Sleep Science

ORIGINAL ARTICLE

# Screening for polymorphisms in the AANAT gene and their association with extreme diurnal preference

## *Rastreamento de polimorfismos no gene AANAT e suas associações à preferência diurna em cronotipos extremos*

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**ABSTRACT**

Arylalkylamine N-Acetyltransferase (*AANAT*) is a rate-limiting enzyme of the melatonin synthesis pathway. Polymorphisms in the *AANAT* gene are likely to alter the rate of melatonin synthesis and thus lead particular variations on the circadian rhythms of individuals. The purpose of this study was to perform a screening of the *AANAT* gene in order to identify the polymorphisms presents in a Brazilian sample and seek associations with diurnal preferences. Individuals that scored extreme diurnal preference according to the Horne-Ostberg questionnaire were selected. The search for SNPs was done using Denaturing High Performance Liquid Chromatography (DHPLC). Segregated association tests were performed for each SNP and for the haplotype blocks. A total of six polymorphisms were found, including two that are still not reported in DNA data banks or in the literature. All polymorphisms in the *AANAT* gene found in this sample, except for one, appeared with very low frequencies, of which the rarer alleles show a maximum frequency 0.06. The most frequent SNP, located in the promoter region (C-263G), showed a borderline association with extreme diurnal preference. It was more frequent in volunteers with an evening preference. The haplotype analysis revealed an association of one haplotype (CACTAC) with diurnal preference. The morning group showed more individuals with the CACTAC haplotype. Thus, it is a very interesting possibility that the C-263G polymorphism, in the context of the other SNPs in the gene, can modulate *AANAT* gene expression and change the speed of enzyme synthesis, with possible consequences for the temporality of the melatonin secretion curve.

**Keywords:** *AANAT*, circadian rhythms, genetics, melatonin, polymorphisms.

**RESUMO**

A arilalquilamina N-acetiltransferase é a enzima passo-limitante na via de síntese da melatonina. Polimorfismos no gene *AANAT* podem alterar a função da enzima e alterar a taxa de síntese da melatonina ocasionando variações particulares nos ritmos circadianos dos indivíduos. A proposta deste estudo é identificar polimorfismos do gene *AANAT* numa amostra da população brasileira e buscar associações com a preferência diurna. Somente indivíduos classificados como vespertinos extremos ou matutinos extremos, segundo o questionário de Horne-Ostberg, foram inclusos neste estudo. A busca pelos polimorfismos foi realizada pela técnica de cromatografia líquida denaturante de alta pressão (DHPLC). Análises de segregação foram feitas para cada um dos polimorfismos e também para os blocos de haplótipos. Um total de seis polimorfismos foram encontrados, incluindo dois ainda não descritos em outras populações. Todos os polimorfismos do gene *AANAT* encontrados nesta amostra, exceto um, apresentaram frequência muito baixa considerando o alelo com menor frequência, na ordem de 0,06% da população. O polimorfismo mais frequente, localizado na região promotora (C-236G), apresentou uma associação limítrofe com a preferência diurna extrema. Observamos maior frequência nos voluntários extremamente vespertinos. A análise haplotípica revelou a associação do haplótipo CACTAC com a preferência diurna. O grupo dos indivíduos extremamente matutinos apresentou mais indivíduos com o haplótipo CACTAC. Há uma interessante possibilidade de que o polimorfismo C-236G, no contexto dos outros polimorfismos no gene, possa modular a expressão do gene *AANAT* e modificar a velocidade da síntese da enzima, com possíveis consequências na temporalidade na curva de secreção da melatonina.

**Descritores:** *AANAT*, genética, melatonina, polimorfismos, ritmos circadianos.

## INTRODUCTION

Melatonin is considered a time-giver hormone. A clear nocturnal increase in circulating melatonin levels leads to a rhythmic pattern of melatonin secretion. This important fact brings up the information about time, which allows them to adapt some of their physiological functions to the daily and seasonal variations of their environment[1].

The nocturnal increase is due to elevated melatonin synthesis in the pineal gland, resulting mainly from enhanced activity of the enzyme arylalkylamine N-acetyltransferase (*AANAT*)[2,3]. *AANAT* converts serotonin to N-acetyl forms by transferring a group acetyl from acetyl coenzyme A[4].

