HAL
open science

Family-based association study of ITGB3 in Autism Spectrum Disorder and its endophenotypes.

Antonio M Persico, Valerio Napolioni, Federica Lombardi, Roberto Sacco, Paolo Curatolo, Barbara Manzi, Riccardo Alessandrelli, Roberto Militerni, Carmela Bravaccio, Carlo Lenti, et al.

To cite this version:

Antonio M Persico, Valerio Napolioni, Federica Lombardi, Roberto Sacco, Paolo Curatolo, et al.. Family-based association study of ITGB3 in Autism Spectrum Disorder and its endophenotypes.. European Journal of Human Genetics, 2010, 10.1038/ejhg.2010.180 . hal-00593670

HAL Id: hal-00593670

https://hal.science/hal-00593670

Submitted on 17 May 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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]

[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, I.R.C.C.S. "Fondazione Santa Lucia", Rome, Italy; [3]Department of Child Neuropsychiatry, University "Tor Vergata", Rome, Italy; [4]Department of Child Neuropsychiatry, II University of 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, U.S.A.; [8]Southwest Autism Research and Resource Center, Phoenix, AZ, U.S.A.; [9]Department of Psychology and Centro "Daniel Bovet", University "La Sapienza", Rome, Italy; [10]Laboratory of Behavioral Neurobiology, Department of Experimental Neurosciences, I.R.C.C.S. "Fondazione Santa Lucia", Rome, Italy; [11]Department of Pediatric Research, Rikshospitalet, University of Oslo, Norway; [12] IntegraGen SA. Genopole, Evry, France.

Short running Title: *ITGB3* and Autism Spectrum Disorder

Correspondence to: Dr. Antonio M. Persico, Laboratory of Molecular Psychiatry & Neurogenetics, University "Campus Bio-Medico", Via Alvaro del Portillo 21, I-00128 Rome, Italy. Tel. +39-06-225419155; Fax +39-06-501703333; E-mail address: a.persico@unicampus.it

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

Abstract:

The integrin beta 3 gene (*ITGB3*), located on human chr. 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; O.R.=2.000), at the expense of haplotype H1 which is under-transmitted (HBAT P=0.018; O.R.=0.725). These two common haplotypes differ only at rs12603582 located in intron 11, which reaches a P=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.

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 behaviour, and disease onset prior to 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 approximately 10% of cases.[4] ASD, like many other complex human disorders, does not display 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 play a role in the aetiological 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 β3 subunit gene (*ITGB3*), located on human chr. 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 chr. 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 human polymorphisms in *ITGB3* are associated with both *ITGB3* and *SLC6A4* expression levels.[14] Secondly, the integrin receptor composed of an αIIb subunit and of the β3 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 due to clinical and genetic heterogeneity in ASD. In particular, different alleles appear 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.

The present 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 (i.e., < 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

behaviours were assessed using the official Italian version of the Autism Diagnostic Observation Schedule (ADOS)[22] and the Autism Diagnostic Interview-Revised (ADI-R);[23] adaptive functioning was assessed using the Vineland Adaptive Behavior Scales (VABS); I.Q. was determined using either the Griffith Mental Developmental Scales, the Coloured 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 I.R.B. of U.C.B.M. (Rome, Italy).

Genotyping

Genomic DNA (gDNA) was extracted from whole blood[24] and quantified in triplicate by PicoGreen®. Based on HapMap phase II (release 21) CEU population data, four independent LD blocks were identified within *ITGB3* (chr 17: 42684-42750 kb) using the 'Solid Spine of LD' algorithm with a minimum D' value of 0.8. Ten tagging SNPs were selected using Tagger from Haploview v4.2[25] [$r^2 > 0.75$ and minor allele frequency (MAF) > 0.05, aggressive tagging, LOD threshold for multi-marker 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, CA, USA) on the ABI Prism 7900HT and analyzed with the SDS software. *SLC6A4* 5-HTTLPR genotyping was performed as previously described.[26]

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 millimetres, 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 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 employed 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 were determined using 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 correction for multiple testing (4 markers x 3 phenotypes, P=0.5/12=0.0041). Gene-gene interaction analyses were performed with the 2-locus transmission/disequilibrium test (TDT) method[34], which has been implemented as a Stata program "pseudocc" (www-gene.cimr.cam.ac.uk/clayton/software/stata). Data are expressed as mean ± S.E.M., except for head circumference which is expressed as median ± semi-interquartilic range (I.Q.R./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) [Suppl.

