# Evidence that ITGB3 promoter variants increase serotonin blood levels by regulating platelet serotonin transporter trafficking

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**Abstract:**

Elevated serotonin (5-HT) blood levels, the first biomarker identified in autism research, has been consistently found in 20-30% of patients with Autism Spectrum Disorder (ASD). Hyperserotonemia is mainly due to greater 5-HT uptake into platelets, mediated by the 5-HT transporter (SERT) located at the platelet plasma membrane. The protein complex involved in platelet SERT trafficking and externalization includes integrin β3, the beta subunit of the platelet membrane adhesive GP IIb/IIIa. Integrin β3 is encoded by the *ITGB3* gene, previously identified as a QTL for 5-HT blood levels in ASD at SNP *rs2317385*. The present study aims to identify the functional *ITGB3* gene variants contributing to hyperserotonemia. *ITGB3* gene sequencing in 20 individuals selected on the basis of *rs2317385* genotypes defined four haplotypes encompassing six SNPs located in the *ITGB3* gene promoter region, all in linkage disequilibrium with *rs2317385*. Luciferase assays in two hematopoietic cell lines, K-562 and HEL 92.1.7, demonstrate that *ITGB3* gene promoter activity is enhanced by the presence of the C allele at *rs55827077* specifically during differentiation into megakaryocytes ($P<0.01$), with modulatory effects by flanking SNPs. This same allele is strongly associated with (a) higher 5-HT blood levels in 176 autistic individuals ($P<0.001$), (b) greater platelet integrin β3 protein expression ($P<0.05$), and (c) enhanced SERT trafficking from the cytosol toward the platelet plasma membrane ($P=4{,}05 \times 10^{-11}$). Our results support *rs55827077* as the functional *ITGB3* gene promoter variant contributing to elevated 5-HT blood levels in ASD and define a mechanistic chain of events linking *ITGB3* to hyperserotonemia.

## Introduction

Autism Spectrum Disorder (ASD, OMIM #209850) is a neurodevelopmental condition characterized by deficits in social interaction and communication, stereotypic behaviors, insistence on sameness and abnormal sensory processing, with onset in early childhood (1). Its prevalence has been progressively increasing over the past few decades from 2-5:10,000 in the 1970s, to 1:59 children in 2010 (2,3). Despite major advances in our understanding of the neurobiological, genetic and developmental processes underlying ASD (4-7), their translational impact into clinical practice remains limited.

The genetic underpinnings of non-syndromic ASD are very heterogeneous, ranging from rare variants endowed with full penetrance, to common variants each contributing modest effects, but collectively enhancing disease vulnerability (6-9). Contributions by rare variants are usually easier to define, whereas the complex scenario of common variants and of gene-environment interactions often benefits from phenotypic dissections based on “endophenotypes”, heritable quantitative traits associated with the disorder and distributed continuously among patients and their first-degree relatives (10,11).

Elevated serotonin (5-HT) blood levels were the first (12) and one of the most consistently replicated biological endophenotypes identified in autism research (13-15). Recently, promising results in gene discovery have been obtained endophenotyping based on 5-HT blood levels (16). Collectively, hyperserotonemia is present in 22.5% and 28.3% of ASD patients, depending on whether measured in platelet-rich plasma or in whole blood, respectively (17). Several studies have addressed the mechanisms underlying elevated 5-HT blood levels in ASD. Hyperserotonemia appears primarily due to enhanced platelet 5-HT, accounting for approximately 99% of whole blood 5-HT content, whereas free 5-HT is barely detectable in plasma and does not seemingly differ between ASD cases and controls (18). Increased platelet 5-HT has been mainly linked to enhanced externalization

of the antidepressant-sensitive 5-HT transporter (increased Vmax). In contrast, SERT affinity (KD), while modulated by rare functional human SERT variation (19,20) that can induce elevated blood serotonin in transgenic mice (21), does not appear to be causal for hyperserotonemia across broader populations (22,23). An important role in this process is seemingly played by the protein complex involved in regulating SERT activity and trafficking at the platelet plasma membrane (11). This complex includes the adhesive protein receptor $\alpha_{IIb}\beta_3$ integrin, protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), Hic5 and the 5-$HT_{2A}$ receptor (15,24-26). In particular, the $\alpha_{IIb}\beta_3$ integrin, encoded by the *ITGB3* gene (chr. 17q21.32), physically interacts with SERT to promote its externalization and transport activity via PP1/PP2A and p38 MAPK signaling (27). This molecular mechanism, regulating SERT trafficking and 5-HT uptake into platelets, is modulated by genetic variation located in the *ITGB3* gene (27-32), as well as by rare variants at *SLC6A4*, the human gene encoding for SERT (19-21,33).

Remarkably, the Central Nervous System (CNS) and other peripheral systems share conserved proteins regulating 5-HT homeostasis. The *SLC6A4* and *HTR2A* genes encode the same SERT and 5-$HT_{2A}$ receptor, respectively, both in platelets and brain (34,35). The same β3 subunit, encoded by the *ITGB3* gene, interacts with either the $\alpha_{IIb}$ subunit or the αvβ3 glycoprotein to form platelet- and brain-specific integrins, respectively, both shown to interact with SERT and to regulate 5-HT uptake into platelets and brain (27,36). Furthermore, *ITGB3* and *SLC6A4* alleles were both identified as quantitative trait loci (QTLs) for 5-HT blood levels (29-32), and/or found genetically associated with ASD in several cohorts (31,33,37-40). We previously identified two independent markers, namely *rs2317385* and *rs12603582* located at opposite ends of the *ITGB3* gene, significantly associated with 5-HT blood levels and autism liability, respectively (28).

