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1 **Family-based association study of *ITGB3* in Autism Spectrum**

2 **Disorder and its endophenotypes.**

3
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23
24 **Short running Title:** *ITGB3* and Autism Spectrum Disorder

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31 **Keywords:** Autism; integrin beta 3; quantitative trait locus; *SLC6A4*; serotonin;
32 serotonin transporter.

1 **Abstract:**

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

20

1 **Introduction**

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

17 Elevated whole blood serotonin (5-HT) levels, one of the most consistent
18 biological endophenotypes in autism research, is recorded in about one third of cases.⁶
19 Autism-associated hyperserotonemia is indeed familial,⁷⁻⁹ and could either play a role in
20 the aetiological processes leading to the disease, or it could at least characterize a
21 relatively homogeneous subgroup of ASD patients. Genes encoding proteins involved in
22 5-HT metabolism and neurotransmission include the integrin β 3 subunit gene (*ITGB3*),
23 located on human chr. 17q21.32, which was identified as a quantitative trait locus
24 (QTL) for 5-HT blood levels in the Hutterites.^{10,11} Interestingly, *ITGB3* maps under a

1 replicated linkage peak for autism.^{12,13} Furthermore, *ITGB3* alleles have been found at
2 least nominally associated with autism in all five studies performed to date,¹⁴⁻¹⁸ either
3 alone or in interaction with allelic variants at the 5-HT transporter gene (*SLC6A4*).
4 Several lines of evidence support functional interactions between *ITGB3* and *SLC6A4*,
5 which also affects 5-HT blood levels and is located on human chr. 17q11.1-q12.¹¹ First,
6 *ITGB3* and *SLC6A4* gene expression levels are correlated in human and mouse tissues.¹⁴
7 In fact, *Slc6a4* mRNA levels map to the *Itgb3* locus using QTL analysis in mouse
8 hematopoietic stem cells, and non-coding human polymorphisms in *ITGB3* are
9 associated with both *ITGB3* and *SLC6A4* expression levels.¹⁴ Secondly, the integrin
10 receptor composed of an α IIb subunit and of the β 3 subunit encoded by the *ITGB3* gene,
11 was recently identified as a novel component of the *SLC6A4* regulatory protein
12 complex.¹⁴ Also the Leu33Pro *ITGB3* SNP (rs5918) modulates *SLC6A4* trafficking and
13 transport activity.¹⁹ Finally, several recently published studies have described
14 significant *SLC6A4* and *ITGB3* interactions for both autism risk and 5-HT blood levels,
15 with a male-specific effect.^{10, 14-17, 20}

16 Despite these positive findings, several inconsistencies complicate their
17 interpretation, possibly due to clinical and genetic heterogeneity in ASD. In particular,
18 different alleles appear associated with autism and/or serotoninemia in independent
19 samples at the *ITGB3* and *SLC6A4* loci.^{16,18} Linkage disequilibrium (LD) blocks
20 associated with autism and/or serotoninemia are not consistent, with different studies
21 pointing toward either the 5' or the 3' ends of the *ITGB3* locus.^{14-18,20} Conceivably,
22 these inconsistencies could stem from different causative variants occurring on distinct
23 marker haplotype backgrounds.

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

8

9 **Materials and methods**

10 *Subjects*

11 A total of 281 simplex and 12 multiplex families with a non-syndromic autistic proband
12 were recruited for this study, including 306 ASD patients, 106 unaffected siblings, and 577
13 parents (total genotyped N=989). Demographic and clinical characteristics of our clinical
14 sample, as well as endophenotypic measures for head circumference, serotonin (5-HT)
15 blood levels and global peptiduria, are summarized in Table 1. The composition by
16 recruiting site is presented in Supplementary Table S1. Diagnostic screening procedures
17 used to exclude syndromic autism have been previously described.²¹ Briefly, patients
18 fulfilling DSM-IV diagnostic criteria for Autistic Disorder¹ were screened for non-
19 syndromic autism using MRI, EEG, audiometry, urinary amino acid and organic acid
20 measurements, cytogenetic and fragile-X testing. Patients with dysmorphic features were
21 excluded even in the absence of detectable cytogenetic alterations. Patients with sporadic
22 seizures (i.e., < 1 every 6 months) were included; patients with frequent seizures or focal
23 neurological deficits were excluded. The M:F ratio in ASD patients is 7.3 : 1. Autistic