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*AANAT*, also known as "timezyme", plays a major role in the regulation of melatonin associated with circadian rhythms in vertebrates because it is the rate-limiting step of melatonin synthesis. The important regulatory role of AANAT has made it of central interest in research on melatonin biochemistry and neurochemical signal transduction[5]. Most circadian variables are heritable, such as diurnal preference (chronotype = morningness/eveningness)[6-9]. This heritability suggests that these markers of circadian gene regulation are driven by genetic factors.

The analysis of the *AANAT* gene may reveal mutations or polymorphisms that could explain, at least in part, the large individual variations in serum melatonin levels and the temporal pattern of the melatonin secretion curve among human subjects. There have been a few studies searching for polymorphisms in the *AANAT* gene associated with circadian parameters[10-14].

Given the importance of *AANAT* in melatonin synthesis, which is closely related to sleep timing, the aim of the present study was to perform a screening of the *AANAT* gene in order to identify the polymorphisms presented in a Brazilian population sample and to seek associations with extreme diurnal preference.

## MATERIAL AND METHODS

### Subjects

The volunteers answered the Horne & Ostberg (HO)[15] questionnaire for diurnal preference. A total of 1289 questionnaires were completed, the scores were distributed in a normal curve and the extreme 10% at each end were selected as the subjects of this study. Individuals who scored up to 33 points were classified as having an extreme evening preference, and individuals who scored over 61 points were classified as having an extreme morning preference. These individuals were invited to donate a blood sample for DNA extraction.

This study was approved by the Committee on Ethics at *Universidade Federal de São Paulo* (UNIFESP Ethics Committee 106/07).

### DNA Extraction

Genomic DNA was directly extracted from 3 mL of whole blood as described in Miller et al.[16], from volunteers, after obtaining their consent information.

### Primer design and PCR conditions

In order to amplify the exons and the promoter region of *AANAT* gene, a total of 5 pair of primers were designed (reference sequence NC_000017.9) using the Primer 3 Program[17]. The sequences of primers that were used and the specific amplification conditions are summarized in Table 1.

### DHPLC analysis

The DNA samples were amplified by PCR and submitted to DHPLC analysis on the WAVE Nucleic Acid Fragment Analysis System equipped with a 3500HT DNASep Cartridge (Transgenomic, Omaha, Neb., USA). The amplified products were first checked on the system at the non-denaturing condition of 50°C to ensure specific and sufficient amplification of the fragments. The samples were heated at 95°C for 10 min and cooled to 37°C at a ramping rate of 2°C/30s. The melting profiles of the fragments were analyzed by Navigator software (Transgenomic). The temperatures at which 40-80% of wild-type DNA was double helical were selected for screening. Eight microliters of the PCR products were injected into a preheated column and eluted at a flow rate of 0.9 ml/min with a linear acetonitrile gradient consisting of buffer A (0.1 M triethylammonium acetate or TEAA) and buffer B (0.1 M TEAA with 25% acetonitrile). The gradient slope was an increase of 2% of buffer B per minute. Each melting domain was screened at the temperatures described in Table 1. For some exons, more than one temperature was necessary in order to detect all alterations in the sequence. In this way, all heterozygous alterations in the fragment could be detected. One homozygous sample was sequenced and considered standard. This was then mixed in equal volume with each homozygous sample to differ the alleles in order to genotype all the samples.

### DNA sequencing

Samples with DHPLC elution profiles indicating the presence of heteroduplex were amplified separately, and direct sequencing was performed with both forward and reverse primers according to the manufacturer instructions on the ABI PRISM 377 using the DYEnamic™ ET Dye Terminator kit (GE Healthcare).

### Statistical analysis

The allelic and genotypic frequencies were compared using the Fisher exact test for low frequency SNPs and $\chi^2$ for the SNP in the promoter region. Pair-wise linkage disequilibrium analysis of all possible combinations and haplotype association was performed by the Haploview software[18].

## RESULTS

A total of 104 volunteers were selected based on the HO questionnaire for diurnal preference and then separated into two groups: 48 classified as extreme morning (mean age 23.5 ± 3.63, 88% Caucasians, 70% women) and 57 classified as extreme evening (mean age 23.45 ± 4.19, 83% Caucasians, 74% women). Both groups are similar in gender, age and ethnicity.

