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ORIGINAL ARTICLE

Juvenile Idiopathic Arthritis Subtype- and Sex-specific Associations with Genetic Variants in the *PSMA6/PSMC6/PSMA3* Gene Cluster

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PSMC6

Background: The ubiquitin proteasome system plays an exceptional biological role in the antigen processing and immune response and it could potentially be involved in pathogenesis of many immunity-related diseases, including juvenile idiopathic arthritis (JIA).
Methods: The *PSMB5* (rs11543947), *PSMA6* (rs2277460, rs1048990), *PSMC6* (rs2295826, rs2295827), and *PSMA3* (rs2348071) proteasomal genes were genotyped on JIA subtype- and sex-specific association; plasma proteasome levels was measured in patients having risk and protective four-locus genotypes and eventual functional significance of allele substitutions was evaluated *in silico*.
Results: Loci rs11543947 and rs1048990 were identified as disease neutral and other loci as disease susceptible ($p < 0.05$). The rs2277460, rs2295826, and rs2295827 loci had the strongest association with oligoarthritis [odds ratio (OR) = 2.024, 95% confidence interval (CI) 1.101–3.722; OR = 2.371, 95% CI 1.390–4.044; OR = 2.183, 95% CI 1.272–2.737, respectively), but the rs2348071 locus was associated with polyarthritis in females (OR = 3.438, 95% CI 1.626–7.265). A strong ($p < 0.001$) association was detected between the rs2277460/rs2295826/rs2295827/rs2348071 four-locus genotypes and the healthy phenotype when all loci were homozygous on common alleles (OR 0.439, 95% CI 0.283–0.681) and with the disease phenotype when the rs2348071 and the rs2295826 and/or rs2295827 loci were represented by risk genotypes simultaneously (OR 4.674, 95% CI 2.096–10.425). Rarely observed in controls, the double rs2277460/rs2348071 heterozygotes were rather frequent in affected males and

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more strongly associated with polyarthritis ($p < 0.05$). Haplotypes carrying the rare rs2295826/rs2295827 and rs2277460 alleles showed a strong ($p < 0.001$) association with oligo- and polyarthritis, respectively. The plasma proteasome level was found to be significantly higher in females having four-locus risk genotypes compared with protective genotypes ($p < 0.001$). Sequence affinity to transcription factors and similarity to splicing signals, microRNAs and/or hairpin precursors potentially depend on allele substitutions in disease susceptible loci.
Conclusion: We demonstrate for the first time evidence of a sex-specific association of *PSMA6/PSMC6/PSMA3* genetic variants with subtypes of JIA and plasma proteasome concentrations. Theoretical models of the functional significance of allele substitutions are discussed.

1. Introduction

Juvenile idiopathic arthritis (JIA) is the most common clinically heterogeneous chronic rheumatic disease in children.[1] Onset-specific clinical features allow discrimination of seven JIA subtypes,[2] with oligoarthritis (JIoA) and polyarthritis (JIpA) being the most frequent.[1] The cause of JIA is complex, involving both environmental and genetic risk factors. The latter could include structural variations in both human leukocyte antigens and non-human leukocyte antigen candidate genes.[3,4,5] Because of the pleiotropic effect, a frequent phenomenon in complex human traits and diseases,[5] some loci of susceptibility may be shared with other autoimmune diseases.[4,5]

An exceptional biological role for the ubiquitin proteasome system (UPS) in antigen processing and immune response, as suggested by Kloetzel,[6] has been increasingly supported this last decade experimentally. A special form of proteasomes, thymoproteasomes, expressed exclusively in the cortex of the thymus and probably involved in positive selection of T cells, has recently been described. This indicates that the role of proteasomes in the immune response might be even more important.[7,8] In patients with systemic autoimmune disease, the concentration of circulating proteasomes has been shown to be strongly increased[8,9]; the core 20S proteasome was identified as a target of the humeral autoreactive immune response.[10–15] The proteasomal inhibitor MG132 has been reported to reduce the severity of arthritis and reverse pain behavior in arthritic rat models.[16]

Highly conserved from an evolutionary standpoint, proteasomal genes appear to be subject to multiple trait purifying selection. Structural variations in proteasomal genes could potentially affect UPS efficiency through modulation of expression of a particular gene, realization of gene and protein networks and metabolic processes that may in the end influence predisposition to and/or development of autoimmune disorders.

The distribution of proteasomal genes over the human genome displays a tendency of clustering in chromosomes. Nine of the proteasomal genes have been localized in the long arm of chromosome 14, including two β (*PSMB5* and *PSMB11*) and two α (*PSMA3* and *PSMA6*) subunits of the core 20S proteasome, ATPases (*PSMC1* and *PSMC6*), 11S non-ATPase activators (*PSME1* and *PSME2*) and the *PSMA3P* pseudogene. The 14q11, 14q13, and 14q22-32 regions carrying the mentioned genes have been reported previously as potentially susceptible to autoimmune,[17–24] and other complex diseases in European and/or Asian populations.[Suppl 1–15] Fine 14q13.2 microsatellite scanning revealed evidence of JIA association with variability in the region encompassing the *PSMA6* gene.[24]

The aim of the current study was to genotype six single nucleotide polymorphisms (SNPs) belonging to the *PSMA6* (rs2277460 and rs1048990), *PSMA3* (rs2348071), *PSMB5* (rs11543947) and *PSMC6* (rs2295826 and rs2295827) proteasomal genes for subtype- and sex-specific association with JIA; to evaluate plasma proteasome levels in JIA patients of different multi locus genotypes, and to perform an *in silico* prediction of eventual functional consequences of nucleotide substitutions, including sequence affinity to transcription factors (TFs) and similarity to splicing signals and microRNAs.