1 behaviours were assessed using the official Italian version of the Autism Diagnostic
2 Observation Schedule (ADOS)²² and the Autism Diagnostic Interview-Revised (ADI-
3 R);²³ adaptive functioning was assessed using the Vineland Adaptive Behavior Scales
4 (VABS); I.Q. was determined using either the Griffith Mental Developmental Scales,
5 the Coloured Raven Matrices, the Bayley Developmental Scales or the Leiter
6 International Performance Scale.²¹ All parents gave written informed consent for
7 themselves and for their children, using the consent form approved by the I.R.B. of
8 U.C.B.M. (Rome, Italy).

9

10 *Genotyping*

11 Genomic DNA (gDNA) was extracted from whole blood²⁴ and quantified in triplicate
12 by PicoGreen[®]. Based on HapMap phase II (release 21) CEU population data, four
13 independent LD blocks were identified within *ITGB3* (chr 17: 42684-42750 kb) using
14 the 'Solid Spine of LD' algorithm with a minimum D' value of 0.8. Ten tagging SNPs
15 were selected using Tagger from Haploview v4.2²⁵ [$r^2 > 0.75$ and minor allele
16 frequency (MAF) > 0.05 , aggressive tagging, LOD threshold for multi-marker test= 3].
17 All SNPs previously associated with autism were comprised by applying the “force
18 include” procedure of Haploview, in addition to rs11650072 which provides further
19 coverage of the 3'-flanking region (Supplementary Table S2). The *ITGB3* genotyping
20 was performed using the Applied Biosystems SNPLext Genotyping System (Applied
21 Biosystems, CA, USA). All samples were electrophoretically separated on a 3730 DNA
22 Genetic Analyzer (Applied Biosystems), and automated allele calls and genotype
23 clustering of each individual sample was performed by Applied Biosystems

1 GeneMapper Software (version 3.5). *ITGB3* SNP rs5918 was genotyped using the
2 TaqMan™ SNP genotyping assay (Applied Biosystems, CA, USA) on the ABI Prism
3 7900HT and analyzed with the SDS software. *SLC6A4* 5-HTTLPR genotyping was
4 performed as previously described.²⁶

5

6 *Endophenotype measures*

7 Serotonin levels were measured in all family members from platelet-rich plasma,
8 obtained by centrifuging whole blood within 20 min of venipuncture at 140 g for 25 min
9 at 4°C; 1 ml of supernatant was stored at -80°C and assessed by HPLC, as described.²⁷

10 Urinary peptide excretion analysis was performed by HPLC in ASD patients and first-
11 degree relatives using the first morning urine samples, as described.²⁸ The total area of
12 peaks under the 215 nm absorption curve (AUC) in the peptide region following the
13 hippuric acid peak was calculated and expressed in μm^2 . Head circumference was
14 measured in ASD patients and unaffected siblings by trained physicians using a non-
15 stretchable plastic measuring tape graded in millimetres, placed over the maximum
16 frontal-occipital head perimeter.²¹

17

18 *Statistical Analysis*

19 Mendelian inheritance was verified using Pedcheck.²⁹ Hardy-Weinberg equilibrium
20 (HWE) was tested using Haploview v4.2 (available at
21 <http://www.broad.mit.edu/mpg/haploview/index.php>),²⁵ applying a Bonferroni
22 correction for multiple testing ($P < 0.05 / 11$ SNPs yields $P < 0.0045$). LD analysis was
23 performed using Haploview, and defining LD blocks based on the solid spine of LD
24 algorithm.²⁵ Differences in LD structure recorded applying the confidence intervals³⁰ and

