

## ORIGINAL ARTICLE

The serotonin-*N*-acetylserotonin–melatonin pathway as a biomarker for autism spectrum disordersC Pagan<sup>1,2,3,4,12</sup>, R Delorme<sup>3,4,5,6</sup>, J Callebert<sup>1,2</sup>, H Goubran-Botros<sup>3</sup>, F Amsellem<sup>4,5</sup>, X Drouot<sup>4,7,13</sup>, C Boudebessé<sup>4,7,8</sup>, K Le Dudal<sup>4,9</sup>, N Ngo-Nguyen<sup>4</sup>, H Laouamri<sup>4</sup>, C Gillberg<sup>10,11</sup>, M Leboyer<sup>4,7,8</sup>, T Bourgeron<sup>3,4,6</sup> and J-M Launay<sup>1,2,4</sup>

Elevated whole-blood serotonin and decreased plasma melatonin (a circadian synchronizer hormone that derives from serotonin) have been reported independently in patients with autism spectrum disorders (ASDs). Here, we explored, in parallel, serotonin, melatonin and the intermediate *N*-acetylserotonin (NAS) in a large cohort of patients with ASD and their relatives. We then investigated the clinical correlates of these biochemical parameters. Whole-blood serotonin, platelet NAS and plasma melatonin were assessed in 278 patients with ASD, their 506 first-degree relatives (129 unaffected siblings, 199 mothers and 178 fathers) and 416 sex- and age-matched controls. We confirmed the previously reported hyperserotonemia in ASD (40% (35–46%) of patients), as well as the deficit in melatonin (51% (45–57%)), taking as a threshold the 95th or 5th percentile of the control group, respectively. In addition, this study reveals an increase of NAS (47% (41–54%) of patients) in platelets, pointing to a disruption of the serotonin–NAS–melatonin pathway in ASD. Biochemical impairments were also observed in the first-degree relatives of patients. A score combining impairments of serotonin, NAS and melatonin distinguished between patients and controls with a sensitivity of 80% and a specificity of 85%. In patients the melatonin deficit was only significantly associated with insomnia. Impairments of melatonin synthesis in ASD may be linked with decreased 14-3-3 proteins. Although ASDs are highly heterogeneous, disruption of the serotonin–NAS–melatonin pathway is a very frequent trait in patients and may represent a useful biomarker for a large subgroup of individuals with ASD.

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## INTRODUCTION

Autism spectrum disorders (ASDs) are complex, heterogeneous and multifactorial disorders characterized by impaired social communication and repetitive/stereotyped behaviors. The diagnosis of ASD currently relies entirely on patient clinical evaluation.<sup>1</sup> Intensive investigations are underway to identify genetic, biochemical, electrophysiological or imaging markers that could contribute to screening and/or subgrouping diagnosis in a clinical setting. Large-scale genetic studies have pointed out a marked heterogeneity of ASD,<sup>2</sup> and, at the present time, routine genetic testing only allows the etiological diagnosis of a minority of cases, although fast technological advances may gradually lead to enlargement of this group. Some imaging,<sup>3,4</sup> electrophysiological<sup>5</sup> and eye-tracking<sup>6</sup> studies have shown promising results as contributors to the diagnosis or to early screening tools, but they have not been translated into clinical practice so far.<sup>7</sup>

Among the different types of paraclinical examinations, biological markers are readily available, as well as are time and cost efficient. Several biological abnormalities have been reported in individuals with ASD, including neurochemical, immunological, endocrine or metabolic variations.<sup>8–10</sup> Among these, elevated

whole-blood serotonin (5-hydroxytryptamine) is the most replicated finding, reported in more than 25 studies.<sup>11–13</sup> A deficit in melatonin (which derives from serotonin) has also been described in several studies on the basis of plasma or urine samples of individuals with ASD.<sup>14–18</sup>

Melatonin, a neurohormone mainly synthesized in the pineal gland during the night, is a biological signal of light/dark cycles and is considered as a major circadian synchronizer. It also displays antioxidant and neuroprotective properties and can directly modulate neuronal networks.<sup>19,20</sup> The phase and amplitude of the nocturnal melatonin peak display marked interindividual variations: although a consensus threshold of 10 ng l<sup>-1</sup> in human plasma is classically used to determine the time of melatonin onset, in some individuals the nocturnal peak remains below this threshold.<sup>21</sup> Pharmacological doses of melatonin (from 0.5 mg) can modulate circadian rhythms;<sup>22</sup> thus it is often used in the treatment of sleep disorders, which are commonly reported in patients with ASD.<sup>23–28</sup>