2. Materials and methods

2.1. Case–control study

Patients were 174 JIA children (108 girls) receiving consultation at the outpatient clinic of P. Stradins Clinical University Hospital and Children Clinical University Hospital Clinic *Gailezers* in Riga, Latvia. JIA was diagnosed and assignment of the JIA patients to subgroups was carried out according to the criteria of the International League of Association for Rheumatology.[2] For association analysis both persistent and extended JIoA, and rheumatoid factor-negative and rheumatoid factor-positive JIpA subgroups were combined in JIoA and JIpA groups of 107 and 55 patients, respectively. Twelve other patients were diagnosed as having systemic ($n = 9$), enthesitis-related ($n = 2$), and psoriatic ($n = 1$) arthritis.

The control group was represented by 191 (117 women) patents of Riga Bikernieki Hospital admitted with a diagnosis of trauma and not diagnosed as having any autoimmune and/or cardiovascular disorders, type 2 diabetes mellitus (T2DM), or obesity.

Informed consent was obtained from all the study participants or their parents. The study was approved by the Central Medical Ethics Committee of the Republic of Latvia Ministry of Health.

2.2. Marker choice

Due to limited data on the genetic diversity and susceptibility to diseases of proteasomal genes, several criteria were taken into account in choosing markers. These included the existence of previously reported findings on locus association with human health status, locus allele-specific potential to be functionally significant, locus variability in Latvians, Hardy–Weinberg expectations and others concerning mainly a genotyping technology. The rs2277460 and rs1048990 of the *PSMA6*, rs2295826 and rs2295827 of the *PSMC6* and rs23480071 of the *PSMA3* were previously studied on disease susceptibility[Suppl 1–12,14–16] and/or genetic diversity[Suppl 17]; rs11543947 of the *PSMB5* gene was previously genotyped on genetic diversity only in HapMap populations. All loci fit all other mentioned criteria of marker choice.

2.3. DNA extraction and genotyping

DNA was extracted using a kit for genomic DNA extraction from nucleated blood cells (Fermentas, Vilnius, Lithuania). Genotyping methods and primer sequences are indicated in Supplementary Table 1. Basic PCR was performed with DreamTaq polymerase (Fermentas) using the following parameters: 94°C for 5 minutes; then 35–40 cycles of 94°C for 45 seconds, appropriate annealing temperature (55–61°C) for 45 seconds, 72°C for 45 seconds and a final extension step at 72°C for 7 minutes. DNA digestion by restriction enzymes was performed according to the manufacturer's protocols (Fermentas).

Amplified and digested products were analyzed by electrophoresis in 1–3% agarose gel for all markers. For quality control, 16 randomly chosen samples for each marker were genotyped in duplicate in different experiments. The concordance of the genotyping was 100%. Genotyping data were verified by direct sequencing of the corresponding DNA fragments in both directions using the Applied Biosystems 3130*xl* Genetic Analyzer. Alleles and genotype frequencies for the rs11543947 (ss69150930), rs2277460 (ss24557113), rs1048990 (ss35076445), rs2295826 (ss3239727 and ss69157456), rs2295827 (ss23619651) and rs2348071 (ss3302481) were obtained for HapMap-CEU (NorthWestern European), YRI (Yoruba), JPT (Japanese), and HCB (Han Chinese) populations from publicly available dbSNP (build 13) entries at NCBI (http://www.ncbi.nlm.nih.gov/snp/). Loci description and nucleotide numbering are given according to the recommended nomenclature system (http://www.genomic.unimelb.edu.au/mdi/mutnomen/recs.html). Sequence information for the chromosome 14 GRCh37.p5 assembly (NCBI reference sequence: NC_000014.8) was used for loci description, nucleotide numbering, and primer design using the Primer 3.0 program.

2.4. Measurement of plasma proteasome concentration

Plasma samples were available only for 23 JIA patients. These plasma samples were obtained from patients randomly chosen for plasma sampling during development of the DNA collection in the JIA study. Therefore, preliminary information on genotypes of these patients did not exist at the moment of the sampling. Blood was harvested on citrate anticoagulant, and plasma stored at −80°C. Plasma proteasome concentration was measured in triplicate for each sample using a standard 20S/26S Proteasome ELISA kit (BML-PW0575; ENZO Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocols. Absorbance was read at 450 nm using a UV-Vis spectrometric plate reader. Results were expressed as concentration of proteasome protein in ng/mL determined by interpolation for the absorbance value using the generated 20S proteasome standard curve.

2.5. Data analysis

Documenting personalized genotyping data allowed determination of rs11543947/rs2277460/rs1048990/rs2295826/rs2295827/rs2348071 six-locus genotype (6-LG) of each individual participant of the study. The 6-LGs, rs2277460/rs2295826/rs2295827/rs2348071 four-locus genotypes (4-LGs), observed haplotypes, single locus genotypes (SLGs), and allele frequencies were estimated by direct counting of genetic variants. Inferred haplotypes prediction, haplotype sorting, estimation of the linkage disequilibrium and probability of recombination were performed using the DnaSP software version 5.10.1 online tool at http://www.ub.es/dnasp.[Suppl 18] Both the two-tailed Fisher's exact test and the χ^2 test were applied to evaluate the linkage between the rs2295826 and rs2295827 polymorphic sites at three *p*-value levels ($p < 0.05$; $p < 0.01$; $p < 0.001$). The Bonferroni correction included in the DnaSP analysis was taken into account to support the significance of the revealed disequilibrium ($\alpha' = 0.05$).

Deviation from the Hardy–Weinberg equilibrium and differences between cases and controls in allele, genotype and haplotype frequencies were evaluated by χ^2 and Cochran–Armitage trend test using XLSTAT 2013 software for Windows. Genetic models for every individual locus were designed according to Lewis.[Suppl 19] Contingency tables were 2×3 for the AA, AB, BB genotypes in the general model; 2×2 for the AA, AB+BB and AA+AB, BB, and AB, AA+BB genotypes in the dominant, recessive, and over dominant models, respectively and A and B alleles in the multiplicative model where A is the major allele and B is the minor allele. Using an additive model, the AA, AB, and BB genotype distribution was analysed using the Cochran–Armitage test for trend. An odds ratio (OR) > 2 and <0.5 was considered to be clinically significant. Stratification was performed by JIoA and JIpA ILAR subtypes and by sex.

