bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Low parental melatonin levels increases autism spectrum disorder risk in children

Wiebe Braam[1,2], Friederike Ehrhart[2,3], Chris T. Evelo[2,3], Anneke P.H.M. Maas[1,2,4], Marcel G. Smits[2,5], Leopold Curfs[2,6]

Affiliations:
[1] 's Heeren Loo, Department Advisium, Wekerom, The Netherlands
[2] Governor Kremers Centre, Maastricht University, Maastricht, The Netherlands
[3] Department of Bioinformatics – BiGCaT, Maastricht University, Maastricht, The Netherlands
[4] Department of Special Education, Radboud University, Nijmegen, The Netherlands
[5] Multidisciplinary expert centre for sleep-wake disturbances and chronobiology, Gelderse Vallei Hospital, Ede, The Netherlands
[6] Department of Clinical Genetics, Maastricht University Medical Centre, Maastricht, The Netherlands

Corresponding author: Wiebe Braam, 's Heeren Loo, Department Advisium, PO Box 6710 BB EDE, The Netherlands (e-mail: braam@planet.nl)

Keywords: autism spectrum disorder, genetics, melatonin, intellectual disability, CYP1A2

Abbreviations: autism spectrum disorder (ASD), intellectual disability (ID), 6-sulfatoxymelatonin (6-SM)

Genes, transcripts and proteins are abbreviated according to human genome name consortium.

Abstract

Background: Low melatonin levels are a frequent finding in autism spectrum disorder (ASD) patients. Melatonin is also important for normal neurodevelopment and embryonic growth. As a free radical scavenger and antioxidant melatonin is highly effective in protecting DNA from oxidative damage. Melatonin deficiency, possibly due to low CYP1A2 activity, could be a major factor, and well a common heritable variation. ASD is already present at birth. As the fetus does not produce melatonin, low maternal melatonin levels should be involved.
Methods: We measured 6-sulfatoxymelatonin in urine of mothers of a child with ASD that attended our sleep clinic for people with an intellectual disability (ID), and asked for parental coffee consumption habits, as these are known to be related to CYP1A2 activity.
Results: 6-Sulfatoxymelatonin levels were significantly lower in mothers than in controls (p = 0.005), as well as evening coffee consumption (p = 0.034). In mothers with a second child with ASD and/or ID, 6-sulfatoxymelatonin levels were lower compared to mothers with one child with ASD (p = 0.084),
Conclusions: Low parental melatonin levels, likely caused by low CYP1A2 activity, seem to be a major contributor to ASD and possibly ID etiology.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

1. Introduction

Autism Spectrum Disorder (ASD) refers to a heterogeneous group of neurodevelopmental disorders, with a complex multifactorial etiology, starting very early in life. The disease is characterized by abnormal social interactions, impaired language development, and stereotyped and repetitive behaviours. ASDs have in common that they each share one or more core symptoms of autism. But at the same time there are large differences between them, both with regard to symptomatology as to etiology (1). Intellectual disabilities (ID) and ASD, although apparently two separate disorders, co-occur much more frequent in the same patient than by chance. While in 40% of persons with ID autism is present, at the same time 70% of people with autism also has an intellectual disability (2). The frequent co-occurrence of ASD and ID, however, raises questions whether this is a true co-occurrence because both share, at least in part, similarities in their etiology. Especially since in more severe cases of ID, the risk of ASD is greater (3), as is the risk of ID in more severe cases of ASD (4).

There is a growing number of literature on the genetic basis of ASD. Over a hundred of chromosomal abnormalities have been found, however, not one of them is responsible for more than 1% of ASD cases. This means that the underlying genetic process in ASD is complex. Recent reviews (5-9) provide a good overview of the current state of knowledge.

The relative risk of a newborn child from parents having a child with ASD is 25-fold increased compared to the general population, suggesting a genetic component (6). However, in only about 10% a specific genetic disorder is diagnosed (7). It is assumed that ASD is caused by a combination of *de novo* inherited variation and common variation. In a recent epidemiological study in Sweden, it was possible to distinguish between both components, estimating liability of *de novo* variations to be 2.6% and common variation to be 49% (10). In a recent large study in twins with ASD, concordance for monozygotic male twins was 77% (females 50%), and for dizygotic male twins 31% (females 36%) (11). Based on their data they were able to calculate that the contribution of genetic factors was smaller (38%) than shared environmental factors (58%).

Epidemiological studies have identified several environmental factors that increase ASD risk (12-16) (see supplementary file). Most important factors are advanced parental age at birth, bleeding, gestational diabetes, having a mother born abroad, fetal distress, birth injury or trauma, multiple birth, maternal haemorrhage, summer birth, low birth weight, small for gestational age, congenital malformation, low 5-minute Apgar score, feeding difficulties, meconium aspiration, neonatal anaemia, ABO or Rh incompatibility, and hyperbilirubinemia. Also subnormal breast feeding increases autism risk (17). Interestingly, many of these environmental factors can be linked to melatonin deficiency.

Disturbances in melatonin turnover leading to different melatonin levels in ASD are found in several studies (18-20). Melatonin levels in ASD are negatively correlated with severity of autistic impairments (21). Melatonin is a hormone with multiple functions and it is synthesized in the pineal gland during the dark phase of the day and involved in sleep induction and circadian rhythm regulation (22). Melatonin also acts as a free radical scavenger and antioxidant and is highly effective in reducing effects of oxidative stress throughout the body (23-25). There are also indications that melatonin protects against oxidative stress-evoked DNA damage (23, 24, 26, 27).

Melatonin is synthesized from serotonin in a two step process, involving the enzymes AANAT and ASMT (see Figure 1) (28). The expression of their mRNAs encoding for these enzymes follows a day/night rhythm.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

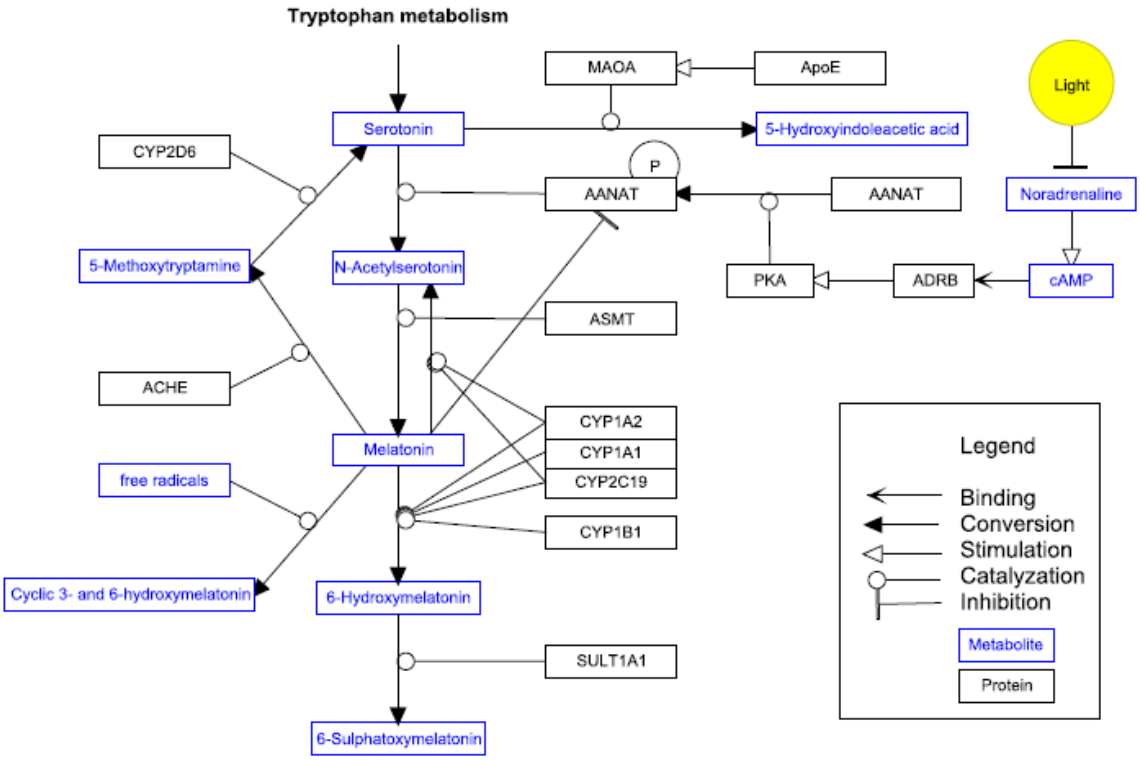

Figure 1 Synthesis and breakdown pathway of melatonin. Figure modified from www.wikipathways.org/instance/WP3298

AANAT is generally considered to be the rate-limiting enzyme in the melatonin synthesis pathway. However, recent studies indicate that the rate limiting role at night is partly taken over by ASMT (29). Exposure to light rapidly decreases AANAT activity. Timing, profile and amount of melatonin secretion display large inter-individual differences, but are in healthy individuals highly reproducible from day to day like a hormonal fingerprint (22). As information on normative data of melatonin levels is scarce and internationally accepted normative data are not available, it is also not clear which melatonin levels are pathologically low. This is remarkable, given the large number of functions of melatonin. Although several positive functions are attributed to melatonin, melatonin deficiency is not regarded as a risk factor or a disease yet.

