Impact of the Phosphodiesterase 8B gene rs4704397 variation on Thyroid Homeostasis in Childhood Obesity.

Running title: PDE8B modulates TSH levels in childhood obesity.

Anna Grandone¹ PHD, Laura Perrone¹MD, Grazia Cirillo¹PHD, Anna Di Sessa¹MD, Alberto Maria Corona¹MD, Alessandra Amato¹MD, Nicoletta Cresta¹MD, Tiziana Romano¹ MD, Emanuele Miraglia del Giudice¹MD.

¹: Department of Pediatrics “F. Fede” Seconda Università degli Studi di Napoli, Via Luigi De Crecchio 2, 80138, Napoli, Italy.

Corresponding Author: Prof. Emanuele Miraglia del Giudice, Dipartimento di Pediatria, Seconda Università di Napoli, Via Luigi De Crecchio N°2, 80138, Napoli, Italy.

Phone: ++39-081-5665420 Fax: ++39-081-5665427

e-mail: emanuele.miraglia@unina2.it

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ABSTRACT

Context: Several studies demonstrated that obese children have higher thyroid-stimulating hormone (TSH) than normal-weight. The polymorphism rs4704397 in the phosphodiesterase 8B (PDE8B) gene showed an association with TSH.

Objectives: i) to assess the effect of PDE8B on TSH in obese children; ii) to dissect the role of obesity degree in modulating this association; iii) to stratify the individual risk to show hyperthyrotropinemia according to PDE8B genotype.

Methods: 867 Italian obese children were investigated. Clinical data and thyroid hormones were evaluated and the PDE8B rs4704397 genotyped.

Results: PDE8B A/A homozygous subjects showed higher TSH (p=0.0005) compared to A/G or to G/G. No differences were found for peripheral thyroid hormones.

Among A/A children 22% had hyperthyrotropinemia, compared to 11.6% of heterozygotes and 10.8% of G/G (p=0.0008). Consistently, A/A had an odds ratio (OR) to show abnormal TSH of 2.25 (p=0.0004). BMI appeared correlated with TSH (p: 0.0001), but the strength of the effect of PDE8B on TSH was independent from BMI (p: 0.1).

Children were subdivided in six groups according to obesity degree and genotypes. PDE8B A/A with BMI-SDS above 3 had the highest OR (OR 2.6, p: 0.0015) to have hyperthyrotropinemia, whereas G/G with BMI-SDS below 3 showed the lowest possibilities (OR 0.3, p: 0.005).

Conclusions: We have i) showed that, in obese children, PDE8B is associated with TSH; ii) demonstrated that the interaction between adiposity and PDE8B on TSH is not synergistic, but follows an additive model iii) highlighted the impact of this association in the stratification of the individual risk to have hyperthyrotropinemia.

Key words: Obesity, thyroid function, PDE8B, children
INTRODUCTION

Attention to thyroid function in obese children is increased in recent years (1). Hypothyroidism has often been thought to be the cause of obesity, and thyroid function test is still one of the most commonly performed laboratory analysis in this group of patients. Several studies have demonstrated that obese children show higher thyroid-stimulating hormone (TSH) levels than normal weight subjects, as well as a positive correlation between TSH levels and body mass index (BMI) (2-7).

Isolated hyperthyrotropinemia is a condition characterized by serum TSH level above the statistically defined upper limit of the reference range, with normal or slightly high serum free T4 (fT4) and free T3 (fT3) concentrations (8). Whether or not increased TSH level affects the metabolic and cardiovascular profile in obese children and adolescents remains unclear, as well as the TSH decrease after weight loss (5,6). Thereby, there is still considerable disagreement regarding treatment (8).

Thyroid-stimulating hormone serum concentration has also genetic determinants, as proven in several populations (9). Recently, in a genome-wide association scan, among more than 350,000 single nucleotide polymorphisms (SNP), the rs4704397 SNP in the phosphodiesterase 8B (PDE8B) gene has been shown to be associated with circulating TSH levels. This SNP, in the general population, explained 2.3% of the variance in TSH (10). Each copy of the minor A allele was found to be associated with an increase in TSH concentration of 0.13 mIU/L. Remarkably, this polymorphism is located in intron 1 of PDE8B, a gene which encodes a high-affinity cAMP-specific phosphodiesterase expressed in the thyroid gland and, likely, regulating TSH signalling (10, 11).