1 the four-gamete rule³¹ algorithms are also reported. Family-based single-marker and
2 haplotype association tests were performed using FBAT (available at
3 <http://www.biostat.harvard.edu/~fbat/fbat.htm>), under an additive model and applying
4 option `-e`, as suggested for candidate genes under known linkage peaks.³² The HBAT
5 procedure in FBAT was also employed to estimate haplotype frequencies, to compute a
6 global P-value, and to provide an ‘exact’ p-value using Monte Carlo tests (option `-p`) for
7 the global test (“ χ^2 sum P”), for each haplotype separately, and for the minimum
8 observed p-value among all haplotypes (“minimal P”).³² Haplotype odds ratios were
9 determined using UNPHASED.³³ Quantitative traits were analyzed by quantitative
10 transmission/disequilibrium test (qTDT), as implemented by the FBAT software³² and
11 by parametric or non-parametric (Kruskal-Wallis) ANOVA, or by Mann-Whitney U-
12 tests based on genotype distributions, applying a stringent Bonferroni correction for
13 multiple testing (4 markers x 3 phenotypes, $P=0.5/12=0.0041$). Gene-gene interaction
14 analyses were performed with the 2-locus transmission/disequilibrium test (TDT)
15 method³⁴, which has been implemented as a Stata program “pseudocc” ([www-
16 gene.cimr.cam.ac.uk/clayton/software/stata](http://www-gene.cimr.cam.ac.uk/clayton/software/stata)). Data are expressed as mean \pm S.E.M.,
17 except for head circumference which is expressed as median \pm semi-interquartilic range
18 (I.Q.R./2). Head circumference measures were transformed into percentiles using sex-
19 and age-specific standard tables, as described.³⁵ Two-tail P values are reported. To
20 correct for multiple comparisons in single-marker analyses, statistical significance was
21 set at $P<0.0016$: this threshold accounts for testing of eight effectively independent
22 markers (seven on *ITGB3* and one on *SLC6A4*), as determined using the Nyholt
23 SNPSpD method³⁶ (available at <http://genepi.qimr.edu.au/general/daleN/SNPSpD/>), and
24 four phenotypes (autism, serotoninemia, peptiduria, and head circumference) [Suppl.

1 Methods]. Nominal P-values obtained by Pearson's χ^2 tests are reported for clinical,
2 patient- and family-history variables, given the exploratory nature of these associations.

3

4

5

6 **Results**

7 *ITGB3* haplotype analysis

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

20 *ITGB3* haplotypes display a statistically significant association with autism
21 (HBAT global P=0.038; whole marker permutation tests yield sum P=0.017 and minimal
22 P=0.011, after 100,000 iterations). Haplotype H3 is transmitted from heterozygous

1 parents to their autistic offspring significantly more often than expected by chance
2 (P=0.005), while haplotype H1 shows the opposite trend (P= 0.018) (Table 2). In terms of
3 odds ratios, haplotype H3 doubles the risk of autism (OR=2.000; $\chi^2=8.426$; P=0.003),
4 while haplotype H1 marginally reduces disease risk (OR=0.725 ; $\chi^2=3.572$; P=0.059).

5

6 *Single-marker analyses*

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

15

16 *Gene-gene interaction between ITGB3 and SLC6A4 in autism*

17 *SLC6A4* 5-HTTLPR genotypes are in HWE and display no association with autism in
18 this sample (TDT LRS=0.562, 1df, P=0.454; FBAT P=0.432). To test for interaction
19 between *ITGB3* and *SLC6A4*, we applied a 2-locus TDT approach,³⁴ crossing *ITGB3*
20 genotypes either at rs5918 (SNP5, leu33pro), rs12603582 (SNP8), or rs3809865
21 (SNP10), with *SLC6A4* genotypes at the 5-HTTLPR. No evidence of epistatic effects on
22 autism risk was detected in our entire sample, in males only, or in simplex families.