Melatonin is metabolised principally by 6-hydroxylation to 6-hydroxymelatonin, which is sulphated to 6-sulfatoxymelatonin (6-SM) and excreted in urine (30, 31) (Figure 1). CYP1A2, and to a lesser extend CYP1A1 and CYP2C19 are the main cytochrome isozymes to be responsible for the plasma clearance of melatonin in the liver (30). CYP1A2 activity in fetal and neonatal liver tissue is very low to undetectable (32). The expression of CYP1A2, as well as other members of the cytochrome P450 gene family, is regulated *i.a.* by the biological clock (33). Melatonin does not influence CYP1A2 activity at physiologic levels. Only at extremely high concentrations of 3 – 300 µM an inhibition was observed (34).

The half life of exogenous melatonin ranges between 28 to 126 minutes (35, 36). Differences in melatonin half-life reflect the wide inter-individual differences (10 to 200-fold) in CYP1A2 activity (37). Several reports indicate that SNPs in the CYP1A2 gene are associated with increased inducibility, decreased activity or inducibility or even loss of activity of the CYP1A2 enzyme as compared to the wild type CYP1A2*1A (38-43). The (sub)variant alleles associated with decreased of absent activity of CYP1A2 are *1C, *1K, *3, *4, *5, *6 and *7. The proportion of individuals with the slow phenotype varies among ethnic populations (41).

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Caffeine clearance is considered as the gold standard for assessment of CYP1A2 activity, because more than 90% of the primary metabolism of caffeine depends on CYP1A2 (44). Caffeine related side effects, i.e. caffeine-induced insomnia, are more frequent in people who are CYP1A2 slow metaboliser (45). Several polymorphisms decrease the metabolic activity of CYP1A2 and are significantly inversely related to coffee intake, so coffee intake is influenced by CYP1A2 genotype (46, 47). In people with caffeine-induced insomnia caffeine clearance is significant slower than in people without sleep onset problems after drinking coffee in the evening (48).

Melatonin and its relation to ASD: Low levels of melatonin are found in the majority of individuals with ASD (18, 21, 49, 50). Studies on genes involved in melatonin metabolism have long been focussed on the melatonin synthesis pathway, mainly of ASMT. Interestingly no studies were performed on the melatonin catabolism by CYP1A2 until in 2013 a possible relationship between slow CYP1A2 polymorphisms in slow melatonin metabolisers and ASD was proposed (51). Even though ASD frequently is associated with low melatonin levels and suspicion exists for an involvement of the ASMT gene in the etiology of autism, several studies could not find any evidence for a significantly higher incidence of abnormalities of the ASMT gene in ASD (52, 53). And if so, ASMT abnormalities were also found in healthy controls.

Another potential link between melatonin and ASD etiology was proposed by us in an earlier report (51). In a study in 15 consecutive patients with ID and sleep problems, presenting with disappearing effect of melatonin treatment after an initial good response, extremely high (>50 pg/ml) melatonin levels at noon and 04:00 pm were found. We hypothesized that the disappearing effectiveness of melatonin treatment was associated with slow metabolisation of melatonin due to a SNP of CYP1A2 and analysed DNA for a CYP1A2 SNP. In 8 patients a SNP was found. Because 13 of 15 patients with CYP1A2 slow metabolism were diagnosed with ASD, we hypothesized that low melatonin levels in autism are caused by a yet unknown mechanism in which CYP1A2 slow metabolism is involved, and that low melatonin levels are possibly part of the mechanisms that *cause* ASD, rather than that low melatonin levels are the *result* of ASD.

The potential mechanism was investigated by Veatch et al. (2014) (54). They found a higher frequency of dysfunctional ASMT polymorphisms than earlier reported (52, 55-57). They also found substantially higher rates of CYP1A2 polymorphism in ASD patients than in our previous report (51). Based on the linkage disequilibrium structure across SNPs in ASMT and CYP1A2 in their subset of individuals, Veatch et al. predicted a potential gene–gene interaction (20).

The embryo and fetus do not secrete melatonin and are totally dependent on maternal melatonin (58, 59). Melatonin secretion begins no earlier than approximately 9 - 15 weeks after birth, depending of gestational age (59). Maternal melatonin passes the placental barrier easily and the fetal circadian melatonin rhythm follows the maternal rhythm (60). Maternal melatonin is also passing easily into breast milk, and levels are a reflection of the circadian melatonin rhythm of the mother (61). ASMT and AANAT gene activity and melatonin levels in disturbed pregnancies (intra uterine growth retardation and in severe preeclamptic women) are lower than in normal pregnancies (62, 63).

Melatonin is important for normal neurodevelopment and embryonic growth and helps regulate synaptic plasticity (18, 19, 58, 64). Melatonin is implicated in neural growth and synapse formation and it can modulate neurite outgrowth in cultured neuronal cells (56). Melatonin guides the differentiation of neuronal stem cells (*in vitro*) as follows (64): It stimulates the mRNA and protein levels of TH, which is a marker for dopaminergic neurons. Dopaminergic neurons are neurons which are able to release dopamine and have therefore depending on their location in the brain 1) a high impact on regulation of cognitive function, emotional behaviour, and reward system or 2) regulate motor function. *In vivo*, melatonin increases dendritogenesis (dendrite formation, enlargement and complexity) in the hilar and mossy neurons of hippocampus in rats (65). The accompanying *in vitro* results indicate that melatonin triggers CALM which stimulates MAP2 via CAMK pathway (Figure 2). Melatonin increases the total

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

cellular level of CALM and fosters the translocation from cytosol to membrane/cytoskeleton interfaces (65).

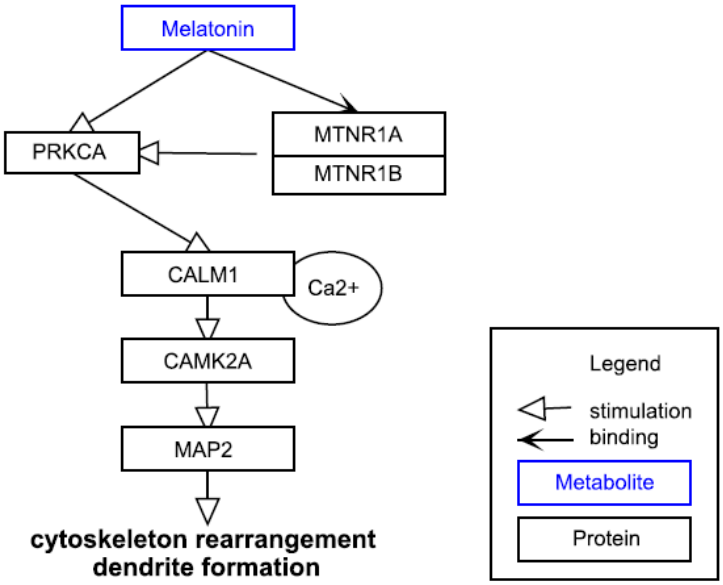

Figure 2: Melatonin influences the cytoskeleton arrangement and dendrite formation in neuronal cells via CALM and CAMK. Figure modified from www.wikipathways.org/instance/WP3298

Although the genetic basis of ASD is not disputed, no single chromosomal abnormality is responsible for >1% of ASD cases. Low melatonin levels are found in the majority of ASD cases, but is regarded as co-morbidity and not considered as part of ASD etiology. All data mentioned above can be linked directly or indirectly with melatonin. As ASD is already present at birth and the fetus does not produce melatonin, low maternal melatonin levels should also be involved. Low melatonin levels are not only caused by disturbances in melatonin synthesis, but are likely also related to low CYP1A2 activity, in view of the finding of CYP1A2 gene SNPs in ASD patients and a potential gene-gene interaction between CYP1A2 and ASMT. We therefore measured 6-SM in urine of mothers of a child with ASD that attended our sleep clinic, and asked for parental coffee consumption habits, as these are known to be related to CYP1A2 activity.

