insulin. Still, the titer of antibodies against bovine insulin spontaneously decrease at the age of 12–18 months in those children in whom other diabetes-associated autoantibodies were not detected, even though they were carrying the HLA DQB1*0302 haplotype. In contrast, in those children who had at least two diabetes-associated autoantibodies at this age, the spontaneous decline in antibovine insulin antibody titer did not occur, instead the titers further increased [108].

The early theory that described viruses and certain environmental triggers as specific causes of T1DM via antigenic mimicry might only be sustained if taken into a more complex view. Viral infections are able to mediate the apoptotic process and some of the candidate genes identified in GWAStudies are coding proteins that are actively involved in a virus-host interplay that may—together in combination—promote the autoimmune process. The β-cells themselves possess pattern recognition receptors (such as IFIH1—interferon induced with helicase C domain 1—, a sensor of double-stranded viral RNA, a candidate gene [10] and TLRs) which, via the activation of NF-κB and

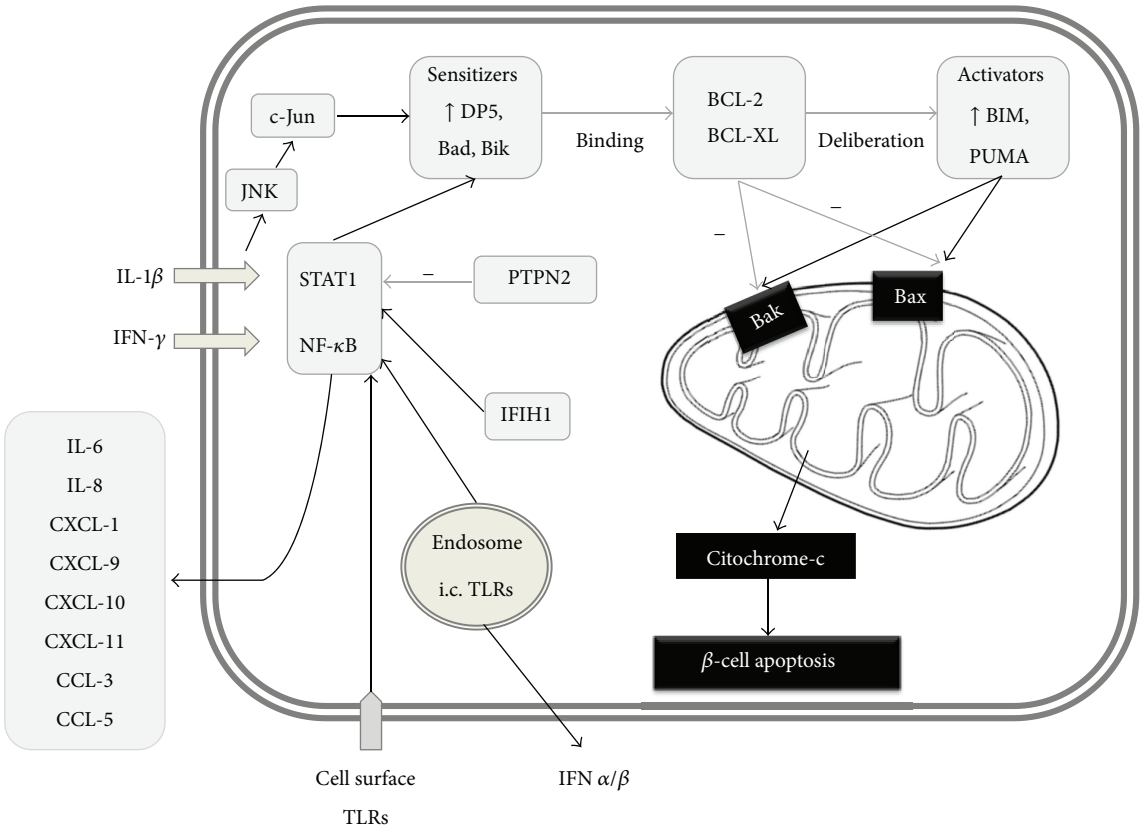

Figure 1: In addition to proinflammatory cytokines such as IL-1 and IFN-γ, the signaling via IFIH1 and various other pathogen recognition receptors mediate β-cell apoptotic death: upregulate certain BH3 proteins and also promote the secretion of numerous chemokines. Certain BH3 "sensitizer" proteins, for example, DP5, bind to BCL2 and BCL-XL which inhibit BAX and BAK activation and at the same time liberate the "activator" proteins (such as BIM and PUMA). PTPN2 is a negative regulator of the pancreatic β-cell apoptosis that reduces the BH3 protein-related apoptotic activation cascade in the β-cell.

STAT1, provide proapoptotic signals for the cell [109]. The cytokines such as IFN-γ and IL-1β, which are known as main mediators of β-cell apoptosis, exert their effect through NF-κB and STAT1 as well [109]. PTPN2 (protein tyrosine phosphatase, non-receptor type 2), which is a candidate gene according to GWAStudies has antiapoptotic activity at least in part through the blockade of JNK1 (c-Jun N-terminal protein kinase 1), which is responsible for the activation of STAT1 and BCL2L11 (BCL2-like 11, BIM; described later). Its inhibition on the translation level both in vitro (human β-cells) and in vivo supported IFN-induced β-cell apoptosis [110]. NF-κB and STAT1 signaling also upregulates MHC-I expression on the cell surface, which might lead to a vicious circle by making the β-cell more "visible" for cytotoxic T-cells [24, 109]. Furthermore, in response to inflammatory cytokines, β-cells are able to express MHC-II as well [111]. Recently, many interesting data have become known on the process linking proapoptotic stimuli to the mitochondrial BAX (BCL2-associated X protein) and BAK (BCL2-antagonist/killer) activation, which is the final common path of cell death via apoptosis. In this intermediate