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## Abnormal melatonin synthesis in autism spectrum disorders

Jonas Melke<sup>1</sup>, Hany Goubran-Botros<sup>1</sup>, Pauline Chaste<sup>1</sup>, Catalina Betancur<sup>2</sup>, Gudrun Nygren<sup>3</sup>, Henrik Anckarsäter<sup>3,4</sup>, Maria Rastam<sup>3</sup>, Ola Ståhlberg<sup>3</sup>, I. Carina Gillberg<sup>3</sup>, Richard Delorme<sup>1</sup>, Nadia Chabane<sup>5</sup>, Marie-Christine Mouren-Simeoni<sup>5</sup>, Fabien Fauchereau<sup>1</sup>, Christelle M. Durand<sup>1</sup>, Fabien Chevalier<sup>1</sup>, Xavier Drouot<sup>6</sup>, Corinne Collet<sup>7</sup>, Jean-Marie Launay<sup>7</sup>, Marion Leboyer<sup>2,8</sup>, Christopher Gillberg<sup>3,9</sup>, Thomas Bourgeron<sup>1,10</sup> and the PARIS study<sup>11</sup>

<sup>1</sup> Human Genetics and Cognitive Functions, Institut Pasteur, 75015 Paris, France; <sup>2</sup> INSERM U513, Université Paris XII, 94000 Créteil, France; <sup>3</sup> Department of Child and Adolescent Psychiatry, Göteborg University, 41119 Göteborg, Sweden; <sup>4</sup> Institute of Clinical Sciences, Lund University, 20502 Malmö, Sweden; <sup>5</sup> Service de Psychopathologie de l'Enfant et de l'Adolescent, Hôpital Robert Debré, Assistance Publique-Hôpitaux de Paris, 75019 Paris, France; <sup>6</sup> Service de Physiologie-Explorations Fonctionnelles, Hôpital Henri Mondor, 94000 Créteil, France; <sup>7</sup> Service de Biochimie, IFR 139, Hôpital Lariboisière, Assistance Publique-Hôpitaux de Paris, EA 3621, Faculté de Pharmacie, 75010 Paris, France; <sup>8</sup> Département de Psychiatrie, Hôpital Henri Mondor et Albert Chenevier, Assistance Publique-Hôpitaux de Paris, 94000 Créteil, France; <sup>9</sup> Saint George's Hospital Medical School, London SW17 0RE, United Kingdom; <sup>10</sup> University Denis Diderot Paris 7, Paris 75005, France

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Correspondence: Professor Thomas Bourgeron, Human Genetics and Cognitive Functions, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France. E-mail: thomasb@pasteur.fr

<sup>11</sup> Paris Autism Research International Sibpair Study: Sweden, Department of Child and Adolescent Psychiatry, Göteborg University, Göteborg: Christopher Gillberg, Maria Råstam, Carina Gillberg, Gudrun Nygren, Henrik Anckarsäter, Ola Ståhlberg, Catrin Håkansson. France, Department of Psychiatry, Hôpital Albert Chenevier et Henri Mondor, Créteil: Marion Leboyer; INSERM U513, Université Paris XII, Créteil: Catalina Betancur; Service de Psychopathologie de l'Enfant et l'Adolescent, Hôpital Robert Debré, Paris: Catherine Colineaux, Deborah Cohen, Nadia Chabane, Marie-Christine Mouren-Siméoni; INSERM U679, Hôpital Pitié-Salpêtrière, Paris: Alexis Brice. Norway, Centre for Child and Adolescent Psychiatry, University of Oslo, Oslo: Eili Sponheim; Department of Pediatrics, Rikshospitalet, University of Oslo, Oslo: Ola H. Skjeldal. USA, Department of Pediatrics, Georgetown University School of Medicine, Washington D.C.: Mary Coleman; Children's National Medical Center, George Washington University School of Medicine, Washington, D.C.: Philip L. Pearl; New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York: Ira L. Cohen, John Tsiouris. Italy, Divisione di Neuropsichiatria Infantile, Azienda Ospedaliera Senese, Siena: Michele Zappella. Austria, Department of General Psychiatry, University Hospital, Vienna: Harald Aschauer. Belgium, Centre de Génétique Humaine, Institut de Pathologie et de Génétique, Gerpennes, Loverval: Lionel Van Maldergem.

**Melatonin is produced in the dark by the pineal gland and is a key regulator of circadian and seasonal rhythms. A low melatonin level was reported in individuals with autism spectrum disorders (ASD), but the underlying cause of this deficit was unknown. The *ASMT* gene, encoding the last enzyme of melatonin synthesis, is located on the pseudo-autosomal region 1 of the sex chromosomes, deleted in several individuals with ASD. In this study, we sequenced all *ASMT* exons and promoters in individuals with ASD (n=250) and compared the allelic frequencies with controls (n=255). Non-conservative variations of *ASMT* were identified, including a splicing mutation present in two families with ASD, but not in controls. Two polymorphisms located in the promoter (rs4446909 and rs5989681) were more frequent in ASD compared to controls ( $P=0.0006$ ) and were associated with a dramatic decrease in *ASMT* transcripts in blood cell lines ( $P=2 \times 10^{-10}$ ). Biochemical analyses performed on blood platelets and/or cultured cells revealed a highly significant decrease in *ASMT* activity ( $P=2 \times 10^{-12}$ ) and melatonin level ( $P=3 \times 10^{-11}$ ) in individuals with ASD. These results indicate that a low melatonin level, caused by a primary deficit in *ASMT* activity, is a risk factor for ASD. They also support *ASMT* as a susceptibility gene for ASD and highlight the crucial role of melatonin in human cognition and behavior.**

