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Association between CLOCK gene polymorphisms and ADHD in Mexican teenagers: A comprehensive assessment

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ABSTRACT

This study aimed to evaluate markers of the CLOCK gene rs1801260 and rs4864548 in Mexican adolescents, addressing clinical and biological aspects previously associated with ADHD. 347 Mexican adolescents were assessed for mental disorders, metabolic disruption and related conditions, circadian preference, as well as genotyping for the CLOCK. We found a significant association between ADHD and the AA and AG genotypes of rs1801260. Also, we identified in the ADHD group that the total Triiodothyronine and total Thyroxine values were respectively 10 ng/dl units and 0.58 ug/dl units lower in females than in males. Previously reported common variations of the CLOCK gene have been associated with ADHD like the Rs1801260 polymorphism hereby we could consider it as risk factor, but genetic, biochemical and clinical studies in the Mexican population are entailed.

1. Introduction

Attention Deficit and Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders in children and teenagers, and it is characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development (American Psychiatric Association, 2013). Its worldwide prevalence throughout life is 5.29%.(Polanczyk et al., 2015) In addition, a higher prevalence has been reported in males in clinical samples (3–4 males: 1 female) than in epidemiological studies (2: 1) (Medina-Mora et al., 2007; Polanczyk et al., 2015). Mexican teenagers' yearly prevalence is at least 1.6% while the male-female ratio is consistent with reports of other populations(Benjet et al., 2009, 2016; Medina-Mora et al., 2007).

There are several clinical courses throughout life; however, three primary presentations can be identified: predominantly inattentive, hyperactive-impulsive, and combined presentations; the onset may be in childhood, adolescence, or adulthood. In the clinical context, people with ADHD could present a greater psychiatric and health comorbidity, poorer performance in a wide range of scenarios, more difficulties in the academic and justice context, as well as increased use of healthcare and public services (Young et al., 2021) due to the proclivity to injuries, legal issues, premature death (Dalsgaard et al., 2015), suicide behavior (Septier et al., 2019), and unplanned parenthood (Skoglund et al.,

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2019), compared with those who do not have this disorder.

About 50% to 80% of children with ADHD may have sleep disturbance, including sleep-onset insomnia, poor or fragmented sleep, daytime sleepiness, and sleep-disordered breathing (Cortese et al., 2009), and these have been linked with an evening chronotype.

A link between sleep quality and thyroid function has been shown (Nazem et al., 2021). Results from prior studies suggest that the prevalence of thyroid abnormalities is higher (5.4%) in children with ADHD than in the general population (<1%) (Weiss et al., 1993) and could be related to the proposed association between maternal thyroid dysfunction and ADHD in their offspring (Drover et al., 2019), and with a higher thyroid-stimulating hormone (TSH) concentration during pregnancy (Ghassabian et al., 2011). However, given the inconsistency of the findings, further analysis is required.

In addition, like in every mammal, our whole physiology, metabolism, and behavior with structured daily rhythms are driven by a circadian system (Buijs et al., 2021; Ikegami et al., 2019). Therefore, circadian desynchrony could enhance the probability of developing behavioral disruptions and conditions associated with metabolic syndrome (MetS), such as abdominal obesity, high triglycerides, and fasting glucose levels, low high-density lipoprotein (HDL) cholesterol, and hypertension (Cortese et al., 2016). Although there is a theoretical debate about a possible relationship between ADHD and MetS, there is an etiological model in which ADHD, from childhood through adulthood, is linked to an increased risk of MetS due to the following symptoms: psychiatric and somatic comorbidities, core ADHD symptoms, and sleep disturbances. When ADHD and Non-ADHD samples were compared in a national Swedish study, adults with ADHD were more likely to have Type 2 Diabetes Mellitus and hypertension (Chen et al., 2018), suggesting that obesity is the main comorbidity associated with MetS. However, this association needs to be further analyzed; it has been suggested by a meta-analysis that the association between ADHD and obesity may not be present in the pre-adolescent years, probably present in teenage girls with comorbid disorders, and present in adults (Nigg et al., 2016). A recent study reported no significant relationship between obesity and ADHD among mostly Hispanic children with a mean age of 11.64 years (6 to 17 years old) in the US-Mexico Border, and contrary to prior research, children with ADHD were more likely to be underweight. These studies highlight the need to analyze ethnicity and sex when evaluating a possible association between ADHD and metabolic disorders such as obesity(Salcido et al., 2021). Previous research has proposed an association between circadian genetic markers and thyroid function, or conditions associated with MetS (Angelousi et al., 2018).