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 eleven *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 (Suppl. 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 display 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), while haplotype H1 shows the opposite trend (P= 0.018) (Table 2). In terms of odds ratios, haplotype H3 doubles the risk of autism (OR=2.000; χ^2=8.426; P=0.003), while haplotype H1 marginally reduces disease risk (OR=0.725 ; χ2=3.572 ; P=0.059).

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 displaying a trend towards 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) (Suppl. 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 display no association with autism in this sample (TDT LRS=0.562, 1df, P=0.454; FBAT P=0.432). To test for interaction between *ITGB3* and *SLC6A4*, we applied a 2-locus TDT approach,[34] crossing *ITGB3* genotypes either at rs5918 (SNP5, leu33pro), rs12603582 (SNP8), or rs3809865 (SNP10), 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 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), while *SLC6A4* 5-HTTLPR reaches marginal significance (P=0.070), with no evidence of gene-gene interactions (P=0.651) (Suppl. 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 df, P=0.008), while the Pro33 allele at rs5918 (SNP5) is nominally associated with obstetric complications in the mother (χ^2=6.40, 2 df, P=0.041), allergies in the patient (χ^2=6.74, 2 df, P=0.034), and modulation of the pain threshold, as reported by parents (χ^2=6.98, 2 df, 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

The present 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 rs2317385, located at the 5' end, is significantly associated with 5-HT blood levels. Conversely, the former SNP displays 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 displayed in Figure 1. This discrepancy between r^2 and LD block definition based on D' is due to 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 autism spectrum disorder, different lines of evidence are starting to converge upon 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 occurrance of obstetric complications and of repeated spontaneous abortions in mothers of autistic individuals come from the Pro33 allele at rs5918. Importantly, Pro33 is in linkage disequilibrium 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, since 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 act 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* displays 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 the present and of previous studies spur strong interest into the identification and functional characterization of *ITGB3* variants functionally implicated in the underpinnings of autism.

Acknowledgements

The authors gratefully acknowledge all the families who participated in this study, Roberto Rigardetto, Marina Gandione, Simona Trillo and Maria Paola Santangelo for contributing to patient recruitment/data collection, and Jerome Carayol for critical reviewing of manuscript. This work was supported by the Italian Ministry for University, Scientific Research and Technology (PRIN n.2006058195), the Italian Ministry of

Health (RFPS-2007-5-640174), the Autism Speaks Foundation (Princeton, NJ), Autism Aid Onlus (Naples, IT), and Fondazione Gaetano and Mafalda Luca (Milan, IT).