Levels of plasma proteasome were expressed as mean ± standard error of the mean for each sample to show the variability associated with the estimation, and as mean ± standard deviation to characterize the spread of a data set within the groups. Both standard error of the mean and standard deviation were calculated using the online NCalculators (http://ncalculators.com/). Differences in plasma proteasome levels between the groups were estimated by nonparametric Mann–Whitney and/or Kruskal–Wallis tests using XLSTAT 2013 software. Results were considered to be of nominal statistical significance at $p < 0.05$, moderate statistical significance at $p < 0.002$, and strong statistical significance at $p < 0.001$.[Suppl 20]

2.6. SNP functional analysis in silico

An eventual functional significance of the SNPs showing evidence of association was analyzed *in silico* on sequence similarity to transcription factors binding sites (TFBSs) using Genomatix software, MatInspector, Release 7.4 online tool, at www.genomatix.de.[Suppl 21] Only parameters with core/matrix similarity of > 1.000/0.800 were taken into account. Splicing signals were predicted by Human Splicing Finder Version 2.4 (http://www.umd.be/HSF)[Suppl 22] with standard threshold values for branch point, donor and acceptor splice sites, enhancer, silencer, heterogeneous nuclear ribonucleoprotein (hnRNP) and other splicing motifs. Sequence similarity to mature microRNAs and hairpin precursors was evaluated, and microRNA targets prediction was done using miRBase (http://www.mirbase.org/index.shtml)[Suppl 23] and miRNAMap (http://mirnamap.mbc.nctu.edu.tw/index.php)[Suppl 24] online tools, respectively.

3. Results

3.1. Genotyping results and single locus association

In both case and control cohorts, the genotyping call rate was 100% and all six markers were found to be in Hardy–Weinberg equilibrium. Allele and genotype spectrum and distributions in Latvians were found to be similar to those of other Europeans (CEU) for all SNPs studied and to the Yoruba population (YRI) for the rs2348071; however, Latvians differ from YRI for resting loci and for all loci from Japanese (JPT) and Han Chinese (HCB) populations (Supplementary Table 2).

The rs11543947 and rs1048990 markers showed similar levels of variation in controls and JIA patients without significant differences between the JIoA and JIpA subtypes and females and males. These markers were considered to be JIA neutral, while other markers were found to be JIA susceptible (Table 1).

The rs2277460 showed nominal association ($P < 0.05$) with JIA, with highest risk effect for JIoA [OR = 2.024, 95% confidence interval (CI) 1.101–3.722]. Alleles and genotypes frequencies of the rs2295826 and rs2295827 were the same between each other in the controls and slightly different in JIoA females with an $r^2 = 0.936$ and D′ of 1.000 [this result is similar to data obtained for Tuscans in Italy (HapMap TSI): $r^2 = 0.923$], suggesting the existence of rare GC haplotype in JIA patients. Both markers were found to be in moderate ($p < 0.002$) association with JIoA (OR = 2.371, 95% CI 1.390–4.044; and OR = 2.183, 95% CI 1.272–2.737 for the rs2295826 and rs2295827 risk genotypes, respectively). Moderate association was also detected for the rs2348071 heterozygous genotype ($p < 0.002$) with risk effect for JIpA in the combined cohort

Table 1 Data on the significant associations between single locus variations and JIA.

Groups of comparison		Marker ID	Genetic model	Risk factor (allele or genotype)	Risk factor number (%)		*p*	OR	CI
Group 1	Group 2				Group 1	Group 2			
JIA (174)	C (191)	rs2277460	Multiplicative	a: A	40 (11.49)	25 (6.54)	<0.05	1.855	1.104–3.114
			Dominant	g: CA	40 (22.99)	25 (13.09)	<0.05	1.982	1.149–3.419
		rs2295826	Multiplicative	a: G	64 (18.39)	40 (10.47)	<0.05	1.927	1.262–2.942
			Dominant	g: AG + GG	55 (31.61)	36 (18.85)	<0.05	1.990	1.230–3.210
		rs2295827	Multiplicative	a: T	61 (17.53)	40 (10.47)	<0.05	1.817	1.186–2.784
			Dominant	g. CT + TT	53 (30.46)	36 (18.85)	<0.05	1.886	1.163–3.057
		rs2348071	Overdominant	g: AG	87 (50.00)	66 (34.55)	<0.05	1.894	1.245–2.882
JIA-F (108)	C-F (117)	rs2295826	Multiplicative	a: G	46 (21.30)	30 (12.82)	<0.05	1.840	1.116–3.033
			Dominant	g: AG + GG	40 (37.04)	27 (23.07)	<0.05	1.932	1.086–3.432
		rs2295827	Multiplicative	a: T	43 (19.91)	30 (12.82)	<0.05	1.690	1.020–2.801
			Dominant	g. CT + TT	38 (35.18)	27 (23.07)	<0.05	1.810	1.013–3.232
		rs2348071	Overdominant	g: AG	57 (52.78)	40 (34.19)	<0.05	**2.151**	1.261–3.672
JIoA (107)	C (191)	rs2277460	Multiplicative	a: A	25 (11.68)	25 (6.54)	<0.05	1.889	1.061–3.362
			Dominant	g: CA	25 (23.36)	25 (13.09)	<0.05	**2.024**	1.101–3.722
		rs2295826	Multiplicative	a: G	45 (21.03)	40 (10.47)	**<0.001**	**2.277**	1.435–3.612
			Dominant	g: AG + GG	38 (35.51)	36 (18.84)	**<0.002**	**2.371**	1.390–4.044
		rs2295827	Multiplicative	a: T	42 (19.63)	40 (10.47)	**<0.002**	**2.088**	1.308–3.333
			Dominant	g. CT + TT	36 (33.64)	36 (18.84)	**<0.002**	**2.183**	1.272–2.737
		rs2348071	Overdominant	g: AG	50 (46.73)	66 (34.56)	<0.05	1.661	1.027–2.687
JIoA-F (63)	C-F (117)	rs2295826	Multiplicative	a: G	31 (24.60)	30 (12.82)	<0.05	**2.219**	1.275–3.861
			Dominant	g: AG + GG	26 (41.27)	27 (23.07)	<0.05	**2.342**	1.216–4.511
		rs2295827	Multiplicative	a: T	28 (22.22)	30 (12.82)	<0.05	1.943	1.105–3.416
			Dominant	g. CT + TT	24 (38.09)	27 (23.07)	<0.05	1.943	1.105–3.416
JIpA (55)	C (191)	rs2348071	Overdominant	g: AG	32 (58.18)	66 (34.56)	**<0.002**	**2.635**	1.434–4.841
JIpA-F (39)	C-F (117)	rs2348071	Overdominant	g: AG	25 (64.10)	40 (34.19)	**<0.002**	**3.438**	1.626–7.265