The present study was undertaken to identify and characterize the functional genetic variants responsible for *ITGB3* contributions to hyperserotonemia in ASD. These variants were predicted to be in linkage disequilibrium (LD) with *rs2317385* and thus presumably located in the *ITGB3* gene promoter region. Applying genetic and molecular strategies, we aimed at: (a) mapping haplotypes involving all SNPs located in the *ITGB3* promoter (*pITGB3*) and in LD with *rs2317385*, by sequencing the genomic DNA of 20 individuals selected on the basis of known *rs2317385* genotypes; (b) identifying functional SNPs by assessing with luciferase assay the influence of *pITGB3* haplotypes on gene expression in two hematopoietic and one neuronal cell lines, under the hypothesis that the former, but not the latter, would have shown genetic effects, since *rs2317385* is associated with hyperserotonemia and not with autistic disorder (28); (c) quantifying the effect of *pITGB3* alleles on 5-HT blood levels in a new cohort of 176 autistic individuals, (d) measuring *pITGB3* genotypic effects on *ITGB3* protein levels in platelets, and (e) assessing genotype-dependent differences in SERT trafficking between the cytosol and the platelet plasma membrane.

## Results

*SNPs in linkage disequilibrium with rs2317385 influence ITGB3 promoter activity*

DNA re-sequencing of the *ITGB3* promoter, exons and exon-intron junctions was performed in 20 individuals selected on the basis of genotypes at *rs2317385*, the SNP previously found associated with 5-HT blood levels (28) (Supplementary Material, Table S3). No protein coding variant was identified, aside from Leu33Pro encoded by *rs5918*, previously found to regulate 5-HT uptake into platelets through SERT surface expression (27), but not associated with 5-HT blood levels in our cohort (28). Sequencing of the *ITGB3* gene promoter region identified a total of six SNPs in tight LD with *rs2317385*, all

listed on dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) (Fig. 1, Table 1). These six SNPs yield four different haplotypes, hereby named *ACGACG-H1, ACGACC-H2, GTAAAG-H3, GTAAAC-H4*, which were then tested for differences in effects on promoter activity using luciferase assays (Fig. 1). Following transfection with four different pITGB3::luc2 pGL4.10 constructs (Fig. 1, bottom), we recorded no modulation of *ITGB3* promoter activity by *H1-H4* haplotypes in undifferentiated K-562 cells [ANOVA, F=2.680 (3 df), P<0.182] (Fig. 2A), and increased gene expression in undifferentiated HEL 92.1.7 cells transfected with *ACGACG-H1* and *GTAAAG-H3*, compared to *ACGACC-H2* (P=0.001) and *GTAAAC-H4* (P=0.006), respectively [ANOVA, F=17.668 (3 df), P<0.01], in the context of relatively low promoter activity (Fig. 2B). TPA-induced differentiation into megakaryocytes produced dramatic five-fold increases in *ITGB3* promoter activity in both hematopoietic cell lines, as expected (41), with more consistent and sizable effects exerted by the *H1-H4* haplotypes [K-562: ANOVA, F=20.688 (3 df), P<0.001; HEL 92.1.7 ANOVA, F=18.982 (3 df), P<0.001] (Fig. 2C and 2D). In both differentiated cell lines, the presence of the *C* allele at *rs55827077* significantly increased luciferase activity compared to the *G* allele, while *GTAAAC-H4* showed the highest luciferase activity (Fig. 2C and 2D), reversing trends present in undifferentiated HEL 92.1.7 cells. The same constructs transfected into neuronal N2A cells did not display any effect of the *H1-H4* haplotypes on *ITGB3* gene promoter activity, despite elevated luciferase activity levels (Supplementary Material, Fig. S1). GTEx data (https://gtexportal.org/home/) relative to these same six SNPs are in line with our results from undifferentiated HEL 92.1.7 cells (Fig. 2B). The C ("alternative") allele at *rs55827077* is indeed associated with significantly lower *ITGB3* gene expression in all tissues tested, including brain anterior cingulate cortex (blue dots in Figure S3). Hence, the expression-promoting effect of the C allele following TPA-induced differentiation (Figs. 2C and 2D) is apparently specific to hematopoietic cells.

Altogether, these results support a primary role for the C allele at *rs55827077* in enhancing *ITGB3* gene promoter activity during megakaryocytic differentiation. This influence appears modulated by the upstream haplotypes *ACGAC* and *GTAAA*, which blunt and boost, respectively, the transcription-enhancing effect of the C allele at *rs55827077*.

*SNPs rs55827077 and rs3809862 significantly influence 5-HT blood levels in ASD individuals*

In order to investigate the potential relationship between these *ITGB3* gene promoter variants and 5-HT blood levels, 566 simplex and 64 multiplex families with a non-syndromic autistic proband were genotyped at *rs55827077.* Among other modulatory SNPs, also *rs3809862* was genotyped in the entire sample in order to extend prior work describing effects by *rs7208055* (-400 bp), *rs62074393* (-425 bp), and *rs7208170* (-468 bp) on *ITGB3* promoter activity in HEL 92.1.7 cells (42), while other data suggest that regulatory elements active in K-562 cells may be mainly located downstream of -146 bp and upstream of -584 bp from the ATG start site (43). Both SNPs were in HWE stratifying by family status (Supplementary Material, Table S4) and were confirmed in tight LD (D'=0.93, P=0.0001). In accordance with *in vitro* results (Fig. 2C and 2D), 5-HT blood levels measured in 176 ASD individuals were significantly higher in carriers of the *C* allele at *rs55827077* (U=2572.0, P<0.001) and the *T* allele at *rs3809862* (U=2469.5, P<0.01) (Fig. 3A and 3B). These two SNPs explain respectively 5.7% and 5.5% of the variance in 5-HT blood levels (both P<0.01) (Supplementary Material, Table S5). Gene-gene interactions were tested in a subset of 112 autistic individuals with known 5-HT blood levels, fully genotyped at *ITGB3 rs55827077* and *rs3809862*, as well as at the *SLC6A4* 5-HTTLPR and intron2 VNTR polymorphisms. No gene x gene interaction reached statistical significance, but both *ITGB3* SNPs displayed their effects on 5-HT blood levels only in the presence of

the SS and SL 5-HTTLPR genotypes (P<0.01), and not in the presence of the LL genotype (P=0.58 and 0.38, respectively) (Supplementary Material, Fig. S2). In line with our previous results (28), using either haplotype or single-marker analyses, no significant association was found between the two promoter SNPs *rs55827077* / *rs3809862* and Autism Spectrum Disorder (Supplementary Material, Table S6).