In another study, this polymorphism has been associated to TSH variations during pregnancy, a condition in which screening for hypothyroidism has been recommended for the possible negative consequences on the foetus of maternal abnormal thyroid function (12).

This result, suggesting a genetic contribution to TSH variation during pregnancy, may have implications in the treatment of pregnant woman considered, under current guidelines, as affected by subclinical hypothyroidism (12).

Finally, a recent meta-analysis confirmed that the PDE8B rs4704397 polymorphism is associated to high TSH in normal subjects, showed that this polymorphism is also weakly associated with low free T4 levels and demonstrated that this association is lost in individuals on levothyroxine treatment (13).
Objectives of the present study were i) to verify the presence of the association between this polymorphism and TSH in obese children, a group of patients at increased risk to show elevate TSH levels; ii) to dissect the potential role of obesity degree in modulating this association; iii) to use this polymorphism in the stratification of the individual risk of obese children to develop abnormally elevated TSH levels.

PATIENTS AND METHODS

Cohort description and clinical evaluation

We examined 927 children and adolescents, referred to our ward (Childhood Obesity Service, Department of Paediatrics, Second University of Naples) for obesity between 1999 and 2008. Subjects with known presence of diabetes or using medications altering blood pressure, glucose or lipid metabolism, with goitre or known thyroid disease were excluded. Sixty patients (60/927: 6%) resulted having autoimmune thyroiditis because of positive auto-antibodies and ultrasonography and were, therefore, excluded from the study. The ethical committee of the Second University of Study of Naples approved the study. Informed consent was obtained by parents and, when appropriate, by children.

Of the 867 subjects enrolled, 425 were girls. Obesity was defined according to the body mass index (BMI) 95th percentile for age and sex, using the definition of the International Task Force for Obesity in Childhood and the charts for Italian population (14). Obesity degree was evaluated using the z-score BMI (BMI SDS), calculated with the LMS method (15).

Waist circumference was measured by the same operator to the nearest centimetre with a flexible steel tape measure while the subjects were standing, after gently exhaling, as the minimal circumference measurable on the horizontal plane between the lowest portion of the rib cage and iliac crest. The intra-operator coefficient of variation was 1.3%.

The average value of 2 waist measurements was obtained and, as indirect measure of the amount of abdominal fat, the ratio between waist and height (W/Hr) was calculated.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured three times while the subjects were seated, the two last measurements were averaged for the analysis, and we calculated SD according to normative values (16).

Pubertal stage was assessed using Tanner criteria (17).
After an overnight fasting, blood sample was obtained for triglycerides, high density lipoprotein (HDL) cholesterol, insulin, serum glucose, thyroid hormones (TSH, fT3, fT4).

Serum fasting glucose level was measured with glucose oxidase method. Triglycerides and HDL cholesterol were measured by an Olympus AU 560 apparatus using an enzymatic colorimetric method.

Immunoreactive insulin was assayed by IMX (Abbott Diagnostics, Santa Clara, CA). The mean intra- and inter-assay coefficients of variations were 4.7% and 7.2%, respectively. The degree of insulin resistance was determined using a homeostasis model assessment (HOMA), [insulin (mU/L) x glucose level (mmol/L) /22.5] (18).

Thyroid hormones (TSH, fT3, and fT4) were determined by high-specific solid-phase technique-chemiluminescence immunoassays (PerkinElmer, Turku, Finland).

Isolated hyperthyrotropinemia was diagnosed when TSH was higher than 4.2 µUI/ml (97.5th for our assay), with normal fT3 and fT4 and no signs or symptoms of hypothyroidism.