$p < 0.002$ and odds ratio > 2 are indicated in bold.
JIA = juvenile idiopathic arthritis; JIoA = juvenile idiopathic oligoarthritis; JIpA = juvenile idiopathic polyarthritis; C = control; F = female; a = risk allele; g = risk genotype.

Table 2 Four-loci genotypes (4-LGs) presentation and results of significant associations with juvenile idiopathic arthritis (JIA).

4-LG configurations					4-LGs number (%) in the groups and association results											
No.	Genotype of individual locus				Controls			JIA			JIoA			JIpA		
	PSMA6	*PSMC6*		*PSMA3*	All $n = 191$	F $n = 117$	M $n = 74$	All $n = 174$	F $n = 108$	M $n = 66$	All $n = 107$	F $n = 63$	M $n = 44$	All $n = 55$	F $n = 39$	M $n = 16$
	L1	L2	L3	L4												
$1^{P;Ref}$	CC	AA	CC	GG or AA	86 (45.03)	52 (44.44)	34 (45.95)	46 (26.44)	24 (22.22)	22 (33.33)	32 (29.91)	17 (26.98)	15 (34.09)	11 (20.00)	6 (15.38)	5 (31.25)
					Association in the groups:	*p*	OR	95% CI								
					JIA vs. C	**<0.001**	**0.439**	0.283—0.681								
					JIA-F vs. C-F	**<0.001**	**0.357**	0.200—0.637								
					JIoA vs. C	<0.05	0.521	0.316—0.859								
					JIoA-F vs. C-F	<0.05	0.462	0.239—0.859								
					JIpA vs. C	**<0.001**	**0.305**	0.150—0.620								
					JIpA-F vs. C-F	**<0.002**	**0.227**	0.091—0.568								
2^{N}	CC	AA	CC	**GA**	48 (25.13)	28 (23.93)	20 (27.03)	44 (25.29)	30 (27.78)	14 (21.21)	20 (18.69)	12 (19.05)	8 (18.18)	19 (34.55)	15 (38.46)	4 (25.00)
3^{N}	CC	**AG** **GG**	**CT** **TT**	GG or AA	22 (11.52)	18 (15.39)	4 (5.41)	19 (10.92)	14 (12.96)	5 (7.58)	11 (10.28)	7 (11.11)	4 (9.09)	7 (12.73)	6 (15.38)	1 (6.25)
4^{N}	**CA**	AA	CC	GG or AA	16 (8.38)	7 (5.98)	9 (12.16)	16 (9.20)	9 (8.33)	7 (10.61)	9 (8.41)	6 (9.52)	3 (6.82)	4 (7.27)	2 (5.13)	2 (12.50)
5^{R}	CC	**AG** **GG** **GG**	**CT** **TT** CC	**GA**	10 (5.24)	7 (5.98)	3 (4.05)	25 (14.37)	19 (17.59)	6 (9.09)	19 (17.76)	14 (22.22)	5 (11.36)	6 (10.91)	5 (12.82)	1 (6.25)
					Association in the groups:	*p*	OR	CI								
					JIA vs. C	**<0.001**	**4.674**	2.096—10.425								
					JIA-F vs. C-F	**<0.001**	**5.881**	2.231—15.500								
					JIoA vs. C	**<0.001**	**5.106**	2.179—11.968								
					JIoA-F vs. C-F	**<0.001**	6.118	2.175—17.210								
					JIpA vs. C	<0.05	4.691	1.477—14.897								
					JIpA-F vs. C-F	<0.05	6.190	1.574—24.343								
6^{R}	**CA**	AA	CC	**AG**	5 (2.62)	3 (2.57)	2 (2.70)	13 (7.47)	5 (4.63)	8 (12.12)	8 (7.48)	2 (3.17)	6 (13.64)	5 (9.09)	3 (7.69)	2 (12.50)
					Association in the groups:	*p*	OR	CI								
					JIA vs. C	<0.05	4.861	1.625—13.940								
					JIA-M vs. C-M	<0.05	6.182	1.370—27.895								

(*continued on next page*)

Table 2 (*continued*)

