ASMT gene expression correlates with cognitive impairment in patients with recurrent depressive disorder

Monika Talarowska
Janusz Szemraj
Marlena Zajączkowska
Piotr Gałecki

Corresponding Author: Monika Talarowska, e-mail: talarowskamonika@wp.pl

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Background: Recurrent depressive disorder is a multifactorial disease; one of the typical features is cognitive impairment. The purpose of this study was analysis of ASMT gene expression both on mRNA and protein levels in patients with recurrent depressive disorder (rDD) and assessment of the relationship between plasma level of ASMT protein, gene expression on mRNA level, and cognitive performance.

Material/Methods: The study included 236 subjects: patients with rDD (n=131) and healthy subjects (n=105, CG). Cognitive function assessment was based on: Trail Making Test, The Stroop Test, Verbal Fluency Test (VFT), and Auditory Verbal Learning Test (AVLT).

Results: Both mRNA and protein expression levels of ASMT gene were significantly higher in healthy subjects when compared to rDD. The average ASMT mRNA expression level measured for the entire group was M=0.21 (SD=0.09), and the protein level was M=12.84 (SD=3.29). In patients with rDD, statistically significant correlations occurred between both mRNA and protein expression levels and part A of the TMT (negative correlation) and verbal fluency test (positive correlation). In the group CG, there was no statistically significant association between the analyzed variables. In the entire group there was a statistically significant correlation between both ASMT mRNA and protein expression levels and all the neuropsychological tests used in the survey.

Conclusions: 1. Our study confirms previous results showing decreased mRNA and protein expression levels of ASMT gene in depression. 2. Our data suggest a relationship between decreased mRNA and protein expression levels of ASMT gene and cognitive impairment.

Keywords: Depressive Disorder • ASMT • Cognitive Impairment

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**Background**

Depressive disorders are among the most commonly diagnosed diseases and are among the diseases most disabling an individual's functioning [1]. The annual prevalence of depression in the adult population ranges from 6% to 12%, and according to various reports, among those over 65 years of age it ranges from 5% to 30% [2].

The etiology of recurrent depressive disorder (rDD) has not yet been fully discovered. For many years, numerous and often competing theories have been published in the literature. Currently, it is assumed that the determinant of the disease is multifactorial, and particular elements are not mutually exclusive, but rather complementary. One of the theories, which are developing intensively at present, is a hypothesis of circadian rhythms disturbance. According to this theory, the reason for recurrence of depression is a disruption of synchronization of basic physiological and metabolic rhythms, which determine adaptation to changes in the outer world (among others, the circadian rhythm of day and night) [3]. Since circadian and neurotransmission systems are tightly connected, circadian and/or sleep-related abnormalities may impact the functioning of the dopamine and serotonin circuits, which in turn affects mood regulation. Circadian rhythm abnormalities have therefore attracted considerable attention as possible biomarkers of these disorders [4].

Cognitive function impairment in a group of patients with depressive disorders can differ in character and severity, from selective, specific, and mild deficits to generalized and intensive changes [5]. Cognitive deficits mainly concern declarative and working memory. Depressive disorders are, in general, associated with deficits in both free and cued recall of episodic information and deficits in short- and long-term memory [6]. The early onset of rDD is associated with a significant volume loss of the hippocampus in patients with geriatric depression and in middle-aged adults [7]. Sheline et al. [8] reported an association between the length of untreated depressive episodes and hippocampal volume reductions in recurrent geriatric major depressive disorders. Dysfunctions of working memory are also observed in patients with rDD, as well as in their first-degree relatives [9]. Working memory impairment is particularly explicit in elderly patients with depression [10], but may also be observed in younger subjects [11]. Deficits in this area exert negative effects on performance levels in psychological tests and on everyday functioning of affected patients, as well as being responsible for decreased remission rates [12].

