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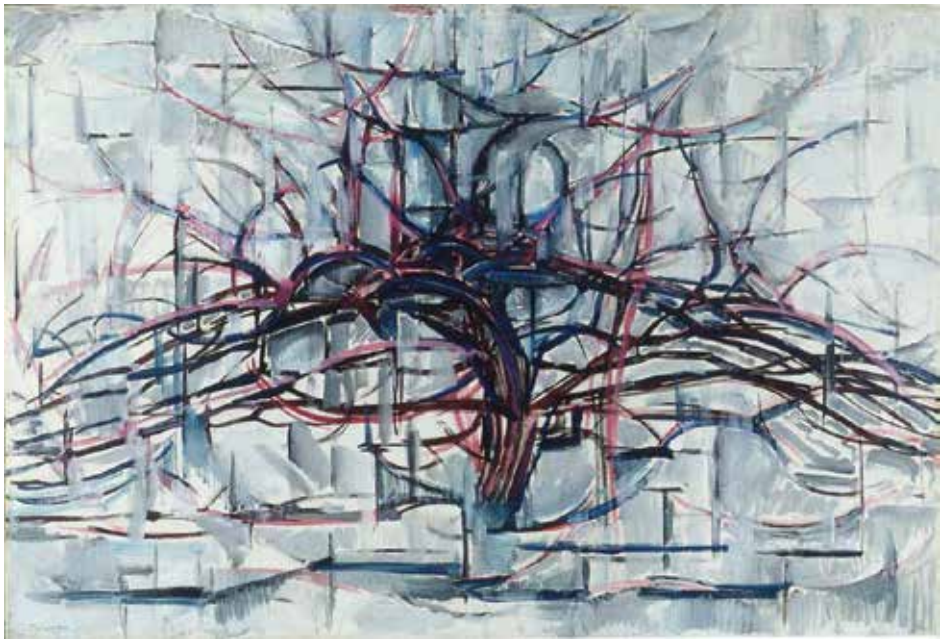

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Diagnosis of acromegaly: black, white… and sometimes gray!

Philippe Chanson[1,2]

Disease definitions often rely on cutoff values chosen to help distinguish a pathological condition from a healthy state. This is particularly true in endocrinology, where hormone hypersecretion or hyposecretion needs to be distinguished from physiological secretion. In general, endocrinological disease states are associated with clearly pathological hormone levels, largely above or below the proposed diagnostic cutoff. In acromegaly for example (1,2), most patients have obvious clinical signs and IGF-I levels markedly above the upper normal limit (ULN). But how is the ULN determined, and what does it signify? In general, the normal range of a biological marker is based on values observed in the healthy general population. If values follow a Gaussian distribution (with as many values above as below the mean), the ULN is generally set at the 97.5th percentile, corresponding more or less to the mean + 2 standard deviations (SD), while the lower limit of normal is the 2.5th percentile, corresponding more or less to the mean - 2SD.

However, it is no simple matter to establish reference values for IGF-I. Indeed, serum IGF-I concentrations rise with age during childhood and puberty, while they fall with age in adults (3). Furthermore, the distribution of IGF-I values in an apparently healthy population is non Gaussian, necessitating the use of complex mathematical transformations to obtain reference intervals for a given age group. For this reason, it is crucial to generate reference values after stratifying a large healthy population into age groups (4). Another problem is that IGF-I concentrations are influenced by many factors other than the GH concentration, including nutritional status and BMI, the use of post-menopausal hormone replacement therapy and its route of administration (5-7), kidney and liver function, and diabetic status (8). Reference IGF-I values may therefore be influenced by the inclusion criteria used to select the reference population. Elsewhere, comparisons of IGF-I assay kits show that, even in the same healthy population, IGF-I reference ranges can differ: as a result, some individuals considered to have "high" IGF-I levels measured with one assay kit may have "normal" levels when another kit is used (9). Finally, by definition, 5% of the healthy population have IGF-I levels either above the 97.5th percentile or below the 2.5th percentile. This means that 2.5% of the normal healthy population may have IGF-I levels above the ULN. All these factors may explain why some of the subjects reported in the article by Rosario and Calsolari were found to have elevated IGF-I levels despite perfectly normal GH secretion (10). The fact that IGF-I levels were above the ULN not only at the first sampling but also at the subsequent measurement five years later suggests that IGF-I levels, like other biological parameters such as TSH, tend to be "set" at an individual level which varies very little, within a range narrower than that of the reference population (11).