No.	4-LG configurations: Genotype of individual locus				4-LGs number (%) in the groups and association results											
					Controls			JIA			JIoA			JIpA		
	PSMA6	*PSMC6*		*PSMA3*	All	F	M	All	F	M	All	F	M	All	F	M
	L1	L2	L3	L4	$n = 191$	$n = 117$	$n = 74$	$n = 174$	$n = 108$	$n = 66$	$n = 107$	$n = 63$	$n = 44$	$n = 55$	$n = 39$	$n = 16$
					JIoA vs. C	<0.05	4.300	1.367–13.524								
					JIoA-M vs. C-M	**<0.002**	6.800	1.405–32.913								
					JIpA vs. C	**<0.002**	6.800	1.405–32.913								
7	CA	AG	CT	GG or AA	1	—	1	6	4	2	5	4	1	1	—	1
		GG	TT		(0.51)		(1.35)	(3.44)	(3.70)	(3.03)	(4.67)	(6.36)	(2.27)	(1.81)		(6.25)
8	CA	AG	CT	GA	3	2	1	5	3	2	3	1	2	2	2	—
					(1.57)	(1.71)	(1.35)	(2.87)	(2.79)	(3.03)	(2.80)	(1.59)	(4.55)	(3.64)	(5.13)	

In statistical analysis the 4-LG1 frequency was compared to sum of all other 4-LGs frequencies; protective 4-LG1 was considered as reference genotype in the risk 4-LGs identification. [P] protective JIA genotype; [R] JIA risk genotype. Risk single locus genotypes are given in bold and underlined.
JIA = juvenile idiopathic arthritis; JIoA = juvenile idiopathic oligoarthritis; JIpA = juvenile idiopathic polyarthritis; F = female; M = male; L1 = rs2277460 locus; L2 = rs2295826 locus; L3 = rs2295827 locus; L4 = rs2348071 locus.

(OR = 2.635, 95% CI 1.434–4.841) and JIpA females (OR = 3.438, 95% CI 1.626–7.265).

In both the control and case groups, risk genotypes were more frequent in males for the rs2277460 locus and in females for the rs2295826 and rs2295827 loci; the rs2348071 heterozygous risk genotype was more frequent in females than in males in JIpA patients (Supplementary Table 2).

3.2. Identification of the risk/protective 4-LGs

Personalized genotyping data documentation allowed analysis of the spectrum and frequencies in the groups of the 4-LGs rs2277460/rs2295826/rs2295827/rs2348071 (Table 2) composed from loci individually susceptible to disease (Table 1). Nineteen 4-LGs observed in both case and control groups were classified by eight categories according to presence/absence of the risk SLG listed in Table 1.

The 4-LG1, having a no risk SLG, was the most frequent in controls (45%) with similar presentation in males and females, but it was significantly less frequent in JIA patients of both JIoA and JIpA subtypes (about 29% and 20% respectively) and appears to be JIA protective with a strong level of association ($p < 0.001$) with healthy phenotype in common cohort and females. The 4-LG5 unites three configurations, all having the rs2348071 risk genotype in combination with risk genotype at the rs2295826 and/or rs2295827 loci. The 4-LG5 was approximately three times more frequent in JIA than in controls and two times more frequent in females than in males of all JIA subtypes. This genotype showed a strong association ($p < 0.001$) with JIA in common and JIoA cohorts and the female phenotype. Rarely observed in controls and JIA females (< 5%), the rs2277460/rs2348071 double heterozygotes (4-LG6) were rather frequent in JIA males (> 12%) and showed an association ($p < 0.002$) with JIpA in common cohort and with JIoA in males.

The 4-LG2, 4-LG3, and 4-LG4 genotypes were observed with similar frequencies in cases and controls and were considered JIA neutral. The two remaining (4-LG7 and 4-LG8) genotypes were rare in controls and only slightly more frequent in JIA patients.

3.3. Four loci haplotype analysis

Table 3 provides information on the four-loci haplotype (4-LH) spectrum and frequencies in the groups. Using the assumption of random assortment of alleles, 24 haplotype configurations were expected for four two-allele loci; however, only 10 variants were identified in cases and controls taken together, and all of them are implicated from the 4-LGs homozygous at all four loci and/or genotypes being heterozygous only at one locus. The 4-LH1–4-LH6 haplotypes were observed in both controls and case groups; the 4-LH7–4-LH10 were identified only in JIoA females. The 4-LH1 (C-C-A-G) having the common alleles at all four loci was found to be the most frequent in all groups and used as reference haplotype in association analysis. The 4-LH4 (C-G-T-A) having the risk alleles at the rs2295826 and rs2295827 and the rs2348071 minor allele A was found to be in strong association ($p < 0.001$) with JIA including both JIoA and JIpA subtypes in female and male cohorts. The 4-LH5 (A-C-A-G)

Table 3 Four-loci haplotypes (4-LHs) presentation and data on significant associations with juvenile idiopathic arthritis (JIA).