**Key words:** autism, melatonin, circadian rhythm, sleep, HIOMT, *ASMT*

## **Introduction**

Melatonin is a powerful antioxidant molecule, involved in the regulation of circadian and seasonal rhythms and immune function.<sup>1-4</sup> It is released mainly by the pineal gland during the night and is produced by the conversion of serotonin to N-acetylserotonin by the rate-limiting enzyme AA-NAT (arylalkylamine N-acetyltransferase; EC 2.3.1.87), followed by the conversion of N-acetylserotonin to melatonin by *ASMT* (acetylserotonin methyltransferase; EC 2.1.1.4), also known as HIOMT (hydroxyindole O-methyltransferase).<sup>5</sup> Melatonin secretion is highly heritable in humans,<sup>6</sup> modulates neuronal plasticity<sup>7-9</sup> and regulates circadian gene expression.<sup>10</sup> It also plays a key role in communication behavior related to seasonal changes, such as song learning in birds.<sup>11</sup> Abnormal melatonin concentrations can have a dramatic effect on human behavior, as shown in patients with Smith–Magenis syndrome, who have an inverted melatonin circadian rhythm and display autistic behavior.<sup>12</sup> In autism spectrum disorders (ASD), low melatonin levels have been reported by three independent groups,<sup>13-15</sup> but the underlying cause of this deficit and its relationship to susceptibility to ASD was unknown. ASD affect at least 6/1000 individuals and are characterized by impairments in communication skills and social interaction, as well as restricted, repetitive and stereotyped patterns of behavior.<sup>16-18</sup> The genes responsible for ASD are largely unknown,<sup>19,20</sup> but cytogenetic abnormalities are observed in at least 3–5% of the affected individuals.<sup>21</sup> The PAR1 of the sex chromosomes, located at the tip of their short arms, has been found to be deleted in several individuals with ASD.<sup>22</sup> Among the 12 PAR1 genes referenced, *ASMT*<sup>23</sup> is an excellent candidate for susceptibility to ASD because it encodes the last enzyme in the melatonin biosynthesis pathway.<sup>24</sup> In this study, using a combination of genetics and functional experiments, we report evidence showing that a low melatonin concentration caused by a primary deficit in *ASMT* activity is a risk factor for ASD.

## Materials and Methods

### *Subjects*

Families with ASD were recruited by the Paris Autism Research International Sibpair study at specialized clinical centers in seven countries (France, Sweden, Norway, Italy, Belgium, Austria, and the United States). Diagnosis was based on clinical evaluation by experienced clinicians, DSM-IV criteria, and the Autism Diagnostic Interview-Revised (ADI-R).<sup>25</sup> In Sweden, the Diagnostic Interview for Social and Communication Disorders (DISCO-10)<sup>26</sup> was used instead of the ADI-R in some cases. Patients with Asperger syndrome were evaluated with the Asperger Syndrome Diagnostic Interview.<sup>27</sup> Patients diagnosed with medical disorders, such as fragile X syndrome or chromosomal anomalies, were excluded from the study.

For mutation screening, the study sample (n=250, 187 men and 63 women) was constituted of 250 independent families (71 subjects from multiplex families and 179 sporadic cases) and included 233 patients with autistic disorder and 11 with Asperger syndrome; six individuals narrowly missed the criteria for autistic disorder and were considered to have atypical autism (pervasive developmental disorder, PDD-NOS). There were 222 Caucasian, nine Black, three Asian, one Hispanic/Latin-American family, and 15 families of mixed ethnicity. For association studies, the ASD sample consisted of 278 patients of Caucasian origin (201 men and 77 women) from 72 multiplex families and 206 sporadic cases. There were 258 patients with autism, 14 with Asperger syndrome and six with atypical autism. The control sample (n=255) comprised 160 French and 95 Swedish individuals. An additional control group of 171 individuals from North Africa was screened for rare variants because one proband carrying the splice-site mutation (IVS5+2T>C) and one proband with the L326F mutation had parents originating from this region.

Blood and platelet biochemical analyses were performed in ASD probands (n=43; 14 female and 29 male patients,  $14.8 \pm 7$  years old), their parents (n=34; 18 females and 16 males:  $44 \pm 9$  years old) and in controls matched for sex and age (n=75; 30 females and 45 males;  $27 \pm 16$  years old). The ASD patients were initially recruited for the analysis of serotonin levels and not chosen on the basis on their ASMT genotype. The controls were recruited at the Department of Orthopedics of the Lariboisière and Robert Debré hospitals in Paris. The control group for the B lymphoblastoid cell lines (BLCL) biochemical analyses comprised 14 French individuals also used in the association study and 19 healthy relatives of patients with Hirschsprung syndrome or mitochondrial diseases. The local research ethics boards reviewed and approved the study. Informed consent was obtained from probands (if possible), parents and controls.

### *Cell culture, DNA and RNA isolation*

BLCL were established from EBV-transformed lymphocytes and grown at 37°C in RPMI-1640 medium (Life Technologies Inc., Grand Island, NY, USA), supplemented with 10% undialyzed fetal calf serum, 2 mM glutamine, 2.5 mM sodium pyruvate, 100 mg/ml streptomycin and 100 IU/ml penicillin, under standard conditions. DNA was extracted by the phenol/chloroform method, and RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). DNA/RNA concentrations were determined by measuring absorbance at 260 nm on a biophotometer (Eppendorf,

Hamburg, Germany). Human pineal gland cDNAs were obtained from the Incyte cDNA library # LHS1565 (BioCat, Heidelberg, Germany).

#### *Mutation screening and genotyping*

Genotyping for the association study and mutation screening were performed by direct sequencing or TaqMan technology. PCR products were sequenced with the BigDye Terminator Cycle Sequencing Kit (V3.1, Applied Biosystems, Foster City, CA, USA). Samples were then subjected to electrophoresis, using an ABI PRISM genetic analyzer (Applied Biosystems). Absence of genotyping errors was controlled by sequencing the PCR product with the opposite primer in a subset of patients. For primers and PCR conditions, see Supplementary Table 1.

#### *Association and statistical analyses*

The linkage disequilibrium (LD) map for *ASMT* was calculated using pairwise LD (D') between the 13 *ASMT* variations in 533 individuals (278 ASD and 255 controls). The LD calculation and the case-control study were performed with Haploview software.<sup>28</sup> To detect population stratification bias, individuals with ASD and controls were screened for three single nucleotide polymorphisms (SNPs) (rs2289311, rs4782053, rs1921361), five ALU insertions (Ya5NBC27, Ya5NBC51, YaNBC102, YaNBC109, YbNBC65), and the mtDNA hypervariable region 1 (HVR1). In addition, all mothers from ASD patients were screened for the androgen receptor microsatellite. No significant genotype difference was observed for any of the markers tested (Supplementary Table 2). The transmission disequilibrium test (TDT) was performed using the family-based association test (FBAT)<sup>29</sup> and haplotype-based association test (HBAT)<sup>30</sup> using the empirical variance ('-e' option). For the TDT, only the four SNPs in promoter B were tested in 278 ASD families in which both parents had been genotyped. All SNPs were at Hardy-Weinberg equilibrium. We evaluated the distribution of the quantitative variables by the Kolmogorov-Smirnov test for Gaussian normality. Because the values for most of the samples were not normally distributed, we used the two-tailed non-parametric Mann-Whitney *U*-test to compare two groups and Spearman's  $\rho$  test to evaluate the correlation between *ASMT* activity and melatonin concentration. We used SPSS version 13 for these tests.