There are several hypotheses attributing to the etiology of depression to melatonin (N-acetyl-5-methoxytryptamine). According to Wetterberg, decreased levels of this hormone are observed in depressive patients (called ‘low melatonin syndrome’) [13]. Low melatonin secretion may be associated with a genetic marker, which also contributes to a higher risk of depression development. Furthermore, elevated level of melatonin in the morning also may be associated with depression, and, according to other authors, concentrations of melatonin that are too low or too high are harmful [14,15]. New evidence suggests that late melatonin elevations may suppress paras tuberalis thyrotroph embryonic factor (TEF), a photoperiodic switch that might control human depression [16]. Confirmation of the high importance of melatonin and melatonin receptor agonists in the pathogenesis of depression is the effectiveness of their use in sleep disorders and circadian rhythms disturbances [17], which in turn are common prognostic factor for the disease development [18]. In addition to sleep induction and circadian rhythms regulation, melatonin is also involved in various other physiologic functions, including immune response, antioxidative defense, metabolic regulations, and memory [19]. Melatonin has a possible effect on improving cognitive function [20–22].

Melatonin is a peptide that is produced at night in the corpus pineal in a rhythmic pattern and is controlled by an endogenous clock in the suprachiasmatic nucleus of the hypothalamus. Its main function is to synchronize the circadian rhythm [23]. Melatonin receptors are expressed in many different cell types, including those of the nervous, cardiovascular, digestive, and endocrine systems. The last step of the metabolic synthesis of melatonin is conversion of N-acetylserotonin to melatonin in methylation reaction, which is catalyzed by N-acetylserotonin O-methyltransferase (ASMT), also known as hydroxyindole-O-methyltransferase (HIOMT) [24]. ASMT is the limiting enzyme in the synthesis of melatonin [25]. ASTM gene is located in the pseudoautosomal region of the X chromosome [26], which is a candidate region for the development of mental illness [27].

The purpose of this study was analysis of ASMT gene both on mRNA and protein levels in patients with rDD, and to investigate the relationship between ASMT expression and cognitive performance. We hypothesized that levels of ASMT gene expression might affect cognitive functions.

**Material and methods**

**Patients**

The study was carried out in a group of 236 subjects aged 20–67 (M=39.79 years, SD=14.02). The participants were divided into 2 groups: patients with rDD (n=131) and healthy subjects (a comparison group, CG, n=105).

All the patients were native Poles, inhabitants of the central Poland, and unrelated. Selection of the individuals to
the test group was random, without replacement sampling. Respondents, before deciding to participate in the study, were informed of its purpose, ensured that the participation is voluntary, and guaranteed that the personal data and the results of the tests will not be distributed, but only used in the overall statistics.

Table 1 presents characteristics of the study group by sex, age, education, and the course of disease (rDD group). Statistically significant differences between the 2 groups were found in terms of sex ($\chi^2 = 1.46$, $p = 0.227$), education ($Z = 3.18$, $p = 0.001$), and age ($Z = 10.44$, $p = 0.001$).

Ethics

An informed, written consent for participation in the study was obtained from each subject, according to the protocol approved by the Bioethics Committee of the Medical University of Lodz (No. RNN/603/08/KB).

Experiment

Patients were selected for the study according to the inclusion criteria of ICD-10 (F32.0–7.32.2, F33.0–F33.8) [28]. All the subjects were examined during the course of their hospitalization. The presence of axis I and II disorders, other than depressive episode, and the diagnosis of somatic diseases and injuries of the central nervous system (CNS), which could have affected the cognitive performance, were regarded as exclusion criteria. Other exclusion criteria were: inflammatory or autoimmune disorders and unwillingness to give informed consent.

In all the included subjects, case history was obtained prior to main study procedure, using the standardized Composite International Diagnostic Interview (CIDI) [29]. Additionally, the number of depression episodes and the disease duration periods were recorded in each patient. During hospitalization, all the patients received antidepressant pharmacotherapy.

The CG consisted of 105 healthy subjects with family history negative for psychiatric disorders. The healthy controls included community volunteers enrolled into the study on the criteria of the psychiatric CIDI interview [29]. Controls with other psychiatric diagnoses concerning axis I and II disorders, neurological disorder, and substance abuse or dependence were excluded from the study.

All subjects were free of medical illness, including infections and inflammatory or allergic reactions. None of the control subjects or depressed patients were treated with drugs known to influence lipid metabolism, immune response, or endocrine function. The control subjects were free of all medication for at least 2 months prior to blood sampling. None of the control subjects were drinkers, heavy smokers, or had ever taken psychotropic drugs.