Regarding genetics, several studies have shown that ADHD is a highly heritable disorder, with up to 76% of the phenotypic variance attributed to genetic factors (Faraone et al., 2005). Susceptibility to ADHD could be associated in part with common variation within the genome, called polymorphisms, located in or close to some genes that encode for receptors and transporters of the catecholaminergic, serotonergic, nicotinic, and glutamatergic neurotransmitter systems (Groenman et al., 2016; Klein et al., 2017; Yadav et al., 2021). However, this genetic variation may only represent a small fraction of the reported genetic variance suggested in some genome-wide association studies (Grimm et al., 2020). Other candidate genes and systems, including the circadian rhythm, have been related to ADHD (Bijlenga et al., 2019).

One of the main genes involved in this circadian system is the *Circadian Locomotor Output Cycles Kaput* (CLOCK) gene that encodes the Clock Circadian Regulator Transcription Factor (Schuch et al., 2018). ADHD has been associated with marker T3111C (rs1801260 A/G) in Caucasians (Kissling et al., 2008) and Taiwanese adults (Xu et al., 2010); in a Brazilian sample, a haplotype encompassing rs1801260 and G276A (rs4864548 G/A) was also associated with ADHD (Carpena et al., 2019). In addition, a study in a non-clinical child population described that sex potentially modified the association between rs1801260 and Body Mass Index (BMI) z-scores. In girls, the G-allele carriers had higher BMI z-scores at baseline and follow-up (Meng et al., 2020), while other studies indicated a possible interaction between rs4864548 and sleep in European children from New Zealand (Krishnan et al., 2017). This suggests that these polymorphisms may be distinctly associated with sleep duration and body weight regulation in clinical non-psychiatric and psychiatric samples.

Along with new genetic discoveries regarding ADHD, it is important to expand our knowledge of the phenotype through clinical and biological assessments, and to determine what aspects of the phenotype may be associated with genetic variants. As previously described, individuals with ADHD may have a higher risk of behavioral and metabolic conditions. Our hypothesis is that in our sample of adolescents that attend a tertiary psychiatric care institution, ADHD may be associated with two polymorphisms in the CLOCK gene (rs1801260 and rs4864548,) but also with conditions such as an evening chronotype, and differences in thyroid and metabolic function (Albrecht et al., 2020; Coogan and McGowan, 2017); this associations may be affected by sex. The aim of the study was to determine if there is an association between rs1801260 and rs4864548 CLOCK polymorphisms, ADHD and their clinical psychiatric profiles, circadian preference, thyroid function markers, and some metabolic markers in Mexican teenagers recruited in a tertiary psychiatric care institution, and if this association is affected by sex.

2. Methods

2.1. Study participants

A comparative cross-sectional study was carried out with a sample of Mexican teenagers who were recruited using a consecutive non-probabilistic sampling. The sample size calculation was calculated with the software OpenEpi v3.01 with a 95% confidence interval and 80% power and expected N of 304 subjects. They were clinically assessed from 2008 to 2016 at a tertiary care psychiatric institution in Mexico City. We included unmedicated teenagers who met the clinical assessment criteria for ADHD and any other psychiatric disorder according to the DSM-IV (American Psychiatric Association, 1994). Exclusion criteria were the diagnosis of any neurological disorders or brain damage, thyroid disorders, diabetes, dyslipidemia, or any other known endocrine dysfunction as well as schizophrenia, bipolar disorder, or any other cognitive condition that, according to the evaluator, could interfere with the ability of the subject to provide a free and informed consent to participate. This study was approved by the Ethics Committee in Research of the National Institute of Psychiatry (internal project identification PAI9487, IC112034.0) It was conducted following the Declaration of Helsinki, the guidelines of The Council for International Organizations of Medical Sciences, and the Mexican General Health Law on Health Research. Parents provided written informed consent and probands a written assent to participate.

2.2. Explanatory covariates

2.2.1. Biochemical diagnostic criteria

Given the lack of reference values in the Mexican pediatric population, we considered the age and sex standardized values reported in the international literature, including the criteria proposed by the consensus of the International Diabetes Federation (Chu et al., 2019) and the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Teenagers (2011) by the Expert Panel on Integrated Guidelines for Cardiovascular health and risk reduction in children and adolescents for the lipid panel and glucose. BMI values by age were reported according to the Growth Tables of the Centers for Disease Control and Prevention (CDC) for boys and girls (Kuczmarski et al., 2002). Reference intervals for thyroid function tests: Thyroid-stimulating hormone (TSH), total Triiodothyronine (T3), and total Levothyroxine (T4) were reported and compared to available international pediatric reference intervals such as the Canadian

Laboratory Initiative on Pediatric Reference Intervals (CALIPER) and the American Association of Clinical Chemistry's (AACC) Pediatric Reference Range Initiative (Kohse, 2015).