Conflict of Interest Statement

The authors declare no conflict of interest

References

1. American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders 4th edition. Washington D.C., American Psychiatric Association; 1994.
2. Persico AM, Bourgeron T: Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci* 2006; **29**: 349-358.
3. Klauck SM: Genetics of autism spectrum disorder. *Eur J Hum Genet*. 2006; **14**: 714-20.
4. Lintas C, Persico AM: Autistic phenotypes and genetic testing: state-of-the-art for the clinical geneticist. *J Med Genet.* 2009; **46**: 1-8.
5. Buxbaum JD: Multiple rare variants in the etiology of autism spectrum disorders. *Dialogues Clin Neurosci.* 2009; **11**:35-43.
6. Cook EH, Leventhal BL: The serotonin system in autism. *Curr Opin Pediatr.* 1996; **8**: 348-54.
7. Abramson RK, Wright HH, Carpenter R *et al*: Elevated blood serotonin in autistic probands and their first-degree relatives. *J Autism Dev Disord* 1989; **19**: 397-407.
8. Cook EH, Leventhal BL, Heller W, Metz J, Wainwright M, Freedman DX: Autistic children and their first-degree relatives: relationships between serotonin and norepinephrine levels and intelligence. *J Neuropsychiatry Clin Neurosci* 1990; **2**: 268–274.
9. Leventhal BL, Cook EH Jr, Morford M, Ravitz A, Freedman DX: Relationships of whole blood serotonin and plasma norepinephrine within families. *J Autism Dev Disord* 1990; **20**: 499–511.

10. Weiss LA, Veenstra-Vanderweele J, Newman DL *et al*: Genome-wide association study identifies ITGB3 as a QTL for whole blood serotonin. *Eur J Hum Genet* 2004; **12**: 949–954
11. Weiss LA, Abney M, Cook EH Jr, Ober C: Sex-specific genetic architecture of whole blood serotonin levels. *Am J Hum Genet* 2005; **76**: 33-41.
12. Stone JL, Merriman B, Cantor RM *et al*: Evidence for sex-specific risk alleles in autism spectrum disorder. *Am J Hum Genet* 2004; **75**: 1117-1123.
13. Cantor RM, Kono N, Duvall JA et al: Replication of autism linkage: fine-mapping peak at 17q21. *Am J Hum Genet* 2005; **76**: 1050–1056
14. Weiss LA, Ober C, Cook EH Jr: ITGB3 shows genetic and expression interaction with SLC6A4. *Hum Genet.* 2006; **120**: 93-100.
15. Coutinho AM, Sousa I, Martins M *et al*: Evidence for epistasis between SLC6A4 and ITGB3 in autism etiology and in the determination of platelet serotonin levels. *Hum Genet.* 2007; **121**: 243-56.
16. Weiss LA, Kosova G, Delahanty RJ *et al*: Variation in ITGB3 is associated with whole-blood serotonin level and autism susceptibility. *Eur J Hum Genet* 2006; **14**: 923–931.
17. Mei H, Cuccaro ML, Martin ER: Multifactor dimensionality reduction-phenomics: a novel method to capture genetic heterogeneity with use of phenotypic variables. *Am J Hum Genet.* 2007; **81**: 1251-61
18. Ma DQ, Rabionet R, Konidari I *et al*: Association and gene-gene interaction of SLC6A4 and ITGB3 in autism. *Am J Med Genet B Neuropsychiatr Genet.* 2009; **153**: 477-483.

19. Carneiro AM, Cook EH, Murphy DL, Blakely RD: Interactions between integrin alphaIIbbeta3 and the serotonin transporter regulate serotonin transport and platelet aggregation in mice and humans. *J Clin Invest.* 2008; **118**: 1544-52.
20. Weiss LA, Abney M, Parry R, Scanu AM, Cook EH Jr, Ober C: Variation in ITGB3 has sex-specific associations with plasma lipoprotein(a) and whole blood serotonin levels in a population-based sample. *Hum Genet* 2005; **117**: 81-87.
21. Conciatori M, Stodgell CJ, Hyman SL *et al*: Morphogenetic effect of the *HOXA1* A218G polymorphism on head circumference in patients with autism. *Biol Psychiatry* 2004; **55:** 413-419.
22. Lord C, Rutter M, DiLavore PC, Risi S: *ADOS, Autism Diagnostic Observation Schedule.* Western Psychological Services: Los Angeles, 2002 (Italian version ed. by Tancredi R, Saccani M, Persico AM, Parrini B, Igliozzi R and Faggioli R. Organizzazioni Speciali: Florence, 2005).
23. Rutter M, Le Couter A, Lord C: ADI-R, Autism Diagnostic Interview - Revised. Western Psychological Services: Los Angeles, 2003 (Italian version ed. by Faggioli R, Saccani M, Persico AM, Tancredi R, Parrini B and Igliozzi R. Organizzazioni Speciali: Florence, 2005).
24. Lahiri DK, Nurnberger JI Jr: A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991; **19**: 5444
25. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
26. Persico AM, Militerni R, Bravaccio C *et al*: Lack of association between serotonin transporter gene promoter variants and autistic disorder in two ethnically distinct samples. *Am J Med Genet.* 2000; **96**: 123-7.

