Original Article

MOLECULAR METABOLISM

# Temporal expression patterns of the melatoninergic system in the human thymus of children

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## ABSTRACT

**Objectives:** To obtain greater knowledge of the extra-pineal sources of melatonin during development, the amount of indolamine and the expression levels of the last two enzymes involved in its biosynthesis, Arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT), were analyzed in the human thymus from children from three different age groups (from days to years). The melatonin membrane and nuclear receptor expression levels also were studied.

**Methods:** Quantitative reverse transcriptase PCR and western blot were performed to investigate the receptor and enzyme expression levels. The results were examined and correlated with the ages of the thymuses.

**Results:** We found high levels of indolamine in the thymuses of newborns (younger than 1 month), which decreased during development; thymuses from the months (from 2 to 11 months) and years (from 1 to 12 years) groups showed lower levels. A similar decline was also observed in the mRNA of the AANAT enzyme and the expression levels of melatonin receptors. However, ASMT expression was exactly the opposite, with low levels in the newborn group and higher levels in the years group. Our results show that the thymic synthesis of melatonin occurs very early in childhood. Additionally, this is the first report that is focused on melatonin receptors expression in the human thymus.

**Conclusion:** Considering the limited melatonin synthesis performed by the newborn pineal gland, we suggest that the high levels of melatonin found in human thymus in this experimental group arise from synthesis in the tissue itself, which could be contributing to the immune efficiency at the thymic level.



**Keywords** Melatonin; Thymus; AANAT; ASMT; Melatonin receptor; Nuclear receptor ROR-alpha

## 1. INTRODUCTION

Extra-pineal melatonin synthesis is not a novelty; many cells and tissues from the immune system have and/or produce melatonin. This is the case in the thymus, which has been described as a tissue with high quantities of melatonin [1]. Its biosynthesis from the amino acid tryptophan requires four enzymatically catalyzed steps [2] that are performed by tryptophan hydroxylase (TPH), aromatic amino acid decarboxylase (AADC), arylalkylamine N-acetyltransferase (AANAT), and hydroxyindole-O-methyltransferase (HIOMT), which is now called N-acetylserotonin-O-methyltransferase (ASMT). The last two steps have been proposed as limiting steps for melatonin production [3]. In general, melatonin from a pineal origin acts as a regular hormone that reaches target cells through the blood stream, but extra-pineal melatonin appears to play a more important function in the tissues where it is produced [4]; thus, it can be retained in these sites. In fact, many extra-pineal tissues have higher melatonin concentrations than those found in plasma, and this amount is not generally released into the circulation [5].

Melatonin production in the pineal gland follows a rhythmic profile, with high nocturnal levels and low diurnal levels. Its secretion not only fluctuates throughout the day but also throughout life. In fact, one of the main factors that influences its secretion is age. It has been observed that after crossing the placenta, maternal melatonin acts as one of the main signals for setting the biological clock of the fetus, since no synthesis of melatonin takes place during the prenatal period. After birth, melatonin synthesis under the control of the circadian rhythm is not discernible until the eighth week of life [6], after which it appears to develop rapidly until reaching its maximum nocturnal levels between 3 and 7 years of age [7]. During the period of time that elapses from birth to the acquisition of a rhythmic melatonin synthesis profile in humans, this deficit is solved by the pronounced circadian

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rhythms associated with melatonin present in breast milk. After puberty, melatonin concentrations show a marked nocturnal drop that gradually continue throughout life; melatonin concentrations appear to be severely attenuated but not absent in centenarian individuals [8]. Thus, pineal and plasma melatonin levels fall with advanced age; however, little is known about indolamine levels in extra-pineal tissues throughout life. In the case of the thymus, most of the work performed in rodents indicates that aging decreases the production of melatonin, although in a study performed by our colleagues [9], they hypothesized that there is some resistance by the thymus to maintain its antioxidant capacity. In humans, there was an interesting study performed in elderly people that showed a decrease in melatonin and AANAT levels in very old participants [10]. However, there is no evidence regarding what happens to the melatoninergic system in the thymus during childhood.

Many of the physiological actions of melatonin have been described to be performed through binding to nuclear and membrane receptors, as well as through receptor-independent pathways. The membrane receptors are called $MT_1$ and $MT_2$, and they are part of the G protein-coupled receptor family [11]. However, the recent demonstration of a fully functional mitochondrial GPCR signaling pathway activated by melatonin in the brain [12] leads us to reconsider that G protein-coupled receptors are not only associated with the plasma membrane. Among the nuclear binding sites for melatonin, also known as ROR orphan receptors, three subtypes ($\alpha$, $\beta$, $\gamma$) and four splicing variants of the $\alpha$-subtype are included [13]. This receptor's family has already been characterized in the thymus from several species, including mouse [14] and rat [15,16]; at present, there is no evidence of melatonin binding sites in the human thymus or how they are influenced by age.