Group		C *n* = 191	C-F *n* = 117	C-M *n* = 74	JIA *n* = 174	JIA-F *n* = 108	JIA-M *n* = 66	JIoA *n* = 107	JIoA-F *n* = 63	JIoA-M *n* = 44	JIpA *n* = 55	JIpA-F *n* = 39	JIpA-M *n* = 16
4-LG		**Number (%)**											
Full homozygote		89 (46.60)	54 (46.15)	35 (47.30)	49 (28.16)	25 (23.15)	24 (36.36)	34 (31.78)	17 (26.98)	17 (38.64)	12 (21.82)	7 (17.95)	5 (31.25)
Single locus heterozygote		65 (34.03)	36 (30.77)	29 (39.19)	66 (37.93)	44 (40.74)	22 (33.33)	34 (31.78)	23 (36.51)	11 (25.00)	24 (43.64)	17 (43.59)	7 (43.75)
Multiple loci heterozygote		37 (19.37)	27 (23.08)	10 (13.51)	59 (33.91)	39 (36.11)	20 (30.30)	39 (36.45)	23 (36.51)	16 (36.36)	19 (34.55)	15 (38.46)	4 (25.00)
Haplotype	**Loci**	**Number (%)**											
	1—2—3—4	382	234	148	348	216	132	214	126	88	110	78	32
4-LH-1^{Ref}	C-A-C-G	231 (60.47)	147 (62.82)	84 (56.76)	178 (51.15)	109 (50.46)	69 (52.27)	117 (54.67)	68 (53.97)	49 (55.68)	53 (48.18)	38 (48.72)	15 (46.88)
4-LH-2	C-A-C-A	86 (22.51)	45 (19.23)	41 (27.70)	72 (20.69)	45 (20.83)	27 (20.45)	32 (14.95)	19 (15.08)	13 (14.77)	27 (24.55)	19 (24.36)	8 (25.00)
4-LH-3	C-**G-T**-G	29 (7.59)	19 (8.12)	10 (6.76)	19 (5.46)	13 (6.02)	6 (4.55)	12 (5.61)	9 (7.14)	3 (3.41)	7 (6.36)	4 (5.13)	3 (9.38)
4-LH-4^{R}	C-**G-T**-A	11 (2.88)	11 (4.70)	—	37 (10.63)	25 (11.57)	12 (9.09)	25 (11.68)	14 (11.11)	11 (12.50)	11 (10.00)	10 (12.82)	1 (3.13)
		Association in the groups	*P*	OR	CI								
		JIA vs. C	**<0.001**	**4.365**	2.192—8.693								
		JIoA vs. C	**<0.001**	**4.487**	2.161—9.318								
		JIpA vs. C	<0.05	4.358	1.826—10.401								
4-LH-5	**A**-A-C-A	15 (3.93)	8 (3.42)	7 (4.73)	12 (3.45)	5 (2.31)	7 (5.30)	11 (5.14)	4 (3.17)	7 (7.95)	—	—	—
4-LH-6^{R}	**A**-A-C-G	10 (2.62)	4 (1.71)	6 (4.05)	22 (6.32)	11 (5.09)	11 (8.33)	9 (4.21)	4 (3.17)	5 (5.68)	12 (10.91)	7 (8.97)	5 (15.63)
		Association in the groups	*P*	OR	CI								
		JIA vs. C	<0.05	2.855	1.338—6.093								
		JIpA vs. C	**<0.001**	**5.230**	2.185—12.517								
4-LH-7	**A-G**-T-G	—	—	—	3	3	—	3	3	—	—	—	—
4-LH-8	**A-G**-T-A	—	—	—	2	2	—	2	2	—	—	—	—
4-LH-9	C-**G**-C-G	—	—	—	1	1	—	1	1	—	—	—	—
4-LH-10	C-**G**-C-A	—	—	—	2	2	—	2	2	—	—	—	—

Superscripts "Ref" and "R" indicate the reference and risk haplotypes respectively. Single nucleotide polymorphism loci in the 1—2—3—4 haplotypes are given in the rs2277460—rs2295826—rs2295827—rs2348071 sequence. Risk alleles are indicated in bold and underlined. Probability of association < 0.002 is indicated in bold. Frequencies of the 4-LH7, 4-LH8, 4-LH9 and 4-LH10 haplotypes rare/absent in all groups (<3%) are not indicated in the table. In statistical analysis the 4-LH1 haplotype was considered as reference.
4-LG = four-loci genotype; JIA = juvenile idiopathic arthritis; JIoA = juvenile idiopathic oligoarthritis; JIpA = juvenile idiopathic polyarthritis; C = control; F = female; M = male.

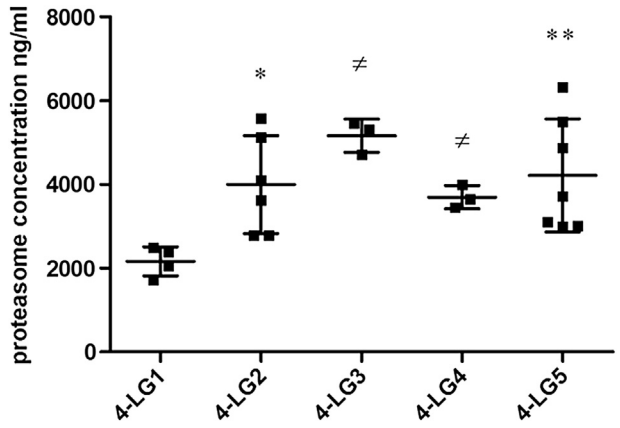

Figure 1 Plasma proteasome level in juvenile idiopathic oligoarthritis patients. Carriers of different four-locus genotypes (4-LGs), 4-LG1, 4-LG2, 4-LG3, 4-LG4, and 4-LG5, were represented by four, six, three, three, and seven patients respectively. * $p < 0.05$, ** $p < 0.001$, # $p > 0.05$.

having the only risk allele at the rs2277460 locus showed strong ($p < 0.001$) association with JIpA.

3.4. Genotype dependent plasma proteasome levels in JIoA patients

Plasma samples were available for 23 JIoA females, carriers of five different 4-LGs (Figure 1). Females of 4-LG1 exhibited a plasma proteasome concentration of approximately 2000 ng/mL, which is similar to previously reported plasma proteasome levels for healthy donors.[25–27] Careers of the 4-LG2 and 4-LG5 genotypes exhibited significantly ($p < 0.05$ and $p < 0.001$) higher plasma proteasome levels. High plasma proteasome levels were also detected in females of 4-LG3; however, the small number of patients in this group did not allow the results to reach statistical significance.

3.5. Eventual functional significance of the SNPs allelic variants

Figure 2 summarizes results of the *in silico* analysis of the functional significance of allele substitutions (only loci detected as JIA susceptible were taken into account) evaluated on the eventual sequence affinity to TFs and splicing signals similarity, and on the homology to known microRNAs and their precursors.