27. Piven J, Tsai GC, Nehme E, Coyle JT, Chase GA, Folstein SE: Platelet serotonin, a possible marker for familial autism. *J Autism Dev Disord* 1991; **21**: 51-59.

28. Reichelt WH, Knivsberg AM, Nodland M, Stensrud M, Reichelt KL: Urinary peptide levels and patterns in autistic children from seven countries, and the effect of dietary intervention after 4 years. *Dev Brain Dysfunct* 1997; **10**: 44-55.

29. O'Connell JR, Weeks DE: PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 1998; **63**: 259–266.

30. Gabriel SB, Schaffner SF, Nguyen H, *et al*: The structure of haplotype blocks in the human genome. *Science* 2002; **296**: 2225–2229.

31. Wang N, Akey JM, Zhang K, Chakraborty R, Jin L: Distribution of recombination crossovers and the origin of haplotype blocks: the interplay of population history, recombination, and mutation, *Am. J. Hum. Genet.* 2002; **71**: 1227-1234.

32. Horvath S, Xu X, Lake SL, Silverman EK, Weiss ST, Laird NM: Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. *Genet Epidemiol* 2004; **26**:61-69.

33. Dudbridge F: Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol.* 2003; **25**: 115-121.

34. Cordell HJ, Barratt BJ, Clayton DG: Case/pseudocontrol analysis in genetic association studies: A unified framework for detection of genotype and haplotype associations, gene-gene and gene-environment interactions, and parent-of-origin effects. *Genet Epidemiol*. 2004; **26**: 167-85.

35. Sacco R, Militerni R, Frolli A *et al*: Clinical, morphological, and biochemical correlates of head circumference in autism. *Biol Psychiatry*. 2007; **62**: 1038-47.

36. Nyholt NR: A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am. J. Hum. Genet.* 2004; **74**: 765-769.

37. Weiss LA, Lester LA, Gern JE *et al*: Variation in ITGB3 is associated with asthma and sensitization to mold allergen in four populations. *Am J Respir Crit Care Med.* 2005; **172**:67-73.

38. Thompson EE, Pan L, Ostrovnaya I, *et al*: Integrin beta 3 genotype influences asthma and allergy phenotypes in the first 6 years of life. *J Allergy Clin Immunol.* 2007; **119**:1423-9.

39. Neale BM: Linkage disequilibrium and tagging; in Neale BM**,** Ferreira MAR, Medland SE, Posthuma D (eds): Statistical Genetics: Gene Mapping Through Linkage and Association, London 2007, pp 451-465

40. Dreyfus M, Kaplan C, Verdy E *et al:* Frequency of immune thrombocytopenia in newborns: a prospective study. Immune Thrombocytopenia Working Group. *Blood* 1997; **89**: 4402-6.

41. Ruzzi L, Ciarafoni I, Silvestri L, Semeraro ML, Abeni D: Association of PLA2 polymorphism of the ITGB3 gene with early fetal loss. *Fertil Steril.* 2005; **83**:511-2.

42. O'Connell PJ, Wang X, Leon-Ponte M, Griffiths C, Pingle SC, Ahern GP: A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. *Blood* 2006; **107**:1010-7.

43. Kundermann B, Hemmeter-Spernal J, Strate P *et al*: Pain sensitivity in major depression and its relationship to central serotoninergic function as reflected by the neuroendocrine response to clomipramine. *J Psychiatr Res*. 2009; **43**:1253-61.

