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ARTICLE

Family-based association study of *ITGB3* in autism spectrum disorder and its endophenotypes

Valerio Napolioni[1,2], Federica Lombardi[1,2], Roberto Sacco[1,2], Paolo Curatolo[3], Barbara Manzi[3], Riccardo Alessandrelli[3], Roberto Militerni[4], Carmela Bravaccio[5], Carlo Lenti[6], Monica Saccani[6], Cindy Schneider[7], Raun Melmed[8], Tiziana Pascucci[9,10], Stefano Puglisi-Allegra[9,10], Karl-Ludvig Reichelt[11], Francis Rousseau[12], Patricia Lewin[12] and Antonio M Persico*[,1,2]

The integrin-β 3 gene (*ITGB3*), located on human chromosome 17q21.3, was previously identified as a quantitative trait locus (QTL) for 5-HT blood levels and has been implicated as a candidate gene for autism spectrum disorder (ASD). We performed a family-based association study in 281 simplex and 12 multiplex Caucasian families. *ITGB3* haplotypes are significantly associated with autism (HBAT, global P=0.038). Haplotype H3 is largely over-transmitted to the affected offspring and doubles the risk of an ASD diagnosis (HBAT P=0.005; odds ratio (OR)=2.000), at the expense of haplotype H1, which is under-transmitted (HBAT P=0.018; OR=0.725). These two common haplotypes differ only at rs12603582 located in intron 11, which reaches a P-value of 0.072 in single-marker FBAT analyses. Interestingly, rs12603582 is strongly associated with pre-term delivery in our ASD patients (P=0.008). On the other hand, it is SNP rs2317385, located at the 5′ end of the gene, that significantly affects 5-HT blood levels (Mann–Whitney *U*-test, P=0.001; multiple regression analysis, P=0.010). No gene–gene interaction between *ITGB3* and *SLC6A4* has been detected. In conclusion, we identify a significant association between a common *ITGB3* haplotype and ASD. Distinct markers, located toward the 5′ and 3′ ends of the gene, seemingly modulate 5-HT blood levels and autism liability, respectively. Our results also raise interest into *ITGB3* influences on feto–maternal immune interactions in autism.
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INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder, characterized by different levels of impairment in social interaction and communication, by stereotypies and rigid patterns of behavior, and disease onset before 3 years of age (OMIM 209850).[1] ASD is believed to primarily stem from genetic factors, based on the observation of 60–92% concordance rates in monozygotic twins *vs* 0–10% in dizygotic twins, with heritability estimated at or above 90%.[2,3] The cause underlying autism in the majority of patients remains unknown, although several known medical conditions account for ~10% of cases.[4] ASD, similar to many other complex human disorders, does not show a simple inheritance pattern, as it may involve multiple common variants, each conveying a modest effect in epistatic interaction, rare variants with high penetrance, or perhaps more likely the coincidence of a rare variant acting upon a genetic background rendered vulnerable by a set of common variants.[2–5] Accordingly, familial aggregation of 'endophenotypes', heritable quantitative traits distributed continuously among ASD patients and first-degree relatives, can promote the search of genetic susceptibility factors in ASD.

Elevated whole-blood serotonin (5-HT) levels, one of the most consistent biological endophenotypes in autism research, is recorded in about one-third of cases.[6] Autism-associated hyperserotonemia is indeed familial,[7–9] and could either have a role in the etiological processes leading to the disease, or it could at least characterize a relatively homogeneous subgroup of ASD patients. Genes encoding proteins involved in 5-HT metabolism and neurotransmission include the integrin-β 3 subunit gene (*ITGB3*), located on human chromosome 17q21.32, which was identified as a quantitative trait locus (QTL) for 5-HT blood levels in the Hutterites.[10,11] Interestingly, *ITGB3* maps under a replicated linkage peak for autism.[12,13] Furthermore, *ITGB3* alleles have been found at least nominally associated with autism in all five studies performed to date,[14–18] either alone or in interaction with allelic variants at the 5-HT transporter gene (*SLC6A4*). Several lines of evidence support functional interactions between *ITGB3* and *SLC6A4*, which also affects 5-HT blood levels and is located on human chromosome 17q11.1-q12.[11] First, *ITGB3* and *SLC6A4* gene expression levels are correlated in human and mouse tissues.[14] In fact, *Slc6a4* mRNA levels map to the *Itgb3* locus using QTL analysis in mouse hematopoietic stem cells, and non-coding