2.2.2. Clinical diagnostic criteria

The Morningness-Eveningness Scale for Children (MESC) score was used to measure circadian preference or chronotype. This 10-item scale evaluates the preferred timing for certain activities such as sleeping habits, self-assessment of the level of alertness, physical achievement, and mood (Adan and Natale, 2002; Díaz-Morales et al., 2007; Díaz-Morales and Escribano, 2013). The instrument was adapted to different cultural contexts and its validity has been compared to a previously reported reliability of $\alpha = 0.70$ in Spanish and Peruvian teenagers (Díaz-Morales et al., 2007). Items have a response scale with four or five options. The total score ranges from 10 (eveningness) to 43 (morningness). To determine the chronotype, extreme percentiles were considered. Scores between 23 and 28 were categorized as undifferentiated, scores >29 as morning chronotype and <22 as evening chronotype.

The clinical assessment of the teenagers with ADHD was performed by a certified psychiatrist with at least fifteen years of clinical experience. The average time of the evaluation was two hours in two separate clinical assessments. Psychiatrists who participated in this study carried out a diagnosis of any mental disorder following DSM-IV and re-assessed using the DSM-5 criteria and the Mexican version of the Brief Psychiatric Rating Scale for Children- 25(BPRS-C)) (De la Peña et al., 2005). This extended version of the original BPRS-C of 21 items includes questions assessing elimination disorders, hyperthymia, alcohol, tobacco, the use and abuse of other drugs, as well as psychological and sexual abuse. Inter-rater and test-retest reliability were $r = 0.824$ and $r = 0.661$, respectively (Lachar et al., 2001; De la Peña et at., 2005; Ulloa et al., 2011).

2.3. DNA isolation and genotyping

The DNA was isolated from saliva or blood samples using standard procedures. We performed genotyping of markers rs1801260 G/A at the 3′ untranslated region of the CLOCK gene (also known as T3111C) and rs4864548 (G276A). For rs1801260, a Bio-Rad ICycler was used, with 3 min at 94 °C followed by 30 cycles (30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C), and 10 min at 72 °C). For rs4864548, a TaqMan probe (C_11821276_10) and an Applied Biosystems 7500 Real-Time PCR thermocycler were used, with 10 min at 95 °C and 40 cycles (15 s at 95 °C and 1 min at 60 °C).

2.4. Statistical analysis

Statistical analyses were performed at the exploratory, descriptive, and inferential levels. Inferential analyses included bivariate and multivariate analyses in a case-pseudo control design. The exploratory analysis included the Kolmogorov-Smirnov test and graphical analyses for quantitative variables for normal distribution. Descriptive representation of variables was established based on the distribution of quantitative data (mean and standard deviation for normally distributed data; median and first/third quartile for non-normally distributed data). Statistical significance was set at 0.05. Bivariate analyses included: Chi-square and Fisher Exact tests to identify possible relationships between gene polymorphisms and psychiatric phenotype; Mann-Whitney tests to identify differences on quantitative measures (like thyroid variables) between psychiatric groups, polymorphisms, and sex.

To evaluate the genetic results, three inheritance models were hypothesized for each polymorphism, performing a forward stepwise regression (Iniesta et al., 2005; Salas and Carracedo, 2007) and allelic frequencies were estimated:

1.- Dominant model: Carrying one (heterozygous) or two copies (homozygous) of a certain allele is associated to the same extent with ADHD or other variables. 2.-Recessive model: Two copies of the allele are required for the association. 3.-Additive model: Each extra copy of the allele increases the probability of association by an additive dose; therefore, homozygotes for the associated allele have a higher risk than heterozygotes.

Haplotype frequencies, linkage disequilibrium (a measure of the non-random association of alleles on the same chromosome, at two regions of the CLOCK gene), and Hardy Weinberg equilibrium were calculated with Haploview 4.2 (Barrett et al., 2005).

We used the listwise deletion of cases with missing values as quality control, whereby five patients were excluded from the multivariate analysis.

In the logarithmic regression analysis, the dependent variable was the presence/absence of ADHD; CLOCK gene polymorphisms (rs1801260 and rs4864548), sex, chronotype, and age were considered as independent variables for multiple logistic regression analyses. For the linear regression, the BMI percentile, Sex, ADHD, chronotype, and polymorphisms were considered. The differences estimated in the bivariate analysis due to the presence/absence of ADHD were controlled by the introduction of the sex variable; therefore, different linear models were created. Nine models correspond either to rs1801260 or rs4864548 polymorphisms, separately, due to the existing linkage disequilibrium and high frequency of common haplotypes of these polymorphisms.

Analyses and data exploration were performed using the IBM SPSS 25.0.0 software (IBM Corp 64 bits edition) and R software.