To assess whether the thymic melatoninergic system shows changes related to development, we studied the enzyme expression required for melatonin synthesis as well as the nuclear and membrane melatonin receptors in normal human thymuses from children of different ages. The results were correlated with the ages of the tissues. Here, we determined both the expression of the enzymatic machinery for the local production of melatonin as well as the expression of its specific receptors in all studied ages.

## 2. MATERIALS AND METHODS

### 2.1. Thymus samples

Thymic tissues from newborns to 12-year-old children were obtained after cardiac surgery procedures (congenital heart diseases). The thymus fragments that made access to the heart more difficult during surgery were removed, rinsed in normal saline solution and frozen at $-80$ °C until RNA and protein extraction was performed. The study followed the Helsinki Declaration for medical research involving human subjects. Legal representatives of the patients signed the written informed consent to participate in the research. The study was approved by the Ethics Committee of the Virgen del Rocio Hospital in Seville, Spain, on February 11th of 2010 (Act n$^0$ 03/2010). The sixty-one (61) samples used in this study were grouped into the following 3 groups of patients: newborns or days (younger than 1 month), months (younger than 1 year), and years (older than 1 year). All the samples' characteristics are described in Table S1.

### 2.2. Measurement of thymus melatonin levels

The tissues were weighed, homogenized in PBS, and centrifuged at 3000 g for 10 min; the supernatants (500 μL) were kept for melatonin determination. The melatonin quantity in the tissue was estimated using an ELISA kit (IBL, Hamburg, Germany) according to the manufacturer's recommendations.

### 2.3. RNA isolation, reverse transcription, and real-time PCR

RNA was extracted from the organs using the TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Single-strand cDNA was synthesized from 3 μg of RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Real-time PCR was performed on a LightCycler 480 (Roche) using the LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). The primer sequences are detailed in Table S2. All PCR reactions included negative controls in which the template cDNA was omitted. The expression level of each gene was normalized to that of β-actin, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

### 2.4. Western blot

The tissue was homogenized and lysed at 4 °C in lysis buffer containing 50 mM Tris, pH 8; 137 mM NaCl; 10% glycerol; and 40% Nonidet with protease inhibitor cocktail (Sigma—Aldrich, St. Louis, MO, USA). The protein content of the lysates was quantified using the Bradford method [17]. Aliquots containing 75 μg of protein were denatured at 85 °C for 5 min in Laemmli's Buffer (Sigma—Aldrich), subjected to SDS—PAGE and transferred to a PVDF membrane. The membranes were blocked with Tris-buffered saline—0.05% Tween 20 (TBST) containing 5% nonfat dry milk for 1 h at RT. The blots were then incubated overnight with primary antibodies against human $MT_1$ (Mel1aR, N-20, sc13179) [Santa Cruz Biotechnology, Santa Cruz, CA] at a dilution of 1:200, human $MT_2$ (Mel1bR, G-20, sc-28453) [Santa Cruz Biotechnology, Santa Cruz, CA] at a dilution of 1:200, and human HIOMT (anti-ASMT/HIOMT, LS-C156543) [LSBiO] at a dilution of 1:500. The blots were then washed 3 times with TBST before incubation for 1 h with secondary antibodies linked to horseradish peroxidase (anti-Rabbit IgG HRP W401; anti-Mouse IgG HRP W402; and anti-Goat IgG HRP V8051) [Promega]. The bound horseradish peroxidase was visualized using the Western Blotting Luminol Reagent sc-2048 (Santa Cruz Biotechnology, Santa Cruz, CA). The bands obtained in the blots were scanned and analyzed using a ChemiDoc-It Imaging System. The amount of protein loaded in each lane was controlled by immunoblotting with the monoclonal anti-GAPDH antibody MAB374 (Millipore) at a dilution of 1:1000.

### 2.5. Statistical analysis

All results are reported as the mean ± SEM. Data were analyzed with the SPSS® v24.0 software. Kruskal—Wallis test was used to determine the overall differences between groups. Spearman correlation coefficient was used to explore possible associations between variables. Values of $p \leq 0.05$ were considered statistically significant.