The major allele of only the rs2295826 locus potentially assists in sequence affinity to TFs. These are proteins of the CREB, MYT1 and PARF families. The rs2295826 minor allele appears to abolish any sequence affinity to TFs. Minor alleles at the rs2277460, rs2295827, and rs2348071 loci potentially assist in the binding of proteins belonging to the BARBIE box, CART, BRN5, LHXF, HOXF, HBOX, and MEF2 families.

Major alleles of both the rs2295826 and rs2295827 loci potentially assist in the generation of additional branch points; the rs2295826 major allele creates a splice site acceptor and targets for the hnRNP A1; the hnRNP A1 target motif is also generated in presence of both the rs2277460 and rs2348071 minor alleles. Major alleles of the rs2295826 and rs2348071 and minor alleles of all loci besides the rs2295826 could potentially change the sequence similarity to a number of splicing enhancers and/or silencers (Figure 2 and Suppl Table 3).

The rs2295827 major and rs2295826 minor allele increase sequence similarity to hsa-miR-603 and hsa-miR-5584-3p, respectively (Figure 2).

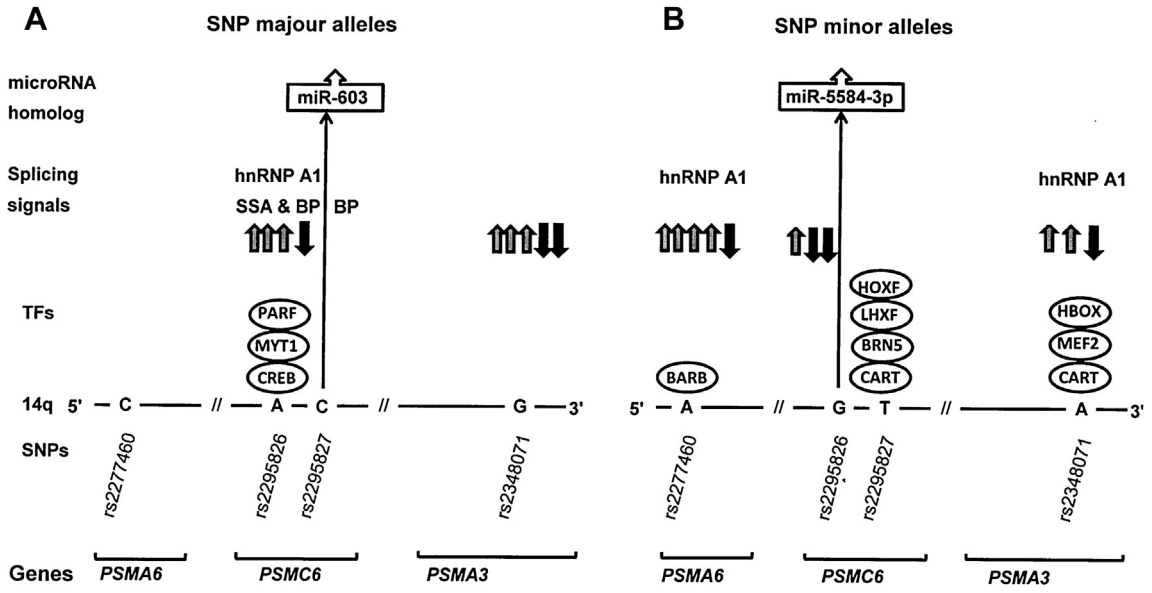

Figure 2 Consequences of the nucleotide substitutions on functional potential of the genome site harboring juvenile idiopathic arthritis associated single nucleotide polymorphisms of the *PSMA6*, *PSMC6*, and *PSMA3* genes. Only family names are indicated for transcription factors. Upward and downward arrows indicate splicing enhancers and silencers, respectively. BP = branch point; SSA = splicing site acceptor. Details are given in Supplementary Table 3.

4. Discussion

The aim of the current study was to evaluate six SNPs belonging to the *PSMA6* (rs2277460 and rs1048990), *PSMA3* (rs2348071), *PSMB5* (rs11543947), and *PSMC6* (rs2295826 and rs2295827) proteasomal genes for association with JIA with adjustment by JIA subtype and sex.

From all SNPs analyzed, the rs1048990 (*PSMA6* c.-8C>G) located in the 5′- untranslated region of the gene is the most studied SNP of proteasomal genes, which has been widely genotyped for association with cardiovascular diseases,[Suppl 1–12,14,15] T2DM,[Suppl 3,14] and children obesity.[Suppl 16] Summarizing the results obtained by different teams, we suggested a potential of the rs1048990 to influence JIA susceptibility. However, we did not find any association between the rs1048990 polymorphism and JIA in Latvians. Similarly, locus did not show any association with obesity in Latvian children.[Suppl 16] In turn, the rs2277460 of the promoter region of the same gene (*PSMA6* c.-110C>A) has been detected as JIA susceptible locus. This conclusion is based on the results of the subtype- and sex-specific disease association with rare allele A, heterozygous SLG, 4-LG6 heterozygous at the rs2277460, and 4-LH6 haplotype both having the rs2277460 rare allele in its structure (Tables 1–3).

It had been reported that the rs2230087 polymorphism of the *PSMB5* gene (3′-untranslated region) is associated with T2DM.[Suppl 9] In our study we were interested in the rs11543947 of the same gene locating in exon 1 or intron 1 (*PSMB5* c.70C>T or *PSMB5* c.-112+300C>T, respectively) depending of transcript variant. This SNP did not show any association with JIA in our study.

The rs2295826 and rs2295827 loci locate in close vicinity to each other in intron 1 of the *PSMC6* gene (*PSMC6* c.86-104A>G and *PSMC6* c.86-46C>T respectively). Linkage between loci is not complete, and rare GC haplotypes were observed in our study similar to data obtained for Tuscans in Italy. Rare alleles of these loci and their risk SLGs showed (dominant model) JIoA subtype-specific association by themselves and as component of risk 4-LG5 genotype and risk 4-LH4 haplotype.