2. Material and methods

2.1. Participants

Approval was obtained through the Ethical Committee of Maastricht University (The Netherlands). Mothers of patients with chronic sleep disturbance, who visited our sleep centre for people with ID between January and July 2015, were asked to participate if her child was diagnosed with ASD. Mothers of children with a genetic ID syndrome and an autistic phenotype were also asked to participate. Out of 97 children seen in this period, 18 were not diagnosed with ASD and 13 mothers were not eligible for several other reasons (five children were adopted or foster child, four children were only seen once, with three mothers we had severe language barriers, and one mother used melatonin supplementary on a daily basis). So, 66 mothers consented to participate. The control group consisted of 15 women who work in the building where the sleep centre is located. These women all had healthy children without signs of autism and siblings of controls also did not have signs of autism.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

2.2. Procedure

After explaining purpose and procedure of the study, participants received an introductory letter with information of the study and an informed consent letter to be returned by return paid mail. After receiving the signed consent letter, a questionnaire, together with a shipping container for a urine sample and instruction sheet was send to participants. Participants were instructed to empty the bladder at bedtime. Time of voiding at bedtime and first voiding in the morning were recorded. The first morning urination, plus any urine voided during the night, was collected and the total urine volume was recorded. A sample of the total urine was poured into a plastic bottle and sent to the sleep centre where it was frozen and stored at minus 20°C until measurements took place. The questionnaire (see supplementary data) contained a total of 17 questions, namely six questions on age, weight, medication use, and number and age of children (oldest and youngest child), seven questions based on the Insomnia Severity Index (66) and four questions about the use of coffee.

2.3. 6-SM concentration measurement

6-SM in urine was analysed by isotope dilution mass spectrometry using online solid phase extraction in combination with liquid chromatography and tandem mass spectrometry (XLC-MS/MS) (Van Faassen et al., manuscript in preparation). In short, 100 µl of urine was used for the analysis, deuterated 6-SM was used as internal standard. Mean intra- and inter-assay coefficients of variation were below 6.0%. Quantification limit for 6-SM was 0.2 nmol/l. As advanced parental age at birth is a factor associated with autism risk (12) and melatonin levels decrease with 2.7% per year (67), we recalculated the urinary 6-SM results in mothers with an ASD child, taking into account the age at which they received this child. In 6-SM levels in controls, we based recalculating on their age at receiving their first-born child. In case controls had more than one child, we took the mean of their age receiving their oldest and their youngest child.

2.4. Statistical analysis

All statistics were performed using IBM SPSS Statistics 20 (IBM, SPSS Inc., Chicago, IL).T-tests for independent samples were conducted to test differences between ASD-groups and controls. To test for differences on sleep questions between the subgroups non-parametric tests (Fisher's exact test and Mann-Whitney test) were used because of low cell frequencies and/or not normally distributed data. To test the relationship between sleep and melatonin bivariate correlation (Kendall's tau-b) was used. Statistical significance was accepted at $p < 0.05$.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

3. Results

Of 66 mothers, who consented to participate in this study, one withdrew without specifying at reason. In five cases the urine samples did not arrive. So urine samples of 60 mothers were analysed. Their mean age was 42.9 (± 5.7) years. The mean age of 15 women in the control group was 44.3 (± 9.7) years. No significant differences with respect to age (p = 0.486) were found between participant and control group. However, mean age of mothers of an ASD child at time of delivery was higher (31.2 years) than the mean age control mothers received their children (28.1 years). This difference was significant (p = 0.01). Other demographic data are shown in Table 1.

Table 1: Demographic data

Demographic data	ASD	SD	Control	SD	*p*
Age of mother at study participation	42.92	5.72	44.27	9.73	0.486
Age of mother at birth child	31.17	4.15	28.07	3.58	0.01
Number of children	2.18	0.89	2.40	0.51	0.37
Weight of mother	73.24	13.04	69.27	8.23	0.27

Data on etiological diagnoses of children with ASD are presented in Table 2. It is noteworthy that there are substantial groups with Angelman syndrome or Smith Magenis syndrome in the group studied. It should however be noted that both syndromes occur relatively frequently (prevalence 1:20.000) and that they are well known for their sleep problems (68, 69).

Table 2: Etiological diagnoses of ASD children

Angelman syndrome		10	(group AS)
Smith Magenis syndrome		18	(group SMS)
Other diagnoses		22	(group Other)
6p25 deletion	2		
15q13.2 duplication	1		
16p11.2 deletion	2		
16p11.2 duplication	1		
16p13.3 deletion	1		
17p11.2 duplication (Potocki syndrome)	1		
17q21 deletion and 16q22.1 deletion	1		
18q12.1 deletion	2		
22q11 duplication	1		
ADNP gene mutation	1		
Congenital cytomegaly	1		
Down syndrom	1		
Isodicentric chromosome 15 syndrome	1		
Phelan McDermid syndrome	2		
SCN2A gene mutation	1		
SOX21 and GPC6 gene mutation	1		
translocation 10:22 (der(10)t(10:23)(qter;qter)	1		
Williams syndrome	1		
Unknown		10	(group Unkown)
Total		60	

In this study we found a significant lower urinary 6-SM excretion in mothers of a child with ASD compared to control mothers (p = 0.012). Group means and SDs of the excretion of 6-SM excretion in the urine in all groups are given in Table 3. Melatonin levels are known to decline

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

after the age of 20 year with 2.7% (67) each year and ages of mothers and children were asked for, we were able to recalculate 6-SM excretion levels in each mother to the year their child was born. Comparing urinary 6-SM levels in the year their child was born is more relevant, given our hypothesis that low melatonin levels in pregnancy are a risk factor for getting a child with ASD. As shown in Table 3 differences in recalculated 6-SM excretion levels between mothers of a child with ASD compared to control mothers were significant (p = 0.002).

Table 3: 6-SM/creatinine ratio in urine measured and recalculated to the age of the mother at child birth

	measured		**recalculated**	
	ASD	**Control**	**ASD**	**Control**
n	60	15	60	15
6-SM/creatinine	7.67±3.62	10.42±6.72	10.52±5.20	16.67±10.72
Min - Max	1.99 – 16.25	2.31 – 25.5	2.81 - 21.93	4.79 - 42.59
p	0.012		0.002	

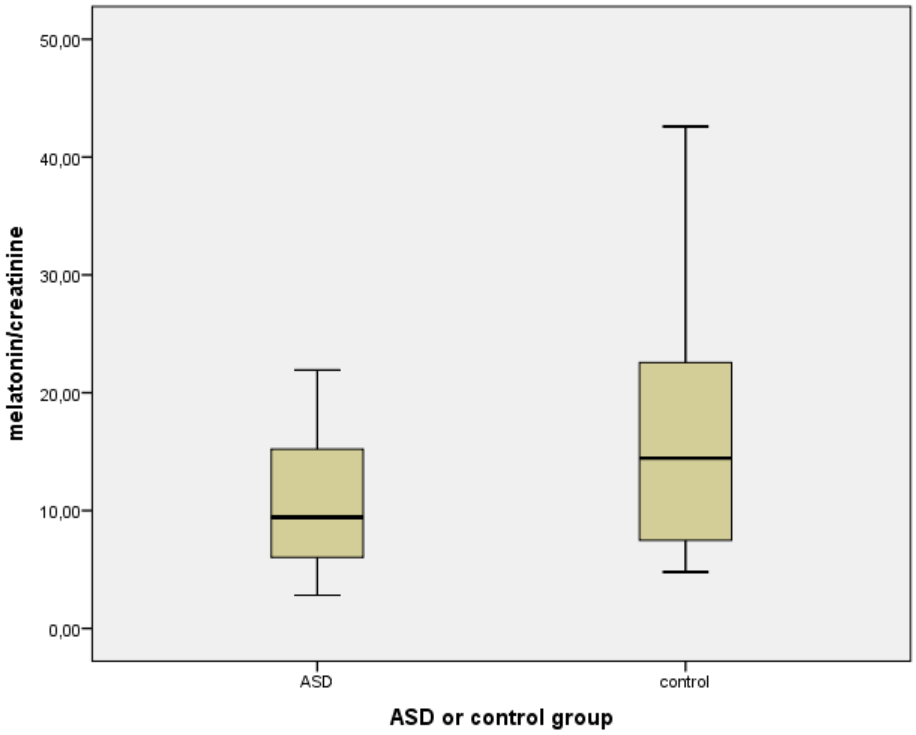

Figure 3: 6-SM/creatinine ratio in urine of mothers. The values have been recalculated to the age of the mother at child birth.