[1]Laboratory of Molecular Psychiatry and Neurogenetics, University 'Campus Bio-Medico', Rome, Italy; [2]Laboratory of Molecular Psychiatry and Psychiatric Genetics, Department of Experimental Neurosciences, IRCCS 'Fondazione Santa Lucia', Rome, Italy; [3]Department of Child Neuropsychiatry, University 'Tor Vergata', Rome, Italy; [4]Department of Child Neuropsychiatry, II University of Naples, Naples, Italy; [5]Department of Pediatrics, University 'Federico II', Naples, Italy; [6]Department of Child Neuropsychiatry, University of Milan, Milan, Italy; [7]Center for Autism Research and Education, Phoenix, AZ, USA; [8]Southwest Autism Research and Resource Center, Phoenix, AZ, USA; [9]Department of Psychology and Centro 'Daniel Bovet', University 'La Sapienza', Rome, Italy; [10]Laboratory of Behavioral Neurobiology, Department of Experimental Neurosciences, IRCCS 'Fondazione Santa Lucia', Rome, Italy; [11]Department of Pediatric Research, Rikshospitalet, University of Oslo, Oslo, Norway; [12]IntegraGen SA Genopole, Evry, France
*Correspondence: Dr AM Persico, Laboratory of Molecular Psychiatry and Neurogenetics, University 'Campus Bio-Medico', Via Alvaro del Portillo 21, Rome I-00128, Italy. Tel: +39 06 225419155; Fax: +39 06 501703333; E-mail: a.persico@unicampus.it
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human polymorphisms in *ITGB3* are associated with both *ITGB3* and *SLC6A4* expression levels.[14] Second, the integrin receptor composed of an αIIb subunit and of the β 3 subunit encoded by the *ITGB3* gene, was recently identified as a novel component of the *SLC6A4* regulatory protein complex.[14] Also the Leu33Pro *ITGB3* SNP (rs5918) modulates *SLC6A4* trafficking and transport activity.[19] Finally, several recently published studies have described significant *SLC6A4* and *ITGB3* interactions for both autism risk and 5-HT blood levels, with a male-specific effect.[10,14–17,20]

Despite these positive findings, several inconsistencies complicate their interpretation, possibly because of clinical and genetic heterogeneity in ASD. In particular, different alleles seem to be associated with autism and/or serotoninemia in independent samples at the *ITGB3* and *SLC6A4* loci.[16,18] Linkage disequilibrium (LD) blocks associated with autism and/or serotoninemia are not consistent, with different studies pointing toward either the 5′ or the 3′ ends of the *ITGB3* locus.[14–18,20] Conceivably, these inconsistencies could stem from different causative variants occurring on distinct marker haplotype backgrounds.

This study was thus undertaken (a) to replicate and extend previous findings, by fine mapping the association between *ITGB3* and ASD using a family-based approach; (b) to determine the effect of *ITGB3* alleles on biochemical and morphological quantitative endophenotypes, including 5-HT blood levels; (c) to test for gene–gene interactions between *ITGB3* and *SLC6A4* in reference to autism risk and 5-HT blood levels, (d) to correlate *ITGB3* and *SLC6A4* genotypes with clinical features, as well as with patient and family history variables.

MATERIALS AND METHODS

Subjects

A total of 281 simplex and 12 multiplex families with a non-syndromic autistic proband were recruited for this study, including 306 ASD patients, 106 unaffected siblings, and 577 parents; total genotyped *N*=989. Demographic and clinical characteristics of our clinical sample, as well as endophenotypic measures for head circumference, serotonin (5-HT) blood levels and global peptiduria, are summarized in Table 1. The composition by recruiting site is presented in Supplementary Table S1. Diagnostic screening procedures used to exclude syndromic autism have been previously described.[21] Briefly, patients fulfilling DSM-IV diagnostic criteria for autistic disorder[1] were screened for non-syndromic autism using MRI, EEG, audiometry, urinary amino acid and organic acid measurements, cytogenetic and fragile-X testing. Patients with dysmorphic features were excluded even in the absence of detectable cytogenetic alterations. Patients with sporadic seizures (ie, <1 every 6 months) were included; patients with frequent seizures or focal neurological deficits were excluded. The M:F ratio in ASD patients is 7.3:1. Autistic behaviors were assessed using the official Italian version of the Autism Diagnostic Observation Schedule[22] and the Autism Diagnostic Interview-Revised;[23] adaptive functioning was assessed using the Vineland Adaptive Behavior Scales; IQ was determined using either the Griffith Mental Developmental Scales, the Colored Raven Matrices, the Bayley Developmental Scales or the Leiter International Performance Scale.[21] All parents gave written informed consent for themselves and for their children, using the consent form approved by the IRB of UCBM (Rome, Italy).