*CC/TT genotypes at rs55827077/rs3809862 increase platelet integrin β3 protein levels and SERT externalization*

Platelets from individuals carrying different genotypes at *rs55827077* and *rs3809862* were analysed to assess integrin β3 protein levels and SERT trafficking among three subcellular compartments: (a) the cytoskeletal insoluble fraction (CS), (b) the membrane skeleton fraction (MS) and (c) the supernatant Triton-X soluble fraction (TS). CS and MS fractions each contain ~20% of total platelet proteins on average and result enriched in filamentous actin and adhesion proteins, respectively; the TS fraction contains mostly soluble cytosolic proteins (26). Individuals homozygous for the "high expression - high 5-HT" C and T alleles at *rs55827077 / rs3809862,* respectively, showed significantly higher integrin β3 protein levels as compared to homozygous GG/CC genotype carriers (P<0.05) (Fig. 4). Importantly, a significant redistribution of SERT was associated with these same genetic backgrounds (Fig. 5). SERT was largely more distributed in the CS and MS fractions of CC/TT individuals, as compared to GG/CC and GC/CT genotype carriers, whose platelet SERT was predominantly internalized in the cytosolic TS fraction (2-way ANOVA, $P=4.05 \times 10^{-11}$) (Fig. 5). Also SERT protein levels displayed a similar trend, with 1.7- and 2.1-fold mean increases in carriers of the GC/CT and CC/TT genotypes compared

to GG/CC individuals (P=0.096), albeit with greater interindividual variability compared to SERT distribution (Suppl. Fig. S4).



**Discussion**

The present study shows that the *ITGB3* gene promoter contains functional common variants able to significantly modulate gene expression in differentiating cells of the megakaryocytic lineage. In particular, the *C* allele at *rs55827077* provides the strongest effect on promoter activation and is associated with hyperserotonemia in our ASD sample. The *T* allele at *rs3809862* provides additional contributions, likely in conjunction with flanking SNPs. In turn, increased *ITGB3* gene promoter activity correlates with enhanced platelet integrin β3 protein levels, and with a striking redistribution of SERT among the membrane and cytosolic compartments, favoring greater SERT externalization at the platelet plasma membrane. Differences in SERT compartimentalization may also shed new light on the link between ITGB3 and SERT protein levels seen here (compare Figure 4 and Supplementary Figure S4) and previously suggested by others (32, 38-40), as protein turnover may target more actively internalized rather than membrane-bound SERT molecules. These results thus largely extend our previous findings concerning the genetic association between 5-HT blood levels in ASD and SNP *rs2317385*, located at -1454 bp from the ATG start site of the *ITGB3* gene (28), as well as prior functional analyses of the *ITGB3* promoter, neither including *rs55827077*, nor evaluating the major influence of differentiation on hematopoietic cell line gene expression (42,43). Indeed the promoter region sequenced in the present study is within the LD block tagged by *rs2317385*. Both *rs55827077* and *rs3809862* are in LD with *rs2317385,* and fall into the regulatory region containing megakaryocytic cell-specific *cis*-acting elements known to positively regulate *ITGB3* promoter activity (43). Most importantly, these data provide a functional link

between *ITGB3* promoter activity and 5-HT blood levels, by documenting a genotype-dependent differential subcellular distribution of platelet SERT.

Direct physical interaction between SERT and the integrin $\alpha_{IIb}\beta_3$ complex has been shown to foster SERT externalization at the plasma membrane, boosting 5-HT uptake in platelets (26,27). In line with this evidence, individuals carrying *ITGB3* promoter-activating alleles at SNPs *rs55827077* and *rs3809862* here display higher 5-HT blood levels, reasonably due to enhanced SERT externalization on the platelet plasma membrane, as promoted by greater synthesis of integrin β3 (Figures 4 and 5). Meanwhile, *SLC6A4* gene variants continue to exert more modest effects on 5-HT blood levels in our sample (44,45), nor do we find significant gene-gene interactions between *SLC6A4* and *ITGB3* gene promoter alleles, although the non-significant trend displayed by the 5-HTTLPR (Supplementary Figure S2) is being currently tested in a larger sample. These results contrast with previous reports of interactions between *SLC6A4* and the *ITGB3* Pro33 allele at *rs5918* (32,36-38,40), a SNP which does not show any effect in our sample (28). These inconsistencies ought to be expected when considering the genetic complexity of 5-HT blood levels (30) and of ASD (6,7). It is nonetheless reassuring to see converging genetic and biochemical evidence support enhanced integrin β3 in platelets as a measurable contributor to hyperserotonemia in autism, despite variable contributions from genetic variants at other loci.

Hyperserotonemia was the first biomarker found associated with ASD, a neurodevelopmental disorder (12). *ITGB3* plays a role both in hyperserotonemia and in autism risk, through at least two distinct gene variants located at the 5' and at the 3' ends of the gene, respectively, as tagged by *rs2317385* and *rs12603582* (Fig. 1) (28). Collectively, published results also fall along this same line of evidence, with hyperserotonemia- and ASD-associated SNPs mainly located towards the 5' and 3' ends of