Looking into subgroups, only mothers in the group with other genetic disorders and with Angelman syndrome had a significant lower urinary 6-SM excretion compared to control mothers (p = 0.012 resp. 0.042) (Table 4). Urinary 6-SM levels in mothers of a child with Smith Magenis syndrome also were lower than in controls, but these differences approached significance (p = 0.05). Five mothers of a child with ASD also had another child with ASD and/or an ID. These children, however, were not treated for sleep problems in our sleep centre. One child was known with Prader Willi syndrome, being genetically of paternal origin. Melatonin levels in the other four mothers were lower (mean 7.05 ± 2.86) compared to the other mothers with one child with ASD (mean 11.61 ± 5.57, p = 0.084).

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Table 4: 6-SM/creatinine in urine (recalculated to age of mother at child birth) for different ID groups; ASD eci (no genetic disorder found yet), ASD syndromal (ASD in child with a chromosomal disorder, but not Angelman of Smith Magenis syndrome), Angelman syndrome and Smith Magenis syndrome.

	ASD eci	ASD syndromal	Angelman syndrome	Smith Magenis syndrome	Controls
n	10	22	10	18	15
6-SM/creatinine	13.37±5.20	9.54±5.63	8.92±4.46	11.03±4.70	16.67±10.72
Min - Max	4.96 - 21.55	3.33 - 21.93	4.46 - 19.91	2.81 - 18.77	4.79 - 42.59
p	0.375	0.012	0.042	0.052	

The number of mothers drinking coffee does not differ between mothers with an ASD child and controls (p = 0.375). However, more mothers with an ASD child do not drink coffee after 06:00 pm than mothers in the control group (p = 0.018). Time of the last cup of coffee was later in control mothers than in mothers with an ASD child (07:10 pm, resp. 05:05 pm: p = 0.058) (Figure 4). Urinary 6-SM levels in mothers that do not drink coffee after 06:00 pm were significantly lower (13.79±9.08) than in mothers that do drink coffee after 06:00 pm (10.39±4.90) (p = 0.039). Also more fathers with an ASD child do not drink coffee after 06:00 pm (p = 0.020).

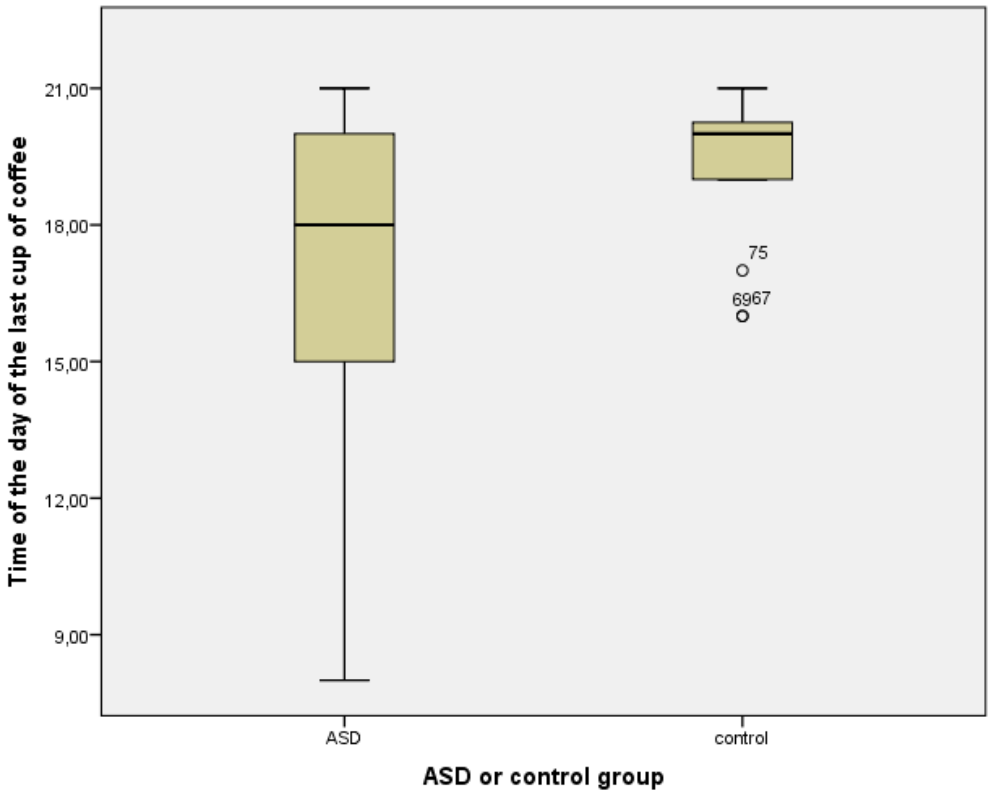

Figure 4: Time of the day of the last cup of coffee for mothers of an ASD child and control.

Of 60 mothers who's urine samples were analysed, one did not fill in the questions on sleep. So questions on sleep of 59 mothers of the patient group and 15 mothers of the control group were analysed. If a mother indicated that she did not experience difficulty with either falling asleep or staying asleep and did not have a problem with waking up too early she was asked to skip three questions and no total score on the Insomnia Severity Index (ISI) could be calculated. A cut-off score of 10 was used to indicate clinical significant insomnia (70). Of the women with an ASD child (n = 33) 67% met the cut-off score of the ISI compared to 29% of the women in the control group (n = 7). This difference was not statistically significant (p = 0.094, Fisher's exact test).

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

In order to compare sleep between all mothers of the patient and the control group we also calculated a shortened ISI (sISI) based on two questions (severity of sleep problems and satisfaction with current sleep pattern) filled in by all mothers (n = 74). Higher scores indicate more severe sleep problems and less satisfaction with current sleep pattern. Scores on sISI in patient group (Mdn = 4) did not differ significantly from the control group (Mdn = 2), U = 542.50, z = 1.366, p = 0.175, r = 0.16.
Furthermore no correlation was found (tau = 0.00) between sISI and the excretion of 6-SM in the urine (*p* = 0.996, two-tailed).

4. Discussion

In this study we found a significant lower 6-SM excretion in mothers of a child with ASD. This difference cannot be explained by the difference in their age at birth of their child. ASD is caused by a mixture of rare and common genetic variation, as well as environmental factors. Melatonin deficiency can play a role in all three of these factors. Epidemiological studies have identified several environmental factors that increase ASD risk. Almost all of the known prenatal, perinatal and neonatal ASD risk factors, found in two meta-analyses (Gardener et al. (2009) (12) and Gardener et al. (2011) (13)) can directly or indirectly be linked to melatonin deficiency (see supplementary files). Additionally, low CYP metabolisation indicated by coffee drinking behaviour was found to be an early indicator. A correlation between ASD and low melatonin levels/slow CYP metabolisers has been found earlier in different experimental settings (see Table 5).

Table 5: Experimental and population data which shows correlation of ASD and low melatonin/CYP1A2

	Correlation of ASD and melatonin/CYP data
Population	Prevalence estimates of ASD ranges from 30.0 : 10 000 to 116.1 : 10 000. Lower prevalence estimates are found in Australia (39.2 : 10 000) and China (11.6 : 10 000), and the highest rates came one Japanese study (181.1 : 10 000) (71). Slow metaboliser phenotype, using substrate probes, has been identified in Australians (5%), Japanese (14%) and Chinese (5%), while Asian and African populations have lower CYP1A2 activity compared with Caucasians (40). See also sup. data Table 1.
Gender difference	Advanced genetic research did not yet identify chromosomal loci that contribute to the gender difference. Perhaps the gender difference in melatonin levels can offer an explanation: Melatonin levels in girls are two to threefold higher than in boys (72). A theoretical explanation could be that the damage to fetal brain development by low maternal pregnancy melatonin levels, after birth may be limited in case melatonin production in the child is normal. As melatonin levels in girls are two to threefold higher than in boys (72), this could ultimately lead to less, or much milder cases of autism in girls than in boys. After puberty the gender difference is smaller. Melatonin amplitude in women is one and a half times higher than in men (73).
Mouse model	Investigation of melatonin supplementation in the BALB/cJ inbred strain of mice on behaviour in off-spring, as well on brain morphology, is advised as this mice strain is already an ASD mouse model because it displays all core behavioural features of ASD (74-76). This mice strain happens to be melatonin deficient (77).

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Finding a significant lower urinary 6-SM excretion in mothers of a child with ASD compared to control mothers, combined with the neurotrophic properties of melatonin, total dependence of the fetus on maternal melatonin and a link between already established ASD risk factors and melatonin, supports our hypothesis that low melatonin levels may be the shared "environmental factor" postulated by earlier twin studies (11).