Genotyping

Genomic DNA was extracted from whole blood[24] and quantified in triplicate by PicoGreen (Molecular Probes, OR, USA). On the basis of HapMap phase II (release 21) CEU population data, four independent LD blocks were identified within *ITGB3* (chromosome 17: 42684–42750 kb) using the 'solid spine of LD' algorithm with a minimum *D*-value of 0.8. In all, 10 tagging SNPs were selected using Tagger from Haploview v4.2[25] (r^2>0.75 and minor allele frequency >0.05, aggressive tagging, LOD threshold for multimarker test=3). All SNPs previously associated with autism were comprised by applying the 'force-include' procedure of Haploview, in addition to rs11650072, which provides further coverage of the 3′-flanking region (Supplementary Table S2). The *ITGB3* genotyping was performed using the Applied Biosystems SNPlext Genotyping System (Applied Biosystems, CA, USA). All samples were electrophoretically separated on a 3730 DNA Genetic Analyzer (Applied Biosystems), and automated allele calls and genotype clustering of each individual sample was performed by Applied Biosystems GeneMapper Software (version 3.5). *ITGB3* SNP rs5918 was genotyped using the TaqMan SNP genotyping assay (Applied Biosystems) on the ABI Prism 7900HT and analyzed with the SDS software (Applied Biosystems). *SLC6A4* 5-HTTLPR genotyping was performed as previously described.[26]

Table 1 Demographic, clinical, and endophenotypic characteristics of the autistic sample

	N	*Mean/median*	*Range*
Age in years (mean ± SEM):	*N*=306	9.18 ± 0.33	2–33
Median VABS scores:	*N*=137		
Communication		69.0	19–128
Daily living skills		67.0	14–170
Socialization		66.0	25–140
Motor skills		80.0	25–128
Composite		60.0	19–137
Head circumference: (median percentile ± IQR/2)	*N*=265	82.5 ± 23.75	2.5–98.5
Serotonin blood levels: (mean ng/ml ± SEM)	*N*=158	329.5 ± 20.9	31.0–987.1
Urinary oligopeptiduria: (mean μm^2 ± SEM)	*N*=231	346.2 ± 16.5	57–1213
	N		*Percent* (%)
Gender:			
Male	269		87.9
Female	37		12.1
M/F ratio	7.3:1		
Family type:			
Simplex	281		95.9
Multiplex	12		4.1
DSM-IV diagnosis:			
Autistic disorder	207		67.6
Asperger syndrome	27		8.8
PDD – NOS	72		23.6
IQ (N=71):			
>70	18		25.4
<70	53		74.6

Abbreviations: IQR/2, semi-interquartilic range; PDD – NOS, pervasive developmental disorder – not otherwise specified.

Endophenotype measures

Serotonin levels were measured in all family members from platelet-rich plasma, obtained by centrifuging whole blood within 20 min of venipuncture at 140 g for 25 min at 4°C; 1 ml of supernatant was stored at −80°C and assessed by HPLC, as described.[27] Urinary peptide excretion analysis was performed by HPLC in ASD patients and first-degree relatives using the first morning urine samples, as described.[28] The total area of peaks under the 215 nm absorption curve (AUC) in the peptide region following the hippuric acid peak was calculated and expressed in μm^2. Head circumference was measured in ASD patients and unaffected siblings by trained physicians using a non-stretchable plastic measuring tape graded in millimeters, placed over the maximum frontal–occipital head perimeter.[21]