Cognitive functions assessment and severity of depression

Assessment of cognitive function was based on the Trail Making Test (TMT), the Stroop Test, the Auditory-Verbal Learning Test (AVLT), and the Verbal Fluency Test (VFT). Depression severity was assessed with the 21-item Hamilton Depression Rating Scale (HDRS). Descriptions of these tests have been presented elsewhere [30].

Regarding the patients with rDD, HDRS, The Stroop Test, TMT, AVLT, and VFT were applied at the therapy onset. All the patients were examined on admission (i.e., at the symptomatic phase, before or shortly after previous antidepressant drug regime modification). In the CG group, neuropsychological tests...
were performed in a single experiment. Examination of pa-

tients by the above-mentioned tests was done by the same

person in each particular case: the same psychologist exam-

ined the patients with neuropsychological tests, including an

evaluation of obtained results, while the HDRS test was per-

formed by the same physician-psychiatrist.

**Determination of protein concentration**

The test protein levels were measured in the patients’ sera. These tests were preceded by the determinations of serum to-
tal protein. Two measuring methods were applied.

**Spectrophotometric protein quantitation assay**

The protein levels were detected with a spectrophotometer GeneQuest (Pharmacia Biotech). Absorbance measurements were performed automatically at wavelengths of 260, 280, and 320 nm, and the protein concentrations were calculat-
ed according to the Warburg formula: protein concentration

\[ \text{mg/cm}^3 = \frac{1.55 (A_{280} - A_{320}) - 0.76 (A_{260} - A_{320})}{\text{sample volume}} \]

**BCA protein assay**

To 0.01 cm\(^3\) of the protein solution (standard and sample),

0.2 cm\(^3\) of BCA reagent (Pierce) was added, and, after thorough mixing, incubated for 30 min at 37°C. After cooling to room temperature, the absorbance was measured at a wavelength of 562 nm using a BCA reagent buffer as a control.

**Determination of serum ASMT protein level**

A commercial kit was used to quantify the ASMT protein levels in the sera (Human acetylserotonin O-Methyltransferase [ASMT] ELISA Kit from Antibodies-on line GmbH Aachen [Germany]).

**Measurement of the mRNA expression**

DNA was extracted from whole blood according to the GTC method [31]. The ASMT promoter B polymorphisms rs4446909 and rs5989681 were analyzed by direct sequencing polymerase chain reaction (PCR) product. Amplification was performed using 0.1 lg genomic DNA, 200 lm each dNTP, 5xGoTaq buffer solution, 1 U GoTaq polymerase (Promega, Madison, WI, USA), 0.5 lm primers 5’GGACCTGCTCGTATCAAGACG3’ and 5’CTAGAAAATTGCTTACCTACGGTA3’ specific for both polymor-

phisms. Amplification product 201 bp was sequenced using specific primer and 5’CCGGCATCAATCTCATAAGACGAT3’ by DNA Sequencing service IBB PAN Warsaw.

The human ASMT and 18S ribosomal RNA gene expression were quantified by real-time PCR using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers’ protocol. Total RNA (1 lg) was extracted from the whole blood using Trizol reagent (Life Technologies Inc., Carlsbad, CA, USA), and was processed direct-