the gene, respectively (Fig. 6). Also in the present study, alleles at *rs55827077* and *rs3809862* significantly contribute to enhance 5-HT blood levels and not autism risk, while influencing *ITGB3* promoter activity in two hematopoietic cell lines and not in N2A neuronal cell lines (Supplementary Material, Fig. S1). Interestingly, GTEx data suggest that in differentiated neural tissues these SNPs may actually modulate gene expression, but in the opposite direction (i.e, decreased expression) compared to differentiated hematopoietic cells (see "brain anterior cingulate cortex BA24" and "tibial nerve" in Supplementary Figure S3). This again underscores the uniqueness of *ITGB3* gene expression regulation in hematopoietic cells, as compared to other cells and tissues which, according to GTEx, may maintain following differentiation the same transcriptional control displayed by undifferentiated HEL 92.1.7 cells (Fig. 2B). Hence genetic variants influencing *ITGB3* transcriptional control appear primarily related to hyperserotonemia rather than to abnormal neurodevelopment. Clearly, this does not imply that 5-HT uptake is not relevant to autism pathogenesis or to social cognition, nor that integrin β3 is not relevant to brain physiology. On the one hand, initial suggestions of possible neurodevelopmental consequences of reduced 5-HT uptake, derived from the observation of minor barrel cortex anomalies in SERT hz mice (46), were later confirmed by studies demonstrating enhanced autism risk in children prenatally exposed to selective serotonin reuptake inhibitors (SSRIs) (47). Furthermore, rare missense variants in the *SLC6A4* gene have been shown to elevate in vitro 5-HT uptake and in vivo 5-HT clearance, as well as contribute to traits of ASD (21,33). On the other hand, integrins play critical roles in neurodevelopment, acting as cell adhesion molecules in the CNS (48,49). In particular, integrin β3 is required for the proper maturation of NMDA-positive excitatory synapses (50), as well as in controlling synaptic strength by regulating AMPA receptors in a subunit-specific manner (51). Moreover, SERT and integrin αvβ3 interact to modulate 5-

HT uptake in presynaptic terminals of serotonergic neurons (36,52). However, our results primarily explain how these *ITGB3* gene variants modulate 5-HT blood levels by playing functional roles in platelet physiology. The modest, yet sizable effects exerted by these *ITGB3* promoter variants on 5-HT uptake (i.e., ~5-6% of the total variance) are within the range of effects on quantitative traits typical of common genetic variants, as compared to the dramatic effects exerted by fully penetrant rare variants able to profoundly influence neurodevelopment and produce deficits in social cognition (21,33). The lack of association between *ITGB3* promoter SNPs and ASD in our cohort likely stems from the presence of hyperserotonemia only in a subset of autistic subjects (17), the *ITGB3* promoter SNPs contributing to only 5% of blood 5-HT variance, and the low LD between the promoter SNPs and *rs12603582*, the SNP located in intron 11 and previously found most associated with ASD (28). Nonetheless, these same promoter SNPs could display some degree of genetic association with ASD in much larger samples, through loose LD with more 3' autism-relevant gene variants (28). Moreover, it will be interesting to assess whether *ITGB3* promoter variants exert subtle influences on the clinical phenotype in ASD, for example contributing to repetitive behaviors, self-injury or to the gastrointestinal abnormalities frequently seen in autistic children (21,33,53-55).

The exact mechanism underlying allele-specific transcriptional regulation of the *ITGB3* promoter remains to be determined. *In silico* analyses of *rs55827077* and *rs3809862* alleles performed using the Alibaba2.1 online tool (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), predict neither gain nor loss of transcription factor binding sites able to fully explain our functional results (Supplementary Material, "Allele-specific Response Element Analysis" file). Preliminary electrophoretic mobility shift assays seemingly confirm this prediction (data not shown). This analysis excludes the transcription factor SP1, a promising candidate for allele-

specific regulation, postulated to augment *ITGB3* gene expression in megakaryocytic cell lines including K562, by binding to response elements located within the regulatory region under scrutiny here (42,43). Furthermore, the G, and not the C allele, at *rs55827077* is predicted to create a functional response element for ETF, another transcription factor relevant to megakaryocytic differentiation (Supplementary Material, "Allele-specific Response Element Analysis" file) (56). ETF binding could thus contribute to higher basal expression by the G allele in HEL 92.1.7 (Fig. 2B), but not to C-allele effects seen following differentiation (Figure 2C and 2D). Also modulatory effects on gene expression by *rs7208055*, *rs62074393* and *rs7208170* were shown not to be paralleled by differential nuclear protein binding profiles (42). Further experiments will thus be necessary to assess whether and through what mechanism larger-scale chromatin remodeling may be involving flanking sequences and possibly the intergenic control region located 2.5 to 7.1 kb upstream of the *ITGB3* gene (57).

The present study displays several limitations: functional evidence on neuronal cells is more limited than data regarding megakaryocytes, as only one neuronal cell line was tested; in addition to SERT trafficking, $\alpha_{IIb}\beta_3$ controls several platelet functions potentially able to modulate 5-HT blood levels, including cell-cell adhesion which can promote vesicular release; future experiments will have to employ direct measures of surface SERT, primarily transporter biotinylation; gene x gene interactions not addressed in this study, involving combinations of SNPs located in genes encoding factors that utilize elevated *ITGB3* to drive SERT more efficiently from the MS fraction to the plasma membrane, could further magnify the functional role of the *ITGB3* gene variants described here. Nonetheless, our results clearly support a functional model whereby the C allele at *rs55827077*, in conjunction with the flanking haplotype GTAAA also encompassing the T allele at *rs3809862*, contribute to enhance 5-HT blood levels in ASD by: (a) increasing

*ITGB3* promoter activity during cell differentiation into megakaryocytes, (b) in turn yielding higher platelet integrin β3 protein levels, (c) ultimately facilitating SERT trafficking toward the platelet plasma membrane and possibly sustaining enhanced SERT protein levels. This mechanism primarily explains *ITGB3* contributions to hyperserotonemia, not to autism as a neurodevelopmental disorder *per se*, although clinically meaningful phenotypic influences in autistic individuals may well be present and will deserve further scrutiny. Identification of a different *ITGB3* gene variant, located toward the 3' end of the gene and in LD with *rs12603582* (Fig. 1), will now be the object of further research, aimed at understanding *ITGB3* effects of greater impact on neurodevelopment and autism risk.

## Materials and Methods

### ITGB3 gene sequencing

PCR amplifications were performed using 40 ng of gDNA and purified products were sequenced using a CEQ8000 apparatus (Beckman-Coulter). Oligonucleotides used to amplify the *ITGB3* gene promoter region, all 15 exons and exon-intron junctions are listed in Supplementary Table S1. Re-sequencing was performed in order to carefully verify LD status between alleles at known common variants.