**Genotyping**

Patients were genotyped for *PDE8B* rs4704397 G to A variant in intron 1. The following primers were used, F: 5’-GGCGCTACTCTATGGTGTGA-3’ and R: 5’-GTCTGCTGCTGGGCTTTCC-3’. The BslI restriction enzyme was used to identify the variant, since the A allele eliminates a BslI restriction site.

Random samples were confirmed by direct genotyping that provided concordant results in all cases.

**Statistics**

A Chi Square test was used to verify whether the genotypes were in Hardy-Weinberg equilibrium and to compare categorical variables. Differences among genotypes for continuous variables were evaluated by a general linear model (GLM). When it was appropriate, age, gender and BMI SDS were used as covariates.

Levene’s test of equality of variance was used to test the differences of the variance of the quantitative trait TSH according to the *PDE8B* genotypes. This test is based on the fact that, under plausible scenarios of gene-gene or gene-environment interaction, the variance of a quantitative trait (e. g.; TSH levels) is expected to differ among the three possible genotypes of a biallelic SNP (e. g.; *PDE8B*) (18). A comparison of regression lines was performed to examine the influence of the genotypes on the relationship between TSH and BMI SDS.
Non-normally distributed variables were log transformed before the analysis, but raw means are shown. A logistic regression was performed to calculate the odds of showing abnormal levels of TSH according to the genotypes. Age, gender and, when appropriate, BMI SDS were used as covariates. Stat-Graph 3.0 software for Windows was used for all the statistical analyses. All data are expressed as means ± SD. P-values less than 0.05 were considered statistically significant and, where appropriate, were adjusted for multiple comparisons.

RESULTS

Distribution of the different PDE8B rs4704397 genotypes was in Hardy-Weinberg equilibrium (p>0.05). Two hundred and fifty-nine patients were homozygous for the wild type allele (G/G), 438 were A/G and 170 were A/A.

The clinical characteristics of the patients involved in the study are shown in Table 1.

Abnormally high TSH level (above 4.2 UI/L) was observed in 117 out of 867 patients (13%).

Difference in circulating TSH levels according to the different PDE8B genotypes was shown, with the higher levels observed in the children carrying the A/A genotype (p= 0.0012). The difference remained statistically significant after adjusting for age, gender, pubertal stage, and BMI SDS (p: 0.0005) (Table 2).

No variations among genotypes were found for BMI SDS, total cholesterol, HDL-C, triglycerides, HOMA, fT3 and fT4 (Table 2).

Thirty-eight out of 170 A/A homozygous patients (22%) had abnormally high TSH levels compared to 11.6% (51 out of 438) of heterozygotes and 10.8 % (28 out of 259) of homozygotes for the G major allele (p=0.0008). Consistently, children homozygotes for the A allele had an Odds Ratio (OR) to show abnormal TSH of 2.25 (95% C.I. 1.5-3.5, p=0.0004) compared to the patients with the other genotypes.

Based on the theoretical observation that the within genotype variance of a quantitative trait will vary when a genetic or an environmental interaction is present, we explored the presence of an interacting covariate on the genetic effect of PDE8B on serum TSH levels. Levene’s test of equality of variance (p: 0.2) did not suggested the presence of an interacting covariate.
Nevertheless, considering that BMI SDS, as expected, correlated with TSH level (p: 0.00001), we focused on the possible modulating role of obesity degree on the effect of the PDE8B polymorphism on TSH levels.

Relationship between BMI SDS and TSH levels was, therefore, analysed in function of the different PDE8B genotypes. Comparison of the slopes of the relative regression lines was not significant (p: 0.1) (Figure 1). This further strengthens the concept that the effect of PDE8B rs4704397 on TSH levels is independent from BMI. Together rs4704397 and BMI SDS explained 8.4% of the variability of TSH. The effect of the genetic component was 2.5% and the specific quantitative contribution of each minor (A) allele to TSH circulating concentration was 0.14 mIU/L, which is in line with what shown in large cohorts of adults previously described (10).

In order to obtain a complete risk stratification of the possibilities to have abnormal TSH levels, we subdivided the population of obese children in six groups according to BMI SDS (below or above 3, the generally accepted cut-off to define severe obesity) and to PDE8B genotypes (G/G, A/G or A/A).