tly to cDNA synthesis using the Oligotex kit (Qiagen, Chatsworth, CA, USA). Briefly, 2.5, 2.0, 1.5, 1.0; 0.5 and 0.25 lL of synthesized cDNA were amplified in triplicate for both 18S ribosomal RNA and ASMT gene to create a standard curve. Likewise, 2 lL of cDNA was amplified in triplicate in all isolated samples for each prim-
er/probe combination and 18S ribosomal RNA. Each sample was supplemented with both respective 0.3 lm forward and reverse primers, fluorescent probe, and made up to 50 lL using qPCR Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium). All of the following PCR primers were designed using PrimerExpress (Applied Biosystem) software and 5’AGCGCTCTGCTGTTCAATGA3’; 5’GGACCTGCTCGTATCAAGACG3’; 5’CTAGAAAATTGCTTACCTACGGTA3’ and 5’CCGGCATCAATCTCATAAGACGAT3’ specific for mRNA of human ASMT and 18S ribosomal RNA, respectively [32]. 18S RNA was used as an active and endogenous control to correct for differences in the amount of total RNA added to the reaction and to compen-
sate for different levels of inhibition during reverse transcription of RNA and during PCR. Each target probe was amplified in separate 96-well plates. All samples were incubated at 50°C for 2 min and at 95°C for 10 min and then cycled at 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 40 cycles. SYBR Green I fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (CT) value. Analyses were performed with ABI Prism 7000 (SDS Software, Foster City, CA, USA). Controls without RT and with no-template cDNA were performed with each assay. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, 18S ribosomal RNA mRNA was quantified and results were normal-

ized to these values. Relative gene expression levels were ob-
tained using the DDCt method [33]. Amplification-specific trans-
scripts were further confirmed by obtaining melting curve profiles.

**Statistical analysis**

Distributions were analyzed using the Shapiro-Wilk test. To com-
pare nonparametric variables in the test groups, the Pearson \(\chi^2\) (qualitative variables) and the Mann-Whitney U test for 2 inde-
pendent groups were used. To evaluate the relations between analyzed variables, Spearman’s R rank order correlation coef-
ficients were estimated. For all analyses, which should be re-

garded as exploratory (without correction for multiple testing),

nominal statistical significance was defined as \(p<0.05\) [34]. All data analyses were performed using STATISTICA PL, version 10.

**Results**

ASMT gene expression at mRNA was significantly lower in the rDD group when compared to the CG (\(p<0.01\)). Likewise, ASMT gene expression at protein level was significantly lower in the
The average level of ASMT gene expression measured at the mRNA level for the whole group is: \( M=0.21 \text{ (SD}=0.09) \), at the protein level: \( M=12.84 \text{ (SD}=3.29) \).

Table 2. Results of the cognitive tests in the rDD group and the CG.

| Variables         | rDD (n=131) | CG (n=105) | U Manna-Whitney test |
|-------------------|-------------|------------|----------------------|
|                   | M (SD)      | M (SD)     | Z        | p        |
| TMT A-time        | 50.14 (36.11) | 26.08 (9.56) | 8.12 | <0.01* |
| TMT B-time        | 107.77 (65.51) | 50.24 (16.15) | 9.95 | <0.01* |
| RCNbt-time        | 32.86 (19.64) | 20.64 (3.35) | 7.81 | <0.01* |
| NCWd-time         | 77.12 (44.53) | 47.97 (10.86) | 8.59 | <0.01* |
| VFT -animals      | 19.15 (6.26) | 26.89 (6.96) | -7.76 | <0.01* |
| VFT-sharp objects | 9.09 (3.31) | 12.15 (4.04) | -5.51 | <0.01* |
| VFT-the letter k  | 14.83 (6.01) | 18.77 (4.58) | -5.59 | <0.01* |
| AVLT-first attempt| 5.06 (1.33) | 6.34 (1.28) | -6.23 | <0.01* |
| AVLT-number of words in 30 min | 6.29 (2.06) | 8.78 (1.45) | -8.37 | <0.01* |
| AVLT-average of 5 tests | 6.72 (1.33) | 8.37 (0.85) | -8.92 | <0.01* |

Table 3. Spearman’s rank correlation coefficients (R) for the variables tested.