## 3. RESULTS

### 3.1. Melatonin content in the human thymus is higher in newborns

Circulating melatonin levels in the blood of mammals reaches concentrations up to 0.5 nM [18], while extra-pineal concentrations vary depending on the tissue. To determine the melatonin contents in the thymus samples from children and to investigate whether these change with age, indolamine levels were measured in tissues from the three age groups (Figure 1). The highest melatonin levels were found in the thymuses from newborns (approximately 40 pg/mg of tissue). This concentration decreased by half in the thymuses from children under 1

 

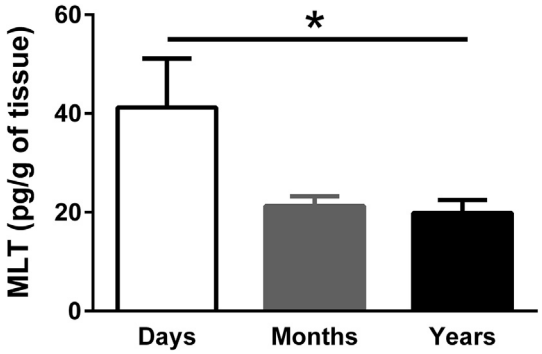


**Figure 1:** Melatonin in the human thymus during childhood. Melatonin contents were measured by ELISA in tissue homogenates from children at several development stages. The data represent the mean ± SEM (n = 34). *p ≤ 0.05.

year old, although statistically significant differences were only found between the days and years groups (p ≤ 0.05).

### 3.2. The expression of the melatonin biosynthetic machinery in the human thymus from children changes with age

Melatonin synthesis is derived from the amino acid tryptophan and is usually found in tissues with AANAT and ASMT activity [2]. To determine the expression of *AANAT* and *ASMT* mRNAs in human thymus from children, cDNAs from the three groups of patients (days, months and years) were subjected to real-time quantitative PCR. The assay revealed that *AANAT* mRNA expression was significantly downregulated with age, with the highest levels of this transcript being measured in the group of neonates (Figure 2A). This decrease was also supported by the negative correlation (rho = −0.4989; p ≤ 0.001) between *AANAT* expression and patient age (Figure 2B). In contrast, a significant increase in *ASMT* mRNA expression was detected in the thymuses of patients older than 1 year (Figure 3A) when compared with the 12 months-younger patients (p ≤ 0.05). This increase was also observed at the translational level (Figure 3B). In western blots, a well-defined band at approximately 52 kDa corresponding to ASMT was found in the three age groups, but the expression was higher in the eldest thymuses when compared with the two other groups (p ≤ 0.001).

### 3.3. The thymus not only synthesizes melatonin but also responds to it

The transcriptional and translational expression of melatonin receptors was determined using qPCR and western blot analysis, respectively. The results revealed that mRNAs of the *MT1*, *MT2* (Figure 4A1 and B1), and *ROR/RZR* receptors (Figure 5) were significantly reduced (more than 60%) in the thymuses from the months and years groups on comparison to newborns. These decreases were also observed at translational level for the membrane receptors (Figure 4A2 and B2), but these results were only statistically significant for subtype 1 (*MT1*).

### 3.4. The melatonin effector system decreases throughout childhood

The variation in mRNA levels for melatonin receptors throughout childhood was supported by statistical analyses using the nonparametric Spearman r test, which showed a negative correlation between melatonin receptor mRNA expression and the age of the thymus (Figure 6). The membrane receptors showed a strong negative correlation (p ≤ 0.001, Figure 6A,B), whereas in the case of nuclear receptors RORα1 and RORα4, the negative correlation was moderate but also significant (p ≤ 0.01 and p ≤ 0.001, respectively, Figure 6C,D), indicating that expression of the melatonin effector system is higher in newborns and decreases throughout childhood. Additionally, to explore whether this expression could also be associated with the melatonin contents in the thymus, we calculated the correlation coefficients; accordingly, only *RORA1* and *RORA4* expressions were slightly correlated (p ≤ 0.05) with intra-thymic melatonin levels (Figure 7).

### 3.5. The expression of genes associated with melatonin synthesis and signaling correlate in the human thymus

The dynamic changes in the melatoninergic system in the natural course of development were analyzed again using the nonparametric Spearman r test (Table 1), which showed strong correlations (rho > 0.8, p ≤ 0.001) between *MT1*, *MT2*, and *AANAT* gene expression. This correlation was also positive and statistically significant between the nuclear receptors RORA1 and RORA4 (rho = 0.642, p ≤ 0.001). Additionally, the nuclear receptors also showed a moderate correlation that was significant with *AANAT*, *MT1*, and *MT2*