Strong evidence points at a substantial contribution of common genetic variation to ASD (10). Low CYP1A2 activity is a common heritable genetic variation which can cause low ASMT transcript production (20) and by that low melatonin levels. Additionally, the SNP reduced enzyme kinetics of CYP1A2 may show substrate saturation or inhibition effects at much lower concentration as wild type. For CYP1A2 and melatonin this has not been measured before and remains speculative. The dual effect of melatonin (neurotropic properties and protection against oxidative DNA damage), provides an explanation for multiple questions about the complex underlying genetic process in ASD. This may explain results of a recent study in quartet families with ASD (parents and two ASD-affected sibs) whole-genome sequencing, which revealed that many (68.4%) of the affected siblings did not share the same mutations, but carried different ASD-relevant mutations (78). It also may explain why in 40% of persons with intellectual disabilities autism is present, and at the same time 70% of people with autism also has an intellectual disability (2). Our finding that in mothers of a child with ASD, who also have another child with ASD and/or an ID, urinary 6-SM excretion was significantly lower compared to mothers with one child with ASD (p = 0.023), is in line with these findings. Low parental melatonin levels can act on two seemingly independent levels: 1) directly by causing disturbances in brain development of the child and 2) indirectly by increasing risk of DNA damage in maternal and paternal germ cells. If melatonin levels in the child with autism are also low, this contributes to greater influence during the further development of the brain.

In a large study, aimed to find normative data on the melatonin excretion in urine, overnight urinary melatonin excretion values in healthy individuals ranged from 0.023 to 0.842 nmol/l and correlated inversely with age (79). There is a decrease in time with 25% in healthy adults aged 36 to 50 years, compared to those in the age group 20 to 35 years (67, 80). Kennaway et al. (67) calculated the yearly decline in melatonin plasma peak levels at night between young age groups (21 to 33 years) and old age groups (49 – 85 years) to be 2.7%, based on data in 11 studies. Melatonin levels did not have a Gaussian distribution, but showed a bimodal distribution, distinguishing two groups of individuals: low and high melatonin excretors. This is the reason why we decided to offer the measured and the recalculated data (Table 3) taking the postulated age dependant melatonin decrease into account.

Caffeine and melatonin are primarily metabolised by CYP1A2. We found that significantly more mothers with an ASD child than in the control group do not drink coffee after 6:00 h pm (p = 0.034), mostly in order to prevent coffee induced insomnia, and that 6-SM levels were also significantly lower than in mothers that do drink coffee after 6:00 h pm (p = 0.039). This is in line with the suggested mechanism that reduced CYP1A2 metabolic activity is connected with lower levels of ASMT transcript production (54) and supports our hypothesis that CYP1A2 slow metabolism due to a CYP1A2 SNP is involved in low melatonin levels in autism ethiology (51). As earlier studies already have shown that caffeine clearance by CYP1A2 is significant slower in people with caffeine-induced insomnia (48), and that metabolic activity of CYP1A2 is significantly inversely related to coffee intake (46, 47), we hypothesize that coffee drinking habits can serve as an indicator for low CYP1A2 activity as well as for low melatonin levels.

Our finding that significantly more fathers with an ASD child than in the control group do not drink coffee after 6:00 pm suggests that they also could be a CYP1A2 slow metaboliser and by that have low melatonin levels. This finding is important and can explain why 76% of the *de novo* mutations, found in a study in quartet families with ASD (parents and two ASD-affected sibs), were of paternal origin (78).

The limitation of our study is that we did not measure CYP1A2 activity, but relied on information on parental coffee consumption habits, as these are known to be related to CYP1A2 activity,

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

and that our control group is not sufficiently large to draw firmer conclusions. Our pilot study needs to be replicated on a much larger scale. It should involve melatonin levels and CYP1A2 activity measurements in ASD patients, as well as both of their parents. The outcomes of this study implicate that in yet to be determined risk groups, melatonin supplementation may be a logical consequence. Nevertheless, melatonin supplementation in pregnant and new-borns that are at risk may possibly enhance brain development but needs careful investigation of possible risks.

5. Conclusion

This is to our knowledge the first experimental evidence of low melatonin levels in mothers being a risk factor for child ASD. It is a comprehensive theory on ASD etiology in which genetic and environmental factors are combined with already known ASD risk factors and results of own melatonin research. It leads to the intriguing hypothesis that low melatonin levels, caused by low CYP1A2 activity - which is again early detectable by coffee drinking behaviour - are a risk factor for ASD as well as ID in their offspring. If so, it is more intriguing that this may lead to policies to detect future parents who are at risk and to treatment strategies to reduce ASD and ID risk.

Competing interests
The authors declare that they have no competing interests.

Acknowledgements
This study was supported by a research grant from ‘s Heeren Loo Zorggroep Steunfonds. We thank the mothers from our patients and women working at ‘s Heeren Loo Wekerom for their participation in this study. We also thank Lars Eijssen and Egon L. Willighagen for helpful discussions and Martijn Faassen for contributions in laboratory measurements.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

References

1. Parellada M, Penzol MJ, Pina L, Moreno C, Gonzalez-Vioque E, Zalsman G, et al. (2014): The neurobiology of autism spectrum disorders. *Eur Psychiatry*. 29:11-19.
2. Matson JL, Shoemaker M (2009): Intellectual disability and its relationship to autism spectrum disorders. *Res Dev Disabil*. 30:1107-1114.
3. Vig S, Jedrysek E (1999): Autistic features in young children with significant cognitive impairment: autism or mental retardation? *J Autism Dev Disord*. 29:235-248.
4. Gotham K, Pickles A, Lord C (2012): Trajectories of autism severity in children using standardized ADOS scores. *Pediatrics*. 130:e1278-1284.
5. Kim YS, Leventhal BL (2015): Genetic epidemiology and insights into interactive genetic and environmental effects in autism spectrum disorders. *Biol Psychiatry*. 77:66-74.
6. Li X, Zou H, Brown WT (2012): Genes associated with autism spectrum disorder. *Brain Res Bull*. 88:543-552.
7. Liu X, Takumi T (2014): Genomic and genetic aspects of autism spectrum disorder. *Biochem Biophys Res Commun*. 452:244-253.
8. Rosti RO, Sadek AA, Vaux KK, Gleeson JG (2014): The genetic landscape of autism spectrum disorders. *Dev Med Child Neurol*. 56:12-18.
9. Sanders SJ, He X, Willsey AJ, Ercan-Sencicek AG, Samocha KE, Cicek AE, et al. (2015): Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. *Neuron*. 87:1215-1233.
10. Gaugler T, Klei L, Sanders SJ, Bodea CA, Goldberg AP, Lee AB, et al. (2014): Most genetic risk for autism resides with common variation. *Nat Genet*. 46:881-885.
11. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. (2011): Genetic heritability and shared environmental factors among twin pairs with autism. *Arch Gen Psychiatry*. 68:1095-1102.
12. Gardener H, Spiegelman D, Buka SL (2009): Prenatal risk factors for autism: comprehensive meta-analysis. *Br J Psychiatry*. 195:7-14.
13. Gardener H, Spiegelman D, Buka SL (2011): Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis. *Pediatrics*. 128:344-355.
14. Polo-Kantola P, Lampi KM, Hinkka-Yli-Salomaki S, Gissler M, Brown AS, Sourander A (2014): Obstetric risk factors and autism spectrum disorders in Finland. *J Pediatr*. 164:358-365.
15. Kuzniewicz MW, Wi S, Qian Y, Walsh EM, Armstrong MA, Croen LA (2014): Prevalence and neonatal factors associated with autism spectrum disorders in preterm infants. *J Pediatr*. 164:20-25.
16. Walker CK, Krakowiak P, Baker A, Hansen RL, Ozonoff S, Hertz-Picciotto I (2015): Preeclampsia, placental insufficiency, and autism spectrum disorder or developmental delay. *JAMA Pediatr*. 169:154-162.
17. Al-Farsi YM, Al-Sharbati MM, Waly MI, Al-Farsi OA, Al-Shafaee MA, Al-Khaduri MM, et al. (2012): Effect of suboptimal breast-feeding on occurrence of autism: a case-control study. *Nutrition*. 28:e27-32.
18. Rossignol DA, Frye RE (2011): Melatonin in autism spectrum disorders: a systematic review and meta-analysis. *Dev Med Child Neurol*. 53:783-792.
19. Tordjman S, Najjar I, Bellissant E, Anderson GM, Barburoth M, Cohen D, et al. (2013): Advances in the research of melatonin in autism spectrum disorders: literature review and new perspectives. *Int J Mol Sci*. 14:20508-20542.
20. Veatch OJ, Pendergast JS, Allen MJ, Leu RM, Johnson CH, Elsea SH, et al. (2015): Genetic variation in melatonin pathway enzymes in children with autism spectrum disorder and comorbid sleep onset delay. *J Autism Dev Disord*. 45:100-110.
21. Tordjman S, Anderson GM, Pichard N, Charbuy H, Touitou Y (2005): Nocturnal excretion of 6-sulphatoxymelatonin in children and adolescents with autistic disorder. *Biol Psychiatry*. 57:134-138.
22. Arendt J (2005): Melatonin: characteristics, concerns, and prospects. *J Biol Rhythms*. 20:291-303.
23. Bejarano I, Monllor F, Marchena AM, Ortiz A, Lozano G, Jimenez MI, et al. (2014): Exogenous melatonin supplementation prevents oxidative stress-evoked DNA damage in human spermatozoa. *J Pineal Res*. 57:333-339.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