Statistical analysis

Mendelian inheritance was verified using Pedcheck.[29] Hardy–Weinberg equilibrium (HWE) was tested using Haploview v4.2 (available at http://www.broad.mit.edu/mpg/haploview/index.php),[25] applying a Bonferroni's correction for multiple testing ($P<0.05/11$ SNPs yields $P<0.0045$). LD analysis was performed using Haploview, and defining LD blocks based on the solid spine of LD algorithm.[25] Differences in LD structure recorded applying the confidence intervals[30] and the four-gamete rule[31] algorithms are also reported. Family-based single-marker and haplotype association tests were performed using FBAT (available at http://www.biostat.harvard.edu/~fbat/fbat.htm), under an additive model and applying option $-e$, as suggested for candidate genes under known linkage peaks.[32] The HBAT procedure in FBAT was also used to estimate haplotype frequencies, to compute a global *P*-value, and to provide an 'exact' *P*-value using Monte Carlo tests (option $-p$) for the global test (χ^2 sum *P*), for each haplotype separately, and for the minimum observed *P*-value among all haplotypes (minimal *P*).[32] Haplotype odds ratios (ORs) were determined using UNPHASED (available at http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/).[33] Quantitative traits were analyzed by quantitative transmission/disequilibrium test (qTDT), as implemented by the FBAT software[32] and by parametric or non-parametric (Kruskal–Wallis) ANOVA, or by Mann–Whitney *U*-tests based on genotype distributions, applying a stringent Bonferroni's correction for multiple testing (4 markers $\times 3$ phenotypes, $P=0.5/12=0.0041$). Gene–gene interaction analyses were performed with the two-locus transmission/disequilibrium test (TDT) method,[34] which has been implemented as a Stata program 'pseudocc' (http://www-gene.cimr.cam.ac.uk/clayton/software/stata/). Data are expressed as mean ± SEM, except for head circumference, which is expressed as median ± semi-interquartilic range (IQR/2). Head circumference measures were transformed into percentiles using sex- and age-specific standard tables, as described.[35] Two-tail *P*-values are reported. To correct for multiple comparisons in single-marker analyses, statistical significance was set at $P<0.0016$: this threshold accounts for testing of eight effectively independent markers (seven on *ITGB3* and one on *SLC6A4*), as determined using the Nyholt SNPSpD method,[36] (available at http://genepi.qimr.edu.au/general/daleN/SNPSpD/), and four phenotypes (autism, serotoninemia, peptiduria, and head circumference; Supplementary Methods). Nominal *P*-values obtained by Pearson's χ^2-tests are reported for clinical, patient and family history variables, given the exploratory nature of these associations.

RESULTS

ITGB3 haplotype analysis

The 11 *ITGB3* SNPs are in HWE both in the entire sample and analyzing separately mothers, fathers, autistic, and unaffected siblings, with the exception of rs3809863, which has been excluded from subsequent analyses (Supplementary Table S2). The results of LD analysis are displayed in Figure 1. All three algorithms applicable for LD block definition consistently identify at the 3′ end one LD block, encompassing the three SNPs located most downstream, and at the 5′ end rs2317385 (SNP1), which is not associated with any other SNP and is part of an independent LD block located upstream of *ITGB3*; in between SNP1 and the 3′ LD block, SNPs 2 to 7 span another LD block showing increasing size when defined according to the confidence intervals, four-gamete rule, or solid spine of LD algorithms, respectively (Figure 1). Mean r^2 is 0.15, confirming a relatively low overall inter-SNP correlation, consistent with the selection of tagging SNPs for genotyping (Figure 1).

ITGB3 haplotypes show a statistically significant association with autism (HBAT global $P=0.038$; whole marker permutation tests yield sum $P=0.017$ and minimal $P=0.011$, after 100 000 iterations). Haplotype H3 is transmitted from heterozygous parents to their autistic offspring significantly more often than expected by chance ($P=0.005$), whereas haplotype H1 shows the opposite trend ($P=0.018$; Table 2). In terms of ORs, haplotype H3 doubles the risk of autism (OR=2.000; $\chi^2=8.426$; $P=0.003$), whereas haplotype H1 marginally reduces disease risk (OR=0.725; $\chi^2=3.572$; $P=0.059$).