phase, the so-called BH3 (BCL2 homologous 3) proteins have a crucial role. Based on their activity, they can be divided into two groups: the sensitizers (e.g., DP5—death protein 5—) bind to BCL2 (B-cell CLL/lymphoma 2) and BCL-XL (BCL2-like protein 1), which inhibit BAX and BAK activation, and at the same time liberate the activators (such as BIM and PUM—p53 upregulated modulator of apoptosis—) from this bond, which activate BAX and BAK [112] (Figure 1). Gurzov and Eizirik describe that in triggering this process endoplasmatic reticulum stress has an important role and in vitro stressed β-cells showed higher DP5 expression [112]. With DP5 inhibition on the RNA level, the apoptotic rate could be, diminished [109]. DP5 did not only have a positive effect on cell survival, but DP5 gene knockout (GKO) mice had larger β-cell mass and turned out to be resistant to high-fat-diet-induced glucose intolerance, directly proving the link between the immunological apoptotic and the metabolic functions [113]. Among the activators, BIM seems to be dominant. The higher apoptotic rate after PTPN2 inhibition could be significantly diminished by BIM inhibition [110]. By the inhibition of PUMA, mitochondrial BAX translocation and both apoptosis could also be diminished [114]. The combination of TNF-α IFN-γ induced DP5, PUMA, and BIM

expression in human islets [115]. The figure summarizes the signaling in the β-cell.

The apoptotic cell remnants that might be further degraded by secondary necrosis as sources of endogenous adjuvants and inflammatory mediators might enhance the local immune response in a nonspecific manner (bystander activation) and are also able to make other previously hidden antigens available (antigenic spreading) [24]. From this view, a viral infection might be able to flare the autoimmunity up or, in the case of insufficient peripheral tolerance, even initiate an autoimmune process. The β-cells are not only targets but also active participants of the inflammation. Eizirik et al. analyzed the transcriptome of human β-cells and found that IFN-γ and IL-1β exposure resulted in several fold elevation of the chemokines CXCL-9, -10, -11, and CCL-2, -3, -5 secreted by the β-cell [116]. The sera of T1DM patients were shown to contain higher levels of CXCL10 compared to healthy individuals and type 2 diabetic patients [117]. The expression of CXCR3, the receptor of CXCL-9, -10, and -11 chemokines is typical of Th1 cells. Some groups were able to effectively block the manifestation of diabetes in prone mice by blocking CXCR3-linked signaling [118, 119]. However, they used the previously mentioned RIP-gp mouse, which is a perfect model of antigenic mimicry but not of the multifactorial human disease. Yamada et al. used a CXCR3 knockout NOD mouse model and expected it to be protected against diabetes. Surprisingly, the CXCR3$^{-/-}$ NOD mouse developed diabetes even earlier, which turned out to be due to impaired navigation of T_{reg} cells to the islets [117].