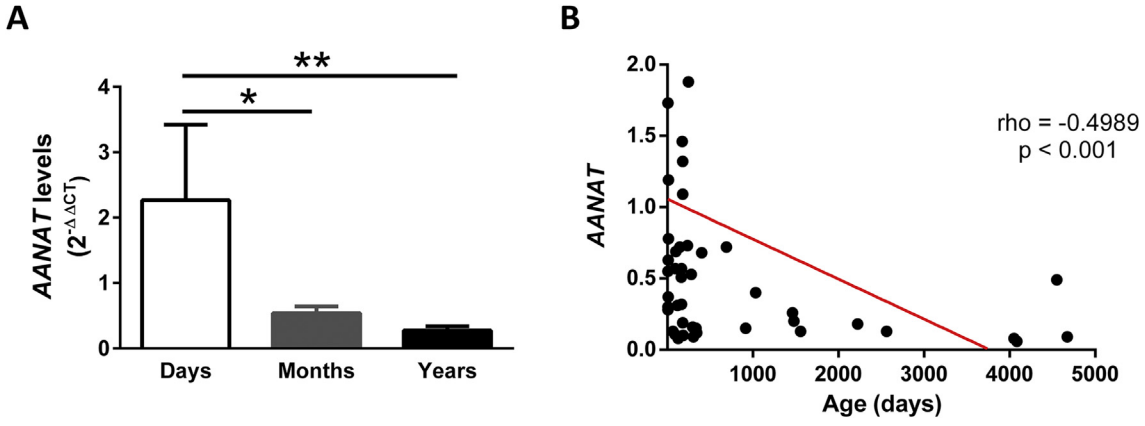


**Figure 2:** *AANAT* is downregulated in the thymus during development. A) *AANAT* mRNA expression in thymus samples from newborn (named "days", white bar), younger than 1-year-old (named "months", gray bar) and younger than 12-year-old (named "years", black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression; values were normalized to *β-actin* expression. The data represent the mean ± SEM (n = 45). *p ≤ 0.05 and **p ≤ 0.01. B) Correlation analysis between *AANAT* mRNA expression and the age (days) of the human thymus. ***p ≤ 0.001.

 

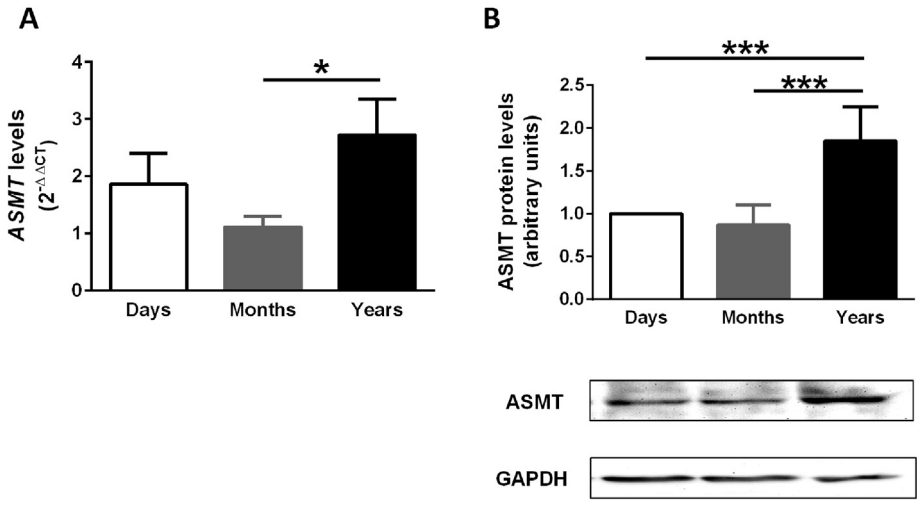


**Figure 3:** ASMT is upregulated in the thymus of children. A) *ASMT* mRNA expression on thymus samples from newborn (named "days", white bar), younger than 1-year-old (named "months", gray bar) and younger than 12-year-old (named "years", black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression; the values were normalized to β-actin expression. The data represent the mean ± SEM (n = 48). *p ≤ 0.05. B) Protein extracts from the thymus were analyzed by western blot for ASMT expression at several ages using GAPDH as a loading control. The quantified results (on the top) are expressed as the optical density of the ASMT signal after normalization to GAPDH (bar graph). The images on the bottom are representative of five independent experiments. ***p ≤ 0.001.