Conversion of serotonin into melatonin involves two sequential enzymatic steps (Figure 1a). The intermediate metabolite, *N*-acetylserotonin (NAS), displays intrinsic biological properties: it is an agonist of the TrkB receptor and may therefore share the

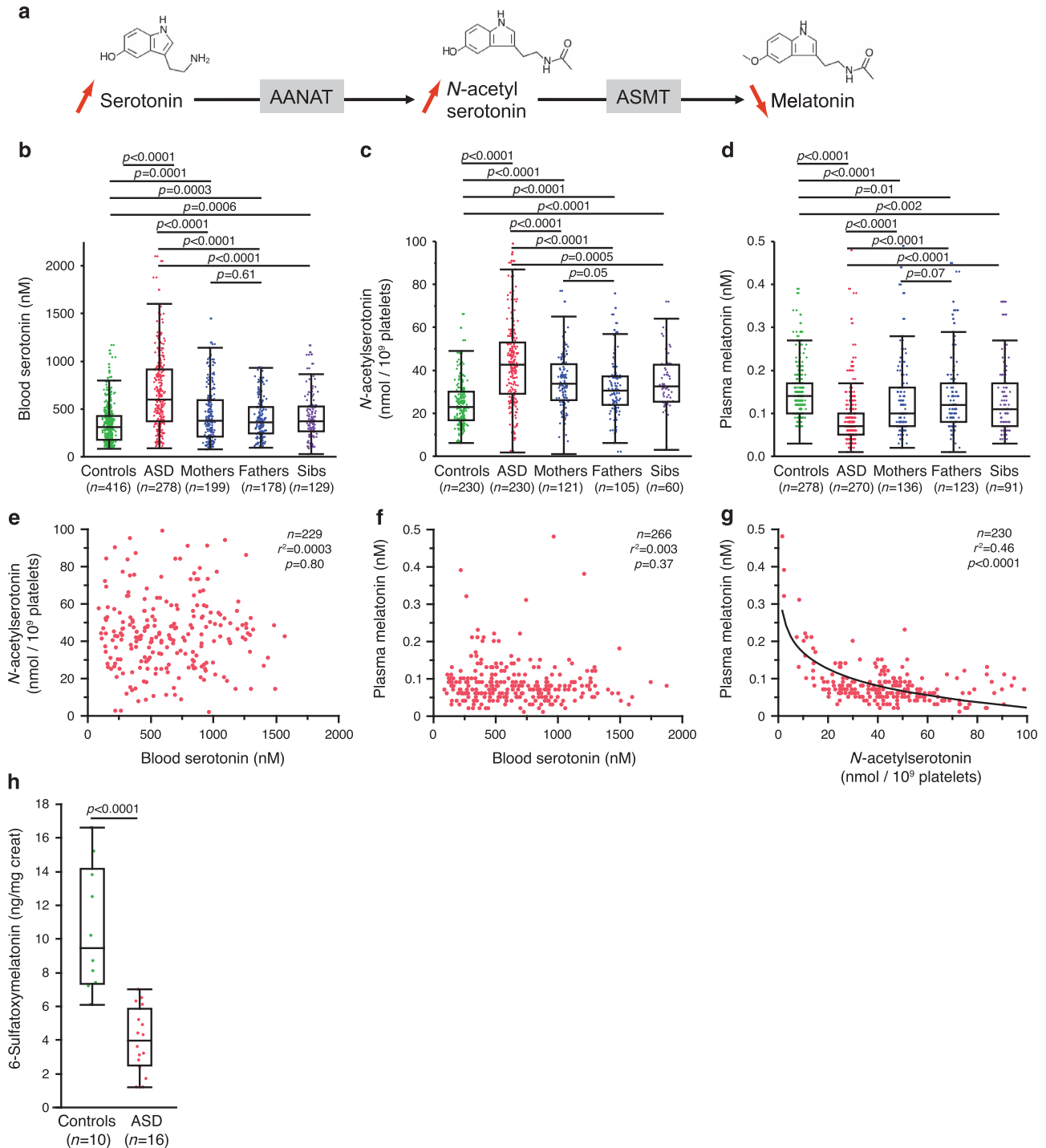
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**Figure 1.** Exploration of the serotonin-NAS-melatonin pathway in the blood. Two hundred and seventy-eight patients with ASD, their first-degree relatives (129 unaffected siblings and 377 parents) and 416 controls were sampled in the morning. Boxes indicate medians and quartiles. Groups were compared using the Wilcoxon two-sample test. **(a)** Overview of the serotonin-NAS-melatonin pathway. Serotonin is converted into NAS (*N*-acetylserotonin) by AANAT (arylalkylamine *N*-acetyltransferase, EC: 2.3.1.87), then converted into melatonin by ASMT (acetylserotonin *N*-methyltransferase, EC: 2.1.1.4). **(b)** Whole-blood serotonin **(c)** Platelet *N*-acetylserotonin **(d)** Plasma melatonin ( $1 \text{ nM} = 232 \text{ ng l}^{-1}$ ). **(e–g)** Correlations between the three parameters in patients with ASD, tested as linear **(e and f)** or log-linear **(g)** correlations. **(h)** 6-Sulfatoxymelatonin measurements in overnight urine from a subgroup of 16 patients with ASD and 10 controls. ASD, autism spectrum disorder.