44. Husebekk A, Killie MK, Kjeldsen-Kragh J, Skogen B: Is it time to implement HPA-1 screening in pregnancy? *Curr Opin Hematol.* 2009; **16**:497-502.

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

		N	Mean/Median	Range
Age in yrs (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%	
I.Q. (N=71):	*>70*	18	25.4%	
	≤ 70	53	74.6%	

Abbreviations: SEM, standard error of the mean; IQR/2, semi-interquartilic range; PDD-NOS, Pervasice Developmental Disorder – Not Otherwise Specified; IQ, intellectual quotient.

Table 2. *ITGB3* haplotypes are associated with autism: (A) Haplotype structure at the *ITGB3* locus. Only haplotypes with estimated frequencies ≥0.005 are listed. (B) Haplotype family-based association tests performed using HBAT, under an additive model (-e).[32] 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 and gray, are significantly under- and over-transmitted, respectively, from heterozygous parents to the affected offspring.

A

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

B

ITGB3 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

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

GENOTYPES		Head circumference		5-HT blood levels		Global peptiduria	
ITGB3, SNP1: *rs2317385*	GG	82.5±47.5 *N=144*	K-W χ^2=3.542 2df, P=0.170	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 2df, P=0.433
	GA	75.0±47.4 *N=60*		468.9±44.0 *N=36*		309.6±32.3 *N=41*	
	AA	97.5 *N=3*		261.0 *N=1*		267.0± 168.0 *N=3*	
ITGB3, SNP5: *rs5918*	TT	82.5±47.5 *N=182*	K-W χ^2=1.377 2df, P=0.502	313.7±23.9 *N=102*	K-W χ^2=1.207 2df, P=0.547	344.3±17.4 *N=151*	K-W χ^2=0.793 2df, P=0.673
	CT	75.0±47.5 *N=74*		314.2±29.2 *N=48*		355.3±37.4 *N=74*	
	TT	82.5±47.5 *N=9*		421.1±104.5 *N=8*		281.2±59.0 *N=6*	
ITGB3, SNP8: *rs12603582*	GG	82.5±47.5 *N=121*	K-W χ^2=0.408 2df, P=0.815	368.4±31.6 *N=77*	K-W χ^2=0.181 2df, P=0.913	331.6±26.1 *N=107*	K-W χ^2=1.586 2df, P=0.453
	GT	78.7±47.5 *N=74*		353.7±35.0 *N=52*		340.6±25.8 *N=74*	
	TT	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 2df, P=0.115	390.3±43.9 *N=41*	K-W χ^2=4.821 2df, P=0.09	325.2±23.7 *N=50*	K-W χ^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*	

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

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 df, 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 df, 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 df, 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 df, 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 df, 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 df, p=0.022
- increased	20.0% (9)	33.3% (15)	46.7% (21)	
Autoimmune disease in 1st degree relatives				
-absent	28.7% (37)	44.2% (57)	27.1% (35)	χ^2=6.05, 2 df, p=0.048
-present	20.0% (5)	28.0% (7)	52.0% (13)	