3. Results

A sample of 347 teenagers was recruited, including 185 males and 162 females with a mean age of 13 and 14 years, respectively. Mean height was 165 and 157 cm in boys and girls, respectively. In male individuals, the mean weight/BMI were 58 kg/21.10 kg/m^2, while in females they were 55 kg/22.7 kg/m^2. In addition, the mean abdominal circumference was 78 cm in boys and 75 cm in females. Body fat percentage was lower (20%) in boys compared to girls (28%), as did the mean fasting cholesterol (145 mg/dl in boys versus 150 mg/dl in girls), HDL (44 mg/dl versus 47 mg/dl), LDL (83.6 mg/dl versus 83.8 mg/dl), and fasting triglycerides (87.5 mg/dl versus 92 mg/dl) (median values,25th and 75th percentiles of the whole sample are shown in Table 1, and by ADHD in Supplementary Table 1).

As shown in Table 2, out of the total sample, 66% were diagnosed with ADHD, primarily boys (53.31%), mostly displaying a combined presentation (80.8%). In females with ADHD, the most common psychiatric comorbidity was major depressive disorder (MDD) in 61.62%, general anxiety disorder (GAD) in 29.06%, anxiety disorder not otherwise specified in 18.60%, panic disorder 16.27%, separation anxiety in 15.11%, and dysthymia in 4.65%; while in males with ADHD we found MDD in 46.15%, anxiety disorder not otherwise specified in 19.58%, GAD in 17.48%, separation anxiety in 11.88%, dysthymia in 8.39%, and panic disorder in 6.29%. Comorbidity was also common in females without ADHD; MDD was present in 89.47%, GAD in 44.73%, panic disorder in 18.42%, anxiety disorder not otherwise specified in 17.10%, separation anxiety in 10.52%, and dysthymia in 5.26%. In males without ADHD, MDD had a frequency of 73.80%, GAD 40.47%, anxiety disorder not otherwise specified in 26.19%, separation anxiety 14.28%, panic disorder 9.52% and dysthymia 4.76%.

No differences were found in the frequency of chronotypes between teenagers with or without ADHD (unadjusted Chi$^2 = 3.28$, $P = 0.194$). However, we did find sex differences in the whole sample (unadjusted Chi$^2 = 20.45$, $P =$ 0–000,036), with higher frequencies of undifferentiated and morning chronotypes in boys. This sex difference was also significant in the ADHD group (unadjusted Chi$^2 = 9.35$, $P = 0.009$) and in the group without ADHD (Fisher test $P = 0.004$). In girls with ADHD, the undifferentiated chronotype was the most common, present in 41 girls (53.95%), followed by the evening chronotype in 22(28.95%), and morning chronotype in 13(17.10%). In males with ADHD the

Table 1
Sample characterization according to demographic and health measures in teenagers.[a].

	Males				Females				Total	
	n	Md	P25	P 75	n	Md	P25	P 75	N(%)	Md
Age $^{(Years)}$	184	14.00	13.00	16.00	162	15.00	14.00	16.00	346(100)	14.0
Weight $^{(Kg)}$*	182	58.00	52.00	68.00	162	55.60	48.50	64.00	344 (99)	57.0
Height $^{(m)}$	182	1.65	1.60	1.70	162	1.57	1.54	1.60	344 (99)	1.60
BMI $^{(kg/m^2)}$*	182	21.25	19.10	24.80	162	22.70	20.40	25.70	344 (99)	21.90
Fat percentage $^{(\%)}$ *	116	19.95	14.15	26.50	108	27.85	23.30	33.10	224 (64.7)	24.60
Abdominal circumference $^{(cm)}$	87	78.00	69.80	87.00	83	75.00	68.00	85.00	170 (49.1)	76.30
Fasting Glucose $^{(mg/dL)\ b,*}$	182	90.00	87.00	95.00	160	88.00	84.00	93.00	342 (98.8)	90.00
Cholesterol $^{(mg/dL)\ b}$	178	145.00	129.00	164.00	154	150.00	134.00	169.00	332 (95.5)	147.00
HDL $^{(mg/dL\ )b}$	79	44.00	36.00	52.00	67	47.00	39.00	54.00	146 (42.2)	44.50
LDL $^{(mg/dL)\ b}$	80	83.60	68.00	96.80	68	83.80	71.00	100.70	148 (42.7)	83.70
Triglycerides $^{(mg/dL)}$	175	87.00	58.00	127.00	153	92.00	67.00	127.00	328 (94.8)	89.50
TSH $^{(ulU/mL)}$	180	2.03	1.56	2.79	160	2.11	1.35	2.85	340(98.2)	2.08
T4 $^{(ug/dL)}$ *	180	7.76	6.83	9.09	160	8.24	7.44	8.99	340(98.2)	8.09
T3 $^{(ng/dL)}$ *	178	132.00	115.00	152.00	159	123.00	108.00	142.00	337(97.3)	127