We found two polymorphisms that had never been described in the literature or genome data banks, and four already known polymorphisms (Table 2). All the polymorphisms that we found are very rare in our sample, except for the one in the promoter region. The linkage disequilibrium analyses reveals that the SNPs found are likely to be randomly associated.

The haplotype association analysis[19] output shows an association of the haplotype CACTAC and extreme diurnal preference (Table 2). The SNP segregated association analysis, reveals a borderline significance (Figure 1, $\chi^2 = 3.404$, $p = 0.06$) the SNP in promoter region (C-236G).

Interesting, as the frequency variations of most of the SNPs are very low, the haplotype composition in fact is highly dependent of the polymorphism in the promoter region, because the SNPs located at exonic and intronic regions are absent in more than 90% of the sample (Table 3 and Figure 2). Thus the haplotypes reflect in a great part the frequency of the SNP in the promoter. The genotype frequency was showed at Table 4.

**Table 1.** PCR conditions: primers, annealing temperature, number of cycles, size of the PCR fragment and DHPLC analysis temperature.

| Region | Primer forward | Primer reverse | PCR TA (°C) | Cycles | Size (bp) | DHPLC temperature (°C) |
|---|---|---|---|---|---|---|
| Éxon 1 | 5'-GTCATCCTGCCTGGGATAGC-3' | 5'-TCCATGTCTAGAAGCTCCAAGT-3' | 58 | 30 | 276 | 60 e 63 |
| Éxon 2 | 5'-GATGAGGATGAGACCCCTGT-3' | 5'-CCAGAGTGGAGCATCCCTCT-3' | 58 | 30 | 320 | 62.5 e 65 |
| Éxon 3 | 5'-CCTAACCCCCATTTTCCTGT-3' | 5'-CCATCTGGCTGACCTCTGTC-3' | 58 | 30 | 335 | 63.8 |
| Éxon 4 | 5'-CTGCCTGGGTTGGTGGTT-3' | 5'-CAGCACCTCCCATTGTCC-3' | 61.3 | 38 | 640 | 61.1, 63.8 e 66 |
| Promoter | 5'-GTTTGGTAGGGCAACCTCCTG-3' | 5'-GCCTGTGGTGCTGTTAGAGG-3' | 65 | 34 | 425 | 65 |

bp: Is base pair.

**Table 2.** Polymorphisms found in the *AANAT* gene.

| | Previously Published | Position | rs | Polymorphism type | Amino acid change | Allelic frequency |
|---|---|---|---|---|---|---|
| Promoter | Yes | C-263G | rs4238989 | - | - | 0.42 |
| Exon 1 | No | A12G | - | untranslated | - | 0.02 |
| Exon 2 | Yes | C244T | rs61739395 | non synonymous | Thr/Met | 0.01 |
| Intron 3-4 | Yes | Intron | rs4646261 | intron | - | 0.06 |
| Exon 4 | Yes | C777A | rs58504104 | synonymous | - | 0.005 |
| | No | C890T | - | untranslated | - | 0.005 |

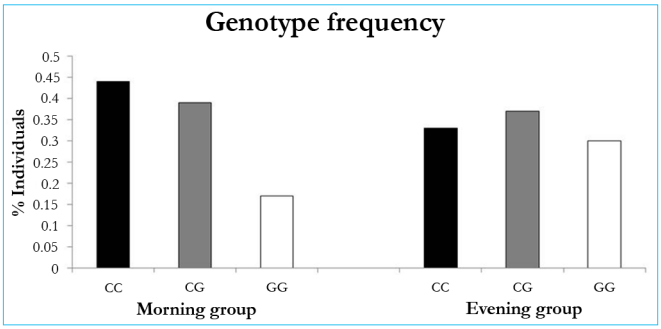


**Figure 1.** Genotypic frequencies of the polymorphism C-263G located at the promoter region of the AANAT gene in individuals that scored at extreme chronotypes. The frequency distribution is marginally different; $p = 0.06$.