23

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

3 Single-marker qTDT analyses show a nominal association of *ITGB3* SNP1, rs2317385,
4 with 5-HT blood levels (P=0.016) and peptiduria (P=0.041), not reaching the
5 significance threshold set by the Nyholt SNPSpD method to control for multiple testing
6 (P<0.0016). However, the association with 5-HT blood levels survives even a stringent
7 Bonferroni correction in quantitative analyses (P=0.001; Table 3). Multiple regression
8 analysis reveals *ITGB3* SNP1, rs2317385 as the only SNP significantly affecting 5-HT
9 blood levels (P=0.010), while *SLC6A4* 5-HTTLPR reaches marginal significance
10 (P=0.070), with no evidence of gene-gene interactions (P=0.651) (Suppl. Figure 1).
11 *SLC6A4* 5-HTTLPR provides negligible contributions to the percentage of variance in
12 5-HT blood levels attributable to *ITGB3* rs2317385 alone, which passes from 5.5% to
13 6.0%.

14

15 *Association of ITGB3 and SLC6A4 genotypes with clinical variables*

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

24

1 **Discussion**

2 The present study reports a significant association between ASD and an *ITGB3* allele
3 marked here by haplotype H3, which doubles the risk of autism in our sample. The
4 autism-associated haplotype is primarily defined by rs12603582, located toward the 3'
5 end of the gene, whereas rs2317385, located at the 5' end, is significantly associated
6 with 5-HT blood levels. Conversely, the former SNP displays no association with
7 serotoninemia, and the latter provides no contribution to autism risk. Hence, multiple
8 functional *ITGB3* polymorphisms located in different parts of the gene are seemingly
9 responsible for contributions to autism liability and to 5-HT blood levels in our sample.

10 The existence of at least two distinct functional genetic variants at the *ITGB3*
11 locus is highly compatible with previous reports on autism and other disorders, such as
12 asthma and allergies.^{37,38} At the 5' end of the gene, rs2317385 is associated with higher
13 5-HT blood levels both in our sample and in a previously-reported healthy population
14 sample recruited in Chicago.¹⁶ This variant was never found associated with autism risk
15 in earlier studies.¹⁴⁻¹⁸ Toward the 3' end, we apparently fail to replicate the positive
16 nominal association between autism and SNPs rs5918, rs15908, and rs3809865 located
17 in exon 3, exon 9, and 3' UTR, respectively. However, at least for rs5918, the initial
18 report of an association with ASD¹⁶ was not replicated in several follow-up studies.^{17,18}
19 On the other hand, rs15908, and rs3809865 are all located at a short distance from our
20 SNP rs12603582 (Figure 1). Conceivably, the association of a single putative functional
21 variant with different markers in different samples, could be well explained by
22 interethnic differences in LD pattern, in the presence of r^2 values as low as those
23 displayed in Figure 1. This discrepancy between r^2 and LD block definition based on D'
24 is due to the very different frequencies of associated alleles at these SNPs. The

1 association of the major allele at each SNP with the minor allele at the other SNP,
2 decreases dramatically the informativeness of major alleles at each SNP in reference to
3 alleles present at the other SNP.³⁹ Regardless, the existence of separate 5' and 3'
4 functional variants contributing to serotoninemia and autism, respectively, remains a
5 consistent observation, closely resembling the association patterns reported for asthma
6 and wheezing vs allergies and IgE levels, also associated with distinct 5' and 3'
7 markers.^{37,38}

8 In spite of the extraordinary challenge posed by the complex pathogenetic
9 processes underlying autism spectrum disorder, different lines of evidence are starting
10 to converge upon some basic mechanisms. The prominent increase in pre-term births
11 detected here among allele T carriers at SNP rs12603582, and the absence of T/T
12 genotype carriers among the autistic offspring, strongly point toward a deleterious effect
13 of the T allele during pregnancy, which would then translate into the preferential
14 transmission of allele G from heterozygous parents to autistic offspring. Additional
15 contributions to the occurrence of obstetric complications and of repeated spontaneous
16 abortions in mothers of autistic individuals come from the Pro33 allele at rs5918.
17 Importantly, Pro33 is in linkage disequilibrium with the G, and not with the T allele, at
18 rs12603582, indicating that the two SNPs may be independently influencing early life
19 liability. This is not entirely surprising, since neonatal alloimmune thrombocytopenia,
20 the most common cause of severe thrombocytopenia in otherwise healthy term infants,
21 is due to a feto-maternal mismatch for human platelet alloantigens encoded by the
22 *ITGB3* gene.⁴⁰ Importantly, the enhanced risk for early fetal loss conferred by the Pro33
23 allele has been previously recorded in the general population,⁴¹ whereas to our
24 knowledge no previous evidence of involvement for rs12603582 has been produced.