6. Epigenetics

Although over the past fifty years there has been a (geo-epidemiologically) significant increase in incidence of T1DM, this is not in parallel with the frequency of the genetic risk. Moreover, the prevalence of the MHC-II genes responsible for approximately 40% of susceptibility has been decreased [120]. Several environmental factors may contribute to complex T1DM pathways and thus to disease manifestation. Epigenetic regulation, as a missing link, has been proposed not only to reflect the influence of environmental exposures, gender, and aging, but also to explain the discordance in monozygotic twins for the development of autoimmunity as well.

Epigenetics is a mechanism defined by mainly heritable changes of gene expression without altering directly the DNA sequence, and thus it affects genotypes to be ultimately manifested in diverse phenotypes. In general, the epigenome can be modified at three main checkpoints, like DNA methylation, posttranslational histone modifications, and expression of noncoding RNAs, such as miRNAs (microRNAs) and lncRNAs (long noncoding RNAs). Epigenetics plays a crucial role in the development and function of different tissues and cells, including the immune system and β-cell mass expansion under stress in the pancreas [121, 122]. Maturation and differentiation of immune cells, and cytokine gene expressions seem to be especially affected [123, 124]. Current lines of evidences suggest the multiple involvement of epigenetics in the pathomechanism of T1DM: epigenetic changes may influence disease outcome by affecting β-cell homeostasis, insulin and glucose metabolism, the gut microbiome, and immune responses.

After detecting a significant increase in β-cell-derived demethylated DNA in the Ins (insulin gene) yet before the onset of hyperglycemia in (prediabetic) NOD mice, Akirav et al. subsequently were able to confirm the increased demethylation of CpG sites within the insulin gene in primary human β-cells and also found increased levels of demethylated insulin DNA in circulating β-cell-derived DNA in patients with new-onset type 1 diabetes. They proposed this observation as an alteration contributing to T1DM pathology, as well as a potentially noninvasive approach for detecting in vivo β-cell death [125].

By using the chromatin immunoprecipitation linked to microarray (ChIP-chip) approach to compare genome wide histone H3 lysine 9 dimethylation (H3K9me2) patterns in peripheral blood lymphocytes and monocytes from T1DM patients, the T1DM candidate gene CTLA4 has been displayed higher H3K9me2 at the promoter region yet standing as an example of interface between genetic and epigenetic information in T1DM [126].

Miao et al. observed marked variations in H3K9-acetylation (H3K9Ac)—that is associated with promoters and active genes—levels at the upstream regions of HLA-DRB1 and HLA-DQB1 in T1DM monocytes and also demonstrated increased expression of HLA-DRB1 and HLA-DQB1 on monocytes in response to interferon and TNF treatment that were accompanied by changes in H3K9Ac at the same promoter regions as those seen in the patients' cells. Therefore, they suggest that the H3K9Ac status may regulate the transcriptional response of HLA-DRB1 and HLA-DQB1 to cytokine stimuli [127].

The complexity of epigenetic mechanisms is well characterized by the recent finding that the promoter of the Ins (insulin) gene is part of an extended "open" chromatin domain and as such is in physical contact with the Syt8, a gene that is located at 300 kb distance in the genome, and interestingly this contact between Ins and Syt8 is strengthened by glucose in pancreatic islets [128].