As shown in Table 3, the prevalence of children with high TSH level is particularly elevated in the group of patients with severe obesity homozygous for the A allele (27%), while children homozygous for the G allele and less severely obese had the lowest prevalence of abnormal TSH levels (5.3%) (p: 0.0001). Logistic regression analysis showed that subjects homozygous for the PDE8B minor allele (A/A) and with BMI SDS above 3 (category I) had the highest OR (OR 2.6 (CI: 1.5-4.7) p: 0.0015) to develop abnormally elevated (> 4.2 UI/L.) TSH and that OR progressively reduced in the other groups of patients according to the simply addictive model of interaction between BMI SDS and PDE8B genotype (Table 3).

In fact, children homozygous for the PDE8B common allele (G/G) and with BMI-SDS equal or below 3 (category VI) showed the lowest possibilities to have abnormal TSH (OR 0.3 (CI:0.1-0.6) p:0.005), (Table 3).
DISCUSSION

Higher serum TSH concentrations are found in obese children and adults compared with normal-weight individuals (4, 5, 20, 21). Furthermore, TSH appears to be positively related to the degree of obesity. A positive correlation has been identified between serum leptin and serum TSH (22, 23), which could reflect the association between TSH and BMI reported in some studies (4, 8, 9, 24). Recent papers, investigating normal subjects or pregnant women, have showed that the rs4704394 polymorphism in the PDE8B gene is associated with variations in TSH levels (12, 13). In line with the data previously reported we have shown in a group of 867 Italian obese children and adolescents, that this polymorphism is associated with TSH levels. Furthermore, we have expanded the knowledge about the clinical impact of this PDE8B variant demonstrating the lack of association with BMI degree, abdominal fat and some component of the metabolic syndrome, such as systolic and diastolic blood pressure, dyslipidemia and insulin resistance. No association has been found with fT3 and fT4. The polymorphism, therefore, appears to be associated with changes in thyroid homeostasis, but not with changes in obesity-linked cardiovascular and metabolic risk factors.

The second question we have faced in this work was the dissection of the interaction between BMI degree and the PDE8B variant in modulating TSH levels in childhood obesity. Particularly, we have analyzed if the phenotypic (BMI increase) and the genetic (PDE8B polymorphism) elements work following a synergistic or an additive model in increasing TSH levels. Both the analysis of equality of variance and the comparison of the slopes of the regression lines between BMI-SDS and TSH in function of the different PDE8B genotypes suggested an additive model of interaction. In other words, the strength of the effect of PDE8B rs4704394 on TSH levels is not modulated by obesity degree. The lack of synergy of the interaction between BMI and PDE8B in increasing TSH levels may be attributable to their different pathophysiologic actions. Adiposity and PDE8B likely modulate thyroid homeostasis at different steps.

PDE8B encodes a cyclic AMP phosphodiesterase enzyme (11,24), strongly expressed in the thyroid gland (26-28) but undetectable in the pituitary gland and its influence on thyroid hormone parameters should be via the hydrolysis and inactivation of cAMP in the thyroid, in response to TSH signalling. Variations in PDE8B may, therefore, influence the thyroid hormone set point by making the thyroid less responsive to TSH (11).
Among the several mechanisms suggested to explain increased TSH levels in obesity, the most favoured one is increased leptin-mediated production of pro-TRH (3, 28, 29). Leptin innervates hypothalamic TRH-synthesizing neurons (31) and stimulates TSH production through the hypothalamic pituitary axis. Therefore, in agreement with our hypothesis, adiposity could exert its action on thyroid homeostasis at a different step (i.e.; central action) compared to the step (i.e.; peripheral action) of PDE8B variant.

Nevertheless, considering that both pregnant women and obese children represent two particular groups of subjects with frequently increased TSH, the possibility that the PDE8B gene is more an amplification gene for mildly failing or challenged thyroid function than a strong determinant of the TSH set-point cannot be a priori excluded. This also considering that in the first paper showing the association between the PDE8B rs4704394 SNP and TSH the first population investigated (Sardinian) is a low iodine intake population, the replication group is an ageing population, and the second replication group is the Amish population (32), a group known to exhibit close to 20% of thyroid autoimmunity features.