### Genotyping and family-based association analysis

SNPs *rs55827077* and *rs3809862* were genotyped using Thermo Fisher TaqMan® SNP Genotyping Real-Time PCR-Based Assays (Applied Biosystems) on a total of 566 simplex and 64 multiplex families with a non-syndromic autistic proband, including 676 ASD patients, 221 unaffected siblings, and 1227 parents (total genotyped N=2124). Diagnostic screening procedures used to exclude syndromic autism have been previously described

(24). Only one patient per multiplex family was randomly recruited for family-based association analyses. Demographic, clinical and endophenotypic characteristics are summarized on Supplementary Table S2. 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).

Mendelian inheritance was verified using PedCheck (58). Hardy–Weinberg equilibrium (HWE) and LD were tested using Haploview v4.2 (available at http://www.broad.mit.edu/mpg/haploview/index.php) (59). Family-based haplotype and single-marker association tests were performed using FBAT (available at https://sites.google.com/view/fbat-web-page), under additive and dominant models (the latter also tests the recessive model for the opposite allele), applying an empirical variance that produces a valid test when linkage is present and the null hypothesis is "no association" (option –e) (60). The HBAT procedure in FBAT was also used to estimate haplotype frequencies and to compute an exact P-value using a Monte Carlo approach (option -p), both for the global whole-marker permutation test ($\chi^2$ sum P) and for each haplotype separately (60). Generalized linear models were used: (a) to test for the interaction between *5-HTTLPR* alleles at *SLC6A4* and *pITGB3* SNPs, and (b) to estimate the total amount of variance in 5-HT blood levels explained by *rs3809862* and *rs55827077*.

**Measurement of 5-HT blood levels**

Serotonin blood levels were measured in 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 (28). Serotonin blood levels

were analyzed as a quantitative trait by Mann-Whitney U-tests based on genotype distributions, applying a stringent Bonferroni correction for multiple testing.

**Generation of ITGB3 reporter constructs**

The *ITGB3* gene promoter region (from +29 to – 1268) was PCR amplified from genomic DNA of individuals carrying different haplotypes of interest. Fragments were introduced into the pGL4.10 vector (Promega) fused with the *LUC2* reporter gene and positive clones were confirmed for proper orientation by restriction and sequencing analyses.

**Cell lines and induction of differentiation**

Two hematopoietic and one neuronal cell lines were employed:

(a) K-562 and HEL 92.1.7 human erythroleukemic pluripotent stem cells were grown in RPMI 1640 medium, supplemented with 10% FBS, 2 mM glutamine and antibiotic mix (Gibco). Megakaryocytic differentiation was induced adding 12-O-tetra-decanoylphorbol-13-acetate (TPA) at a final concentration of 16 nM (10 ng/ml) (41,61);

(b) murine Neuroblastoma 2A (N2A) cells were grown in Advanced D-MEM/F-12 supplemented with 10% FBS, 2mM glutamine, 0.1 mM MEM Non-Essential Amino Acids Solution (Gibco) and antibiotic mix.

**Luciferase reporter assay**

Cells were transiently co-transfected using Lipofectamine™ LTX Reagent, with 1 μg of pITGB3::luc2 pGL4.10 vector and 5 ng Renilla Luciferase-TK (pRL-TK) vector (Promega), as internal control. For transfections, 1 x $10^5$ K-562 and HEL 92.1.7 cells and 1

x $10^4$ N2A cells were seeded in 24-multiwell plate, incubated a 37°C and 5% $CO_2$ for 24 h or 48 h prior to reporter gene analysis. Luciferase reporter gene analysis was performed using the Dual-Luciferase Reporter Assay system (Promega). Transfected cells were pelleted by centrifugation, washed and lysed with 100 µl Passive Lysis Buffer (PLB). Luciferases were determined in 20 µl of lysate using a Wallac 1420 Multilabel Counter (Perkin-Elmer). Firefly luciferase activity was normalized to Renilla luciferase. Each construct was assessed in triplicate in every transfection and three-four independent sets of transfections were performed. Results are expressed as Luciferase/Renilla ratio mean values ± S.E.M. Statistical analysis was performed using either parametric ANOVA or non-parametric Kruskal-Wallis (K-W) test, depending on whether the assumption of variance homogeneity was satisfied according to Levene's test.

**Isolation of platelet fractions, protein extracts and Western blotting**

Blood was collected from healthy blood donors who gave written consent to the use of their biomaterials for research purposes. Platelet fractions were isolated using standard protocols (26). Briefly, $10^8$ platelets were obtained from Platelet-Rich Plasma (PRP) after centrifugation at 2,500 g for 15 min at room temperature. After a quick rinse in phosphate-buffered saline (PBS) buffer, pH 7.4, platelets were lysed with 100 µl of 1% Triton X-100, 21 µM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 µM pepstatin A, and 0.56 trypsin inhibitor unit/ml aprotinin in PBS, pH 7.4. Samples were resuspended and spun at 15,600 g for 4 min at 4°C to collect cytoskeletal pellets (CS). Supernatants were then centrifuged at 100,000 g for 2 h (Sorvall™ MTX 150 Micro-Ultracentrifuge) to separate membrane skeleton (MS) pellets from the Triton-soluble (TS) supernatant. Each pellet was resuspended in 100 µl of RIPA buffer and the total content immediately loaded onto 10% SDS-PAGE for Western blot analysis. SERT amounts were assessed using monoclonal

anti-serotonin transporter antibodies (ab181034 by Abcam, 1:5000). Figures show SERT amounts in each fraction, whereby CS + MS + TS = 100%. Genotypic effects on SERT distribution among the three fractions were analyzed by 2-way ANOVA.

Western blot analysis was performed using a monoclonal anti-integrin β3 antibody (ab34409 by Abcam, 1:10000), paired with monoclonal anti-β Actin (ab6276 by Abcam, 1:10000), after separation and blotting of total platelet lysates in RIPA buffer. Statistical analysis was performed by parametric ANOVA.

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**Conflict of Interest Statement**

The authors declare no conflict of interest

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**Figure Legends**

**Figure 1:** Schematic representation of (*top panel*) the *ITGB3* promoter region (*pITGB3*) and the exon-intron structure of the *ITGB3* locus, with the position of SNPs analysed in this study, and (*bottom panel*) the four constructs cloned into the pGL4.10 vector and used for luciferase assays.