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GH levels are also clearly elevated in the vast majority of patients with acromegaly, both at baseline and in the oral glucose tolerance test (OGTT), making the biochemical diagnosis of acromegaly quite straightforward (2,12). However, it must be kept in mind that a few patients with clear clinical signs and high IGF-I levels may also have authentic acromegaly despite very low GH secretion, including a nadir of < 1 µg/l in the OGTT (13-18). These patients generally have a microadenoma, which can be difficult to visualize or may even have questionable pituitary MRI. Moreover, when GH output is low (basal level < 4 µg/l), the OGTT may sometimes be misleading, as GH levels can be suppressed below 0.3 µg/l in some patients with true acromegaly (17,19). The existence of these very rare cases means that all patients with clinical signs of acromegaly and elevated IGF-I levels should have the OGTT. If GH is suppressed to below 0.3 µg/l, acromegaly is unlikely but cannot be ruled out. As stated by Rosario and Calsolari, the most reasonable attitude is to monitor the patient and to repeat laboratory tests after a few months or years.

For the diagnosis of acromegaly, as in all fields of medicine, one must accept that not everything is black and white, and that there may be many shades of gray. As Osler put it, "*medicine is a science of uncertainty and an art of probability*" (20).

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Is body mass index still a good tool for obesity evaluation?

Erika Bezerra Parente[1]

Obesity has been increasing worldwide during the last decades (1,2). According to the World Health Organization (WHO 2014), 17.3% of Brazilians are obese (3). The diagnosis of obesity has huge consequences as this disease is associated with several comorbidities like diabetes, cancer, cardiovascular diseases and also increased mortality (4). Considering the pandemic of obesity, there is a need of a simple, reliable and low-cost tool for obesity evaluation.

The definition of obesity is based on the percentage of fat mass excess related to the total body weight (5). The body fat changes along aging and gender. Mean percentage of body fat may ranges from 22.9% at age 16-19 years to 30.9% at age 60-79 years in males, while mean percentage of body fat may ranges from 32.0% at age 8-11 years to 42.4% at age 60-79 years, in females (6). Therefore, how to perform this specific diagnosis of body fat excess?

Skinfold measurement is globally used for obesity diagnosis since it is quite simple and inexpensive method. The correlation of skinfolds to dual-energy-X-ray (DXA), the gold-standard, is better in non-obese people as the first method underestimates fat mass in obese ones (7).

The bioelectrical impedance analysis (BIA) is another useful method to calculate percentage of body fat and has an advantage over skinfold as it can estimate trunk fat. It is not so expensive and shows a good correlation to DXA (7).

Another recognized and validated tool is the whole-body air displacement plethysmography (ADP). Unfortunately, it is expensive and not available in many centers. Compared to DXA, ADP can overestimate body fat percentage in thinner people and underestimate body fat percentage in heavier ones (8).

The most accurate technique is the analysis of body composition by DXA, however it is also expensive and the patient is exposed to radiation. Even though it is an accurate method, the estimation of fat and lean mass by DXA software depends on levels of hydration, potassium content or tissue density (9).

Computed tomography (CT) and magnetic resonance imaging (MRI) may be useful to evaluate visceral fat or intramyocellular fat, respectively. Nevertheless they are expensive and not practical (10,11).

And what about body mass index (BMI)? In this issue of *Archives of Endocrinology and Metabolism* (AE&M), two studies addressed the issue of efficacy of BMI for obesity diagnosis and hyperglycemia screening.

BMI has limitations regarding the ability to discriminate fat mass from lean mass, which can drives to obesity over diagnosis in well trained people with high percentage of lean mass. Porto and cols. (12) studied 3,822 military firefighters divided in groups according to abdominal strength by sit-up test, cardiorespiratory fitness and age, since the

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percentage of body fat changes along aging and fitness. They found a similar prevalence of obesity estimated by BMI (13.3%) compared to percentage of body fat (%BF) (15.9%) measured by skinfold method. They verified an agreement of 85.8% between BMI and skinfold, although BMI underestimated the prevalence of obesity with high specificity (≥ 81.2%) and a low sensitivity (≤ 67.0) in all subgroups. They also found that a BMI over 30 kg/m^2 was highly specific to exclude obesity; however BMI misclassification occurred on intermediate BMI (27.0 to 30.0 kg/m^2). A limitation of this trial was the use of skinfold as the reference method for %BF instead of DXA. Skinfold have limitations like poor reproducibility and variation among different populations (8); nevertheless the authors were careful to use Brazilian references. Probably would be better to have DXA as the reference for body composition; however its high cost is the main barrier to be used in a large sample size.