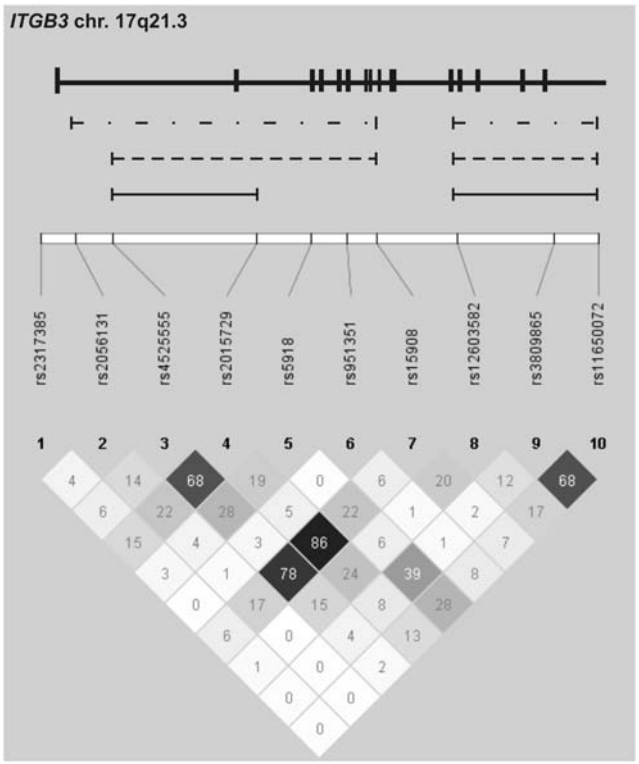

Figure 1 *ITGB3* exon–intron structure, genotyped SNPs, and linkage disequilibrium expressed in r^2. Haplotype blocks defined according to the confidence interval, four-gamete rule, and solid spine of LD algorithms are shown above by solid, broken, and dotted/broken lines, respectively.

Single-marker analyses

Interestingly, haplotypes H1 and H3 differ only at SNP rs12603582 located in intron 11. Using single-marker FBAT and TDT analyses, rs12603582 was the only marker showing a trend toward the preferential transmission of allele G in the overall sample (FBAT additive model, $P=0.072$; TDT, $P=0.057$), in autistic males only ($N=236$, FBAT $P=0.049$) and in simplex families ($N=281$, FBAT $P=0.053$; Supplementary Table S3). No significant evidence of protective alleles, preferentially transmitted from heterozygous parents to unaffected siblings, was found using both haplotype and single-marker analyses (data not shown).

Gene–gene interaction between *ITGB3* and *SLC6A4* in autism

SLC6A4 5-HTTLPR genotypes are in HWE and show no association with autism in this sample (TDT LRS=0.562, 1 d.f., $P=0.454$; FBAT $P=0.432$). To test for interaction between *ITGB3* and *SLC6A4*, we applied a two-locus TDT approach,[34] crossing *ITGB3* genotypes either at rs5918 (SNP5, Leu33Pro), rs12603582 (SNP8), or rs3809865 (SNP9), with *SLC6A4* genotypes at the 5-HTTLPR. No evidence of epistatic effects on autism risk was detected in our entire sample, in males only, or in simplex families.

Quantitative endophenotypes: single-gene effects and gene–gene interactions for *ITGB3* and *SLC6A4*

Single-marker qTDT analyses show a nominal association of *ITGB3* SNP1, rs2317385, with 5-HT blood levels ($P=0.016$) and peptiduria ($P=0.041$), not reaching the significance threshold set by the Nyholt SNPSpD method to control for multiple testing ($P<0.0016$). However, the association with 5-HT blood levels survives even a

Table 2 *ITGB3* haplotypes are associated with autism: (a) haplotype structure at the *ITGB3* locus. (b) Haplotype family-based association tests performed using HBAT, under an additive model ($-e$)[32]

(a)[a]

	ITGB3 SNPs										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	Estimated
Haplotype	*rs2317385*	*rs2056131*	*rs4525555*	*rs2015729*	*rs5918*	*rs951351*	*rs15908*	*rs12603582*	*rs3809865*	*rs11650072*	*Frequency*
H1	**G**	**G**	**C**	**G**	**T**	**G**	**C**	**T**	**A**	**C**	**0.158**
H2	G	G	T	A	C	G	A	G	T	T	0.125
H3	**G**	**G**	**C**	**G**	**T**	**G**	**C**	**G**	**A**	**C**	**0.103**
H4	G	G	T	A	T	G	A	G	A	C	0.102
H5	A	G	T	A	T	G	A	G	A	C	0.097
H6	G	A	C	G	T	G	C	G	A	C	0.083
H7	G	A	C	G	T	G	C	G	T	T	0.074
H8	G	A	C	G	T	G	C	T	A	C	0.058
H9	G	G	C	A	T	A	A	G	A	T	0.041
H10	A	G	C	A	T	G	C	G	T	T	0.027
H11	A	G	C	G	T	G	C	T	A	C	0.011
H12	G	A	C	G	T	G	C	G	A	T	0.011
H13	G	G	T	A	C	G	A	G	A	C	0.010
H14	A	G	T	A	T	G	A	T	A	C	0.009
H15	A	A	T	A	T	G	A	G	A	C	0.007
H16	A	G	C	A	T	A	A	G	A	T	0.006
H17	G	G	T	A	T	G	A	G	T	T	0.005
H18	G	G	C	G	T	G	C	G	T	T	0.005
H19	A	G	T	A	T	G	A	G	A	T	0.005