*rs2317385 and ** rs12603582 are the SNPs previously associated with 5-HT blood levels and with autism spectrum disorder, respectively (24).

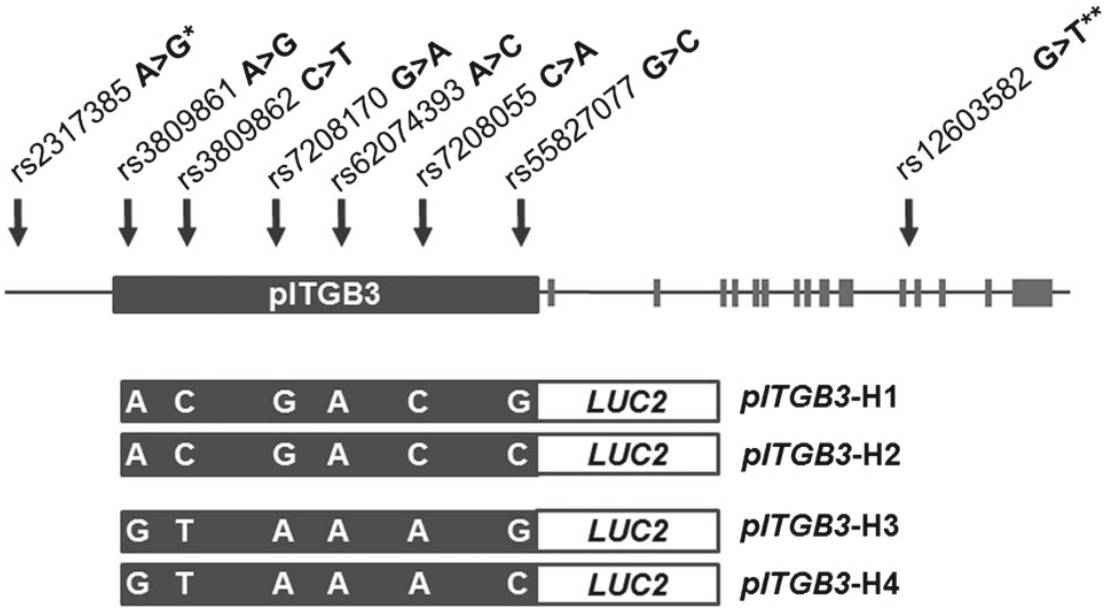

**Figure2**: Luciferase assays performed on (A) undifferentiated K-562 cells, (B) undifferentiated HEL 92.1.7 cells, (C) K-562 and (D) HEL 92.1.7 cells treated with TPA 10 ng/ml for induction of megakaryocytic differentiation. All experiments were performed after 48h incubation in the presence/ absence of the differentiation-promoting agent TPA. Data are presented as mean Firefly/Renilla relative luminescence units (RLU) ± SEM. N=4 transfections per group; all four constructs assessed in triplicate in each transfection.

* P<0.05; ** P<0.01; *** P<0.001

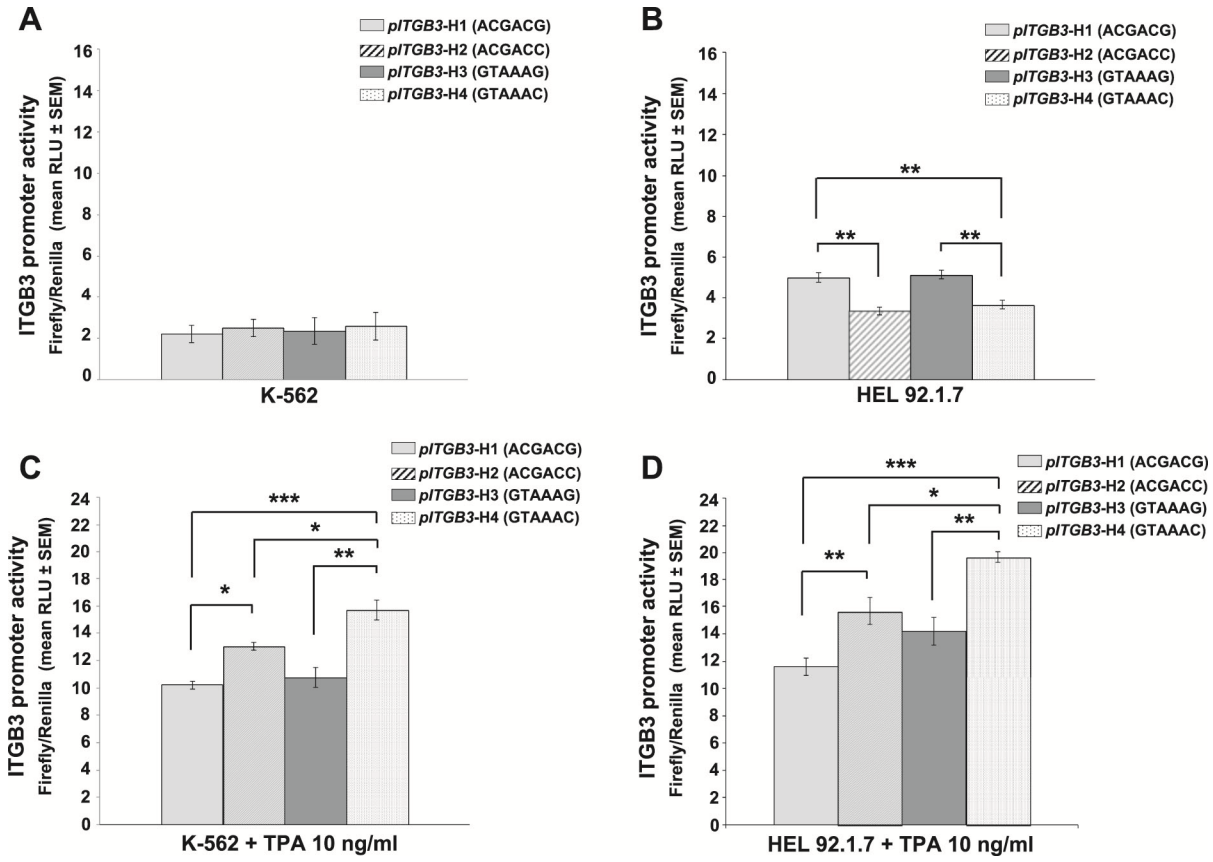

**Figure 3:** Serotonin (5HT) blood levels (ng/ml) in 176 ASD individuals by genotype at *ITGB3* promoter SNPs (A) *rs55827077* and (B) *rs3809862*.