Continuous variables are shown as median (Md), quartiles (P25 and P75) . Categorical variables are shown as number of subjects and percentages. Sample sizes are based on observed values and may vary because of missing data. Abbreviations: BMI = body mass index, HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TSH, thyroid-stimulating hormone; T3, total triiodothyronine; T4, total levothyroxine. P values calculated using chi-squared test or Fisher exact test if percentages were compared, or Mann-Whitney test if quantitative measures were compared.
Age and sex standardized rates. b. Mean/standard deviation . *. $p \leq 0.05$.

Table 2
Frequency of Comorbid Conditions in teenagers with and without ADHD.*

	ADHD**			**Non-ADHD**		
	Males*n* = 143	**Females***n* = 86	**Total** *N* = 229	**Males***n* = 42	***Females****n* = 76	**Total*N* = 118**
Combined ADHD	116	65	181	–	–	–
Hyperactive ADHD	2	1	3	–	–	–
Inattentive ADHD	26	20	46	–	–	–
Major Depressive disorder	66	53	119(55%)	31	68	99(45%)
Dysthymia	12	4	16(73%)	2	4	6(27%)
Generalized anxiety disorder	25	25	50(49%)	17	34	51(51%)
Anxiety disorder not otherwise specified	28	16	44(65%)	11	13	24(35%)
Panic disorder	9	14	23(56%)	4	14	18(44%)
Separation anxiety disorder	17	13	30(68%)	6	8	14(32%)
Post-traumatic stress disorder	6	7	13(62%)	2	6	8(38%)
Oppositional defiant disorder	105	59	164(82%)	14	20	34(18%)
Conduct disorder	33	22	55(83%)	6	5	11(17%)

Categorical variables are shown as number of subjects and percentages. Abbreviations: ADHD, attention-deficit/ hyperactivity disorder. . P values calculated using chi-squared test or Fisher exact test if percentages were compared.
*Same individual could have more than two diagnoses. ** ≤p00.05.

undifferentiated chronotype was also the most common, in 76(66.67%), followed by the morning chronotype in 25(21.93%) and the evening chronotype in 13(11.40%). In non-ADHD females, we found 27 girls (42.86%) with undifferentiated chronotype, 22(34.92%) with the evening chronotype, and 14(22.22%) with the morning chronotype. In Non-ADHD males, the frequencies were 22(64.71%) for the undifferentiated, 10(29.41%) for the morning and 2(5.88%) for the evening chronotypes, respectively.

The genetic marker Rs1801260, at position 55435202 from the p telomere on chromosome 4, had heterozygosity of 32.5%. G was the minor allele with a frequency of 20.9%, and in Hardy-Weinberg equilibrium ($p = 0.82$). Rs4864548, at position 55547636, had heterozygosity of 49.6%, being A the minor allele with a frequency of 45.9%, and in Hardy-Weinberg equilibrium ($p = 1.00$). Genotype and allele distribution of the rs1801260 and rs4864548 markers are shown in Table 3. Bivariate unadjusted analysis showed a significant genotype association between marker rs1801260 and ADHD. An allelic association was only observed in girls. Regarding rs4864548, genotype and allelic associations with ADHD were found for boys in a similar analysis. The most common haplotype was rs1801260/A-rs4864548/G, present in 52.9% of our sample, followed by A-A in 26.2%, G-A in 19.7%, and G-G in only 1.2%. There was a strong linkage disequilibrium between the markers (D' = 0.89, confidence bounds from 0.66 to 0.95; $r^2 = 0.25$).

The logarithmic regression analysis showed a significant association ($P < 0.001$) between rs1801260 and the presence of ADHD. In addition, when 342 participants were included, we confirmed that there is an association between ADHD and the AA and AG genotypes of rs1801260 (dominant model for allele A) with an OR = 3.27, 95% confidence interval 1.14–10.17 ($p < 0.001$), adjusting for sex and age. No statistical or clinical relevance was detected when the models were adjusted by other variables such as, BMI percentile and thyroid function values.

The frequency of haplotype rs1801260/A-rs4864548/G was 55.5% in individuals with ADHD and 48% in the comparison group (Chi2 = 3.53, $P = 0.06$); the rest of the haplotypes were more common in individuals without ADHD, but this difference was not statistically significant ($P \geq 0.1$). In addition, linkage disequilibrium between both markers was stronger in individuals with ADHD (D' = 0.91, confidence bounds of 0.78 to 0.97, R2 = 0.25) than in individuals without ADHD (D' = 0.86, confidence bounds of 0.66 to 0.95, R2 = 0.23).