(b)[b]

ITGB3 Haplotypes	*Estimated frequency*	*No. of families*	*S*	*E(S)*	*Var(S)*	*Z*	P-*value*
H1	**0.158**	**79.2**	**53.241**	**66.215**	**30.170**	**−2.362**	**0.018**
H2	0.125	60.2	55.239	53.866	19.295	0.313	0.754
H3	**0.103**	**55.9**	**54.993**	**41.941**	**21.999**	**2.783**	**0.005**
H4	0.102	48.0	37.016	38.468	13.996	−0.388	0.698
H5	0.097	55.2	37.996	36.582	15.763	0.356	0.721
H6	0.083	42.0	36.000	34.000	13.500	0.544	0.586
H7	0.074	45.2	37.000	34.458	12.627	0.715	0.474
H8	0.058	36.4	30.375	27.188	10.520	0.983	0.326
H9	0.041	22.5	14.250	14.750	8.281	−0.174	0.862
H10	0.027	17.1	7.000	9.562	4.254	−1.242	0.214

[a]Only haplotypes with estimated frequencies $\geq$0.005 are listed.
[b]Haplotype global *P*-value for HBAT is *P*=0.038; whole marker permutation tests yield χ^2 sum *P*=0.017 and minimal *P*=0.011, after 100 000 iterations.
Haplotypes H1 and H3 highlighted in bold are significantly under- and over-transmitted, respectively, from heterozygous parents to the affected offspring.

stringent Bonferroni correction in quantitative analyses (*P*=0.001; Table 3). Multiple regression analysis reveals *ITGB3* SNP1, rs2317385 as the only SNP significantly affecting 5-HT blood levels (*P*=0.010), whereas *SLC6A4* 5-HTTLPR reaches marginal significance (*P*=0.070), with no evidence of gene–gene interactions (*P*=0.651; Supplementary Figure 1). *SLC6A4* 5-HTTLPR provides negligible contributions to the percentage of variance in 5-HT blood levels attributable to *ITGB3* rs2317385 alone, which passes from 5.5 to 6.0%.

Association of *ITGB3* and *SLC6A4* genotypes with clinical variables
Allele T at rs12603582 (SNP8 in *ITGB3*) is strongly associated with a shorter pregnancy duration ending in pre-term delivery (χ^2=9.78, 2 d.f., *P*=0.008), whereas the Pro33 allele at rs5918 (SNP5) is nominally associated with obstetric complications in the mother (χ^2=6.40, 2 d.f., *P*=0.041), allergies in the patient (χ^2=6.74, 2 d.f., *P*=0.034), and modulation of the pain threshold, as reported by parents (χ^2=6.98, 2 d.f., *P*=0.030; Table 4). On the other hand, *SLC6A4* 5-HTTLPR is nominally associated with several immune-related clinical variables and with parent-reported elevated pain thresholds (Table 4). No association with any clinical variable was found for rs2317385 (SNP1).

DISCUSSION

This study reports a significant association between ASD and an *ITGB3* allele marked here by haplotype H3, which doubles the risk of autism in our sample. The autism-associated haplotype is primarily defined by rs12603582, located toward the 3′ end of the gene, whereas

Table 3 Head circumference, serotonin blood levels, and global peptiduria by *ITGB3* and *SLC6A4* genotypes