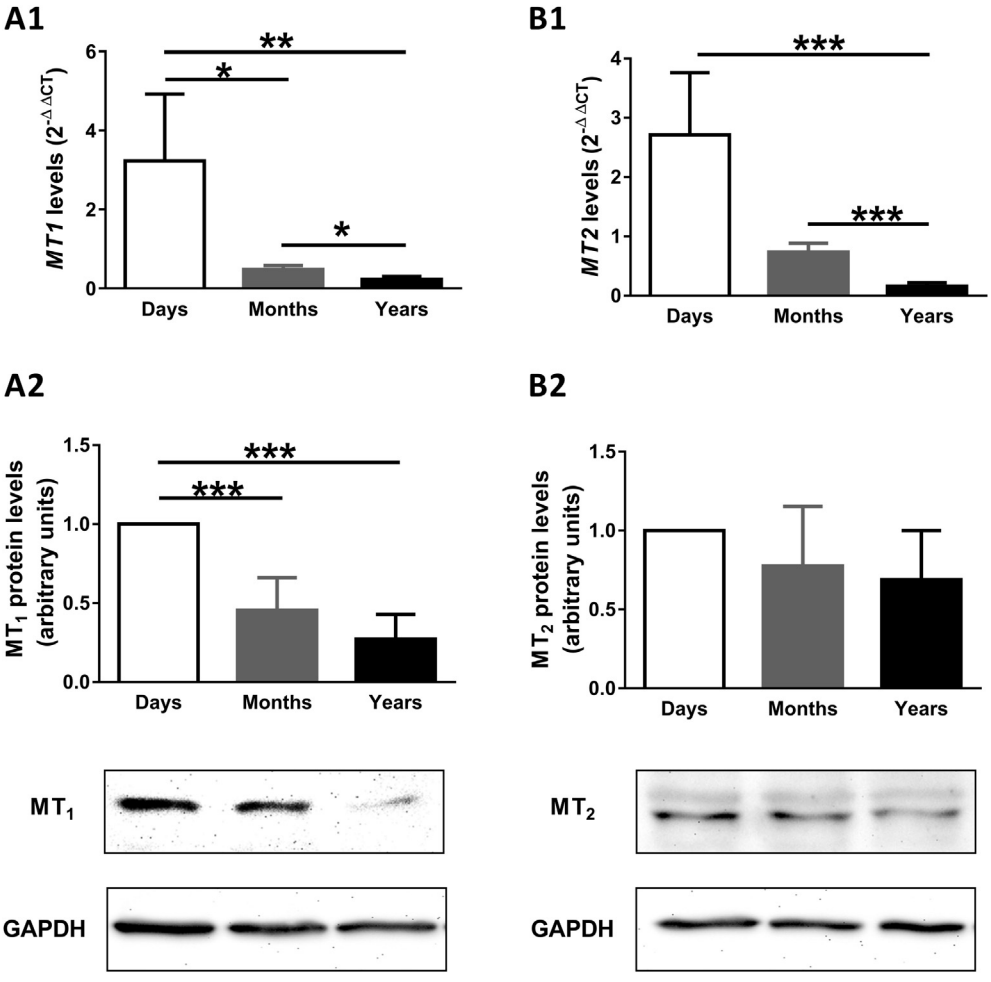


**Figure 4:** Expression of membrane melatonin receptors in human thymus during childhood. MT1 (A1) and MT2 (B1) mRNA expression in thymus samples from newborn (white bar), younger than 1-year-old (gray bar) and younger than 12-year-old (black bar) patients was analyzed by qRT-PCR. The 2-ΔΔCt equation was applied to calculate the relative expression; the values were normalized to B-ACTIN expression. The data represent the mean ± SEM (n = 46). *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001. Protein extracts from the thymus were analyzed by western blot for the expression of MT1 (A2) and MT2 (B2) at several ages using GAPDH as a loading control. The optical densities of the signal from both receptors after normalization to GAPDH are shown on the top. The images on the bottom are representative of five independent experiments. ***p ≤ 0.001.

 

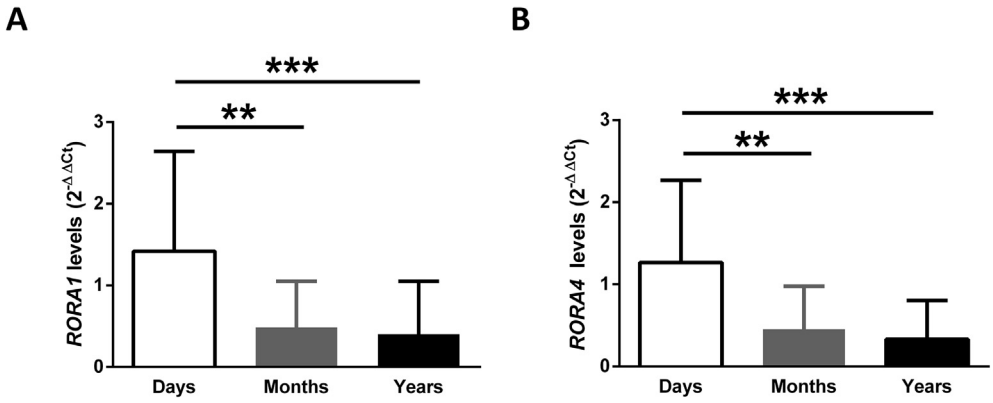


**Figure 5:** The human thymus expresses nuclear melatonin receptors. *RORA1* (A) and *RORA4* (B) mRNA expression in thymus samples from newborn (white bar), younger than 1-year-old (gray bar) and younger than 12-year-old (black bar) patients was analyzed by qRT-PCR. The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression; the values were normalized to β-actin expression. The data represent the mean ± SEM ($n = 60$). **$p \leq 0.01$ and ***$p \leq 0.001$.