24. Reiter RJ, Tan DX, Galano A (2014): Melatonin reduces lipid peroxidation and membrane viscosity. *Front Physiol*. 5:377.
25. Korkmaz GG, Uzun H, Cakatay U, Aydin S (2012): Melatonin ameliorates oxidative damage in hyperglycemia-induced liver injury. *Clin Invest Med*. 35:E370-377.
26. Ferreira SG, Peliciari-Garcia RA, Takahashi-Hyodo SA, Rodrigues AC, Amaral FG, Berra CM, et al. (2013): Effects of melatonin on DNA damage induced by cyclophosphamide in rats. *Braz J Med Biol Res*. 46:278-286.
27. Reiter RJ, Tan DX, Manchester LC, Tamura H (2007): Melatonin defeats neurally-derived free radicals and reduces the associated neuromorphological and neurobehavioral damage. *J Physiol Pharmacol*. 58 Suppl 6:5-22.
28. Ackermann K, Stehle JH (2006): Melatonin synthesis in the human pineal gland: advantages, implications, and difficulties. *Chronobiol Int*. 23:369-379.
29. Liu T, Borjigin J (2005): N-acetyltransferase is not the rate-limiting enzyme of melatonin synthesis at night. *J Pineal Res*. 39:91-96.
30. Ma X, Idle JR, Krausz KW, Gonzalez FJ (2005): Metabolism of melatonin by human cytochromes p450. *Drug Metab Dispos*. 33:489-494.
31. Yu AM, Idle JR, Byrd LG, Krausz KW, Kupfer A, Gonzalez FJ (2003): Regeneration of serotonin from 5-methoxytryptamine by polymorphic human CYP2D6. *Pharmacogenetics*. 13:173-181.
32. Sonnier M, Cresteil T (1998): Delayed ontogenesis of CYP1A2 in the human liver. *Eur J Biochem*. 251:893-898.
33. Froy O (2009): Cytochrome P450 and the biological clock in mammals. *Curr Drug Metab*. 10:104-115.
34. Chang TK, Chen J, Yang G, Yeung EY (2010): Inhibition of procarcinogen-bioactivating human CYP1A1, CYP1A2 and CYP1B1 enzymes by melatonin. *J Pineal Res*. 48:55-64.
35. Fourtillan JB, Brisson AM, Gobin P, Ingrand I, Decourt JP, Girault J (2000): Bioavailability of melatonin in humans after day-time administration of D(7) melatonin. *Biopharm Drug Dispos*. 21:15-22.
36. Harpsoe NG, Andersen LP, Gogenur I, Rosenberg J (2015): Clinical pharmacokinetics of melatonin: a systematic review. *Eur J Clin Pharmacol*. 71:901-909.
37. Gunes A, Dahl ML (2008): Variation in CYP1A2 activity and its clinical implications: influence of environmental factors and genetic polymorphisms. *Pharmacogenomics*. 9:625-637.
38. Sachse C, Brockmoller J, Bauer S, Roots I (1999): Functional significance of a C-->A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. *Br J Clin Pharmacol*. 47:445-449.
39. Zhou SF, Chan E, Zhou ZW, Xue CC, Lai X, Duan W (2009): Insights into the structure, function, and regulation of human cytochrome P450 1A2. *Curr Drug Metab*. 10:713-729.
40. Zhou SF, Yang LP, Zhou ZW, Liu YH, Chan E (2009): Insights into the substrate specificity, inhibitors, regulation, and polymorphisms and the clinical impact of human cytochrome P450 1A2. *AAPS J*. 11:481-494.
41. Zhou SF, Wang B, Yang LP, Liu JP (2010): Structure, function, regulation and polymorphism and the clinical significance of human cytochrome P450 1A2. *Drug Metab Rev*. 42:268-354.
42. Nakajima M, Yokoi T, Mizutani M, Kinoshita M, Funayama M, Kamataki T (1999): Genetic polymorphism in the 5'-flanking region of human CYP1A2 gene: effect on the CYP1A2 inducibility in humans. *J Biochem*. 125:803-808.
43. Chevalier D, Cauffiez C, Allorge D, Lo-Guidice JM, Lhermitte M, Lafitte JJ, et al. (2001): Five novel natural allelic variants-951A>C, 1042G>A (D348N), 1156A>T (I386F), 1217G>A (C406Y) and 1291C>T (C431Y)-of the human CYP1A2 gene in a French Caucasian population. *Hum Mutat*. 17:355-356.
44. Hartter S, Korhonen T, Lundgren S, Rane A, Tolonen A, Turpeinen M, et al. (2006): Effect of caffeine intake 12 or 24 hours prior to melatonin intake and CYP1A2*1F polymorphism on CYP1A2 phenotyping by melatonin. *Basic Clin Pharmacol Toxicol*. 99:300-304.
45. Carrillo JA, Benitez J (1996): CYP1A2 activity, gender and smoking, as variables influencing the toxicity of caffeine. *Br J Clin Pharmacol*. 41:605-608.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

46. Josse AR, Da Costa LA, Campos H, El-Sohemy A (2012): Associations between polymorphisms in the AHR and CYP1A1-CYP1A2 gene regions and habitual caffeine consumption. *Am J Clin Nutr*. 96:665-671.
47. Rodenburg EM, Eijgelsheim M, Geleijnse JM, Amin N, van Duijn CM, Hofman A, et al. (2012): CYP1A2 and coffee intake and the modifying effect of sex, age, and smoking. *Am J Clin Nutr*. 96:182-187.
48. Levy M, Zylber-Katz E (1983): Caffeine metabolism and coffee-attributed sleep disturbances. *Clin Pharmacol Ther*. 33:770-775.
49. Nir I, Meir D, Zilber N, Knobler H, Hadjez J, Lerner Y (1995): Brief report: circadian melatonin, thyroid-stimulating hormone, prolactin, and cortisol levels in serum of young adults with autism. *J Autism Dev Disord*. 25:641-654.
50. Kulman G, Lissoni P, Rovelli F, Roselli MG, Brivio F, Sequeri P (2000): Evidence of pineal endocrine hypofunction in autistic children. *Neuro Endocrinol Lett*. 21:31-34.
51. Braam W, Keijzer H, Struijker Boudier H, Didden R, Smits M, Curfs L (2013): CYP1A2 polymorphisms in slow melatonin metabolisers: a possible relationship with autism spectrum disorder? *J Intellect Disabil Res*. 57:993-1000.
52. Melke J, Goubran Botros H, Chaste P, Betancur C, Nygren G, Anckarsater H, et al. (2008): Abnormal melatonin synthesis in autism spectrum disorders. *Mol Psychiatry*. 13:90-98.
53. Pagan C, Delorme R, Callebert J, Goubran-Botros H, Amsellem F, Drouot X, et al. (2014): The serotonin-N-acetylserotonin-melatonin pathway as a biomarker for autism spectrum disorders. *Transl Psychiatry*. 4:e479.
54. Veatch OJ, Veenstra-Vanderweele J, Potter M, Pericak-Vance MA, Haines JL (2014): Genetically meaningful phenotypic subgroups in autism spectrum disorders. *Genes Brain Behav*. 13:276-285.
55. Toma C, Rossi M, Sousa I, Blasi F, Bacchelli E, Alen R, et al. (2007): Is ASMT a susceptibility gene for autism spectrum disorders? A replication study in European populations. *Mol Psychiatry*. 12:977-979.
56. Jonsson L, Ljunggren E, Bremer A, Pedersen C, Landen M, Thuresson K, et al. (2010): Mutation screening of melatonin-related genes in patients with autism spectrum disorders. *BMC Med Genomics*. 3:10.
57. Wang L, Li J, Ruan Y, Lu T, Liu C, Jia M, et al. (2013): Sequencing ASMT identifies rare mutations in Chinese Han patients with autism. *PLoS One*. 8:e53727.
58. Merchant NM, Azzopardi DV, Hawwa AF, McElnay JC, Middleton B, Arendt J, et al. (2013): Pharmacokinetics of melatonin in preterm infants. *Br J Clin Pharmacol*. 76:725-733.
59. Kennaway DJ, Goble FC, Stamp GE (1996): Factors influencing the development of melatonin rhythmicity in humans. *J Clin Endocrinol Metab*. 81:1525-1532.
60. Reiter RJ, Rosales-Corral SA, Manchester LC, Tan DX (2013): Peripheral reproductive organ health and melatonin: ready for prime time. *Int J Mol Sci*. 14:7231-7272.
61. Cohen Engler A, Hadash A, Shehadeh N, Pillar G (2012): Breastfeeding may improve nocturnal sleep and reduce infantile colic: potential role of breast milk melatonin. *Eur J Pediatr*. 171:729-732.
62. Tamura H, Nakamura Y, Terron MP, Flores LJ, Manchester LC, Tan DX, et al. (2008): Melatonin and pregnancy in the human. *Reprod Toxicol*. 25:291-303.
63. Lanoix D, Guerin P, Vaillancourt C (2012): Placental melatonin production and melatonin receptor expression are altered in preeclampsia: new insights into the role of this hormone in pregnancy. *J Pineal Res*. 53:417-425.
64. Kong X, Li X, Cai Z, Yang N, Liu Y, Shu J, et al. (2008): Melatonin regulates the viability and differentiation of rat midbrain neural stem cells. *Cell Mol Neurobiol*. 28:569-579.
65. Dominguez-Alonso A, Valdes-Tovar M, Solis-Chagoyan H, Benitez-King G (2015): Melatonin stimulates dendrite formation and complexity in the hilar zone of the rat hippocampus: participation of the Ca++/Calmodulin complex. *Int J Mol Sci*. 16:1907-1927.
66. Bastien CH, Vallieres A, Morin CM (2001): Validation of the Insomnia Severity Index as an outcome measure for insomnia research. *Sleep Med*. 2:297-307.
67. Kennaway DJ, Lushington K, Dawson D, Lack L, van den Heuvel C, Rogers N (1999): Urinary 6-sulfatoxymelatonin excretion and aging: new results and a critical review of the literature. *J Pineal Res*. 27:210-220.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