GENOTYPES		*Head circumference*		*5-HT blood levels*		*Global peptiduria*	
***ITGB3**, SNP1: rs2317385*	G/G	82.5 ± 47.5 *N*=144	K–W χ^2=3.542	317.3 ± 25.0 *N*=99	**Pairwise *U*-test: GG *vs* GA+AA U=1087.0, *P*=0.001**	345.3 ± 23.0 *N*=145	K–W χ^2=1.676
	G/A	75.0 ± 47.4 *N*=60	2 d.f., *P*=0.170	468.9 ± 44.0 *N*=36		309.6 ± 32.3 *N*=41	2 d.f., *P*=0.433
	A/A	97.5 *N*=3		261.0 *N*=1		267.0 ± 168.0 *N*=3	
***ITGB3**, SNP5: rs5918*	T/T	82.5 ± 47.5 *N*=182	K–W χ^2=1.377	313.7 ± 23.9 *N*=102	K–W χ^2=1.207	344.3 ± 17.4 *N*=151	K–W χ^2=0.793
	C/T	75.0 ± 47.5 *N*=74	2 d.f., *P*=0.502	314.2 ± 29.2 *N*=48	2 d.f., *P*=0.547	355.3 ± 37.4 *N*=74	2 d.f., *P*=0.673
	C/C	82.5 ± 47.5 *N*=9		421.1 ± 104.5 *N*=8		281.2 ± 59.0 *N*=6	
***ITGB3**, SNP8: rs12603582*	G/G	82.5 ± 47.5 *N*=121	K–W χ^2=0.408	368.4 ± 31.6 *N*=77	K–W χ^2=0.181	331.6 ± 26.1 *N*=107	K–W χ^2=1.586
	G/T	78.7 ± 47.5 *N*=74	2 d.f., *P*=0.815	353.7 ± 35.0 *N*=52	2 d.f., *P*=0.913	340.6 ± 25.8 *N*=74	2 d.f., P=0.453
	T/T	86.2 ± 47.5 *N*=10		311.2 ± 97.5 *N*=5		366.1 ± 171.9 *N*=8	
***SLC6A4**, 5-HTTLPR*	S/S	82.5 ± 47.3 *N*=69	K–W χ^2=4.329	390.3 ± 43.9 *N*=41	K–W χ^2=4.821	325.2 ± 23.7 *N*=50	K–W χ^2=0.260
	S/L	82.5 ± 47.5 *N*=123	2 d.f., *P*=0.115	282.0 ± 21.4 *N*=80	2 d.f., *P*=0.09	364.4 ± 28.4 *N*=114	2 d.f., *P*=0.878
	L/L	90.0 ± 35.0 *N*=82		385.0 ± 41.0 *N*=49		329.6 ± 22.9 *N*=76	

Abbreviation: K–W, Kruskal–Wallis test (non-parametric ANOVA).
Data are expressed as mean ± SEM, except for head circumference, which is expressed as median ± semi-interquartilic range (IQR/2). Nominal *P*-value are reported; highlighted in bold, statistically significant results surviving Bonferroni's correction (significance set at $P<0.0041$).

Table 4 Association of *ITGB3* and *SLC6A4* genotypes with clinical variables

Clinical variable	*Genotype*			*Statistics*
		***ITGB3* rs12603582**		
Pregnancy duration	*G/G*	*G/T*	*T/T*	
At term	60.9% (126)	33.3% (69)	5.8% (12)	χ^2=9.78, 2 d.f., *P*=0.008
Pre-term	38.7% (12)	61.3% (19)	0.0% (0)	
		***ITGB3* rs5918 (Leu33Pro)**		
Pain tolerance	*T/T*	*C/T*	*C/C*	
Normal	60,3% (35)	29.3% (17)	10.4% (6)	χ^2=6.98, 2 d.f., *P*=0.030
Increased	84.8% (28)	15.2% (5)	0.0% (0)	
Allergies in the patient				
Absent	74.5% (117)	24.2% (38)	1.3% (2)	χ^2=6.74, 2 d.f., *P*=0.034
Present	65.6% (42)	26.6% (17)	7.8% (5)	
Obstetric complications in the mother				
Absent	76.7% (112)	20.6 % (30)	2.7% (4)	χ^2=6.40, 2 d.f., *P*=0.041
Present	61.0% (47)	32.5% (25)	6.5% (5)	
		***SLC6A4* 5-HTTLPR**		
Food allergies in family members	*S/S*	*S/L*	*L/L*	
Absent	29.0% (51)	45.5% (80)	25.6% (45)	χ^2=10.98, 2 d.f., *P*=0.004
Present	15.8% (6)	31.6% (12)	52.6% (20)	
Increased pain tolerance				
Normal	40.0% (28)	35.7% (25)	24.3% (17)	χ^2=7.60, 2 d.f., *P*=0.022
Increased	20.0% (9)	33.3% (15)	46.7% (21)	
Autoimmune disease in first-degree relatives				
Absent	28.7% (37)	44.2% (57)	27.1% (35)	χ^2=6.05, 2 d.f., *P*=0.048
Present	20.0% (5)	28.0% (7)	52.0% (13)	
Immune and/or allergic disease in the family				
Absent	28.0% (37)	47.0% (62)	25.0% (33)	χ^2=4.98, 2 d.f., *P*=0.083
Present	23.8% (20)	36.9% (31)	39.3% (33)	