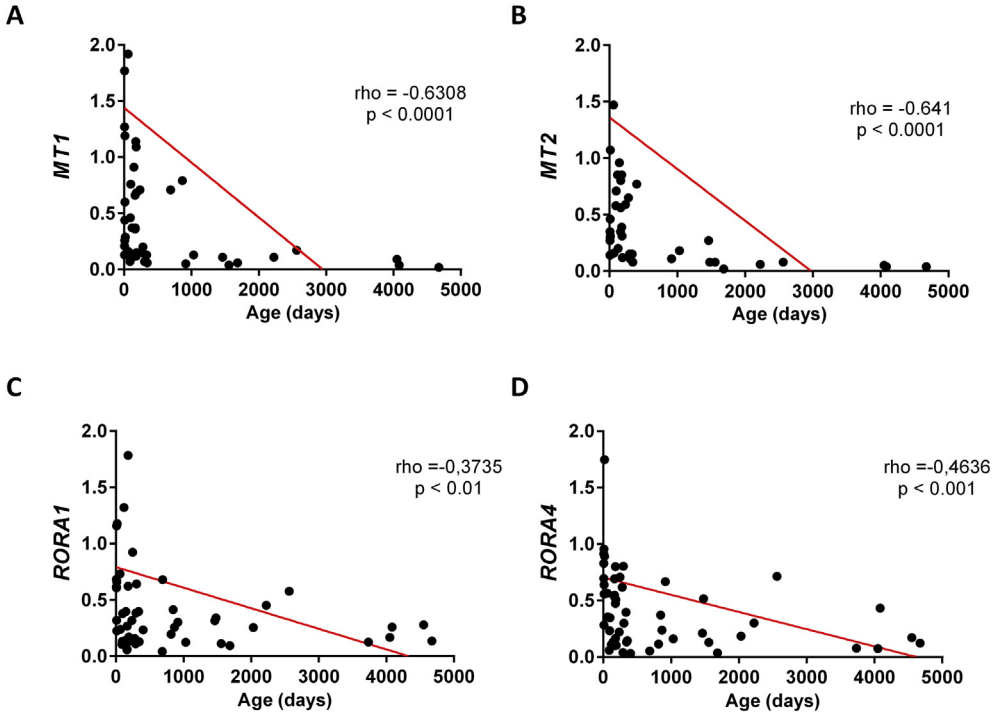


**Figure 6:** The melatonin receptor system correlates inversely with age. Correlation analysis between age (days) and mRNA expression ($2^{-\Delta\Delta Ct}$) for $MT_1$ (A), $MT_2$ (B), *RORA1* (C), and *RORA4* (D) in the human thymus. Each dot represents one thymus, and the straight-line represents the best-fit line obtained by linear regression analysis. ($n = 46$ for *MT1* and *MT2*; $n = 58$ for *RORA1*; and $n = 60$ for *RORA4*).

expression. Interestingly, only the *RORA1* subtype was positively correlated with ASMT expression ($p \leq 0.05$).

## 4. DISCUSSION

The leading site for T-cell development in the human body is the thymus; consequently, this is one of the organs involved in the generation and preservation of the adaptive immune system [19]. In terms of the main cellular subpopulations, the human thymus develops fully before birth and begins to atrophy after puberty, when the number of thymocytes decreases and the thymic stroma is gradually replaced by adipose fat throughout a lifetime. However, according to evidence, residual T lymphopoiesis continues until adulthood [20]. It is well-documented that melatonin influences both the morphology and function of this vital organ. The first study that showed a connection between the thymus and melatonin was performed almost 50 years ago, when a pinealectomy caused a reduction in the size of the thymus from 130 to 70 mg in mice [21]. In 1975, other authors also showed a loss in the proliferation of thymic cells after pinealectomy [22]. However, several studies have shown that the age-related thymic involution can be delayed or even reverted. In this sense, the grafting of a pineal gland from young animals into an old one or the administration of melatonin (the pineal secretory product) maintained thymus function and cellularity [23,24]. Moreover, indolamine improves the proliferative

 