Another BMI limitation is its lack of ability to identify visceral fat which is relevant to metabolic diseases and cardiovascular risk. In this *AE&M* issue, Quadros and cols. (13) studied 1,139 schoolchildren aged from six to seventeen years in order to evaluate the ability of BMI, waist circumference (WC) and waist-to-height ratio (WHR) to discriminate hyperglycemia. Hyperglycemia prevalence was 6.6% and it was more commonly present in young people with excess of weight, high WC and high WHR. The accuracies to discriminate hyperglycemia were significant, but low, for the individual (BMI = 0.56; WC = 0.53; WHR = 0.55) and combined indicators (BMI + WC = 0.55; BMI + WHR = 0.55). In addition, it was shown that adding WC and WHR measurement did increase the accuracy of BMI to diagnosis hyperglycemia in this pediatric population. Albeit the correlation between BMI and metabolic diseases, WC and cardiovascular risk is well established in adult population, these relationships in children and adolescents are still controversial. Other authors as Kuba and cols. (14), who studied children from six to ten years old, verified different results: significant correlations between WHR and BMI z score with cardio metabolic risk markers. Differences among ethnics, age and fat distribution may change the correlation between anthropometric indicators and metabolic disease. These variables could be more important among children and adolescents as BMI, WC and fat distribution change along growth. Quadros and cols. (13) did not demonstrate advantage to add WC and WHR to BMI for hyperglycemia screen, however they performed only one measurement of fasting glucose and this can be a bias for under diagnosis. Although anthropometric indicators correlation to metabolic diseases in children and adolescents is still in discussion, they should continue be used because of their simplicity, low cost and noninvasive method.

Therefore, is body mass index still a good tool for obesity evaluation? The answer is: yes, it is. Albeit BMI does not define fat distribution, it is simple, reliable, with low cost and a good correlation with metabolic disease in adults; even though with some limitations in the pediatric population.

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Elevated IGF-1 with GH suppression after an oral glucose overload: incipient acromegaly or false-positive IGF-1?

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ABSTRACT

Objective: To report the evolution of patients with a suggestive clinical scenario and elevated serum insulin-like growth factor-1 (IGF-1), but growth hormone (GH) suppression in the oral glucose tolerance test (OGTT), in whom acromegaly was not initially excluded. **Subjects and methods:** Forty six patients with a suggestive clinical scenario, who had elevated IGF-1 (outside puberty and pregnancy) in two measurements, but GH < 0.4 µg/L in the OGTT, were selected. Five years after initial evaluation, the patients were submitted to clinical and laboratory (serum IGF-1) reassessment. Patients with persistently elevated IGF-1 were submitted to a new GH suppression test and magnetic resonance imaging (MRI) of the pituitary. **Results:** Four patients were lost to follow-up. During reassessment, 42 patients continued to show no "typical phenotype" or changes in physiognomy. Fifteen of the 42 patients had normal IGF-1. Among the 27 patients with persistently elevated IGF-1 and who were submitted to a new OGTT, GH suppression was confirmed in all. Two patients exhibited a lesion suggestive of microadenoma on pituitary MRI. In our interpretation of the results, acromegaly was ruled out in 40 patients and considered "possible" in only 2. **Conclusion:** Our results show that even in patients with a suggestive clinical scenario and elevated IGF-1, confirmed in a second measurement and without apparent cause, acromegaly is very unlikely in the case of GH suppression in the OGTT. Arch Endocrinol Metab. 2016;60(6):510-4