1 Hence, the latter may act specifically in families carrying an autism-predisposing
2 genetic background. Finally, contrary to previous studies,^{14,15,17,18} our sample provides
3 no evidence of significant gene-gene interaction between *SLC6A4* and *ITGB3*. Instead,
4 the L/L genotype at *SLC6A4* displays nominal associations with immunological
5 conditions and increased pain tolerance, a result quite compatible with well-known 5-
6 HT roles in adaptive immune responses and in determining the sensitive threshold to
7 noxious stimuli.^{42,43}

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

17

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3

4 **Conflict of Interest Statement**

5 The authors declare no conflict of interest

6

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

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

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 4 Abbreviations: SEM, standard error of the mean; IQR/2, semi-interquartile range; PDD-
 5 NOS, Pervasive Developmental Disorder – Not Otherwise Specified; IQ, intellectual
 6 quotient.
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1 **Table 2.** *ITGB3* haplotypes are associated with autism: (A) Haplotype structure at the
 2 *ITGB3* locus. Only haplotypes with estimated frequencies ≥ 0.005 are listed. (B)
 3 Haplotype family-based association tests performed using HBAT, under an additive
 4 model (-e).³² Haplotype global P-value for HBAT is P=0.038; whole marker
 5 permutation tests yield χ^2 sum P=0.017 and minimal P=0.011, after 100,000 iterations.
 6 Haplotypes H1 and H3, highlighted in bold and gray, are significantly under- and over-
 7 transmitted, respectively, from heterozygous parents to the affected offspring.

8 **A**

Haplotype	<i>ITGB3</i> SNPs										Estimated Frequency
	1	2	3	4	5	6	7	8	9	10	
	<i>rs2317385</i>	<i>rs2056131</i>	<i>rs4525555</i>	<i>rs2015729</i>	<i>rs5918</i>	<i>rs951351</i>	<i>rs15908</i>	<i>rs12603582</i>	<i>rs3809865</i>	<i>rs11650072</i>	
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	A	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

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B

<i>ITGB3</i> Haplotypes	Estimated Freq	N. 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

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1 **Table 3.** Head circumference, serotonin blood levels, and global peptiduria by *ITGB3* and *SLC6A4* genotypes. Data are expressed as mean \pm
 2 S.E.M., except for head circumference which is expressed as median \pm semi-interquartile range (I.Q.R./2). Nominal P-value are reported;
 3 highlighted in bold, statistically significant results surviving Bonferroni's correction (significance set at $P < 0.0041$).

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GENOTYPES		Head circumference		5-HT blood levels		Global peptiduria	
<i>ITGB3</i> , SNP1: <i>rs2317385</i>	GG	82.5 \pm 47.5 <i>N</i> =144	K-W χ^2 =3.542 2df, P=0.170	317.3 \pm 25.0 <i>N</i> =99 ^	Pairwise U test: GG vs GA+AA U=1087.0, P=0.001	345.3 \pm 23.0 <i>N</i> =145	K-W χ^2 =1.676 2df, P=0.433
	GA	75.0 \pm 47.4 <i>N</i> =60		468.9 \pm 44.0 <i>N</i> =36		309.6 \pm 32.3 <i>N</i> =41	
	AA	97.5 <i>N</i> =3		261.0 <i>N</i> =1		267.0 \pm 168.0 <i>N</i> =3	
<i>ITGB3</i> , SNP5: <i>rs5918</i>	TT	82.5 \pm 47.5 <i>N</i> =182	K-W χ^2 =1.377 2df, P=0.502	313.7 \pm 23.9 <i>N</i> =102	K-W χ^2 =1.207 2df, P=0.547	344.3 \pm 17.4 <i>N</i> =151	K-W χ^2 =0.793 2df, P=0.673
	CT	75.0 \pm 47.5 <i>N</i> =74		314.2 \pm 29.2 <i>N</i> =48		355.3 \pm 37.4 <i>N</i> =74	
	TT	82.5 \pm 47.5 <i>N</i> =9		421.1 \pm 104.5 <i>N</i> =8		281.2 \pm 59.0 <i>N</i> =6	
<i>ITGB3</i> , SNP8: <i>rs12603582</i>	GG	82.5 \pm 47.5 <i>N</i> =121	K-W χ^2 =0.408 2df, P=0.815	368.4 \pm 31.6 <i>N</i> =77	K-W χ^2 =0.181 2df, P=0.913	331.6 \pm 26.1 <i>N</i> =107	K-W χ^2 =1.586 2df, P=0.453
	GT	78.7 \pm 47.5 <i>N</i> =74		353.7 \pm 35.0 <i>N</i> =52		340.6 \pm 25.8 <i>N</i> =74	
	TT	86.2 \pm 47.5 <i>N</i> =10		311.2 \pm 97.5 <i>N</i> =5		366.1 \pm 171.9 <i>N</i> =8	