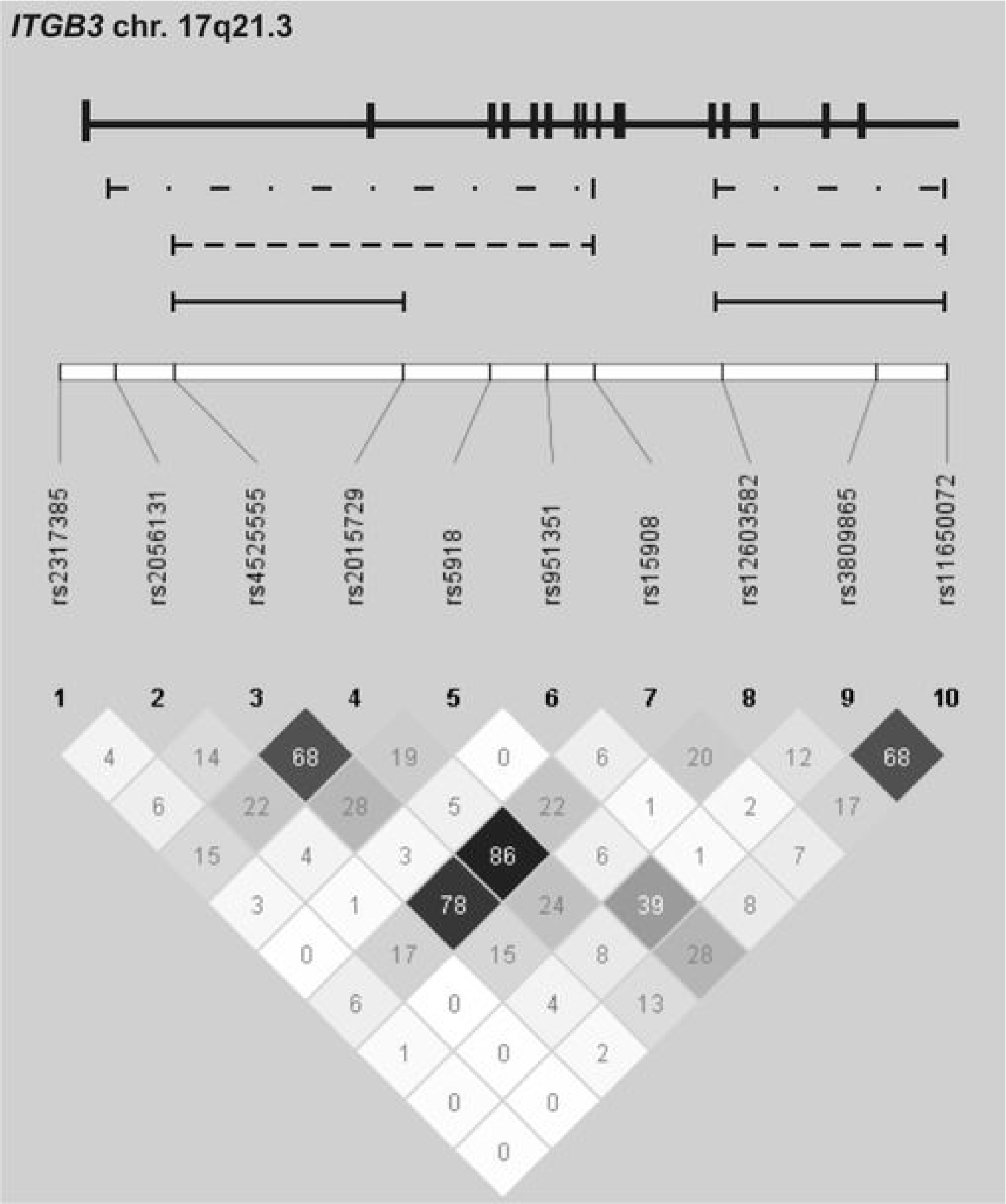
1

Immune and/or allergic disease in the family				
-absent	28.0% (37)	47.0% (62)	25.0% (33)	χ^2=4.98, 2 df, p=0.083
-present	23.8% (20)	36.9% (31)	39.3% (33)	

2

Figure Legends

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.


ITGB3 chr. 17q21.3
rs2317385
rs2056131
rs4525555
rs2015729
rs5918
rs951351
rs15908
rs12603582
rs3809865
rs11650072
1 2 3 4 5 6 7 8 9 10
4 14 68 19 0 6 20 12 68
6 22 28 5 22 1 2 17
15 4 3 86 6 1 7
3 1 78 24 39 8
0 17 15 8 28
6 0 4 13
1 0 2
0 0
0