neurotrophic properties of brain-derived neurotrophic factor, the canonical TrkB ligand.<sup>29,30</sup>

In a previous study,<sup>17</sup> we reported alterations of peripheral serotonin and melatonin levels in patients with ASD. In the present research, we hypothesized that (i) the intermediate NAS

might also be altered, (ii) alterations of the serotonin-NAS-melatonin pathway might constitute a possible biomarker for a subgroup of individuals with ASD and that (iii) they would be associated with specific clinical profiles. Thus, to address the issue of diagnostic validity and clinical correlates of impairments of the

serotonin-NAS–melatonin pathway, our study was extended to a larger cohort of patients with ASD and their relatives, including the assessment of NAS as well as serotonin and melatonin.

## MATERIALS AND METHODS

### Ethics statement

The local Institutional Review Boards approved the study. Written informed consents were obtained after oral and written information from all adult participants of the study and from the children's parents when subjects were under 18 years of age.

### Subjects and clinical evaluations

Unrelated patients with ASD ( $n=278$ ), their first-degree relatives (129 unaffected siblings and 377 parents, involving 171 trios, 28 index-mother pairs and 7 index-father pairs) and 416 sex- and age-matched controls were recruited into the Paris Autism Research International Sib-pair (PARIS) study at specialized centers in France. The patient characteristics and parameters are presented in Supplementary Table 1 and Supplementary Figure 2b. All individuals, including controls, were enrolled after a medical and psychiatric assessment using the Diagnostic Interview for Genetic Studies for adults and Kiddie-Schedule for Affective Disorders and Schizophrenia (K-SADS) for children. Proband with a total score  $>30$  at the Social Responsiveness Scale (SRS)<sup>31</sup> or for which a diagnosis of ASD was suspected, underwent additional screening with the Autism Diagnostic Interview-Revised with a parent<sup>32</sup> and/or the Autism Diagnostic Observation Schedule.<sup>33</sup> The final diagnosis of ASD was made when clinical assessment diagnosis coincided with algorithm-suggested diagnosis of autism on either or both of the Autism Diagnostic Interview-Revised and the Autism Diagnostic Observation Schedule. Autistic traits were measured using SRS in first-degree relatives of probands and in controls. Repetitive behaviors were assessed using the RBS (Repetitive Behavior Scale) for probands and their relatives.<sup>34</sup> General medical history (including neurological, gastro-enterological and other conditions) was checked during interviews. For probands with ASD, a cognitive level was assigned using appropriate tests (Wechsler scales or Raven's progressive matrices for nonverbal individuals). Intellectual disability was defined as verbal and performance IQ  $<70$ . Sleep difficulties were assessed in probands, relatives and controls by self-report (and/or parent questionnaire for probands who were themselves not sufficiently verbal to describe sleep behaviors) during interviews. Standard karyotyping, fragile X testing, MRI and electroencephalography were performed whenever possible. Individuals diagnosed with medical disorders related to ASD, such as fragile X syndrome, Rett syndrome or tuberous sclerosis, were excluded from the study.

### Biochemical measurements

Individuals were asked to avoid food with a high content of tryptophan and/or serotonin (for example, chocolate, bananas) for 2 days preceding the blood draw. Individuals receiving exogenous melatonin or psychotropic drugs were excluded from the study. Blood samples were collected in the morning between 8:30 and 10:30 and fractionated, as previously published.<sup>17</sup> Whole-blood serotonin was measured by high-performance liquid chromatography.<sup>35</sup> Plasma melatonin was measured using a radioimmunoassay (RK-MEL, Bühlmann, Switzerland). NAS and 14-3-3 were determined in platelet pellets by radioenzymology<sup>36</sup> and ELISA,<sup>37</sup> respectively. Urine samples were collected overnight (2000–0800 hours) from 16 adult patients with high-functioning ASD and 10 adult controls. 6-Sulfatoxymelatonin was measured by a radioimmunological method as in Tordjman *et al.*<sup>16,18</sup>