Keywords
Acromegaly; elevated IGF-1; GH suppression

INTRODUCTION

As emphasized by some authors, acromegaly is not always accompanied by a typical phenotype: "acromegaly is a clinical syndrome that may not manifest with clear diagnostic features" (1), "some patients with acromegaly have mild or absent clinical features" (2), "the diagnosis does not require the presence of typical phenotypic features" (3), and "we suggest the measurement of IGF-1 in patients without the typical manifestations of acromegaly, but who have several associated conditions" (4). Therefore, patients with a suggestive clinical scenario should be investigated even in the absence of typical phenotypic features (1-5). The finding of elevated IGF-1 outside puberty and pregnancy strongly supports the hypothesis of acromegaly. Although the diagnosis is confirmed traditionally by the lack of GH suppression in the oral glucose tolerance test (OGTT), cases of acromegaly in the presence of nadir GH < 1 µg/L (6-9) and even < 0.4 µg/L (8,9) have been reported. Many authors therefore consider that the disease should not be readily excluded based on GH suppression in patients with a suggestive clinical scenario and elevated IGF-1 (2,8-12). "Recognition that acromegaly can be accompanied by apparently normal GH concentrations and dynamics, and mild or absent clinical features indicates the importance of IGF-I measurements for diagnosis" (2).

The objective of the present study was to report the evolution of patients with a suggestive clinical scenario (1-5) and elevated IGF-1 (in two measurements and in the absence of another apparent cause), but GH suppression in the OGTT, in whom acromegaly was not initially excluded (2,8-12).

SUBJECTS AND METHODS

Patients

First, 4,350 adults (age between 18 and 70 years, excluding pregnant women and patients with known

pituitary disease) underwent acromegaly screening: 2,270 patients with type 2 diabetes mellitus or glucose intolerance (13), 178 patients who reported "enlargement of their extremities" (14), and 1,902 patients with two or more comorbidities related to acromegaly [including arterial hypertension in 1,806 patients (15)]. In patients with elevated IGF-1, a new measurement was obtained and combined with the measurement of GH during an OGTT. For this study, 46 patients with a suggestive clinical scenario (1-5) according to the definition below (1,4,5), who had elevated IGF-1 (outside puberty and pregnancy) in two measurements, but GH < 0.4 μg/L in the OGTT (1,11,12,16), were selected. The study and its respective protocol were approved by the Ethics Committee of our institution.

Follow-up

For this study (5 years after initial evaluation), the patients were submitted to clinical and laboratory (serum IGF-1) reassessment. The aim of clinical examination was to identify typical phenotypic features (see below) and changes in physiognomy by comparing current photographs with those obtained at the time of initial evaluation. Patients with persistently elevated IGF-1 were submitted to a new GH suppression test and magnetic resonance imaging (MRI) of the pituitary.

Definitions

A typical acromegalic phenotype was defined i) by an endocrinologist with experience in the disease (P.W.R.); ii) based on ectoscopy, and iii) considering acral enlargement and maxillofacial changes (4).

A suggestive clinical scenario was defined in the presence of two or more comorbidities related to acromegaly according to the Canadian Consensus (5), American Association of Clinical Endocrinologists (1), and Endocrine Society (4). The comorbidities considered were (1,4,5): i) nonspecific chronic headache (for example, migraine and hypertensive headache were not considered); ii) generalized and persistent excessive sweating; iii) diffuse arthralgias associated with some radiologic alteration (17) in the absence of known rheumatological disease (reported by the patient, suspected, or confirmed in the medical record); iv) chronic fatigue not explained by any other underlying disease (among the diagnoses reported by the patient or present in the medical record); v) bilateral paresthesias (Carpal tunnel syndrome); vi) recently diagnosed diabetes mellitus; vii) recently diagnosed arterial hypertension requiring antihypertensive medication.

METHODS

The samples were collected in the morning after an approximately 10-h fast, with the subject resting for 20 min before and during the OGTT. For the OGTT, GH was measured before and 30, 60, 90 and 120 min after the oral administration of 75 g anhydrous glucose.

GH was measured with a chemiluminescence assay (Immulite, Diagnostic Products Corporation, Los Angeles, CA) with an analytical sensitivity ≤ 0.05 μg/L. The standard provided by the kit was calibrated against the World Health Organization (WHO) 2nd International Standard (IS) 98/574. The results are expressed as μg/L. IGF-1 was also measured with a chemiluminescent assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA) (analytical sensitivity of 25 μg/L) using antibodies highly specific for IGF-1 and standards calibrated against the WHO IS 87/518 preparation and previously established reference values stratified by age based on a sample of 1,000 subjects rigorously selected in the same town where the study was conducted (18). "Functional separation" (acidification followed by saturation with IGF-II) was the technique used to exclude interference from IGF-binding proteins (IGFBPs) (18).