There were no metabolic differences between teenagers with ADHD and without ADHD. However, T3 levels were higher in the group of ADHD. Analysis by sex also showed differences between these groups (Table 1). When the whole sample, regardless of the presence or not of ADHD, was analyzed, significant differences were found in the T3 levels by sex, which were 9.73 ± 3.78 ng/dl higher in male probands than in females ($p = 0.001$), and the T4 levels were 0.62 ± 0.24 ug/dl higher in females than in males ($P < 0.04$). In the linear regression, the mean of T3 and T4 values were respectively 10 units (95% CI 3.07–17.77 units) and

Table 3
Genotype and allele frequencies of rs1801260 and rs4864548 in teenagers with ADHD and without ADHD.

	ADHD ($n = 227$)			**Non-ADHD $n = 116$**			**Total $n = 343$**
Rs1801260 Genotypes	Male **n (%)**	Female **n (%)**	Total **n (%)**	Male **n (%)**	Female **n (%)**	Total **n (%)**	**n (%)**
AA	88 (61.97)	59 (69.41)	**147 (64.76)**[a]	28 (68.29)	41 (54.67)	**69 (59.48)**[a]	216 (62.97)
AG	50 (35.21)	24 (28.24)	**74 (32.60)**[a]	10 (24.39)	27 (36)	**37 (31.90)**[a]	111 (32.36)
GG	4 (2.82)	2 (2.35)	**6 (2.64)**[a]	3 (7.32)	7 (9.33)	**10 (8.62)**[a]	16 (4.67)
Alleles							
A	226 (79.58)	**142 (83.53)**[b]	368 (81.06)	66 (80.49)	**109 (72.67)**[b]	175 (75.43)	543 (79.15)
G	58 (20.42)	**28 (16.47)**[b]	86 (18.94)	16 (19.51)	**41 (27.33)**[b]	57 (24.57)	143 (20.85)
Rs4864548 Genotypes							
GG	**36 (25.53)**[c]	32 (37.64)	68 (30.09)	**10 (24.39)**[c]	22 (29.33)	32 (27.59)	100 (29.24)
GA	**84 (59.58)**[c]	35 (41.18)	119 (52.65)	**15 (36.59)**[c]	36 (48)	51 (43.96)	170 (49.71)
AA	**21 (14.89)**[c]	18 (21.18)	39 (17.26)	**16 (39.02)** [c]	17 (22.67)	33 (28.45)	72 (21.05)
Alleles							
G	**156 (55.32)**[d]	99 (58.24)	255 (56.42)	**35 (42.68)**[d]	80 (53.33)	115 (49.57)	370 (54.09)
A	**126 (44.68)**[d]	71 (41.76)	197 (43.58)	**47 (57.32)**[d]	70 (46.67)	117 (50.43)	314 (45.91)

Categorical variables are shown as number of subjects and percentages. Sample sizes are based on observed values and may vary because of missing data. Abbreviations: ADHD, attention-deficit/ hyperactivity disorder.

[a] Unadjusted differences between ADHD and non-ADHD for rs1801260; Chi2 = 6.23, $p = 0.044$.

[b] Unadjusted differences between females with and without ADHD and females for rs1801260; Chi2 = 5.56, $p = 0.018$.

[c] Unadjusted differences between males with and without ADHD for rs4864548; Chi2 = 12.20, $p = 0.002$.

[d] Unadjusted differences in allelic frequencies between males with and without ADHD for rs4864548; Chi2 = 4.07, $p = 0.044$.

0.58 units (95% CI, 0.11–1.04) lower in females than in males, with a mean of 0.134 units increase in BMI percentile (95% CI 0.01–0.26 units) in females when compared to males. There were no significant differences in TSH according to ADHD and sex ($p > 0.05$).

4. Discussion

Although many reports have associated ADHD susceptibility to various biological, social, and environmental phenomena, these aspects have been addressed in isolation. In this comprehensive study we sought to analyze a broader ADHD phenotype in Mexican teenagers and its possible association with two CLOCK gene polymorphisms, taking into consideration clinical-psychiatric profiles, circadian preferences, thyroid function, and metabolic markers. Our most significant result was that allele A of the rs1801260 polymorphism is associated with ADHD, as previously reported in other populations (Carpena et al., 2019; Kissling et al., 2008; Schuch et al., 2018; Xu et al., 2010) but now reported in Mexican teenagers. The association was confirmed with a dominant model between ADHD and the AA/AG genotypes of rs1801260. Earlier clinical studies were conducted mainly in males with ADHD and linked the chronotype with the CLOCK gene, specifically the rs1801260 polymorphism in ADHD and non-ADHD samples (Carpena et al., 2019; Kissling et al., 2008; Meng et al., 2020; Xu et al., 2010). In contrast, our sample provides a broader representation of the ADHD teenager population. There was a larger proportion of girls when compared to other studies, and the fact that a bivariate analysis showed a higher frequency of allele A in girls with ADHD could imply a possible role of rs1801260 in both boys and girls.