### Statistical analyses

Statistical analyses and graphs were performed using JMP Pro 9 (SAS, Toronto, ON, Canada), R-statistical software (<http://www.r-project.org/>) and Stata 11.0 (StataCorp, College Station, TX, USA). Because the studied biochemical parameters were not normally distributed, categorical and nonparametric statistical tests were preferred. Error type I was defined as 0.05. ROC curves were analyzed and the areas under the curves compared according to the Hanley and McNeil method.<sup>38</sup>

## RESULTS

Disruption of the serotonin-NAS–melatonin pathway in patients with ASD and their relatives

Whole-blood serotonin, plasma melatonin and platelet NAS were assessed in individuals with ASD, their first-degree relatives and controls (Supplementary Table 1 for groups' characteristics). To gain statistical power efficiency, the original serotonin and melatonin data collected for the present study were pooled with a smaller, independent set published in a previous report.<sup>17</sup> Separate analysis indicated no significant differences between these two data sets (Supplementary Figure 1).

Individuals with ASD displayed elevated whole-blood serotonin (Figure 1b), as previously reported.<sup>8,11–13</sup> Analyses stratified by age (two groups: below and over 16 years old) were also performed, because serotoninemia is known to be age-dependent.<sup>13,39</sup> On the basis of this stratification, hyperserotonemia remained significant for patients within the two age groups (Supplementary Figure 2a). Taking as a threshold the 95th percentile of the control group (830 nM for children  $<16$  years old, 655 nM for individuals of 16 years or more), hyperserotonemia was observed in 40% of individuals with ASD. By contrast whole-blood serotonin was significantly elevated in ~15% of their first-degree relatives (17% for mothers, 14% for fathers and 14% for unaffected siblings—frequencies with 95% confidence interval and comparisons with controls and patients with ASD are summarized in Supplementary Table 2).

Plasma melatonin was significantly decreased in individuals with ASD and their relatives compared with controls (Figure 1d). Taking as a threshold the 5th percentile of the control group (0.07 nM or 16 ng l<sup>-1</sup>), melatonin deficit was observed in 51% of individuals with ASD, 26% of parents with a trend for a gender effect (31% of mothers and 21% of fathers,  $P=0.08$ ) and 25% of unaffected siblings.

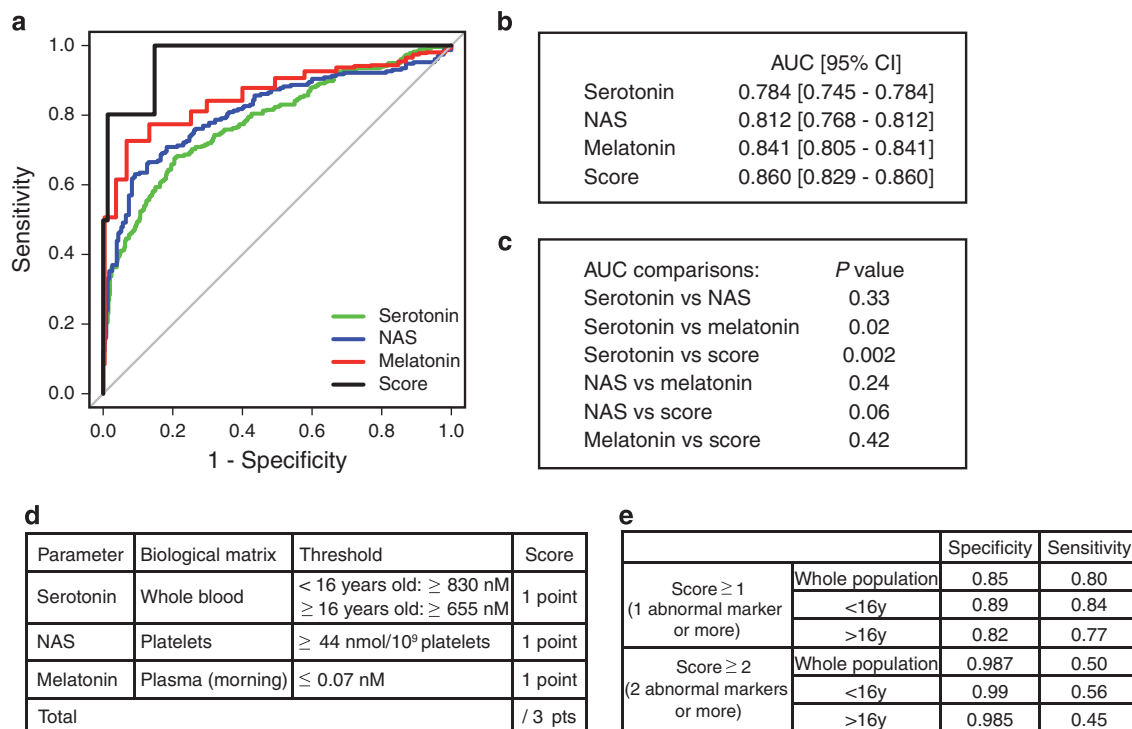
For the first time to our knowledge, the intermediate metabolite NAS, measured in blood platelets, was found to be significantly elevated in individuals with ASD and their relatives compared with controls (Figure 1c). Taking as a threshold the 95th percentile of the control group (44 nmoles/10<sup>9</sup> platelets), NAS was elevated in 47% of individuals with ASD, 19% of parents (24% of mothers and 15% of fathers,  $P=0.23$ ) and 17% of unaffected siblings.