** P<0.01; *** P<0.001

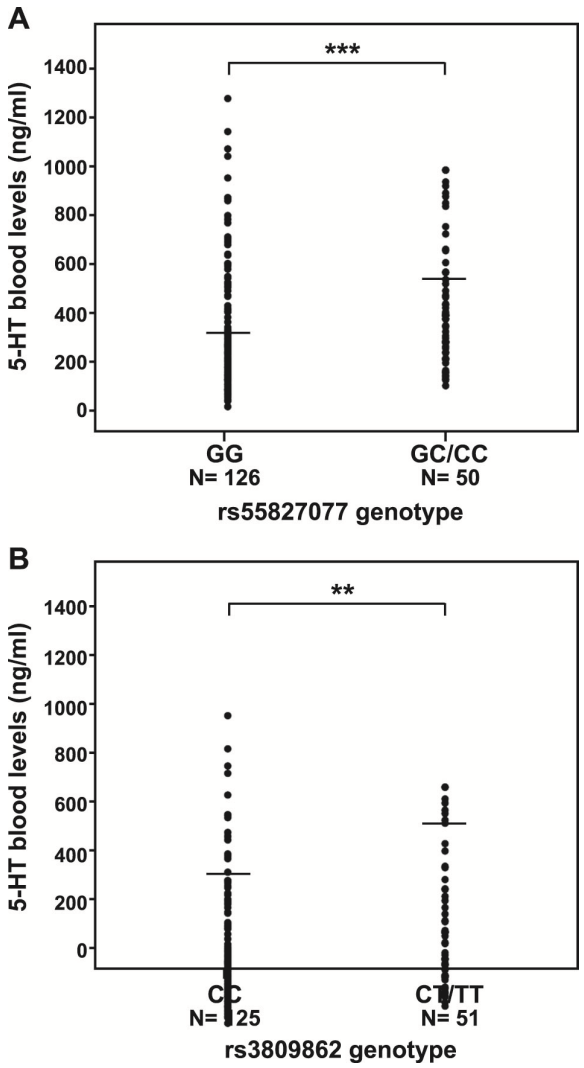

**Figure 4:** Integrin β3 protein levels normalized over β-actin in platelet lysates of healthy control individuals carrying different *rs55827077*/ *rs3809862* genotypes (N=3 per group).

* P<0.05

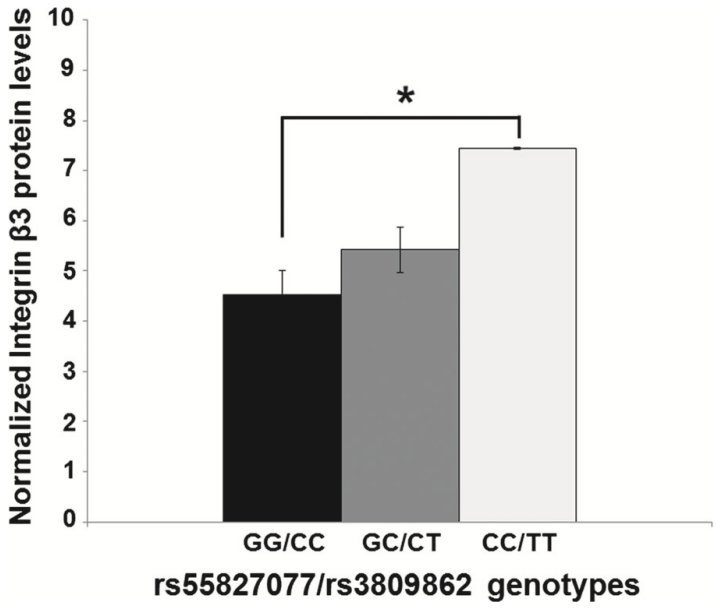

**Figure 5**: Subcellular SERT distribution in platelets of healthy control individuals carrying different *rs55827077*/*rs3809862* genotypes. N=5 per group. Two-way ANOVA: $P=4.05x10^{-11}$