We did not find an association between rs1801260, and thyroid function or conditions associated with MetS. A recent systematic review and meta-analysis showed that, although marker rs1801260 was associated with MetS risk in Asian samples, it was not associated in Caucasians or Hispanics (Škrlec et al., 2021).

Among populations, the most common allele for marker RS1801260 is A, with a frequency as high as 93.2% in Han Chinese in Beijing and 92.9% in Southern Han Chinese, and as low as 54% in a sample of Gujarati Indians in Houston and 58.1% in Finish. The frequency in our sample (81.1% in the ADHD group and 75.4% in the comparison group) was close to the frequency in individuals with Mexican ancestry in Los Angeles, California (71.9%) (Auton et al., 2015). The ancestral allele A of the Rs1801260 or a genetic variant close to it may have been evolutionarily favorable or simply has not been selected against. An alternative hypothesis is that allele G of the same marker or a variant nearby could have a protective effect against ADHD. Indeed, allele A has been associated with ADHD using different methods. For example, an increased transmission of Allele A from parents to children/adolescents with ADHD was identified in a sample from Taiwan (Xu et al., 2010).

Other common variants have been associated with ADHD in children, such as the 10-repeat allele of the 40-base pair polymorphism at the 3′UTR region of the dopamine transporter gene, SLC6A3 (Grünblatt et al., 2019) or the 7-repeat allele of the 48-base pair polymorphism in exon 3 of the dopamine receptor 4 gene, DRD4 (Bonvicini et al., 2020). A hypothesis stated that long alleles of the DRD4 gene were selected because they could have adaptive value in migratory societies characterized by novelty-seeking personality, hyperactivity, and risk-taking behaviors, traits that we can clinically see and measure in the ADHD population. In a genomic analysis of the natural history of ADHD using Homo neanderthalensis and ancient Homo sapiens samples, it was found that the ancestral allele tended to be the risk variant (significant at $p \leq$ 1–8) (Esteller-Cucala et al., 2020).

We suggest that the once-adaptive components of ADHD nowadays could become problematic in highly structured societies, and these characteristics could no longer be considered adaptive and valuable. Furthermore, it could lead to chrono- and metabolic disruptions and be a

response to those changes in environments. For example, it has been suggested that Homo neanderthalensis had higher daily energy requirements than Homo sapiens (Froehle and Churchill, 2009). The International Study of Childhood Obesity, Lifestyle, and the Environment (ISCOLE) reported the correlates of obesity and lifestyle behaviors in individuals, their locality and schools confirming epidemiological transitions in obesity and physical activity, but not for other variables (Katzmarzyk et al., 2019). In countries such as Mexico, there is evidence of a nutritional transition from a mainly plant-based diet to a highly caloric and poor in nutrients diet (Roman et al., 2013).

Regarding haplotype analysis, the strong linkage disequilibrium between rs1801260 and rs4864548 indicates that their alleles are very frequently co-inherited, especially in the group of teenagers with ADHD, which should be further investigated. Different circadian genes may be involved in ADHD or in metabolic conditions associated with ADHD. In Han Chinese children, ADHD was found to be associated with a combined effect of variants of PER1, ARNTL2, and NR1D1 circadian genes (Wang et al., 2020). In addition, metabolic phenotypes such as diabetes, have been associated with PER2 circadian genes in Mexican samples (Below et al., 2011). CLOCK genes interact with each other, suggesting that variation in different CLOCK genes could be linked with molecular changes associated with ADHD and metabolic phenotypes. Future research in larger samples will help increase our knowledge about the precise implications of these results. Circadian/sleep disorders have been linked to body weight regulation in animal models (Schuch et al., 2018), and in human ADHD or non-ADHD-related pathophysiology (Ciarleglio et al., 2008; Serretti et al., 2003; Yadav et al., 2021).