Platelet NAS was strongly correlated with plasma melatonin in patients with ASD (Figure 1g) and to a lesser extent in parents and controls (Supplementary Figure 3), suggesting common factors of deregulation. In contrast, a correlation between whole-blood serotonin and platelet NAS or plasma melatonin was observed neither in patients (Figures 1e and f) nor in controls (Supplementary Figure 3), pointing toward an independence of mechanisms leading to serotonin impairment on one hand, NAS and melatonin alterations on the other hand.

One limitation of our study is the assessment of melatonin only from plasma sampled in the morning, as melatonin displays marked nyctohemeral variations with a peak occurring at night.<sup>40</sup> Thus, we extended melatonin assessment in a subgroup of 16 ASD patients and 10 controls by measuring 6-sulfatoxymelatonin (the main melatonin catabolite) in overnight urine. Consistent with previous studies,<sup>16,18</sup> nighttime 6-sulfatoxymelatonin excretion was significantly lower in patients than in controls, thus confirming a melatonin deficit in ASD (Figure 1h).

### Serotonin, NAS and melatonin as biomarkers of ASD

Given that increased whole-blood serotonin and platelet NAS and decreased plasma melatonin are reported in approximately half of the patients with ASD in our study, we tested their accuracy to discriminate patients with ASD from controls. Figures 2a and b show that each potential biomarker performed well with areas under the curves  $>0.75$ . Platelet NAS was as powerful as blood serotonin in discriminating ASD patients from controls ( $P=0.33$  for



**Figure 2.** Diagnostic performances of the serotonin-NAS-melatonin pathway parameters in ASD. **(a)** ROC curve analysis for serotonin, NAS, melatonin and the score combining all three as diagnostic markers of ASD. **(b)** AUC with confidence interval for each parameter. **(c)** Comparisons of AUC. **(d)** Design of the score combining impairments of serotonin, NAS and melatonin. The pathological thresholds were defined as the 95th percentile (for serotonin and NAS) or the 5th percentile (for melatonin) of the control group. **(e)** Diagnostic performances for ASD of the score. This score is based on  $n = 230$  controls and 229 patients, described for the whole population and after stratification by age category. ASD, autism spectrum disorder; AUC, area under the curve; NAS, *N*-acetylserotonin; ROC, receiver operating characteristic.

serotonin vs NAS). Plasma melatonin performed better than serotonin ( $P = 0.02$  for serotonin vs melatonin; Figure 2c).

Considering that alterations of serotonin, NAS and melatonin are partially independent (Figures 1e–g), we tested whether a combination of these parameters could improve diagnostic performances. We designed a score taking into account serotonin, NAS and melatonin, with the 95th or 5th percentile of the control group as pathological thresholds (Figure 2d). Using this score, 80% sensitivity for 85% specificity was achieved for subjects displaying at least one abnormal parameter (score  $\geq 1$ ), and the specificity reached 98.7% for subjects displaying two abnormal parameters or more (score  $\geq 2$ ) with a 50% sensitivity; interestingly, the performance for this score was slightly better in children than in adults (Figure 2e).

Thus, the combination of serotonin, NAS and melatonin allows a good discrimination between ASD patients and controls.

#### Clinical correlates of the disruptions of the serotonin-NAS-melatonin pathway in ASD

We evaluated the association of serotonin, NAS and melatonin impairment with the clinical features of patients with ASD (Table 1). Overall, we did not observe a significant association between biochemical alterations and the severity of autistic symptoms or a comorbid intellectual disability. A trend was observed for an association between high blood serotonin in patients and lower social abilities (higher SRS score), but this trend was not consistent with the analysis of the 'social' (B) item of Autism Diagnostic Interview-Revised. Similarly, a nonsignificant trend was observed between low melatonin and higher scores on the repetitive behavior (D) item of Autism Diagnostic Interview-Revised, but this trend was not consistent with the analysis of the Repetitive Behavior Scale (RBS-R) scores. No association was observed between biochemical impairments and autistic traits

(assessed by SRS and RBS-S) in first-degree relatives of patients (details not shown).