#### *RT-PCR and quantitative RT-PCR*

Oligo(dT)-primed cDNA was prepared from 5  $\mu$ g of BLCL RNA, using Superscript II (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. The cDNAs were used directly in TaqMan assays, using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Samples were run in duplicate or triplicate on 96-well optical PCR plates (ABgene, Surrey, UK). *ASMT* mRNA was quantified using commercially available assays. Two different assays were used, one covering the boundary between exon 1B and exon 2 (Hs00946625\_m1), and the other covering the boundary between exon 8 and exon 9 (Hs00187839\_m1). The two assays gave similar results and only the data obtained with Hs00946625\_m1 are presented. Relative values of expression were determined for each sample, using the standard curve method (ABI user's manual), and these values were normalized to the threshold cycle (Ct) values of *GAPDH*, using the Hs99999905\_m1 assay. For *ASMT* and *GAPDH*, the thresholds were set at 0.2 and 0.25, respectively, within the linear region of

the semi-logarithmic plot in all assays (data not shown). The consequence of the splice site mutation was investigated by sequencing the abnormal transcript after cloning the RT-PCR product.

### *Biochemical analyses*

Blood samples were collected in the morning, between 0900 and 1100 h. The procedures for collecting and processing blood samples were designed to prevent release reactions. The anticoagulant was ACD-A (1 vol to 9 vol of whole blood). For the melatonin profile of family ASD 1, blood samples were collected every 2 h, from 1800 h to 1600 h the following day. Overnight, blood samples were collected in dimly lit conditions (<20 lux). Samples were drawn from an indwelling forearm catheter into Becton-Dickinson plastic tubes, centrifuged and frozen at  $-20^{\circ}\text{C}$ . Platelets were counted with a Coulter ZBI electronic counter. ASMT enzymatic activities were determined, at least in duplicate, by radioenzymology,<sup>31</sup> on the platelet pellet obtained by centrifugation of the platelet-rich plasma at 800 g for 20 min at room temperature and lysis with 100 hemolytic units of a purified SH-activated toxin (streptolysin O or alveolysin, generously provided by Prof. J. Alouf, Pasteur Institute, Paris). Melatonin content was measured in the resulting supernatant (*i.e.*, platelet-poor plasma or plasma) by HPLC, with random controls by mass spectrometry.<sup>32</sup>

### *Sleep analysis*

The three individuals from family ASD 1 were taking no medication that could interfere with melatonin secretion. Sleep analysis was performed by standard polysomnography (two EEG, two electro-oculograms, one submental electromyogram, two anterior tibialis muscle electromyograms and respiratory signals, Embla N7000, Flaga, Iceland), 1 week before blood was collected for the melatonin assay.

## **Results**

### *Rare ASMT variations in ASD and ADHD*

We investigated whether variations in *ASMT* were associated with ASD and ADHD by directly sequencing all *ASMT* exons and the two promoters, A and B, in 250 affected individuals. Several *ASMT* variants were identified (Figure 1), including a splice-site mutation (IVS5+2T>C), four non-synonymous variations (N17K, K81E, G306A, L326F) and two synonymous variations (N167N, Q205Q). Two of these variations, N17K (rs17149149) and L326F, were observed in the general population. N17K was found in one family with ASD from China (ASD 3) and is present in the SNP database at a frequency of 0.4-0.7% in the Han Chinese population. L326F was found in two ASD families (ASD 6 and ASD 7) and in 3 out of 426 controls (two from Sweden and one from North-Africa). The splice-site mutation (IVS5+2T>C) was present in two ASD families (ASD 1 and ASD 2) (Figure 1a), but not in controls (n=426). Using BLCL RNA from the ASD 1 proband, we detected abnormal *ASMT* transcripts, encoding a putative truncated *ASMT* protein, lacking the methyltransferase domain (Figure 1a). Biochemical analysis of the IVS5+2T>C and L326F variations indicated that these mutations were associated with very low levels of *ASMT* activity and melatonin (Figure 2a, 2b). We could not analyze the functional consequences of the remaining mutations due to a

lack of blood samples or cell lines. Interestingly, the nine individuals with ASD carrying rare mutations were also hyperactive and several had sleep problems (see clinical description of the patients in Supplementary Table 3).

#### *ASMT polymorphisms in ASD and ADHD*

We investigated whether frequent polymorphisms of the *ASMT* gene were associated with ASD by studying one insertion/deletion located in promoter A and 12 SNPs with a minor allele frequency greater than 5%, capturing most of the haplotype diversity (Figure 3a). ASD patients (n=278) differed significantly from controls (n=255) in terms of the allelic frequency of two SNPs — rs4446909 ( $P=0.006$ ) and rs5989681 ( $P=0.007$ ), located in promoter B (Figure 3b, Table 1 and Supplementary Figure 1). The H1 GGGC haplotype in promoter B was more frequent in ASD ( $P=0.002$ ) than in controls, whereas the haplotype H3 ACGC was more frequent in controls than in ASD ( $P=0.005$ ). We carried out a TDT using FBAT and HBAT on 278 families (Supplementary Table 4), and observed an overtransmission of haplotype H1 GGGC to probands (additive model  $P = 0.05$ ; dominant model  $P = 0.02$ ) and overtransmission of alleles G and C of the SNPs P1BC (dominant model  $P = 0.02$ ) and rs6644635 (dominant model  $P = 0.04$ ), respectively.

We then explored the relationship between these frequent variations of the *ASMT* gene and *ASMT* expression. Promoter A activity is restricted to the retina,<sup>24</sup> whereas promoter B is active in BLCL and in the pineal gland. In view of the results of quantitative RT-PCR, *ASMT* transcript level was found to be significantly associated with the two SNPs linked to ASD — rs4446909 and rs5989681 (Figure 3c). Interestingly, the G alleles of both SNPs were more frequent in ASD patients and were associated with a decrease in *ASMT* transcript levels by a factor of 4 to 20, respectively (rs4446909  $P=2 \times 10^{-8}$  and rs5989681  $P=2 \times 10^{-10}$ ).