68. Dagli A, Buiting K, Williams CA (2012): Molecular and Clinical Aspects of Angelman Syndrome. *Mol Syndromol*. 2:100-112.
69. De Leersnyder H (2006): Inverted rhythm of melatonin secretion in Smith-Magenis syndrome: from symptoms to treatment. *Trends Endocrinol Metab*. 17:291-298.
70. Morin CM, Belleville G, Belanger L, Ivers H (2011): The Insomnia Severity Index: psychometric indicators to detect insomnia cases and evaluate treatment response. *Sleep*. 34:601-608.
71. Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcin C, et al. (2012): Global prevalence of autism and other pervasive developmental disorders. *Autism Res*. 5:160-179.
72. Fideleff HL, Boquete H, Fideleff G, Albornoz L, Perez Lloret S, Suarez M, et al. (2006): Gender-related differences in urinary 6-sulfatoxymelatonin levels in obese pubertal individuals. *J Pineal Res*. 40:214-218.
73. Cain SW, Dennison CF, Zeitzer JM, Guzik AM, Khalsa SB, Santhi N, et al. (2010): Sex differences in phase angle of entrainment and melatonin amplitude in humans. *J Biol Rhythms*. 25:288-296.
74. Blanchard DC, Summers CH, Blanchard RJ (2013): The role of behavior in translational models for psychopathology: functionality and dysfunctional behaviors. *Neurosci Biobehav Rev*. 37:1567-1577.
75. Meyza KZ, Defensor EB, Jensen AL, Corley MJ, Pearson BL, Pobbe RL, et al. (2013): The BTBR T+ tf/J mouse model for autism spectrum disorders-in search of biomarkers. *Behav Brain Res*. 251:25-34.
76. Meyza K, Nikolaev T, Kondrakiewicz K, Blanchard DC, Blanchard RJ, Knapska E (2015): Neuronal correlates of asocial behavior in a BTBR T (+) Itpr3(tf)/J mouse model of autism. *Front Behav Neurosci*. 9:199.
77. Vollrath L, Huesgen A, Manz B, Pollow K (1988): Day/night serotonin levels in the pineal gland of male BALB/c mice with melatonin deficiency. *Acta Endocrinol (Copenh)*. 117:93-98.
78. Yuen RK, Thiruvahindrapuram B, Merico D, Walker S, Tammimies K, Hoang N, et al. (2015): Whole-genome sequencing of quartet families with autism spectrum disorder. *Nat Med*. 21:185-191.
79. Wetterberg L, Bratlid T, von Knorring L, Eberhard G, Yuwiler A (1999): A multinational study of the relationships between nighttime urinary melatonin production, age, gender, body size, and latitude. *Eur Arch Psychiatry Clin Neurosci*. 249:256-262.
80. Mahlberg R, Tilmann A, Salewski L, Kunz D (2006): Normative data on the daily profile of urinary 6-sulfatoxymelatonin in healthy subjects between the ages of 20 and 84. *Psychoneuroendocrinology*. 31:634-641.

Additional data:

1. Questionaire (original in Dutch)
2. Sup. Table 1. ASD risk factors

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Hoeveelheid urine ……..ml

Tijdstip in bed …….. **CODE ……..**

Tijdstip uit bed ……..

Onderzoek melatoninespiegels in nacht-urine bij moeders

Persoonlijke informatie

Uw leeftijd en gewicht	..... jaar en ……..kg
Hoeveel kinderen heeft u?	..... kinderen
Wat is de leeftijd van uw oudste kind?	..... jaar
Wat is de leeftijd van uw jongste kind?	..... jaar

Beoordeling van uw eigen slaap

Gebaseerd op de Insomnia Severity Index (Bastien, Vallières, Morin, 2001)

1. **Heeft u moeite met in slaap vallen, doorslapen en/of problemen met te vroeg wakker worden?**

   O nee ***(sla de vragen 4, 5 en 6 over)***

   O ja ***(vul de volledige vragenlijst in)***

2. **Beoordeel aub de *ERNST* van uw huidige slaapproblemen (in de laatste 2 weken)**

	Geen	Licht	Matig	Ernstig	Zeer ernstig
Moeite met in slaap vallen:	(0)	(1)	(2)	(3)	(4)
Moeite met doorslapen:	(0)	(1)	(2)	(3)	(4)
Problemen met te vroeg wakker worden:	(0)	(1)	(2)	(3)	(4)

3. **Hoe *TEVREDEN* of ontevreden bent u over uw huidige slaappatroon?**

Erg tevreden				Erg ontevreden
(0)	(1)	(2)	(3)	(4)

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

4. In welke mate vindt u dat uw slaapprobleem *INVLOED* heeft op uw dagelijks functioneren (zoals vermoeidheid overdag, al dan niet goed kunnen functioneren op werk of bij dagelijkse bezigheden, concentratievermogen, geheugen, stemming, etc)

Geen enkele invloed	Een beetje	Enigszins	Veel	Heel veel invloed
(0)	(1)	(2)	(3)	(4)

5. Hoe *OPVALLEND* voor anderen is het volgens u dat uw slaapprobleem een negatieve invloed heeft op uw kwaliteit van leven?

Niet opvallend	Nauwelijks	Enigszins	Erg	Zeer erg opvallend
(0)	(1)	(2)	(3)	(4)

6. In welke mate maakt u zich *ONGERUST* over uw slaapprobleem?

Helemaal niet	Een beetje	Enigszins	Erg	Heel erg
(0)	(1)	(2)	(3)	(4)

7. Als u iets wilt toevoegen over uw eigen slaap, dan kunt u dat hier doen.

Medicijngebruik

8. Wilt u hier aangeven welke medicijnen u gebruikt? *(naam, sterkte, aantal per dag)*

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Koffiegebruik

9. Drinkt u ('gewone' cafeïnehoudende) koffie?

O Ja

O Nee

Indien Nee, wat is daarvan de reden (meerdere antwoorden mogelijk)?

O Dat lust ik niet

O Daar krijg ik last van (zoals hartkloppingen, trillerig gevoel etc)

O Dan krijg ik last van mijn maag

O Anders, namelijk ……..