In the third part of this work we have stratified the entire population of obese children on the basis of their PDE8B genotypes and of the severity of their obesity (below or above 3 BMI SDS) in six groups with different possibilities to show abnormally elevated TSH levels. The prevalence of patients with altered TSH levels ranged from 27% to 5% according to PDE8B genotype and BMI measure.

Endocrinologists and paediatricians frequently face the decision of what to do about an obese child who has a normal fT3 and fT4 levels and TSH levels slightly above the upper range (1). For these clinicians may be of interest to know more about the intriguing relationship between thyroid and obesity and, therefore, about the individual risk of obese children to have abnormal TSH levels.

In fact, in patients whose TSH level is shifted toward the upper limits, such as in obese patients, the clinical impact of PDE8B polymorphism is more relevant as it increases the number of patients recognized as having hyperthyrotropinemia.

Concluding, we have studied for the first time the rs4704394 PDE8B polymorphism in a group of obese patients and, particularly, in a cohort of obese children and we have i) confirmed, also in this kind of patients, the association with TSH levels; ii) demonstrated that the interaction between adiposity and PDE8B in elevating TSH levels is not synergistic, but follows an additive model and, iii) highlighted the
clinical impact of this association, stratifying the individual risk to develop abnormally elevated TSH levels on the basis of the interaction between the \textit{PDE8B} genotype and the degree of obesity.

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Conflict of interest: Authors have no conflict of interest to declare.
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**Figure 1**

**Title:** Association between TSH levels and BMI SDS according to the PDE8B genotypes.

**Legend:** Regression analysis describing in 867 obese children the relationship between TSH levels and BMI SDS in patients homozygous for PDE8B G variant, heterozygous and homozygous for PDE8B A variant. The three regression lines are not significantly different as to slopes (p: 0.7).
Figure 1.

149x94mm (150 x 150 DPI)
Table 1: Clinical and laboratory characteristics of the children involved in the study