NAS might display neurotrophic properties as a TrkB agonist<sup>29,30</sup> and early brain overgrowth was reported during infancy and the toddler years in ASD.<sup>41,42</sup> We then explored the associations between biochemical impairments and abnormal head sizes: 'normal' NAS tended to be associated with a lower frequency of small heads (Table 1).

Sleep abnormalities are frequently associated with ASD, and melatonin has a major role in synchronizing circadian rhythms. Thus, we assessed sleep difficulties in patients with ASD (Supplementary Figure 4) and explored the associations between alterations of serotonin, NAS and melatonin and sleep difficulties. Patients with melatonin deficit reported sleep-onset and sleep-maintenance insomnia significantly more frequently than patients with normal melatonin levels (Table 1). Conversely, patients reporting sleep abnormalities displayed significantly lower melatonin levels than patients without such difficulties (Supplementary Figure 5a). This association was not observed in first-degree relatives of patients.

In addition to self-reporting, we assessed sleep disorders by a Children's Sleep Habit Questionnaire<sup>43</sup> in a subgroup of ASD and control children, and by actigraphy and specific sleep questionnaires<sup>44–46</sup> in a subgroup of high-functioning ASD and control adults (Supplementary Figure 4). The ASD patients displayed a high frequency of sleep disorders, including mostly insomnia (reported by 44% of patients) and delayed sleep onset. Children's Sleep Habit Questionnaire analysis revealed no associations between sleep parameters and biochemical impairments in the subgroup of children with ASD (data not shown). In contrast, according to actigraphic recordings, elevated NAS was associated with lower diurnal motor activity (M10) and tended to be associated with lower amplitude between minimal and



**Table 1.** Clinical correlates of impairments of serotonin, NAS and melatonin in patients with ASD

Patients with	High serotonin	Normal serotonin	P	High NAS	Normal NAS	P	Low melatonin	Normal melatonin	P
ADI scores, <i>n</i>	43	78		64	55		60	60	
B (social) median (quartiles)	23 (19–27)	24 (17–27)	0.50	23 (19–27)	25 (16–29)	0.97	23 (17–26)	23 (18–27)	0.57
C (verbal communication median (quartiles)	16 (9–20)	15 (12–18)	0.28	16 (12–19)	15 (9–19)	0.31	16 (12–20)	14.5 (9–18)	0.33
C (nonverbal communication) median (quartiles)	12 (6–14)	10 (7–14)	0.60	11 (8–14)	10 (5–14)	0.38	11 (8–14)	11 (5–14)	0.73
D (repetitive behavior) median (quartiles)	6 (3–8)	6.5 (5–8)	0.12	7 (4–9)	6 (4–7)	0.38	7 (5–9)	6 (4–8)	0.07
<i>n</i>	32	49		40	40		47	30	
RBS total score median (quartiles)	14 (8–33)	23.5 (14–34)	0.21	19 (9–34)	26 (14–34)	0.34	20 (9–32)	26 (13–38)	0.17
SRS total score median (quartiles)	91 (76–102)	79 (71–92)	0.07	82 (70–97)	85 (73–104)	0.34	83 (74–102)	83 (65–95)	0.24
<i>n</i>	41	77		64	52		60	57	
Intellectual disability ( <i>n</i> %)	11 (27%)	17 (22%)	0.65	15 (23%)	12 (23%)	1	13 (22%)	14 (25%)	0.83
Head circumference, <i>n</i>	47	80		68	56		61	65	
Small (< 10th percentile) ( <i>n</i> %)	4 (9%)	4 (5%)	0.73	7 (10%)	1 (2%)	0.07	4 (7%)	4 (6%)	0.17
Large (> 90th percentile) ( <i>n</i> %)	17 (36%)	30 (38%)		27 (40%)	18 (32%)		27 (44%)	19 (29%)	
Insomnia (self-report), <i>n</i> ( <i>n</i> %)	50 (36%)	96 (48%)	0.22	78 (49%)	65 (38%)	0.24	77 (52%)	68 (35%)	<b>0.05</b>

Abbreviations: ADI-R, Autism Diagnostic Interview-Revised; ASD, autism spectrum disorder; B, social interactions; C, verbal and nonverbal communication; D, stereotyped behavior; NAS, *N*-acetylserotonin; RBS, Repetitive Behavior Scale; SRS, Social Responsiveness Scale. For each biochemical parameter, patients with ASD were classified into normal and pathological group (high or low) on the basis of the threshold of the 95th or 5th percentiles of the control group. The two groups were compared using the Fisher's exact test for categorical items (*n* and % of individuals with diagnosis are indicated) and the Wilcoxon two-sample test for quantitative items (medians and quartiles are indicated). Because the number of individuals investigated displays mild variations across biochemical parameters and across clinical items, the total sample size (*n*) is indicated separately for each test. Intellectual disability is defined as verbal IQ and performance IQ < 70. The italics refer to *P*-values which are not primary data. Bold character is for significant *P*-value.