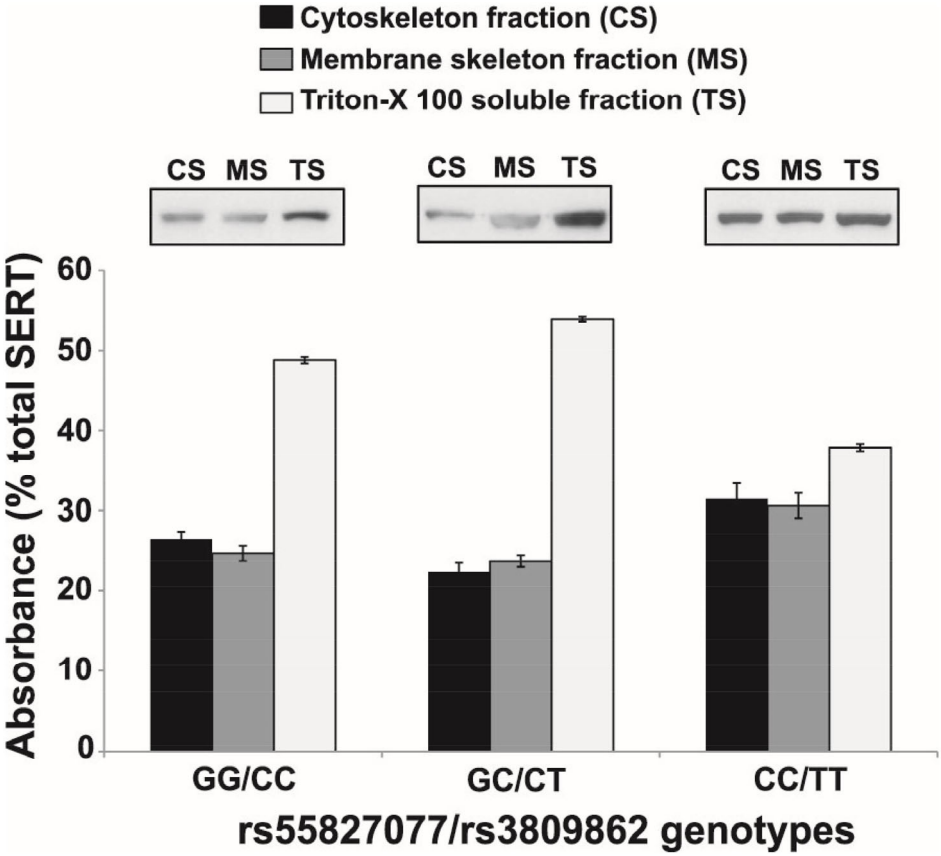

**Figure 6:** Schematic map of the distribution of ITGB3 SNPs found associated with ASD (empty triangles) or with 5-HT blood levels (black triangles) in previous studies involving outbred populations. SNPs analysed in this study are depicted at the top.

* Only statistically significant associations from the outbred Chicago population are displayed;

**Associated with ASD in gene x gene interaction with the SLC6A4 5-HTTLPR long/short polymorphism

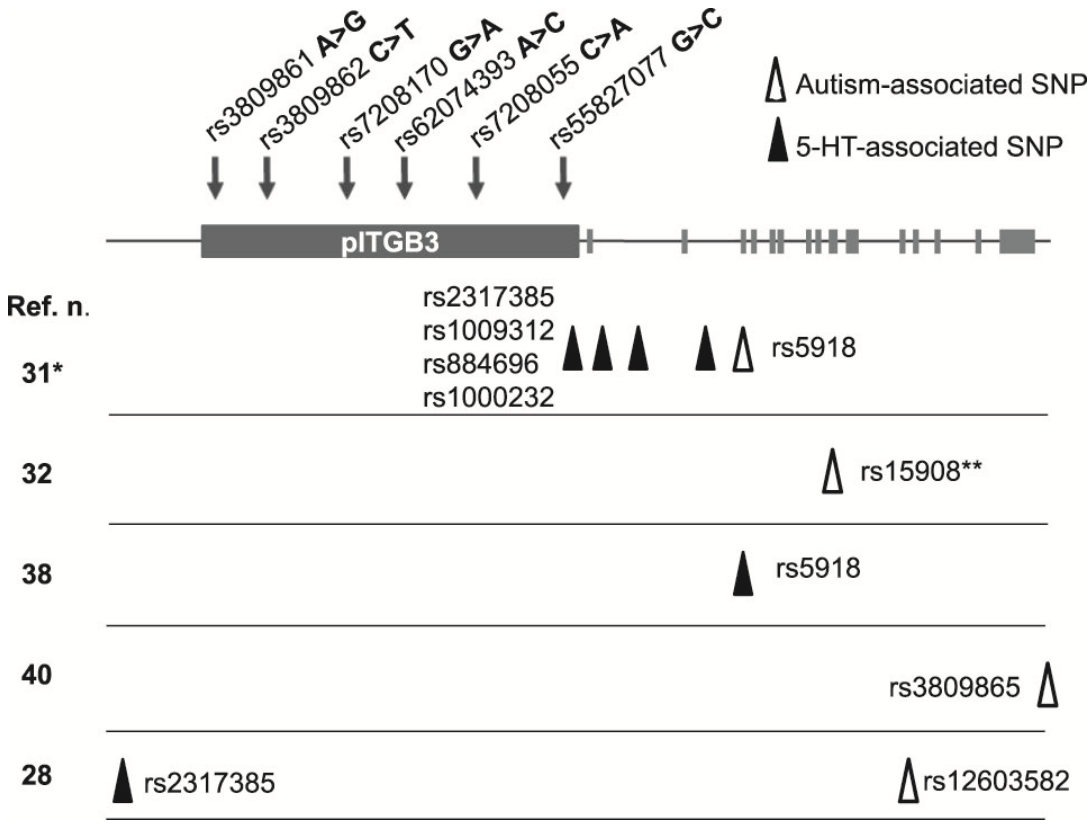

**Table 1.** SNPs located in the *ITGB3* gene promoter region, listed in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) and resequenced here for haplotyping. SNPs genotyped in the family-based association study are highlighted in bold.

| SNP id. | A1>A2 | Position in contig* | bp from ATG |
|---|---|---|---|
| rs3809861 | A>G | 45,330,066 | -1161 |
| **rs3809862** | C>T | 45,330,428 | -799 |
| rs7208170 | G>A | 45,330,759 | -468 |
| rs62074393 | A>C | 45,330,802 | -425 |
| rs7208055 | C>A | 45,330,827 | -400 |
| **rs55827077** | G>C | 45,331,083 | -144 |

*Contig Label GRCh37; NT_010783.15.

**Abbreviations:**

5-HT - serotonin

5-HT transporter (SERT)

ASD - Autism Spectrum Disorder

CNS - Central Nervous System

CS - Cytoskeletal insoluble fraction

HBAT - Haplotype-based association test

HWE - Hardy–Weinberg equilibrium

FBAT - Family-based association test

pITGB3 - ITGB3 promoter

K-W – Non-parametric Kruskal-Wallis ANOVA

LD - Linkage disequilibrium

MS - Membrane skeleton fraction (MS)

PBS - Phosphate-buffered saline

PRP - Platelet-Rich Plasma

PP1 - Protein phosphatase 1

PP2A - Protein phosphatase 2A

QTLs - Quantitative trait loci

SNPs – Single nucleotide polymorphisms

SSRIs - Selective serotonin reuptake inhibitors

TS - Supernatant Triton-X soluble fraction