| Variables         | rDD (n=131) | CG (n=105) | The whole group (N=236) |
|-------------------|-------------|------------|-------------------------|
|                   | ASMT mRNA  | ASMT protein | ASMT mRNA  | ASMT protein  |
|                   | \( (2^{-\Delta Ct}) \) | (pg/ml) | \( (2^{-\Delta Ct}) \) | (pg/ml) |
|                   | M=0.149 (SD±0.06) | M=10.897 (SD±2.53) | M=0.268 (SD±0.07) | M=15.264 (SD±2.41) |
| TMT A-time        | -0.179*     | -0.139     | 0.042    | 0.033    | 0.307* | 0.239* |
| TMT B-time        | -0.108     | -0.076     | -0.004   | -0.007   | 0.154  | 0.346* |
| RCNbt-time        | -0.108     | -0.112     | 0.071    | 0.084    | 0.154  | 0.307* |
| NCWd-time         | -0.033     | -0.033     | -0.018   | -0.035   | 0.047  | 0.235* |
| VFT -animals      | 0.173*     | 0.154      | 0.071    | 0.091    | 0.154  | 0.126  |
| VFT-sharp objects | 0.232*     | 0.222*     | 0.007    | 0.014    | 0.154  | 0.015  |
| VFT-the letter k  | 0.007      | 0.041      | 0.075    | 0.078    | 0.007  | 0.041  |
| AVLT-first attempt| 0.045      | 0.047      | -0.085   | -0.086   | 0.047  | 0.047  |
| AVLT-average of 5 tests | 0.015 | 0.039 | -0.026 | -0.025 | 0.015 | 0.039 |

rDD – recurrent depressive disorder; CG – control group; TMT – Trail Making Test; RCNbt – reading color names in black; NCWd – naming color of word-different; AVLT – Auditory-Verbal Learning Test; VFT – Verbal Fluency Test; M – mean; ±SD – standard deviation; * p statistically significant.

Statistically significant differences were found in the cognitive performance in all tests between the rDD group when compared to the CG (Table 2).
Table 3 presents the correlation between both ASMT mRNA and protein expression levels and the results of neuropsychological tests, separately for rDD and CG test group. In patients with rDD, statistically significant correlations occurred between both mRNA and protein expression levels and the following tests: part A of the TMT (negative correlation), and verbal fluency test (positive correlation). In the CG group, there was no statistically significant association between the analyzed variables.

Table 3 also presents the results of testing correlation between both ASMT mRNA and protein expression levels and the neuropsychological tests for the entire group. There was a statistically significant correlation between both ASMT gene expression levels and all the neuropsychological tests used in the survey.

**Discussion**

Obtained results demonstrate that at both mRNA and protein expression levels of ASMT gene were higher in controls than in patients with rDD. The increase of these parameters in healthy controls in comparison with depressed subjects indicates the importance of melatonin in the etiology of recurrent depression.

The presented results are in agreement with reports by Galecki et al. [35] and Etain et al. [36], showing that a single-nucleotide polymorphism (SNP) in ASMT gene expression was associated with depression (a group of 181 patients with rDD and 149 controls). The presence of AA genotype of rs4446909 polymorphism and of GG genotype of rs5989681 polymorphism was associated with lower risk for having rDD (odds ratio=0.195%, CI=-0.03-0.58, p=0.007). One or 2 G alleles were observed in 99% of cases and 92% of controls. Etain et al. [36] examined 345 patients with bipolar disorder (BD) and 220 healthy controls, and showed that rs4446909 polymorphism of the ASMT gene was significantly associated with BD (p=0.01) and associated with a lower mRNA level (p<0.001) and a lower enzymatic activity (p<0.05) of ASMT. A reduced concentration of melatonin is also reported in dementia [37,38], in schizophrenia [39], in children with autism spectrum disorders (ASD) [40], and in patients with attention-deficit hyperactivity disorders (ADHD) [41].

An important factor for the development and course of depressive disorders may be the neuroprotective effect of melatonin [42], which results from the reduction of glutamatergic cytotoxicity, oxidative stress, and inflammation (including neuroinflammation) [43]. Melatonin decreases levels of proinflammatory cytokines, which have a significant influence on the occurrence of neurobiological changes in depression [42].

Melatonin enhances cell survival and dendrite maturation of new neurons in the dentate gyrus (DG) of adult mice [44]. Moreover, Monje et al. [45] investigated whether long-term light deprivation in the constant darkness (DD) paradigm affects depression-like behavior in mice and concomitantly modulates the levels of proinflammatory cytokines. They found that after 4 weeks of DD, mice display depression-like behavior, which is paralleled by reduced hippocampal cell proliferation. This chronobiologically-induced depressive state is associated with elevated levels of plasma interleukin-6 (IL-6) and interleukin 1 receptor, type I (II1-R1) protein levels in the hippocampus, and also alters hippocampal protein levels of the clock genes per2 and npas2. Furthermore, there were observed morning elevations of plasma IL-6 and a reversal of its circadian rhythm in MDD patients (the circadian rhythm of IL-6 was shifted by 12 h, and its physiological complexity was reduced) [46].