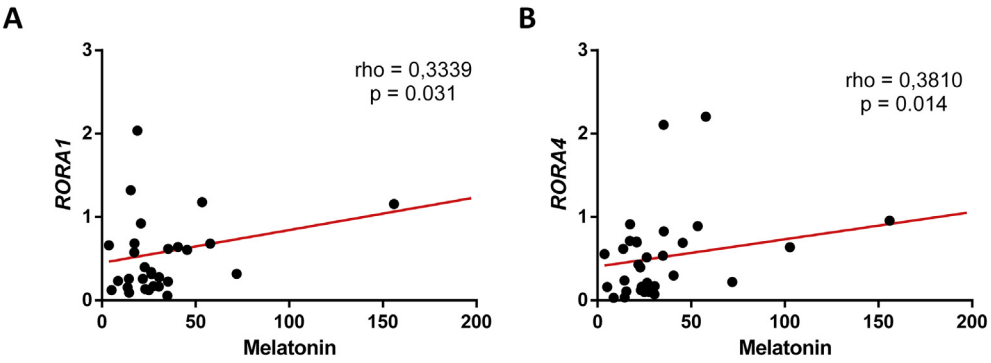


**Figure 7:** Nuclear melatonin receptor expression correlates positively with thymic melatonin contents. Correlation analysis between the melatonin contents and the mRNA expression ($2^{-\Delta\Delta Ct}$) of *RORA1* (A) and *RORA4* (B) in the human thymus. Each dot represents one thymus, and the straight-line represents the best-fit line obtained by linear regression analysis. (n = 32 for RORA1 and n = 33 for RORA4).

**Table 1** — Spearman's rank correlation coefficients between the mRNA expression of melatonin synthesis and signaling genes.

| Genes | *AANAT* | *ASMT* | *MT1* | *MT2* | *RORA1* | *RORA4* |
|---|---|---|---|---|---|---|
| *AANAT* | 1 | −0.1363 | 0.8775*** | 0.8892*** | 0.4219** | 0.3751* |
| *ASMT* | | 1 | −0.0407 | −0.177 | 0.3399* | 0.205 |
| *MT1* | | | 1 | 0.8676*** | 0.4318** | 0.4469** |
| *MT2* | | | | 1 | 0.4284** | 0.3434* |
| *RORA1* | | | | | 1 | 0.642*** |
| *RORA4* | | | | | | 1 |

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

capacity and degree of DNA synthesis in lymphocytes from the thymus in old rats [25]. Therefore, there is no doubt about the relationship between the thymus and melatonin, which is reinforced by the presence of melatonin receptors in this immune organ from mice and rats [14,15]. However, until now, there was no evidence regarding the expression of these receptors in human thymus. Naranjo et al. detected great quantities of melatonin as well as the expression and activity of the AANAT and ASMT enzymes in human thymuses in adults [1]. Our study is the first to evaluate the entire melatoninergic system in the human thymus in children of several ages. Our results show that the melatonin biosynthetic machinery in the human thymus is highly expressed from the beginning of birth. We detected the mRNA and protein expression of both enzymes in thymuses in newborns (from 6 to 19 days of age) as well as high levels of melatonin. Although we cannot rule out that part of this melatonin comes from the circulation, based on the high levels of both enzymes in the thymuses from the youngest age group, it is logical to think that its main source is local synthesis. Taking into account that pineal melatonin synthesis is minimal for the first six weeks of life [26], the thymus could show a compensatory increase in melatonin production, as has been previously shown [16]. The role of higher melatonin production in the human thymus at early stages might be related with two main functions: on one hand by improving the morphology and thymic function and, on the other, by protecting against the oxidative damage in the tissue [27]. According to the first hypothesis, some authors state that the thymic function gradually decreases in thymopoietic function as from year 1 of life, coinciding with the decrease in thymic epithelial space [28]. In fact, a very recent article showed that the thymic index to weight ratio (cm3/Kg) was largest at early infancy (1−8 weeks) and smallest at 1 year of age [29]. These findings would match with the higher levels of melatonin found in the youngest thymuses. Furthermore, since newborns are especially susceptible to oxidative stress [30], another possible role of melatonin at such an early age would be its cytoprotective ability [31]. In fact, the recent evidence about melatonin is mainly synthesized in the mitochondria from several tissues lead us to think that the thymus would not be an exception [32], especially if we consider that in eukaryotes, mitochondria are the major source of reactive oxygen species (ROS) and they require specific onsite protection [32]. Another fact to contemplate is the potential effect of surgery on increasing local melatonin synthesis as part of the acute inflammatory response [33], which increases TNF and leads to the transcription and activation of AANAT. According to this, the presence of melatonin might help to protect thymocytes against an oxidative and inflammatory damage. However, this effect would not seem explain the differences found among the three group of ages since all samples were obtained from the surgical intervention.