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SLC6A4, 5-HTTLPR	S/S	82.5±47.3 N=69	K-W $\chi^2=4.329$ 2df, P=0.115	390.3±43.9 N=41	K-W $\chi^2=4.821$ 2df, P=0.09	325.2±23.7 N=50	K-W $\chi^2=0.260$ 2df, P=0.878
	S/L	82.5±47.5 N=123		282.0±21.4 N=80		364.4±28.4 N=114	
	L/L	90.0±35.0 N=82		385.0±41.0 N=49		329.6±22.9 N=76	

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3 Abbreviations: K-W= Kruskal-Wallis test (non-parametric ANOVA).

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

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Clinical Variable	Genotype			Statistics
	<i>ITGB3</i> rs12603582			
	<i>G/G</i>	<i>G/T</i>	<i>T/T</i>	
<i>Pregnancy Duration</i>				
-at term	60.9% (126)	33.3% (69)	5.8% (12)	$\chi^2=9.78$, 2 df, P=0.008
-pre-term	38.7% (12)	61.3% (19)	0.0% (0)	
	<i>ITGB3</i> rs5918 (Leu33Pro)			
	<i>T/T</i>	<i>C/T</i>	<i>C/C</i>	
<i>Pain Tolerance</i>				
- normal	60,3% (35)	29.3% (17)	10.4% (6)	$\chi^2=6.98$, 2 df, P=0.030
- increased	84.8% (28)	15.2% (5)	0.0% (0)	
<i>Allergies in the patient</i>				
-absent	74.5% (117)	24.2% (38)	1.3% (2)	$\chi^2=6.74$, 2 df, P=0.034
-present	65.6% (42)	26.6% (17)	7.8% (5)	
<i>Obstetric complications in the mother</i>				
- absent	76.7% (112)	20.6 % (30)	2.7% (4)	$\chi^2=6.40$, 2 df, P=0.041
- present	61.0% (47)	32.5% (25)	6.5% (5)	
	<i>SLC6A4</i> 5-HTTLPR			
	<i>S/S</i>	<i>S/L</i>	<i>L/L</i>	
<i>Food Allergies in family members</i>				
- absent	29.0% (51)	45.5% (80)	25.6% (45)	$\chi^2=10.98$, 2 df, p=0.004
- present	15.8% (6)	31.6% (12)	52.6% (20)	
<i>Increased Pain Tolerance</i>				
- normal	40.0% (28)	35.7% (25)	24.3% (17)	$\chi^2=7.60$, 2 df, p=0.022
- increased	20.0% (9)	33.3% (15)	46.7% (21)	
<i>Autoimmune disease in 1st degree relatives</i>				
-absent	28.7% (37)	44.2% (57)	27.1% (35)	$\chi^2=6.05$, 2 df, p=0.048
-present	20.0% (5)	28.0% (7)	52.0% (13)	

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<i>Immune and/or allergic disease in the family</i>				
-absent	28.0% (37)	47.0% (62)	25.0% (33)	$\chi^2=4.98, 2 \text{ df}, p=0.083$
-present	23.8% (20)	36.9% (31)	39.3% (33)	

2

Figure Legends

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

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