Certain miRNAs are also related to T1DM: miR-21a and miR-93 were shown to be downregulated in peripheral blood mononuclear cells of T1DM patients. Moreover, a population of T_{reg} cells of T1DM patients showed a higher expression of miR-146 that is crucial in maintaining the suppressor function of T_{reg} cells, while a lower expression of eight other miRNAs (20b, 31, 99a, 100, 125b, 151, 335, and 365) were found [129, 130].

Furthermore, a role for long noncoding (lnc) RNAs, both in the *cis* and *trans* regulation of transcription via interaction with chromatin modifying complexes to target epigenetic marks to particular genomic loci, has only been recently described. An islet-specific lncRNA expressed from the Pdx1 locus regulates Pdx1 activity, a master gene of β-cell differentiation and regulation, therefore has a potential impact on maintaining glucose homeostasis. Long noncoding RNA molecular studies might open a yet largely unrevealed novel

layer of transcribed but not translated genetic information and new dimensions in diabetes research [131–134].

7. Summary

Type 1 diabetes mellitus is a prototype disorder of both endocrine and organ-specific autoimmune diseases. There is a growing amount of evidence suggesting that autoimmunity cannot be interpreted as a binary state. It rather becomes pathologic through impaired regulation. From the view of the congenital susceptibility, the role of environmental factors might be revised as some might be considered rather enhancers than triggers. The early and adequate nature of the response with optimal limitation of autoimmunity might be the difference between the physiologic and the pathologic conditions [5]. The impaired elimination of apoptotic remnants may create a less tolerogenic inflammatory environment favorable for the initiation of autoimmunity [53]. The transient local inflammation due to, for example, a viral infection in combination with the impaired immune regulatory functions that are in part determined by the genetic background may eventually lead to the generation and expansion of autoreactive T-cells [24, 135]. Data from the World Health Organization Diabetes Mondiale (WHO Diamond) Project suggested the role of late enhancers: as the later the disease onset was, the more the pronounced seasonality evaluators could find [136]. Therefore, the possibility of an early immunization as a preventive approach might be raised.

GWAStudies made real breakthrough in identifying non-HLA genetic variations that participate in the establishment of the genetic susceptibility. In addition, the identification of a number of candidate genes and their risk polymorphisms GWAS contributed significantly to the understanding of the molecular pathology of certain immune mechanisms. Epigenetics has also been reported to contribute significantly to the pathology of this autoimmune disease.

The most recent national and international epidemiologic data still show elevation in T1DM incidence in all age groups but especially among the youngest, under 4 years [137, 138]. Although the role of certain viral infections cannot be excluded in the priming, such epidemics have become even less frequent, which is the theoretical basis of the hygiene hypothesis. Yet, a germ-free environment might not only exclude an immunomodulatory effect of various infections [139], but also, if the time of the first infections is delayed, it may more easily enhance latent autoimmunity. Interactions of metabolic and immunologic processes are also likely to be considered as between potential cause of the epidemiological tendencies. According to the results of the search for diabetes in the youth (USA), 35.4% of those diagnosed with diabetes under the age of 20 are insulin resistant, and 19.5% also have autoantibodies [140].

A number of clinical trials have been conducted or are still under clinical investigation, and the framework of this review limited us to comprehensively report all trials. Effector and regulatory T-cells are known to differ in their affinity to IL-2 and therefore theoretically provide a narrow range where T_{reg} induction by IL-2 is feasible without the induction of effector T-cells (as summarized earlier) [41]. Induction of immune tolerance via nasal, or oral whole insulin antigen, intramuscular insulin B-chain and subcutaneous GAD65 (with alum) administration or subcutaneous $hsp60_{437–460}$ (p277) administration either failed to demonstrate clinically significant improvement or resulted in controversial outcomes [141]. Studies administering D-vitamin based on its immunomodulatory properties have also failed to demonstrate clear clinical benefit [142, 143]; nevertheless, a recent study found that T1DM risk was highest among individuals whose 25(OH)D vitamin levels were in the lowest 20% of those measured and concluded that low 25(OH)D-vitamin levels may predispose young adults to the development of T1DM [144].