**Table 3.** Haplotypic frequencies of polymorphisms found in the *AANAT* gene. In order: C-236G, A12G, C244T, A/T íntron 3-4, C777A and C890T (* $p = 0.03$).

| Haplotype | Frequency | Association ($X^2$; $p$) |
|---|---|---|
| GACTAC | 0.558 | 1.82; 0.17 |
| CACTAC | 0.350 | 4.44; 0.03* |
| CACAAC | 0.047 | 0.08; 0.77 |
| CGCTAC | 0.012 | 0.69; 0.40 |
| GACAAC | 0.011 | 1.37; 0.24 |

## DISCUSSION

In this study, we screened the *AANAT* gene in a Brazilian population sample and found six polymorphisms; two of these had never been described before (Figure 3). We observed that, except for one SNP (C-236G in the promoter region), the frequencies of the remaining five SNPs are extremely low, which indicates that the *AANAT* gene is highly conserved. Segregated association tests for each SNP did not reveal significance, except a marginal significance for the SNP in the promoter region. The haplotype analysis revealed an association of one haplotype (CACTAC) with diurnal preference. This haplotype was more frequent in the morning group.

The comparison between the morning and evening groups reveal a significant association of one specific haplotype with extreme diurnal preference, suggesting that the polymorphisms found in *AANAT* gene may influence the temporality of the melatonin secretion curve and modulate diurnal preference through melatonin signaling. In the SNP segregated analyses we found a borderline association between the promoter region SNP C-263G and the extreme chronotypes. Wang et al.[12] showed that the -263C variant is associated with a late onset/short sleep pattern phenotype and suggested that this variant in the promoter region may disrupt a transcription binding site and consequently alter the transcription of the *AANAT* gene. Concurrently, Ying et al.[20] were not able to detect an effect of the C-263G SNP in the temporality of excretion rate of 6-hydroxymelatonin sulfate (melatonin metabolite). There are controversial data at the literature; it makes more difficult the polymorphism analysis and correlations.

In a wide and elegant study, the C-263G polymorphism was associated with major depression[21], where the authors had suggested the melatonin pathway and circadian clock mechanisms work in contribution to the pathophysiology of major depression. These data together with ours, suggest that the haplotype effect on phenotype is highly dependent of the SNP C-236G in the promoter region (Table 3).

Several recent studies demonstrated that eveningness was associated with more frequent depressive states[22-24].

An association of polymorphism in the promoter region of the melatonin receptor type 1gene with schizophrenia has been reported[25], meanwhile no studies with the *AANAT* gene nor *HIOMT* gene associated with schizophrenia has been reported even though most affected individuals show circadian rhythm disturbances[26]. However, a list of clock genes and clock controlled genes has been associated either with schizophrenia, as bipolar disorders and schizoaffective disorders[27].

At the coding region of the *AANAT* gene, SNPs appears in very low frequencies, i.e., no polymorphisms are present in

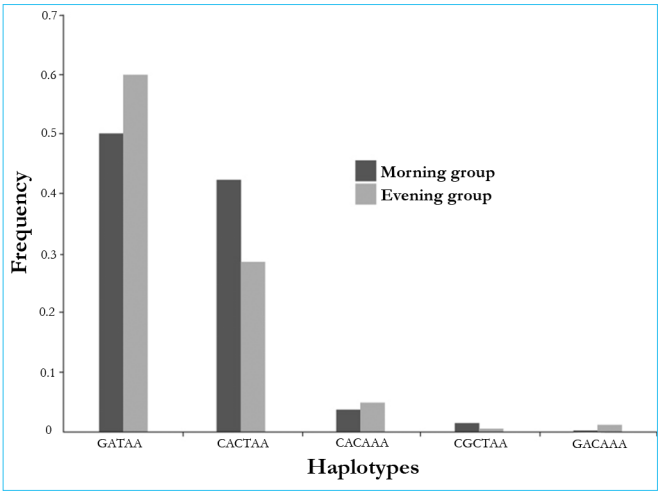


**Figure 2.** Haplotype analysis considering the polymorphisms found in this study. The frequency has been showed separately for the groups.