#### *Serotonin, ASMT activity and melatonin concentration in ASD*

We then measured the serotonin concentration, the *ASMT* activity and melatonin concentration in blood platelets from 43 ASD patients, 34 parents of ASD patients and 48 control individuals. Consistent with previous studies,<sup>33</sup> the serotonin level was significantly higher in individuals with ASD ( $P=2 \times 10^{-11}$ ) and their parents ( $P=10^{-8}$ ) than in controls (Figure 4a). In contrast, the *ASMT* activity levels were significantly lower in individuals with ASD ( $P=2 \times 10^{-12}$ ) and their parents ( $P=10^{-5}$ ) than in controls (Figure 4b). This deficit in *ASMT* activity was accompanied by a lower plasma melatonin concentration in patients with ASD ( $P=3 \times 10^{-11}$ ) and their parents ( $P=9 \times 10^{-5}$ ) than in controls (Figure 4c). Platelet *ASMT* activity and plasma melatonin levels were not correlated in controls (Figure 4d), whereas they were strongly correlated in patients with ASD ( $\rho=0.83$ ;  $P=10^{-6}$ ). Thus, the decreased *ASMT* activity in ASD patients acted as a limiting factor for the production of melatonin by the pineal gland. We investigated this *ASMT* deficiency further by analyzing BLCLs from 53 individuals with ASD (for 15 of whom blood samples had already been tested) and 33 new independent controls (Figure 4e). We found that ASD patients had lower levels of *ASMT* activity than controls ( $P=7 \times 10^{-8}$ ), as shown previously with platelets. These results obtained with cultured cells replicate our previous finding and exclude possible effects of environmental factors or regulation acting at a higher physiological level. We found no significant correlation between the severity of the

deficit and clinical phenotype (IQ, language level or ADI-R scores in the three major domains of impairment: Reciprocal Social Interaction, Communication, and Repetitive Behaviors and Stereotyped Patterns) (data not shown).

#### *Melatonin cycle and sleep pattern in family ASD 1*

Finally, we investigated circadian melatonin synthesis *in vivo* and sleep patterns in family ASD 1, in which the unaffected mother and the son with ASD carry the splice-site mutation. Neither of the individuals carrying the *ASMT* mutation — the unaffected mother and the son with ASD — showed the normal increase in melatonin during the night (Figure 2c). They displayed modest sleep abnormalities, with poor sleep efficiency (proband 70% and mother 82%; controls > 85%) and a moderately high arousal index (22 and 17/h; controls <10/h), but had normal proportions of rapid eye movement sleep (26% and 24%; control range 15%-30%) and a normal amount of slow wave sleep (61 and 104 min; control range: 60–120 min).

#### **Discussion**

Abnormal melatonin concentration was observed in individuals with ASD by three independent groups using different methodological approaches.<sup>13-15</sup> Our results confirm that low plasma melatonin concentration (half the mean of the control values) is a frequent trait in ASD patients, as observed in 65% of the patients tested, a proportion very similar (63%) to that previously reported by Tordjman *et al.*<sup>15</sup> We show for the first time that abnormal melatonin levels are also present in the unaffected parents of ASD patients, suggesting a genetic origin. Indeed, the melatonin deficit observed in the patients was associated with low *ASMT* activity, suggesting that variations in the *ASMT* gene could be the cause of this deficit. This hypothesis was supported by the identification of genetic variations, which probably contribute to the enzymatic deficit by decreasing transcript levels, or altering the sequence of the *ASMT* protein. However, other unidentified genetic or epigenetic factors are contributing to the *ASMT* deficit since non-conservative mutations were observed only in a limited number of patients and the genetic association with the polymorphisms located in the *ASMT* promoter does not solely contribute to the enzymatic deficiency. Furthermore, we found unaffected relatives and controls with *ASMT* mutations and/or low melatonin concentration in the blood. Therefore, low *ASMT* activity cannot be considered as a direct cause of ASD, but as a susceptibility factor for this condition (less than 100 pmol melatonin/10<sup>9</sup> platelets/ 30 min; odds ratio: 77; 95% confidence interval: 19 <OR< 320).

Individuals with ASD frequently show irregularities in the circadian sleep–wake cycle<sup>34-36</sup> and some show a free-running pattern, which is suppressed by melatonin treatment.<sup>37</sup> In view of these clinical observations, it was postulated that one alteration at the origin of autism may occur as the child is entering into the day–night cycle.<sup>38</sup> In agreement with this hypothesis, the deficit in melatonin may cause abnormal sleep–wake cycle in affected individuals. Additionally, since melatonin influences synaptic plasticity,<sup>5,7-9,11,39,40</sup> a deficit in this molecule may also weaken neuronal networks, thereby increasing the effect of other pathological processes, such as abnormal synaptogenesis.<sup>41-44</sup>

Taken together, these findings indicate that a subgroup of individuals with ASD and low melatonin levels could benefit from the use of melatonin as a therapeutic compound. Melatonin treatment seems

to help patients with ASD to fall asleep and to sleep through the night,<sup>37,45–48</sup> but it remains unknown if melatonin could have a more beneficial effect if given before 3 years of age. Further studies are required to determine the role of the melatonin deficit in the affected individuals, and more generally of circadian and seasonal rhythms, in the susceptibility to neuropsychiatric disorders.

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**Table 1** Frequencies of the polymorphisms located in *ASMT* promoter B in ASD patients and controls

	<i>ASD</i> (n= 278)	<i>Controls</i> (n= 255)
<i>SNPs</i>		
rs4446909		
f(G)	0.77	0.70
P value (P <sub>c</sub> value <sup>a</sup> )	<b>0.006 (0.10)</b>	
OR [95% CI] <sup>b</sup>	<b>1.5 [1.1-2]</b>	
rs5989681		
f(G)	0.73	0.65
P value (P <sub>c</sub> value)	<b>0.007 (0.12)</b>	
OR [95% CI]	<b>1.4 [1.1-2]</b>	
<i>P1BC</i>		
f(G)	0.90	0.90
P value	0.78	
rs6644635		
f(C)	0.65	0.63
P value	0.66	
<i>Haplotypes<sup>3</sup></i>		
H1 GGGC	0.36	0.27
P value (P <sub>c</sub> value)	<b>0.002 (0.04)</b>	
H2 GGGT	0.26	0.28
P value	0.54	
H3 ACGC	0.21	0.29
P value (P <sub>c</sub> value)	<b>0.005 (0.08)</b>	
H4 GGAT	0.097	0.09
P value	0.78	
H5 GCGC	0.055	0.057
P value	0.88	