10. Indien u (overdag) koffie drinkt, drinkt u dan ook 's avonds koffie?

O Ja

O Ja, maar 's avonds alleen cafeïne vrije koffie

O Nee

O Niet van toepassing (Ik drink overdag ook geen koffie)

11. Indien u 's avonds geen koffie drinkt, of alleen cafeïne vrije koffie, wat is daarvan de reden?

O Niet van toepassing, ik drink 's avonds gewoon koffie

O Ik kan niet goed in slaap vallen als ik koffie drink

O Anders, namelijk ……

12. Hoe laat drinkt u uw laatste kop (cafeïne bevattende) koffie?

O Om …… uur

O Niet van toepassing (ik drink geen koffie)

Hartelijk dank voor het invullen van deze vragenlijst.

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Sup. Table 1: ASD risk factors

		title article	findings/statement	possible explanation relation with ASD	explanation by author(s)
Prenatal risk factors					
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	The factors with the strongest evidence against a role in autism risk included previous fetal loss and maternal pre-eclampsia, proteinuria, hypertension and swelling	Placental dysfunction is linked to lower melatonin levels.	Pregnancy-related factors related to hypoxia.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Increased maternal age at birth is associated with an elevated risk of autism.	Melatonin protects against damage due to fetal hypoxia.	Maternal age has been shown to be associated with an increased risk of obstetrical complications. Maternal age may be associated with autism because of the increased risk of chromosomal abnormalities in ova of increased age.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Increased paternal age at birth is associated with an elevated risk of autism.	Melatonin levels decrease with age.	Maternal and paternal age at birth are likely correlated. It is possible that advanced age of both parents plays a role in the susceptibility to autism.There is evidence that paternal age may be more important.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	The factor that was most commonly associated with the risk of autism in the literature was birth order.	Melatonin protects against damage due to fetal hypoxia.	Possibly attributed to parents deciding not to have additional children after one has developed autism.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Gestational bleeding	Melatonin protects against damage due to fetal hypoxia.	Fetal hypoxia may underlie a potential relationship between gestational bleeding and autism.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Fetal distress, maternal hypertension, are other pregnancy-related factors that are believed to be related to hypoxia and have been associated with autism risk in some, but not all, studies.	Melatonin protects against damage due to fetal hypoxia.	
	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article).	Advanced parental age at birth is associated with an elevated risk of autism.	Melatonin levels decrease with age.	
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Advanced maternal age is associated with an elevated risk of autism.	Melatonin levels decrease with age.	Nucleotide repeat instability.
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Advanced paternal age is associated with an elevated risk of autism.	Melatonin levels decrease with age.	The most widely proposed mechanism underlying these congenital anomalies is known as the “copy error” hypothesis. Paternal age remained a significant risk factor for autism and ASDs.
	Mann 2009	Pre-Eclampsia, Birth Weight, and Autism Spectrum Disorders.	Children with ASD had older mothers.	Melatonin levels decrease with age.	

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

	Dodds 2010	The role of prenatal, obstetric and neonatal factors in the development of autism (database cohort study).	Increased risk of autism: pre-pregnancy weight 90 kg or more, pregnancy weight gain of 18 kg or more.	Melatonin level in diabetes is lower, diabetes in pregnancy is related to weight gain.	It has been shown that the concentration of leptin is correlated with the percentage of body fat and that obese subjects have higher leptin levels than normal weight subjects AND elevated leptin levels are suggested to be predictive of placental dysfunction.
Perinatal risk factors					
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Caesarean delivery.	Melatonin protects against damage due to fetal hypoxia.	Unable to untangle the true effect of cesarean section from the underlying indications to this operative procedure.
	Gardener 2009	Prenatal risk factors for autism: comprehensive meta-analysis.	Prolonged labour, cord complications, low Apgar score and Caesarean delivery.	Melatonin protects against damage due to fetal hypoxia.	
	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article)	Breech presentation and planned cesarean section	Melatonin protects against damage due to fetal hypoxia.	
	Mann 2009	Pre-Eclampsia, Birth Weight, and Autism Spectrum Disorders.	Maternal pre-eclampsia/eclampsia was significantly associated with greater odds of ASD.	Placental dysfunction is linked to lower melatonin levels.	
Premature birth					
	Al-Farsi, 2012	Effect of suboptimal breast-feeding on occurrence of autism: A case–control study.	The percentage of premature deliveries was three times higher in ASD cases than in control participants.	Maternal melatonin in breastfeeding protects against melatonin deficiency in child, as endogenous melatonin production only begins 9 - 14 weeks after birth.	Premature babies tend to have lower amounts of polyunsaturated fatty acids and docosahexaenoic acid in their brains and central nervous systems than full-term babies.
	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article).	Adjusted odd ratio for preterm birth increased by as much as 5 in a group of girls born before 33 weeks.	Endogenous melatonin production in prematures begins later than in a terme born children (between 46-52 weeks postconception).	
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Birth at less than 35 weeks was associated with increased risk of autism.	Endogenous melatonin production in prematures begins later than in a terme born children (between 46-52 weeks postconception).	
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Being small for gestational age or having LBW or slow growth was associated with a 2-fold increase in risk in 2 studies	Placental dysfunction is linked to lower melatonin levels.	
Neonatal risk factors					
	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article).	Low birth weight and size.	Melatonin protects against damage due to fetal hypoxia.	It is difficult to consider preterm birth and LBW as truly being independent from other potential risks, such as being small for gestational age at birth. The phenomenon of being small for gestational age has heterogeneous etiologies, including genetic factors and placental insufficiency.
	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article).	Poor condition at birth (low Apgar, hypoxia).	Melatonin protects against damage due to fetal hypoxia.	

bioRxiv preprint doi: https://doi.org/10.1101/046722; this version posted April 2, 2016. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

	Guinchat 2012	Pre-, peri- and neonatal riskfactors for autism (review article).	Hyperbilirubinemia increases ASD risk..	Melatonin acts as a free radical scavenger and antioxidant and is highly effective in reducing oxidative stress.	Hyperbilirubinemia is thought to exert toxicity on the basal ganglia and cerebellum, two structural brain regions that have been identified as important in the development of autism.
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Being small for gestational age or having LBW or slow growth was associated with a 2-fold increase in risk in 2 studies.	Placental dysfunction is linked to lower melatonin levels.	
	Kolevzon (2007)	Prenatal and perinatal risk factors for autism: a review and integration of findings.	Fetal hypoxia (including low Apgar score, fetal distress, cesarean delivery).	Melatonin protects against damage due to fetal hypoxia.	3 regions in the brain are especially vulnerable to perinatal insult, including the basal ganglia, the hippocampus, and the lateral ventricles. Neuroimaging studies have shown that the lateral ventricles in particular are larger in patients with autism compared with controls. Brains of individuals with autism have also been shown to exhibit morphological hippocampal abnormalities.
	Mann 2009	Pre-Eclampsia, Birth Weight, and Autism Spectrum Disorders.	Birth weight was significantly inversely associated with the odds of ASD (OR = 0.78, p = .001 for each additional kilogram).	Placental dysfunction is linked to lower melatonin levels.	
Breastfeeding					
	Al-Farsi, 2012	Effect of suboptimal breast-feeding on occurrence of autism: A case–control study.	ASD was found to be associated with a shorter durtion of breast-feeding (≤3 month).	Maternal melatonin in breastfeeding protects against melatonin deficiency in child, as endogenous melatonin production only begins 9 - 14 weeks after birth.	Breastfeeding and skin-to-skin contact between a mother and her child stimulates the secretion of oxytocin, which enhances emotional bonding. Also premature babies tend to have lower amounts of polyunsaturated fatty acids and docosahexaenoic acid (DHA) in their brains and central nervous systems than full-term babies
	Schultz, 2006	Breastfeeding, infant formula supplementation, and Autistic Disorder: the results of a parent survey	Children who were not breastfed or were fed infant formula without docosahexaenoic acid/arachidonic acid supplementation were significantly more likely to have autistic disorder.	Maternal melatonin in breastfeeding protects against melatonin deficiency in child, as endogenous melatonin production only begins 9 - 14 weeks after birth.	Infant formula supplementation protects against immune system dysfunction.
	Tanoue, 1988	Weaning time of children with infantile autism.	24.8% of the patients and 7.5% of the controls were weaned by the end of 1 week, a significant difference.	Maternal melatonin in breastfeeding protects against melatonin deficiency in child, as endogenous melatonin production only begins 9 - 14 weeks after birth.	Historical studies on infantile autism revealed that the disease developed more prevalently in the socioeconomic status where the incidence of breast-feeding was less frequent. These results suggest that early weaning may contribute to the etiology of infantile autism.