The rs2348071 locus belongs to intron 7 of the *PSMA3* gene (PSMA3 c.543+138G>A or c.522+138G>A depending of transcript variant). Heterozygous genotype GA was found to be associated mostly with JIpA. In 4-LG structures, the rs2348071 heterozygotes were involved in both risk 4-LG5 and 4-LG6. Interestingly, the rs2348071 heterozygotes were implicated previously as an obesity risk factor in Latvian children with a family history of obesity.[Suppl 16]

It is important to note that strength of association with the disease was much stronger for combination of several risk SLGs than for any individual risk SLG. Therefore, we have reported here for the first time evidence of an association between JIA and genetic variants in the *PSMA6/PSMC6/PSMA3* gene cluster represented by combinations of at least two risk SLGs in a particular 4-LG, namely 4-LG5 (risk rs2295826/rs2295827/rs2348071) and 4-LG6 (risk rs2277460/rs2348071). The 4-LG1 having no risk SLGs in its composition showed strong association with healthy phenotype ($p < 0.001$).

The JIA-associated SNPs discovered in our study potentially could be themselves primarily susceptible to disease or linked with other primary genetic variations linked to disease. It appears that both scenarios are possible. Concerning chromosome 14, several loci potentially susceptible to autoimmune diseases have been reported in different human populations.[17–24] The functional significance of the discovered allele substitutions is to be clarified. We attempted to shed more light on the problem using two approaches.

First, we evaluated plasma proteasome level in JIoA females of different 4-LGs and found significantly increased levels of plasma proteasomes in JIoA female carriers of the 4-LG5 risk genotype in comparison to carriers of protective 4-LG1 ($p < 0.001$) genotype. Earlier, circulating proteasomes were suggested as markers in autoimmune diseases.[8] Concentration of circulating proteasomes was shown to be substantially elevated in patients with rheumatoid[8] and psoriatic[9] arthritis. The 20S proteasome has been identified as a target of the humoral autoreactive immune response in patients with systemic inflammatory diseases including autoimmune myositis,[10] primary Sjögren's syndrome,[11] dilated cardiomyopathy,[12] systemic lupus erythematosus,[10,13,14] multiple sclerosis,[15] and psoriatic arthritis.[14] The proteasomal inhibitor MG132 has been reported to reduce the severity of arthritis and reverse the pain behavior in the arthritic rat models.[16] To our knowledge, plasma levels of factors within the UPS have not been yet evaluated in JIA, and here we report data on that for the first time.

Second, we have evaluated eventual functional significance of allele substitutions on sequence affinity to TFs, splicing signals similarity and on the homology to known microRNAs and their precursors.

The major allele of the rs2295826 potentially assists to sequence affinity for TFs of CREB, MYT1, and PARF families known to be involved in regulation of multiple physiological processes and control of the circadian clock.[28–30] CREB-related TFs are especially interesting with respect of JIA pathogenesis, as they are known to be essential for osteoblast differentiation and function,[28] and they have been implicated in immune response.[29] It is of interest that expression of CREB, MYT1, and PARF proteins potentially could share the same epigenetic mechanism of regulation by hsa-miR-1264 originated from the X chromosome and potentially be differently expressed and differently involved in epigenetic network in females and males (data not shown).

The presence of a minor allele at the rs2277460 locus creates a binding site to the BARBIE box proteins reported to be involved in signal transduction pathways during development[31] and modulation of innate immunity.[32] Sequences having minor alleles at the rs2295827 and rs2348071 sites can potentially bind CART proteins responsible for bone and cartilage development.[33] Moreover, the rs2295827 and rs2348071 minor alleles could assist in sequence affinity to BRN5, LHXF, MEF2, and HBOX factors known to mediate transcriptional control of neuronal differentiation[34–37] and HOXF family NANOG.01 factor generally involved in signal transduction pathways during development.[38]

Similar to TFBSs, patterns of predicted splicing signals are allele specific. The rs2295826 and rs2348071 loci create a number of allele-specific targets for splicing enhancers and silencers. Only minor allele of the rs2277460 and only major allele of the rs2295827 is functional in this respect. Nucleotide substitutions at the rs2277460, rs2295826, and

rs2348071 define affinity of corresponding sequences to the hnRNP A1 known as alternative splicing repressor[39] and factor facilitating processing of specific microRNAs.[40] The above mentioned allele-specific differences in spectra of splicing signals could potentially significantly affect genes splicing activity and affect UPS efficiency.

Therefore, all of the above types of associations revealed and data on functional significance of allele substitutions are in good agreement between themselves and provide evidence that: (1) variations at the rs2277460, rs2295826, rs2295827, and rs2348071 loci could assist JIA susceptibility; (2) combination of the rs2348071 and rs2295826 and/or rs2295827 risk genotypes (4-LG5) represents the genetic module highly associated with both JIoA and JIpA and JIA female phenotype and plasma proteasome level in JIoA females; (3) combination of the rs2348071 and rs2277460 risk genotypes (4-LG6) represents the genetic module presumably associated with JIpA and male phenotype; (4) nucleotide substitutions affect the potential of encompassing sequences to create splicing signals, TFBSs and microRNAs; and (5) the *PSMA6/PSMC6/PSMA3* genetic variants and multiloci genetic modules could be suggested as JIA subtype- and sex-specific risk factors.

In conclusion it should be mentioned that, despite the rather small number (174/191 of cases/controls), this study can be considered as representative for the small Latvian population (< 2 million). Keeping in mind that JIA unites several clinically different subtypes, and that this disease tends to affect females more than males, we have applied stratification by JIA-subtype and sex. Due to the small subgroups, we sometimes could not reach significance. However, when significance was achieved, we obtained interesting results to be investigated with reference to other populations.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pedneo.2014.01.007.

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