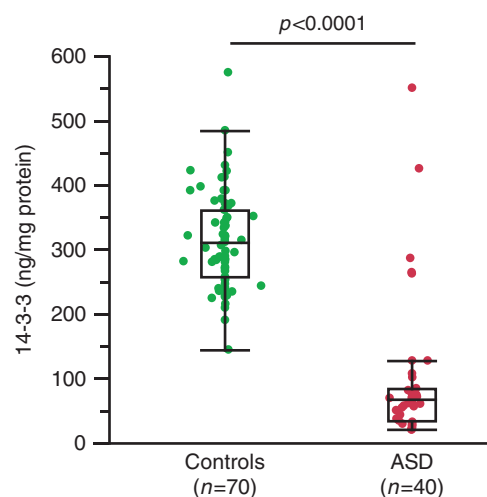
maximal activity (Supplementary Figure 5). A trend for an association between the melatonin deficit and the increased sleep latency was also observed. These results need further replication in larger groups. No association was observed between biochemical impairments and other comorbid psychiatric or somatic conditions.

Disruption of 14-3-3 proteins in platelets of patients with ASD  
Melatonin synthesis involves two enzymes, AANAT and ASMT, known to form protein complexes with 14-3-3 scaffolding proteins.<sup>47</sup> Interaction with 14-3-3 regulates this process by stabilizing and activating AANAT.<sup>48</sup> In a previous study, we reported a decrease of ASMT activity in platelets of patients with ASD.<sup>17</sup> Here, 14-3-3 was measured in platelets and found significantly decreased in patients with ASD (Figure 3). Thus, although obtained from peripheral tissues, these results suggest that the disruption of melatonin synthesis in patients with ASD may be linked with impairments of protein complex formation involving 14-3-3.

## DISCUSSION

The exploration of the serotonin-NAS-melatonin pathway in our study confirmed the hyperserotonemia<sup>8,11–13</sup> and the melatonin deficit<sup>14–18</sup> in ASD that has been reported in several studies, and it also revealed an increase of NAS in platelets, pointing towards a global disruption of this pathway in ASD.

Consistent with previous reports on serotonin and melatonin, impairments of the serotonin-NAS-melatonin pathway were frequent (40 to 51%) in this cohort of patients with ASD, and may thus be considered as possible biomarkers for a large subgroup of patients. In line with this idea, we found that combining the three parameters was sensitive and specific for discrimination of probands from controls.



**Figure 3.** 14-3-3 protein measurement in platelets. Forty patients with ASD and 70 controls were assessed. Boxes indicate medians and quartiles. Groups were compared using the Wilcoxon two-sample test. ASD, autism spectrum disorder.

The specificity of the combination of these biochemical biomarkers regarding other neurodevelopmental conditions should be further evaluated in future studies. However, previous reports<sup>49</sup> and our preliminary data (Supplementary Figure 6) indicate that patients with intellectual disability not associated with autism (ID) or patients with attention-deficit hyperactivity disorder (ADHD) do not display as high blood serotonin levels as patients with ASD. To our knowledge, plasma melatonin has been documented neither in ID nor in ADHD patients. Our preliminary data suggest that ADHD or ID patients display a decreased level of

plasma melatonin, but less pronounced than in ASD. ROC curve analysis indicated that whole-blood serotonin and plasma melatonin were discriminant between ADHD or ID patients and ASD patients, but not between ADHD or ID patients and controls (Supplementary Figure 6).

Disruption of the serotonin-NAS–melatonin pathway was also observed in first-degree relatives of patients with ASD, suggesting a genetic liability. This finding is consistent with previous reports of (i) increased serotonin among parents and siblings of patients with ASD,<sup>13,50–52</sup> (ii) decreased melatonin among parents<sup>17</sup> and (iii) strong heritability of serotonin<sup>53</sup> and melatonin<sup>54</sup> reported in the general population. However, in our cohort, disruption of the serotonin-NAS–melatonin pathway does not always segregate in the families with ASD and rarely with a simple recessive/dominant model, suggesting a complex inheritance. Interestingly, mothers of patients with ASD would tend to display more pronounced biochemical impairments (elevated platelet NAS and low melatonin) than fathers. Considering that melatonin crosses the placental barrier, melatonin deficit in a mother might result in insufficient fetal exposure to melatonin *in utero*, and might thus be a prenatal, environmental susceptibility factor to ASD.