Another important function of melatonin is modulation of neuronal plasticity and regulation of behavior control and mood [47]. Melatonin has an effect on proper functioning [48] and protection from oxidative stress of the hippocampus [43], the area of the brain important for the development and course of depression. Our presented results support findings of previous reports. Another finding of our study is the link between cognitive impairment in rDD patients and reduced mRNA and protein expression levels of the ASMT gene. To our knowledge this is the first study investigating the relationship between cognitive function and mRNA and protein expression levels. In rDD patients, there were statistically significant associations of ASMT mRNA and protein expression level and TMT test part A (negative correlation), and verbal fluency test (positive correlation). In the group of patients with depression, reduced ASMT gene expression is related to decreased efficiency of those cognitive functions attributed to frontal lobes and hippocampus: working memory, attention, and verbal fluency. For the entire group, the analysis revealed a significant correlation between mRNA and protein expression levels of the ASMT gene and all the results of neuropsychological tests. Both mRNA and protein expression levels of the ASMT gene affect: auditory-verbal and visual-spatial working memory, verbal fluency, verbal memory, auditory-verbal immediate and deferred memory, the effectiveness of learning, and attention span. These results are in line with the data obtained in studies based on animal models [49]. Laborie [50] showed a positive effect of phototherapy and melatonin medication on the improvement of cognitive functioning in 189 patients (mean age 85.8 years) with symptoms of dementia. These 2 therapies help to resynchronize the circadian rhythm and thus might improve cognition and abnormalities of mood and behavior, as well as independence and sleep disturbance. The obtained results demonstrated a slower cognitive decline and less diminution of autonomy in those who received phototherapy and melatonin. According to Baydas et al. [51], melatonin has a modulatory effect on the expression of neural cell adhesion molecule (NCAM) in brain areas involved with cognitive plasticity and regulation of behavior control and mood.
function. Melatonin may be involved in structural remodeling of synaptic connections during memory and learning processes. Moreover, results obtained by Ramirez-Rodriguez et al. [52] indicate that melatonin also modulates the neurogenic process in the hippocampus during normal aging of mice. Moreover, recent data have shown that neurodegenerative diseases (e.g., Alzheimer’s disease [AD] or Parkinson’s disease [PD]) are often associated with sleep disturbances beyond what is observed in normal aging [53]. Disturbed regulation of sleep often occurs early in the course of AD and PD, and may contribute to the cognitive and motor symptoms [54]. Baydas et al. [55] observed that chronic administration of melatonin significantly reduced lipid peroxidation and restored the decreased glutathione levels induced by chronic hyperhomocysteinemia in mice. They also found that learning and memory deficits were reversed by long-term treatment with antioxidant melatonin.

Taken together, these findings indicate that a subgroup of individuals with rDD and low melatonin levels could benefit from the use of melatonin as a therapeutic compound. Melatonin treatment seems to help patients with rDD to fall asleep and to sleep through the night. Melatonin’s effect on improving cognitive functioning in patients with rDD remains unknown. Further studies are required to determine the role of melatonin deficit in affected individuals, and, more generally, of circadian and seasonal rhythms, in the susceptibility to neuropsychiatric disorders.

There are some limitations of our study. One of them is the relatively low number of patients participating in the study. Although the study was performed in a homogeneous group of patients, a possible stratification effect should be taken into account. Another limiting factor may be the presence of statistically significant differences between the 2 test groups, especially in terms of age, education, and number of schooling years. These differences should be taken into consideration in the interpretation of the outcomes. The results of our preliminary study require further validation in subsequent research.

Conclusions

1. Our study confirms previous results showing decreased mRNA and protein expression levels of the ASMT gene in depression.
2. Our data suggest a relation between decreased mRNA and protein expression levels of the ASMT gene and cognitive impairment.

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