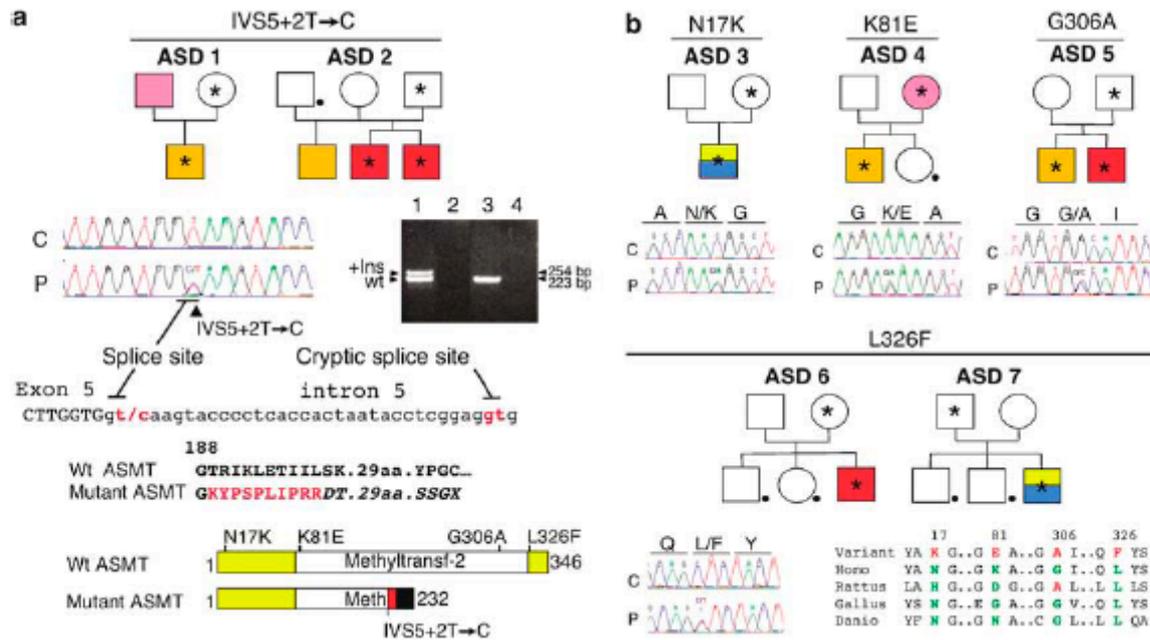
Abbreviations: ASD, autism spectrum disorders; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

<sup>a</sup>P<sub>c</sub> value: Significance levels corrected for multiple comparisons using a step-down permutation procedure (comprising 100,000 permutations).

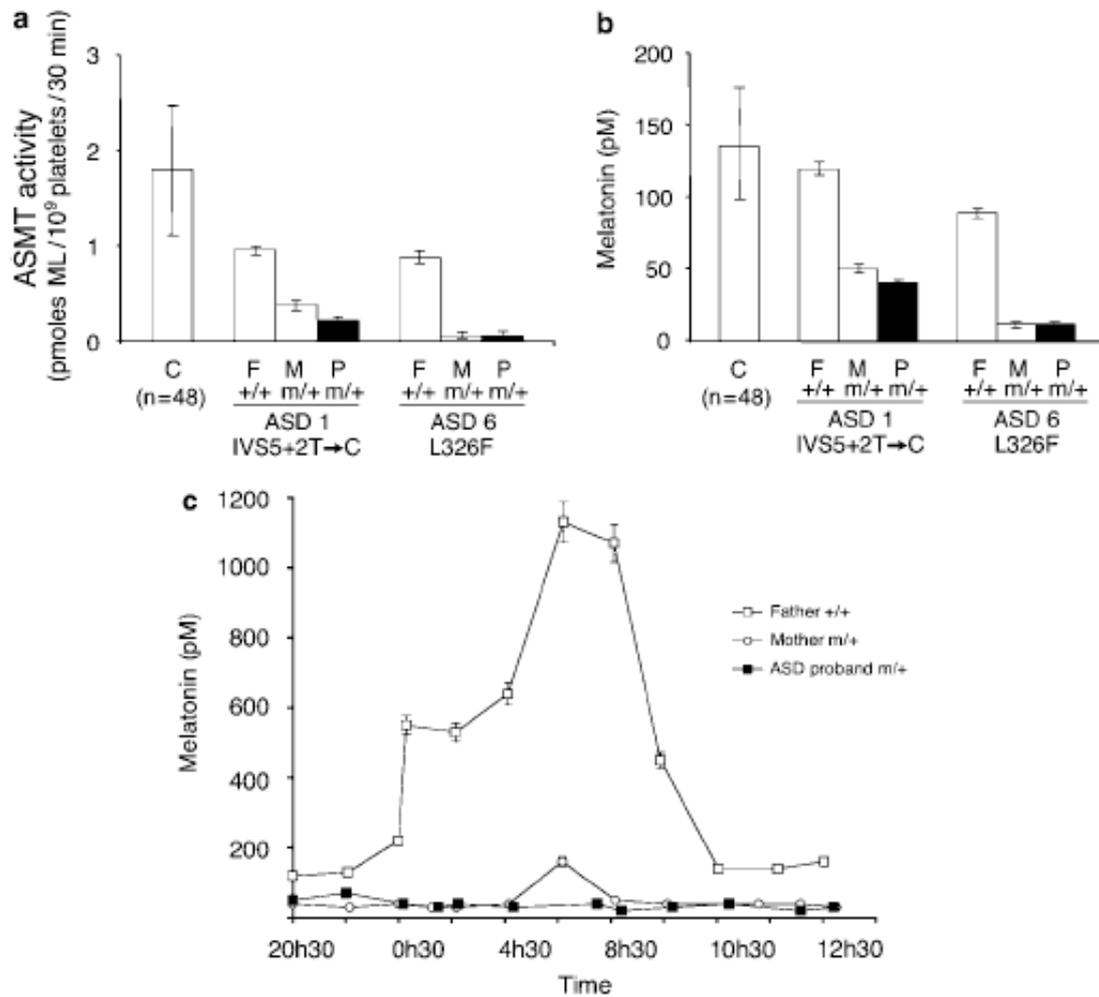
<sup>b</sup>Odds ratio: major allele vs minor allele.

<sup>c</sup>Haplotype using rs4446909, rs5989681, P1BC and rs6644635.