AANAT mRNA expression levels decreased over the course of growth, both in the months (from 2 to 11 months of age) and years (from 1 to 12 years) groups. In fact, the expression of this enzyme is inversely correlated with the age of the thymuses. In contrast, the expression pattern for ASMT was exactly the opposite, with the highest levels found in the years group. The unexpected result of high levels of AANAT and low levels of ASMT in the newborn samples might suggest that ASMT would not play an important role in the thymic synthesis of melatonin during the early days of birth, with AANAT being primarily responsible for melatonin levels in the organ. As the thymus matures, AANAT levels decrease, and ASMT levels rise instead. This increase in ASMT expression could be a mechanism to overcome the decrease in AANAT so as to maintain sufficient melatonin levels in the organ. Another possibility, albeit far-fetched, is that the last two enzymes involved in melatonin synthesis are inversed, such that the ASMT enzyme acts first and the AANAT afterwards. This alternate pathway has been observed in plants [34] and it is worthy of further investigation.

We found that melatonin receptor expression supports the effects of melatonin produced locally. The presence of melatonin receptors in the thymus of several species had been previously reported [14,16,35]. However, this is the first description of the expression of a melatonin effector system in human thymus samples. We detected mRNA and protein expression of both membrane receptors subtypes. The highest levels were found in the days group but the expression decreased throughout childhood. The same mRNA expression pattern was observed for the nuclear receptors (RORα1 and RORα4 variants), while the highest levels were observed in newborns. Therefore, we found

 

that the expression of the $MT_1$, $MT_2$, and RORα receptors in the thymus decreased with age. In line with this, an age-dependent decrease in $MT_1$ and $MT_2$ receptor expression was also reported in several tissues by Sanchez-Hidalgo et al. [15] and later by Hill et al. [36]; they suggested that this reduction led to the early onset of senescence. In our study, the correlation coefficients showed that the expression of membrane receptors is inversely related with the age of the thymuses, but the expression of nuclear receptors appears to also be associated with melatonin levels. Although a correlation does not indicate causality, in this case, it is not illogical to think that melatonin levels may influence the expression of the nuclear receptor that has already been described to directly interact with it so as to exert its transcriptional functions [37]. We were unable to find a relationship between the melatonin content in the thymus and the other genes in the melatoninergic system, which could be due to the reduced sample size or problems with the melatonin quantification technique.

A logical but not less interesting finding from our study was the highly statistically significant association between the expression of the melatonin membrane receptors and AANAT; at lower enzymes levels, there were also lower receptors levels. These receptors are largely responsible for mediating the downstream effects of melatonin [38], and AANAT is the major enzyme in melatonin synthesis [39]. Thus, an increase in local synthesis of melatonin via AANAT during the early age coincides with an increase in the action of melatonin via $MT_1$ and $MT_2$ receptors. The expression of the two RORα isoforms studied was also associated with the expression of AANAT and the membrane receptors, however, these correlations were lower. This is not the first time that we suggest interplay between nuclear and melatonin receptors, as this was previously shown in human lymphocytes [40]. However, the way in which these interactions take place in the thymus and how they are regulated are still poorly understood and require further studies.

## 5. CONCLUSION

The thymic melatoninergic system is present and especially active from the first days of human life; on excluding ASMT, the thymic melatoninergic system is inversely correlated with the age of the human thymus. We ignored whether these variations are related to changes in the composition and cellularity as described by Weerkamp et al. [41], but we believe that the decrease in the receptors themselves as well as the melatonin content might have an altering effect on the architecture of the thymus, consequently affecting the main function of this organ. Consistent with our conclusion, Odinokov and Hamblin suggest that photo-biomodulation can revert age-associated thymic involution through stimulation of extra-pineal synthesis, thus improving the immune function [42]. Although additional studies are necessary to better understand the network between the neuroendocrine and immune systems within the human thymus, our findings suggest that melatonin locally produced may participate in intra-thymic maturation and T-cell differentiation, which leads to the development of cell-mediated immunity in humans.

## STATEMENT OF ETHICS

Legal representatives of the patients have given their written informed consent.

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## AUTHOR CONTRIBUTIONS

Drs Lardone and Molinero conceptualized and designed the study, contributed to the interpretation of the data analysis, drafted the initial manuscript, and revised the manuscript; Drs Cruz-Chamorro, Álvarez-Sanchez and Ms Escalante-Andicoechea designed the data collection instruments, collected data, carried out the initial analyses, and reviewed the manuscript; Drs Guerrero, Carrillo-Vico, and Rubio coordinated and supervised data collection and critically reviewed the manuscript for important intellectual content; all authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.07.007.

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