The search for clinical correlates of disruptions of the serotonin-NAS–melatonin pathway yielded no significant associations with ASD symptom severity or presence of intellectual disability. In addition, no association was found between biochemical impairments and diagnostic categories of ASD according to the DSM-IV (autism, Asperger syndrome, PDD-NOS—data not shown), which suggests that a defect in the serotonin-NAS–melatonin pathway is a shared feature across the spectrum.

Attempts to link whole-blood serotonin levels to clinical symptoms have been inconsistent. However, the overall evidence implicates the serotonin system in ASD: serotonin is crucial for embryonic brain development<sup>55,56</sup> and regulates the release of oxytocin whose signal alterations are important for ASD<sup>57</sup> and which promotes social behavior in high-functioning ASD patients.<sup>58</sup> Of note, oxytocin and serotonin interact in the human brain<sup>59</sup> and their blood levels are negatively correlated in humans.<sup>60</sup>

One study reported an association between melatonin deficit and language difficulties in patients with ASD,<sup>16</sup> but we were not able to replicate this finding. In our study, we observed a significant association between melatonin deficit and sleep problems in patients. Sleep difficulties are frequently associated with ASD and are a major concern for the patients and their families.<sup>61,62</sup> These results provide a rationale for the therapeutic use of melatonin in sleep disorders associated with ASD.<sup>23–28,63</sup>

A possible pitfall of this study is that melatonin levels were assessed only at one time point (over a 2-h window well after sleep offset) and not under dim light conditions. Obviously these conditions are not adequate for assessing the endogenous phase or amplitude of nocturnal melatonin secretion. However, they allow a good discrimination between patients and controls and are more convenient in a clinical setting than nocturnal melatonin assessment. Furthermore, nighttime 6-sulfatoxymelatonin excretion in a subset of patients confirmed a nocturnal melatonin deficit, consistent with previous studies.<sup>16,18</sup> To the best of our knowledge the circadian rhythm of platelet NAS has not been reported, whereas whole-blood or platelet serotonin only displays minor (< 10%) circadian variations.<sup>64,65</sup>

We reported for the first time an increase of the intermediate metabolite NAS in platelets of patients with ASD. Interestingly, NAS displays proper biological functions. At a metabolic level, it is an inhibitor of tetrahydrobiopterin synthesis,<sup>66</sup> a cofactor of several pathways such as nitric oxide formation<sup>67</sup> and tyrosine/bioamine synthesis. Thus, in addition to the possible consequences of alterations of serotonin and melatonin,<sup>6,20</sup> NAS accumulation may have implications for the pathophysiology of ASD.

The mechanisms of these impairments of the serotonin-NAS–melatonin pathway in ASD remain to be elucidated. Various studies have not succeeded in revealing a convincing mechanism for hyperserotonemia.<sup>68</sup> Considering that melatonin synthesis represents only a small fraction (about 1%) of serotonin catabolism, it is unlikely that an impairment of melatonin synthesis would be sufficient to provoke a significant increase of serotonin. Our previous study<sup>17</sup> suggested that melatonin deficit results from impaired synthesis as a consequence of a decrease in ASMT activity. This enzymatic alteration may be associated with genetic factors, including coding mutations in a subset of patients with ASD—although with a low frequency—and polymorphisms in the ASMT promoter (rs4446909 and rs5989681) with functional effects. The association finding was not fully replicated in an independent study, although a similar trend was observed.<sup>69</sup> Interestingly, these SNPs have also been associated with bipolar disorder,<sup>70</sup> depression<sup>71</sup> and depressive symptoms in individuals with sleep phase delay.<sup>72</sup> Here we show that 14-3-3 scaffolding protein levels are decreased in patients with ASD. As protein complex formation involving 14-3-3 regulate melatonin synthesis,<sup>47,48</sup> we propose that impairments of posttranslational regulations of AANAT and/or ASMT may participate in the mechanisms of the disruption of melatonin synthesis in ASD.

Although ASDs are considered as highly heterogeneous, this study reports disruptions of the serotonin-NAS–melatonin pathway as a highly sensitive and specific biomarker of ASD. Associations with sleep problems provide a rationale for melatonin therapy. Finally, these results open new hypotheses for the mechanisms of impairments of melatonin synthesis in ASD.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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