rs2317385, located at the 5′ end, is significantly associated with 5-HT blood levels. Conversely, the former SNP shows no association with serotoninemia, and the latter provides no contribution to autism risk. Hence, multiple functional *ITGB3* polymorphisms located in different parts of the gene are seemingly responsible for contributions to autism liability and to 5-HT blood levels in our sample.

The existence of at least two distinct functional genetic variants at the *ITGB3* locus is highly compatible with previous reports on autism and other disorders, such as asthma and allergies.[37,38] At the 5′ end of the gene, rs2317385 is associated with higher 5-HT blood levels both in our sample and in a previously reported healthy population sample recruited in Chicago.[16] This variant was never found associated with autism risk in earlier studies.[14–18] Toward the 3′ end, we apparently fail to replicate the positive nominal association between autism and SNPs rs5918, rs15908, and rs3809865 located in exon 3, exon 9, and 3′ UTR, respectively. However, at least for rs5918, the initial report of an association with ASD[16] was not replicated in several follow-up studies.[17,18] On the other hand, rs15908, and rs3809865 are all located at a short distance from our SNP rs12603582 (Figure 1). Conceivably, the association of a single putative functional variant with different markers in different samples, could be well explained by interethnic differences in LD pattern, in the presence of r^2 values as low as those shown in Figure 1. This discrepancy between r^2 and LD block definition based on D value is because of the very different frequencies of associated alleles at these SNPs. The association of the major allele at each SNP with the minor allele at the other SNP, decreases dramatically the informativeness of major alleles at each SNP in reference to alleles present at the other SNP.[39] Regardless, the existence of separate 5′ and 3′ functional variants contributing to serotoninemia and autism, respectively, remains a consistent observation, closely resembling the association patterns reported for asthma and wheezing *vs* allergies and IgE levels, also associated with distinct 5′ and 3′ markers.[37,38]

In spite of the extraordinary challenge posed by the complex pathogenetic processes underlying ASD, different lines of evidence are starting to converge on some basic mechanisms. The prominent increase in pre-term births detected here among allele T carriers at SNP rs12603582, and the absence of T/T genotype carriers among the autistic offspring, strongly point toward a deleterious effect of the T allele during pregnancy, which would then translate into the preferential transmission of allele G from heterozygous parents to autistic offspring. Additional contributions to the occurrence of obstetric complications and of repeated spontaneous abortions in mothers of autistic individuals come from the Pro33 allele at rs5918. Importantly, Pro33 is in LD with the G, and not with the T allele, at rs12603582, indicating that the two SNPs may be independently influencing early life liability. This is not entirely surprising, as neonatal alloimmune thrombocytopenia, the most common cause of severe thrombocytopenia in otherwise healthy term infants, is due to a feto–maternal mismatch for human platelet alloantigens encoded by the *ITGB3* gene.[40] Importantly, the enhanced risk for early fetal loss conferred by the Pro33 allele has been previously recorded in the general population,[41] whereas to our knowledge no previous evidence of involvement for rs12603582 has been produced. Hence, the latter may function specifically in families carrying an autism-predisposing genetic background. Finally, contrary to previous studies,[14,15,17,18] our sample provides no evidence of significant gene–gene interaction between *SLC6A4* and *ITGB3*. Instead, the L/L genotype at *SLC6A4* shows nominal associations with immunological conditions and increased pain tolerance, a result quite compatible with well-known 5-HT roles in adaptive immune responses and in determining the sensitive threshold to noxious stimuli.[42,43]

In conclusion, our results confirm and extend previous findings, supporting the existence of relevant influences by *ITGB3* gene variants on autism liability and on 5-HT blood levels. We further describe a significant association between early fetal loss, preterm delivery, and obstetric complications in the mothers of autistic children, with *ITGB3* gene variants active either in the general population, as previously reported,[40,41,44] or possibly affecting the feto–maternal unit only in autism spectrum families. Collectively, the results of this and of previous studies spur strong interest into the identification and functional characterization of *ITGB3* variants functionally implicated in the underpinnings of autism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)