**Table 4.** Genotypic frequency of polymorphisms found in the *AANAT* gene.

| SNP region | Morningness | | | Eveningness | | |
|---|---|---|---|---|---|---|
| Éxon 1 (A12G) | AA | AG | GG | AA | AG | GG |
| | 0.96 | 0.04 | 0 | 0.95 | 0.05 | 0 |
| Éxon 2 (C244T) | CC | CT | TT | CC | CT | TT |
| | 0.96 | 0.04 | 0 | 1.0 | 0 | 0 |
| Éxon 3 (rs4646261) | TT | TA | AA | TT | TA | AA |
| | 0.89 | 0.08 | 0.03 | 0.91 | 0.09 | 0 |
| Éxon 4 (C777A e C890T) | TT | TC | CC | TT | TC | CC |
| | 0.98 | 0.02 | 0 | 1.0 | 0 | 0 |
| | CC | CT | TT | CC | CT | TT |
| | 0.98 | 0.02 | 0 | 1.0 | 0 | 0 |
| Promoter Region (C-236G) | CC | CG | GG | CC | CG | GG |
| | 0.44 | 0.39 | 0.17 | 0.33 | 0.37 | 0.30 |

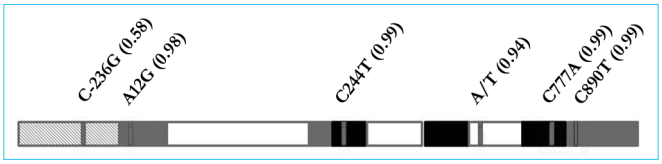


**Figure 3.** Representative scheme of the *AANAT* gene and the polymorphism that we have found. The hatched area represents the gene promoter region, the introns are represented by the white areas. The grey retangles are the exons non-coding region and black represents the coding regions. Grey vertical bars indicate the polymorphisms positions. The numbers between parentheses are the allele frequencies found in this study

the majority of the sample. This fact indicates that the *AANAT* gene is highly conserved, and suggests that the possible variations in this gene would have occurred during human evolution either were not selected or had negative selection. The AANAT enzyme has a well established and fundamental function in organisms, transferring an acetyl radical from AcetylCoA to other molecules, and polymorphisms are likely to affect this function. Phylogenetic analysis of the *AANAT* protein shows that few changes can be observed among the phylum and those that do occur are mainly located flanking the regulatory regions, with some changes also occurring in the catalytic core[28].

The very low frequency of most of the *AANAT* SNPs in our sample corroborates data in the literature. These same SNPs were reported in very low frequencies in Chinese[10] and Japanese[11] populations. In 2003, Hohjoh et al.[11] reported an association between a polymorphism in the *AANAT* gene (G619A) and Delayed Sleep Phase Syndrome (DSPS) in a Japanese sample. The researchers reported an increased frequency of the 619A allele in the group of DSPS patients. A similar approach has been adopted by Pedrazzoli's group[13]. They have demonstrated that this polymorphism is extremely rare (0.2%) in the Brazilian population with no association with DSPS. In our present sample no one subject carries the allele 619A. A more drastic picture can be observed in a study with a Caucasian population sample[14], in which the polymorphisms observed in the Asian populations were not found at all. In addition we have found two new SNPs never been described in genome data banks or in the literature before. This may indicate that these polymorphisms are exclusive to the Brazilian population or they were present in populations never been studied before, such as native indigenous Brazilian populations. Thus, these studies reinforce our idea that the genetic variation of *AANAT* gene seems to be related to ethnic differences.

Interestingly, the polymorphism C-263G which has shown weak signs of association with circadian parameters in our present study and others, seems to have capital importance when considering SNPs in the gene as a whole.

From a functional point of view, this polymorphism is located exactly at a putative sequence for the binding site of transcription factor SP1[26]. Thus potentially regulate the transcriptional rate of the *AANAT* gene. Also, the SP1 played an important role in the up-regulation of the *CREB* gene expression[29] (cAMP response element - binding protein) and, which in the case of the *AANAT* gene, SP1 binding site is located very close to the CREB binding sequence in the promoter region and maybe can play a role in modulating CREB function at the *AANAT* gene transcription. It is a very interesting possibility that the C-263G polymorphism, in the context of the other SNPs in the gene, may modulate *AANAT* gene expression and could change the speed of enzyme synthesis, with possible consequences for the temporality of the melatonin secretion curve. Even thought, the enzymes involved in the melatonin synthesis pathway has been associated with the Clock/+ mutant mice, altering their phenotype and affecting the mammalian circadian system[30].

We have described here the first screening of *AANAT* gene polymorphisms in a Brazilian sample and their association with diurnal preference. One next step will be to consider the effects variations in the *AANAT* gene in combination with variations in the *HIOMT* gene on melatonin circadian profile since these genes have a clear pattern of gene interaction (epistasis) in the formation of melatonin.

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