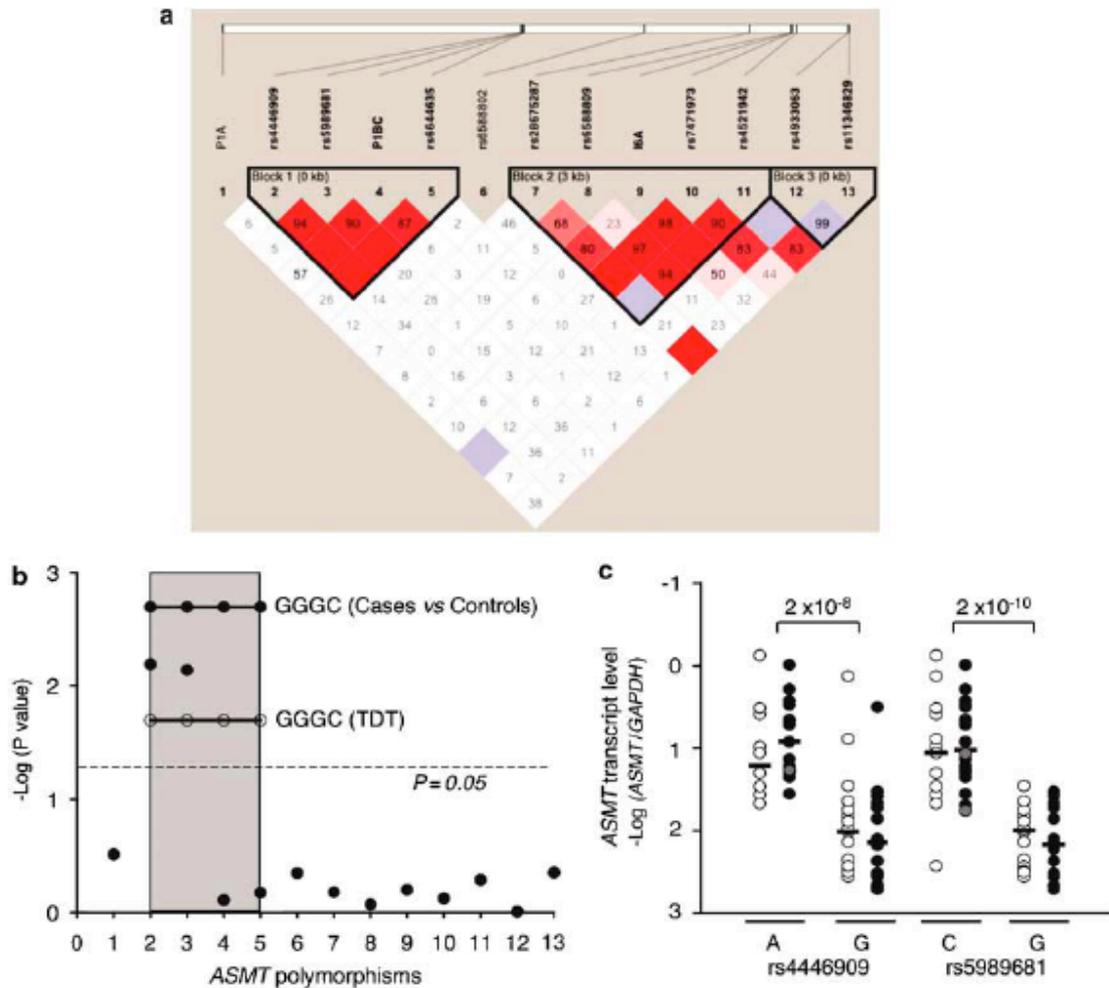
Results with significance < 0.05 are indicated in bold.



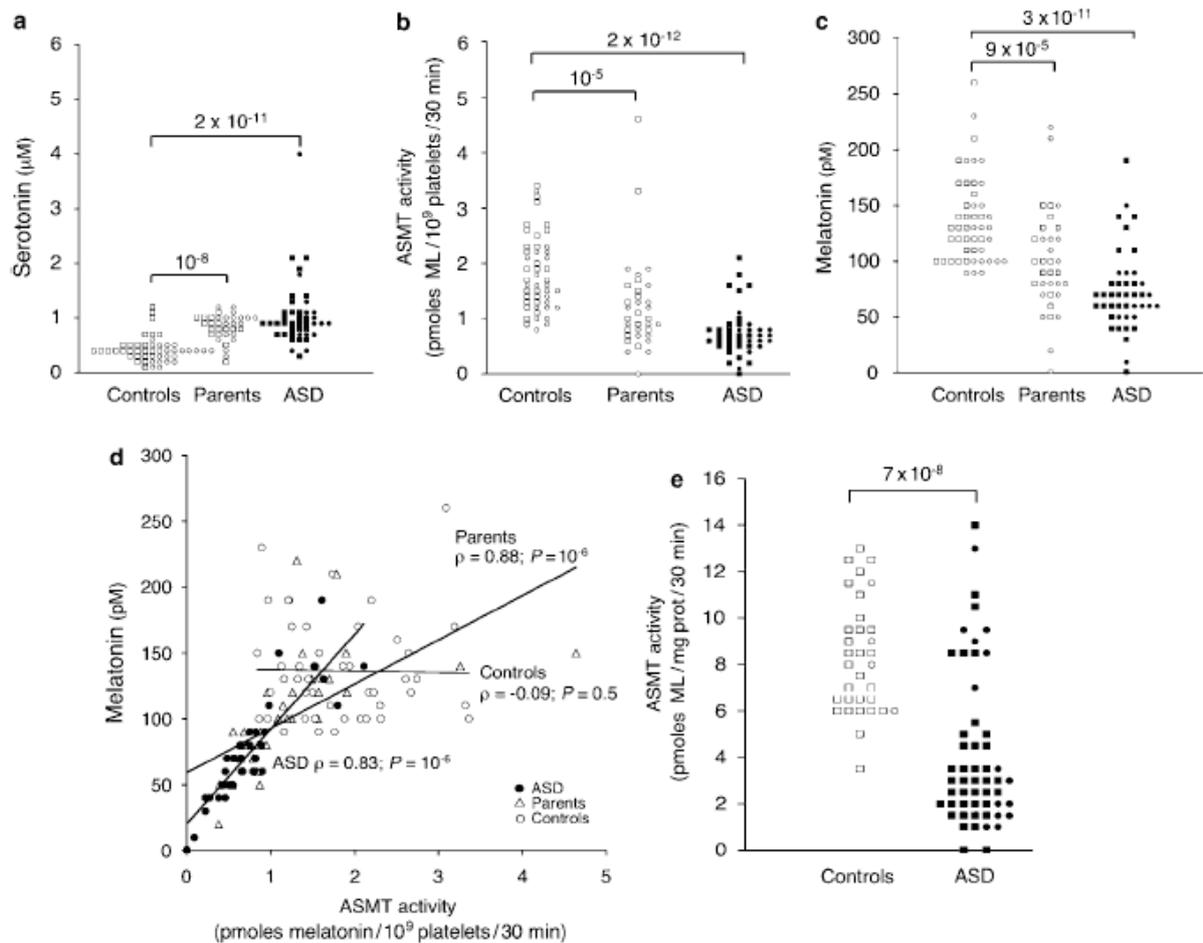
**Figure 1** Non-synonymous *ASMT* variations in autism spectrum disorders (ASD) families. **(a)** Pedigree structure of the families carrying the splice-site mutation IVS5+2T>C; reverse transcriptase-polymerase chain reaction (RT-PCR) amplifying exons 4 to 6 of the *ASMT* cDNA from B lymphoblastoid cell lines of the ASD 1 proband carrying the splice-site mutation IVS5+2T > C (lane 1 +RT, lane 2 -RT) and a control (lane 3 +RT, lane 4 -RT). The insertion (+Ins) of 31 bp in the *ASMT* transcript originates from a cryptic donor splice-site downstream from exon 5. This insertion should lead to the additional sequence indicated in red and to a frame shift (characters in italics), causing premature truncation of the protein, lacking the methyl-transferase domain. Wt, wild-type. **(b)** Pedigree structure of the families carrying rare non-synonymous *ASMT* variations and conservation of the variant amino-acid in different species. Color codes in the pedigrees: autism with mild (orange) or severe (red) mental retardation, Asperger syndrome or high-functioning autism (yellow), attention-deficit/hyperactivity disorder ADHD (light blue) and depression (pink). The proband ASD 3 fulfilled diagnostic criteria for both high functioning autism and ADHD. The proband ASD 7 fulfilled diagnostic criteria for both Asperger syndrome and ADHD. The asterisk and the dot indicate the presence of the mutation and the absence of a DNA sample for analysis, respectively.