An important role of CLOCK in neuronal function, mainly in the dopamine output regulation, has been shown (Faraone et al., 2005; Groenman et al., 2016; Klein et al., 2017; Yadav et al., 2021). Therefore, dopamine-related medication such as methylphenidate, atomoxetine, and amphetamines, could be related to the expression of the CLOCK gene and the circadian system regulation in ADHD (Coogan and McGowan, 2017; Meng et al., 2020; Weiss and Salpekar, 2010; Yadav et al., 2021). The rs1801260 polymorphism has also been associated with Bipolar Disorder, MDD, and schizophrenia (Benedetti et al., 2003; Lee et al., 2010; Serretti et al., 2003; Zhang et al., 2011) However, no association between this polymorphism and depression was found in Mexican adults (Calati et al., 2010).

Regarding chronotypes, modifiable and non-modifiable factors such as age have been linked as important determinants. In children, the morning chronotype is more prevalent, with a shift towards eveningness being observed during puberty. By contrast, a shift towards morningness is seen around middle age (Adan and Natale, 2002; Paine et al., 2006). Higher rates of evening chronotype were also previously found in children and adults with ADHD (Coogan and McGowan, 2017), but others suggested that the association was only clear in adults. We found that the undifferentiated chronotype was the most common in teenagers with or without ADHD, especially in boys, which was also reported in a sample from Spain (Martinez-Cayuelas et al., 2022) as same as Mexican sample of adults with depression (Calati et al., 2010). However, we could not replicate an association between ADHD and the evening chronotype, but we confirmed differences by sex in a bivariate analysis. It is possible that the sex distribution (with a higher number of girls in the non-ADHD group) and the fact that girls were older than boys influenced the lack of association between ADHD and the evening chronotype. In addition, there may have been a selection bias in our sample, if girls who seek attention at a tertiary care unit are relatively older and have more severe symptoms than boys.

The chronotype may also be affected by other aspects such as those related to modern life. Additional research with larger subgroups by age may take into consideration other biological and environmental factors and standardized criteria to determine chronotypes in teenagers, in well-characterized ADHD samples in specific populations.

Conditions associated with MetS did not vary according to the presence of ADHD. Although we found differences in thyroid T3 levels between the ADHD and non-ADHD groups, the sex differences may explain this result. As well, differences by sex in T3 and T4 were also found in German children, with a positive association between the functional fraction of T3 (fT3) and ADHD symptoms ($\beta = 0.08$; 95% CI 0.03–0.14) (Albrecht et al., 2020), closely related to the fT3 difference of 0.4 ± 0.5 pg/mL between overweight children with and without ADHD found in a similar population (Langrock et al., 2018). We found an association between the thyroid function test and the BMI percentile, but this was not strong enough to support a clinical or laboratory phenotype as previously suggested (Carpena et al., 2019; Kissling et al., 2008; Meng et al., 2020; Xu et al., 2010). Well-defined studies on clinical and laboratory assessments in healthy and psychiatric conditions may be needed.

In conclusion, in our sample of Mexican teenagers, allele A of Rs1801260 CLOCK polymorphism was associated with ADHD, however we were unable to fully determine its impact on metabolic conditions likely due to our limited sample size. This supports the need for further studies to better elucidate the complex pathophysiology of ADHD and how the environment could be influencing its expressed characteristics.

5. Limitations

We recognize that a cross-sectional design limits the interpretation of the clinical and metabolic values since we cannot establish causality and severity between the studied variables. Given that these teenagers are from a clinical sample from a tertiary-care institution, the results are not generalizable. The differences of these values among the diverse ethnic groups in the world are well known (Ciarleglio et al., 2008). Our study was likely underpowered to detect small to moderate changes. Lastly, as for the precision and interpretation of the anthropometric, clinical and laboratory characteristics, we are faced with the controversial lack of reference values specific to our Mexican pediatric population, however, recent efforts of our local scientific community are being made (Costa-Urrutia et al., 2019), and we are looking to incorporate these into our future work.

CRediT authorship contribution statement

Alfonso Cabrera Lagunes: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. **Adriana Díaz-Anzaldúa:** Conceptualization, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **Gustavo Rojas Andrade:** Conceptualization, Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Vanessa-Giselle Peschard:** Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Adriana Arias Caballero:** Investigation, Project administration, Resources, Writing – review & editing. **César Enrique Gaspar-Barba:** Investigation, Methodology, Writing – review & editing. **Arlette Yunes Jimenez:** Investigation, Methodology, Writing – review & editing. **Francisco Rafael De la Peña Olvera:** Investigation, Validation, Writing – review & editing. **Carlos Sabas Cruz Fuentes:** Methodology, Project administration, Resources, Writing – review & editing. **Miriam Feria-Aranda:** Investigation, Writing – review & editing. **Liz Sosa Mora:** Investigation, Writing – review & editing. **Armando Pérez Molina:** Investigation, Writing – review & editing. **Diana Guizar Sanchez:** Investigation. **Lino Palacios-Cruz:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None of the authors have any conflicts of interest to report.

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Supplementary materials

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