Our study group have reported earlier that the dipeptidyl-peptidase-4 (DPP-4)-incretin axis might be dysregulated and the serum DPP-4 enzymatic activity is higher in patients with T1DM than in healthy controls [145]. Blandino-Rosano et al. found that in vitro GLP-1 (glucagon-like peptide-1) is able to reverse the inhibition of extracellular signal-regulated kinase 1 and 2 (ERK1 and 2) phosphorylation and the β-cell antiproliferative effect of proinflammatory cytokines IL-1β, IFN-γ, and TNF-α [2]. In addition to the β-cell protective effect of GLP-1, the incretin agonists might have a role in the maintenance of the peripheral T_{reg} cell population [146]. The results of a few pilot studies indicated a lower amount of insulin needed to gain similar (or even better) glycemic control using a DPP-4 inhibitor or a GLP-1 agonist in combination with the insulin therapy and also significantly less time spent in hypoglycemia when the GLP-1 agonist Liraglutide therapy was applied with insulin in T1DM patients with residual β-cell function [147, 148]. Therefore, lowering the time spent with the glucose levels below 3.9 mmol/L might be an advantage in the everyday clinical praxis because hypoglycaemia is still an existing problem.

Either the early immunization against the potential enhancers in a susceptible population that might be a realistic approach in prevention programs or the immune tolerance induction in combination with parallel therapies targeting β-cell recovery that might provide future alternatives for the therapy of the already developed disease will enter a highly competitive field where the already existing standard care provides T1DM patients with good quality of life and acceptable life expectancy [149].

Abbreviations

AIP: Autoimmune pancreatitis
APC: Antigen-presenting cell
BAFF: B-lymphocyte activating factor
BAK: BCL2-antagonist/killer
BAX: BCL2-associated X protein
BCL2: B-cell CLL/lymphoma 2
BCL2L11: BCL2-like 11
BCL-XL: BCL2-like protein 1
BH3: BCL2 homologous 3
CBV: Coxsackie-B virus
CD: Cluster of differentiation
CCL: Chemokine (C-C motif) ligand
CTLA-4: Cytotoxic T-lymphocyte antigen 4

CXCL: Chemokine (C-X-C motif) ligand
CXCR: C-X-C chemokine receptor
CMV: Cytomegalovirus
DC: Dendritic cell
DIPP: Diabetes prediction and prevention
DP5: Death protein 5
DPP-4: Dipeptidyl-peptidase-4
ERK: Extracellular signal-regulated kinase
Foxp3: Forkhead box P3
FT1DM: Fulminant type 1 diabetes
GAD: Glutamic acid decarboxylase
GKO: Gene knockout
gp: Glycoprotein
GWAStudies: Genomewide association Studies
HLA: Human leukocyte antigen
IDDM2: Insulin-dependent diabetes mellitus 2 locus
IFIH1: Interferon induced with helicase C domain 1
IFN: Interferon
IL: Interleukin
Ins: Insulin gene
iT_{reg}: Induced regulatory T-cell
JNK1: c-Jun N-terminal protein kinase 1
LCMV: Lymphocytic choriomeningitis virus
lncRNA: Long noncoding RNA
LPS: Lipopolysaccharide
miRNA: Micro-RNA
MHC: Major histocompatibility complex
NF-κB: Nuclear factor κB
NOD: Nonobese diabetic
nT_{reg}: Natural regulatory T-cell
PTPN: Protein tyrosine phosphatase, nonreceptor type
PUMA: p53 upregulated modulator of apoptosis
RAG: Recombination-activating gene
RIP-gp: Rat insulin promoter glycoprotein
SNP: Single-nucleotide polymorphism
STAT: Signal transducer and activator of transcription
T1DM: Type 1 diabetes mellitus
TCR: T-cell receptors
TGF: Transforming growth factor
Th: Helper T-cell
TLR: Toll-like receptor
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
Eu: Indicates participating individuals with European ancestry in the GWAStudies.

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