**Figure 2** Impact of the ASMT mutations on enzyme activity and melatonin concentration. **(a)** ASMT activity, measured in platelets of the members of families autism spectrum disorder (ASD) 1 and ASD 6 carrying the splice-site IVS5+2T>C and the L326F ASMT mutations, respectively. **(b)** Blood melatonin concentration in the same individuals. **(c)** Nocturnal melatonin profile of family ASD 1. The proband (male, 24 years old) and his mother (53 years old) are heterozygous (m/+) for the splice-site mutation IVS5+2T>C. The proband's father (55 years old) has no ASMT mutation (+/+). Error bars represent s.d. C: controls; F: father; M: mother; P: proband.



**Figure 3** Association studies and transcript analyses of the *ASMT* gene. **(a)** Haplotype block structure of the *ASMT* gene. The relative physical position of each single nucleotide polymorphism (SNP) is given in the upper diagram, and the pairwise LD ( $D_0$ ) between all SNPs is given below each SNP combination. **(b)** Plot of the case–control P-values ( $-\log_{10}$ ) for all variations studied within *ASMT*. 1: E1A; 2: rs4446909; 3: rs5989681; 4: P1BC; 5:rs6644635; 6: rs6588802; 7: rs28675287; 8: rs6588809; 9: I6A; 10: rs7471973; 11: rs5431942; 12: rs4933063; 13: rs11346829. SNPs located in promoter B are included in the shaded box. P-values for the risk haplotype GGGC are indicated as straight lines with close (cases vs controls) or open circles (transmission disequilibrium test (TDT)). **(c)** Quantification of *ASMT* transcripts relative to rs4446909 and rs5989681 genotypes (A represents the individuals with an A/A or A/G genotype; C represents the individuals with C/C or C/G genotype; G represents the individuals homozygous G/G). Black and white circles indicate individuals with autism spectrum disorders (ASD) and controls, respectively. The gray symbols indicate individuals homozygous A/A and C/C for rs4446909 and rs5989681, respectively. Real-time reverse transcriptase (RT)-PCR was performed with B lymphoblastoid cell lines from 38 ASD probands and 29 controls. Horizontal bars indicate medians. No statistical difference was observed between ASD and controls (Mann–Whitney *U*-test).



**Figure 4** ASMT activity and melatonin concentration in patients, parents and controls. **(a)** Serotonin levels, measured in the platelets of 43 ASD patients (mean $\pm$ s.d.;  $1 \pm 0.6 \mu\text{M}$ ), 34 parents ( $0.8 \pm 0.2 \mu\text{M}$ ) and 48 controls ( $0.4 \pm 0.2 \mu\text{M}$ ). **(b)** ASMT activity, measured in the platelets of 43 ASD patients ( $0.73 \pm 0.43 \text{ pmol}/10^9 \text{ platelets}/30 \text{ min}$ ), 34 parents ( $1.20 \pm 0.85 \text{ pmol}/10^9 \text{ platelets}/30 \text{ min}$ ) and 48 controls ( $1.81 \pm 0.68 \text{ pmol}/10^9 \text{ platelets}/30 \text{ min}$ ). **(c)** Plasma melatonin concentration in 43 autism spectrum disorders (ASD) patients ( $73 \pm 36 \text{ pmol}$ ), 34 parents ( $99 \pm 46 \text{ pmol}$ ) and 48 controls ( $136 \pm 39 \text{ pmol}$ ). **(d)** Blood melatonin concentration expressed with respect to ASMT activity in platelets. ASMT activity and melatonin concentration were not correlated in controls (white circles). In contrast, in ASD patients (black circles), and their parents (white triangles), a significant correlation was observed. Spearman's rho test was used to evaluate the correlation between ASMT activity and melatonin concentration. **(e)** ASMT activity in B lymphoblastoid cell lines from 53 ASD patients ( $3.9 \pm 3.4$ ) and 33 controls ( $8.3 \pm 2.4$ ). Circles and squares indicate female and male subjects, respectively. Statistical significance was assessed with the Mann–Whitney *U*-test.

**Supplementary Table 1. *ASMT* primers used in this study**

Exon	Exon size (bp)	PCR fragment size (bp)	Primers	Annealing (°C)
<b>Sequencing</b>				
1A	78	875	ASMT1AF: GAGCGATTCTTCTGCCTCAGC ASMT1AR: TCTGCGCACACTCCCAGGTG	67
1B/C	136 (coding 69)	491	ASMT1BF: GAGGCAGGAGAATCGCTTGAA ASMT1BR: CAACAATGGAACGTGAGTGTG	65
2	175	552	ASMT2F: TGGTGCAATCTCATTGACTCTG ASMT2R: GGGTTCATGCCATTCTCTG	58
3	130	950	ASMT3F: CAGCTGTACAAGGCAAGAGGA ASMT3R: CTTTCACCTCCTCCACTGCCA	55
4	69	283	ASMT4F: GCCTGGGCTACAGAGCTGAAA ASMT4R: CTCCTGGGTTGTGCCATTTG	55
5	119	331	ASMT5F: CCTGTGGGGTATAGCTCCGTTC ASMT5R: CGCACATGTCAAAGCATCAGA	64
6	84	342	ASMT6F: AGCTTGCAGTGAGCGGAAATC ASMT6R: GCACCCATCGACTCGTCATTT	64
7	141	352	ASMT7F: TGGGTTGGACCTTCATGAGT ASMT7R: GTGTTTCCGGGAGTGAGAGGA	64
8	123	338	ASMT8F: AGCCTGGAAGACCTGGGAAAG ASMT8R: CCTGTGGGATGATTTCAGTGC	64
9	279 (coding 212)	506	ASMT9F: GGTGCCCTGACTGTCTCTGA ASMT9R: CCATCAGCGTGGTCCTCAGTA	64
<b>Expression</b>				
E 4-6		223	ASMTsplice4F: CCAGTACCTGGAGACGTTTGG ASMTexp6R1: TCCTTGCGATAGTTTGCTGA	55

All PCRs were performed with Qiagen HotStarTaq at the following temperatures: 15 min at 95°C, 35 cycles of: 30 s at 95°C, 30 s at annealing temperature, 0.5-1 min at 72°C, followed by a final extension step of 10 min at 72°C. One 28 bp insertion/deletion located 194 bp upstream from exon 1A was used for the association study. bp, base pairs.