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>867</td>
</tr>
<tr>
<td>Male/Female</td>
<td>412/455</td>
</tr>
<tr>
<td>Prepuberal (%)</td>
<td>50.3</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.5 ± 2.8 (4-16)</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>2.9 ± 0.7 (1.5-8.5)</td>
</tr>
<tr>
<td>Waist to height ratio</td>
<td>0.61 ± 0.06 (0.42-0.89)</td>
</tr>
<tr>
<td>SBP-SDS</td>
<td>0.6 ± 1.1</td>
</tr>
<tr>
<td>DBP-SDS</td>
<td>0.1 ± 0.8</td>
</tr>
<tr>
<td>HOMA</td>
<td>5.3 ± 3.7 (0.3-25)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>95 ± 52 (18-344)</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>43 ± 26 (26-147)</td>
</tr>
<tr>
<td>TSH mIU/L</td>
<td>2.7 ± 1.3 (0.5-10)</td>
</tr>
<tr>
<td>fT4 (pg/ml)</td>
<td>9.9 ± 2.2 (0.93-15.9)</td>
</tr>
<tr>
<td>fT3 (pg/ml)</td>
<td>3.9 ± 0.7 (1.9-6.8)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviations (ranges). Abbreviations: BMI SDS: Body Mass Index Standard Deviation Scores; DPB-SDS: diastolic blood pressure standard deviations score; SBP-SDS: systolic blood pressure standard deviations score; HOMA-IR: homeostatic model of assessment of insulin resistance index; HDL-C: high density lipoprotein-cholesterol; SD: Standard deviation
<table>
<thead>
<tr>
<th></th>
<th>GG (%)</th>
<th>GA  (50.5%)</th>
<th>AA  (19.6%)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>259 (29.9%)</td>
<td>438 (50.5%)</td>
<td>170 (19.6%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.6 ± 2.9</td>
<td>10.5 ± 2.8</td>
<td>10.7 ± 2.7</td>
<td>0.7</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>3 ± 0.7</td>
<td>2.9 ± 7</td>
<td>2.9 ± 0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Waist to height ratio</td>
<td>0.60± 0.05</td>
<td>0.61±0.05</td>
<td>0.60± 0.06</td>
<td>0.7</td>
</tr>
<tr>
<td>SBP-SDS</td>
<td>0.76 ± 1</td>
<td>0.67±1</td>
<td>0.63 ± 1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>DBP-SDS</td>
<td>0.29 ± 0.8</td>
<td>0.15±0.7</td>
<td>0.2 ± 0.7</td>
<td>0.06</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.7 ± 4.8</td>
<td>5.5 ± 3.6</td>
<td>5.1 ± 3.8</td>
<td>0.7</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>41 ± 16</td>
<td>43± 34</td>
<td>45 ± 14</td>
<td>0.35</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>96± 46</td>
<td>97± 44</td>
<td>107 ± 73</td>
<td>0.1</td>
</tr>
<tr>
<td>TSH (mUI/L)</td>
<td>2.6 ± 1.16</td>
<td>2.7±1.25</td>
<td>3±1.5</td>
<td>0.0012</td>
</tr>
<tr>
<td>fT4 (pg/ml)</td>
<td>10±2.3</td>
<td>9.9±2.3</td>
<td>9.9±3.5</td>
<td>0.9</td>
</tr>
<tr>
<td>fT3 (pg/ml)</td>
<td>3.9± 0.7</td>
<td>3.9± 0.8</td>
<td>3.8 ± 0.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviations. GLM analysis including gender, age and pubertal stage as covariates has been used to compare continuous variables. Abbreviations: BMI SDS: Body Mass Index Standard Deviation Scores; DPB-SDS: diastolic blood pressure standard deviations score; SBP-SDS: systolic blood pressure standard deviations score, HOMA: homeostatic model of assessment of insulin resistance index; HDL-C: high density lipoprotein-cholesterol
Table 3: Risk to show abnormal TSH levels in 867 obese children stratified in six categories according to BMI SDS and PDE8B genotype.

<table>
<thead>
<tr>
<th>Categories</th>
<th>BMI SDS</th>
<th>PDE8B Genotype</th>
<th>Patients number</th>
<th>TSH (mUI/L)</th>
<th>Patients (%) with TSH&gt;4.2 mUI/L</th>
<th>OR (C.I.) p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>&gt;3</td>
<td>AA</td>
<td>70</td>
<td>3.3±1.6</td>
<td>27.1%</td>
<td>2.6 (1.5-4.7) 0.0015</td>
</tr>
<tr>
<td>II</td>
<td>&gt;3</td>
<td>AG</td>
<td>278</td>
<td>2.7±1.2</td>
<td>11.5%</td>
<td>0.8 (0.5-1.5) 0.5</td>
</tr>
<tr>
<td>III</td>
<td>&gt;3</td>
<td>GG</td>
<td>110</td>
<td>2.8±1.3</td>
<td>18.8%</td>
<td>1.5 (0.9-2.5) 0.1</td>
</tr>
<tr>
<td>IV</td>
<td>≤3</td>
<td>AA</td>
<td>160</td>
<td>2.8±1.4</td>
<td>19%</td>
<td>1.6 (0.9-2.7) 0.1</td>
</tr>
<tr>
<td>V</td>
<td>≤3</td>
<td>AG</td>
<td>278</td>
<td>2.7±1.2</td>
<td>11.8%</td>
<td>1.1 (0.7-2) 0.5</td>
</tr>
<tr>
<td>VI</td>
<td>≤3</td>
<td>GG</td>
<td>149</td>
<td>2.4±1.1</td>
<td>5.3%</td>
<td>0.3 (0.1-0.6) 0.005</td>
</tr>
</tbody>
</table>

Thyroid-stimulating hormone values are expressed as means ± standard deviations. Logistic regression analysis has been used to calculate the Odds Ratios (OR) to have TSH>4.2 mUI/L for each category of patients (from I to VI), compared to the entire cohort of children.