**Supplementary Table 2. Stratification of the ASD and control populations used for the case control study.**

Variations	Allele	ASD	Control	Chi2	df	p value
<b>SNPs</b>						
RS2289311	A	0.66	0.66	0.06	1	0.8
RS4782053	G	0.67	0.69	0.4	1	0.5
RS1921361	C	0.64	0.62	0.1	1	0.7
<b>Alu</b>						
Ya5NBC27	Empty	0.71	0.76	3.4	1	0.07
Ya5NBC51	Empty	0.57	0.58	0.07	1	0.8
YaNBC102	Empty	0.64	0.64	0.1	1	0.8
YaNBC109	Full	0.60	0.56	1.7	1	0.2
YbNBC65	Empty	0.51	0.54	0.8	1	0.4
<b>Microsatellite<sup>1</sup></b>						
Androgen receptor	Multiple alleles			15	19	0.7
<b>MtDNA<sup>2</sup></b>						
HVR1	Multiple Haplotypes			1.6	3	0.6

<sup>1</sup>Mothers of children with ASD and affected girls were compared to control females. <sup>2</sup>The test concerns the haplotype matching the control reference sequence and those containing the variations 16126, 16223 located in the hypervariable region 1 (HVR1) of the mtDNA. df, degrees of freedom.

Supplementary Table 3. Clinical features of patients with rare variants of the *ASMT* gene

Family and patient	Location and nucleotide change	Protein change	ASMT activity deficit	Inheritance	Sex	Age at last evaluation (years)	Ethnic origin	Diagnosis	Cognitive level	Language	Attention/ Hyperactivity/ Sleep	Seizures	Family history
<b>ASD 1</b>													
Patient 1	IVS5+2T>C	Frameshift mutation	yes	Maternal	M	25	European	autism	mild MR	language delay (started to speak at 5y)	ADHD symptoms present until the age of 12 y. Abnormal sleep pattern, falls asleep very late, nocturnal awakenings.	—	The father has depression, the mother is healthy but she has a particular sleep organization, sleeping only when she's very tired, at any time, otherwise sleeping few hours.
<b>ASD 2</b>													
Patient 1	—	—	nd	—	M	12	North Africa	autism	mild MR	no language delay	Hyperactivity during childhood; no longer present at 12 y	—	Three affected children from two fathers; the mother and the father of the two younger sibs are healthy, no information about the father of the first child
Patient 2	IVS5+2T>C	Frameshift mutation	nd	Paternal	M	7	North Africa	autism	severe MR	non verbal	Severe hyperactivity during childhood; still present at 7 y	+	
Patient 3	IVS5+2T>C	Frameshift mutation	nd	Paternal	M	4	North Africa	autism	severe MR	non verbal	Hyperactive in the evening	+	
<b>ASD 3</b>													
Patient 1	exon 1B, C51A	N17K	nd	Maternal	M	9	China	autism + ADHD combined type	low normal	first words 14 m sentences 36 m	ADHD; no sleep problems	—	Only child. Negative family history.
<b>ASD 4</b>													
Patient 1	exon 2, A241G	K81E	nd	Maternal	M	8	African-American	autism	mild MR	first words 19 m, sentences 40 m	Slept very little during the first years but his sleep is now normal. Marked hyperactivity and difficulty maintaining attention	—	Father: negative medical and psychiatric history; mother: depressive episode during first pregnancy
<b>ASD 5</b>													
Patient 1	exon 9, G917C	G306A	nd	Maternal	M	12	European	autism	mild MR	first words 14 m, sentences at 36 m, followed by language regression	Motor hyperactivity; very active during night	—	The parents have no medical or psychiatric history.
Patient 2	exon 9, G917C	G306A	nd	Maternal	M	9	European	autism	severe MR	language development stopped at 15 m; very limited language at 9 y	Motor hyperactivity	—	
<b>ASD 6</b>													
Patient1	exon 9, C976T	L326F	yes	Maternal	M	11	North Africa	autism	severe MR	first words 18 m, never acquired any sentences	Motor hyperactivity; normal sleep.	—	The parents have no medical or psychiatric history
<b>ASD 7</b>													
Patient 1	exon 9, C976T	L326F	nd	Maternal	M	6	European	Asperger + ADHD combined type	normal	first words 24 m, sentences 36 m	ADHD, no sleep problems.	—	The parents have no medical or psychiatric history

MR, mental retardation; nd, not determined

**Supplementary Table 4. TDT of the polymorphisms located in *ASMT* promoter B in 278 families with ASD using FBAT and HBAT.**

	freq	Additive model						Dominant model					
		fam#	S	E(S)	Var(S)	Z	P	fam#	S	E(S)	Var(S)	Z	P
<b>SNPs</b>													
rs44446909													
A	0.25	104	81	84	55	-0.4	0.68	104	66	68	35	-0.4	0.7
G	0.75	104	163	160	55	0.4	0.68	39	31	30	13	0.2	0.8
rs5989681													
C	0.3	110	93	96	62	-0.4	0.66	114	78	79	40	-0.12	0.9
G	0.7	110	171	167	62	0.4	0.66	55	46	43	15	0.7	0.48
<b>P1BC</b>													
A	0.12	67	38	45	29	-1.29	0.2	73	44	48	28	-0.8	0.42
G	0.88	67	128	121	29	1.29	0.2	12	13	10	1.3	2.4	<b>0.02</b>
rs6644635													
C	0.64	119	206	193	73	1.54	0.12	68	66	57	18	2	<b>0.04</b>
T	0.36	119	104	117	73	-1.54	0.12	128	90	95	50	-0.7	0.51
<b>Haplotypes</b>													
H1 GGGC	0.34	116	197	183	52	1.97	<b>0.05</b>	115	164	150	34	2.3	<b>0.02</b>
H2 GGGT	0.24	105	133	137	52	-0.47	0.63	105	121	120	44	0.2	0.9
H3 ACGC	0.23	102	114	115	50	-0.21	0.83	92	101	100	33	0.07	0.9
H4 GGAT	0.11	63	55	57	25	-0.42	0.67	62	54	56	23	-0.3	0.7
H5 GCGC	0.05	38	26	28	12	-0.69	0.49	38	26	28	12	-0.7	0.5

Freq, frequency; fam#, number of informative families; S, observed transmission; E(S), expected transmission; Var(S) Variance of the transmission observed. P values below 0.05 are indicated in bold.