MRI of the pituitary (at 3 tesla) was obtained using gadolinium as contrast agent.

RESULTS

The study included 24 women and 22 men aged 30 to 60 years (median 45 years) with initial IGF-1 ranging from 1.05 to 1.5 times the upper limit of normal range (ULN) for age (18). Thyroid dysfunction and pregnancy (in premenopausal women) were excluded in all patients on initial assessment and 5 years later.

Four patients were lost to follow-up. During reassessment, 42 patients continued to show no "typical phenotype" or changes in physiognomy.

Fifteen of the 42 patients had normal IGF-1 (confirmed in two measurements); none of the patients had kidney or liver failure, malnutrition, uncontrolled hypothyroidism or used oral estrogen; six patients had diabetes mellitus, but were compensated at the time of

IGF-1 measurement. The body mass index change of these patients ranged from – 1.2 to + 2 kg/m^2 (initial assessment versus 5 years later).

Among the 27 patients with persistently elevated IGF-1 and who were submitted to a new OGTT, GH suppression was confirmed in all. Comparing the final and initial concentrations, none of the patient exhibited a significant increase in IGF-1, i.e., increment > 20% [limit defined based on the variation found in 100 healthy (rigorously selected) subjects in stable conditions, in whom IGF-1 was measured at an interval of 3 months using the same assay as employed in this study (18)]. The last IGF-1 ranged from 1.12 to 1.63 times the ULN, already considering the current age of the patient. IGFBP-3 was also measured in these 27 patients, and was normal in 17 patients and slightly elevated in 10 (ranging from 1.02 to 1.2 times the ULN). Two patients exhibited a lesion suggestive of microadenoma on pituitary MRI (hypointense nodule measuring 4 and 5 mm in diameter and showing no contrast enhancement after the administration of gadolinium). Details of these two patients are shown in Table 1. It should be noted that other hormone hypersecretions were also excluded in these two cases.

In our interpretation of the results, acromegaly was ruled out in 40/42 patients and considered "possible" in only 2/42 (with persistently elevated IGF-1 and microadenoma detected by MRI, but with GH suppression and without clinical or laboratory progression).

DISCUSSION

There is consensus that not only patients with typical phenotypic features should be investigated for acromegaly (1-5). The patients included in this study had two or more comorbidities commonly found in "active" acromegaly (1,4,5), and additional criteria were required so that they were considered compatible with this condition (see Methods). Moreover, the age range of the patients (30-60 years) coincides with that of a higher incidence of the disease. Consequently, there was a suggestive clinical scenario justifying investigation for acromegaly (1-5).

Elevated IGF-1 does not always indicate acromegaly, but its specificity increases when measured outside puberty and pregnancy (situations characterized by physiological elevation of this hormone). Furthermore, the results should be confirmed in a subsequent measurement. One cause of falsely elevated IGF-1 are inadequate limits of normality. When defined using an inadequately selected sample or an insufficient number of subjects, the upper limit may be underestimated and, consequently, an individual with normal IGF-1 may be erroneously classified as having elevated IGF-1. In the present study, IGF-1 was considered elevated based on the limits established from a sample of 1,000 subjects from the same town as the patients included in this study, who were selected rigorously (exclusion of interfering conditions and medications and extremes of body mass index) and stratified by decade of life (18) according to current recommendations (16,19,20). Hence, in the present study "elevated IGF-1" refers to the measurement obtained outside puberty and pregnancy, confirmed in two measurements, and based on adequate normative information.

Although theoretically possible, heterophile antibodies are not cited as possible agents that interfere with serum IGF-1 (19,20). Moreover, the only case report in the literature mentioning interference of these antibodies with the Immulite assay inexplicably found a reduction in IGF-1 (21). The assay used does not show cross-reactivity to insulin or IGF-II and is highly specific for IGF-1. Finally, "functional separation" (acidification followed by saturation with IGF-II) was used to exclude interference from IGFBPs. Nevertheless, using this assay, eventual interference from IGFBPs would cause a reduction in IGF-1 (19,20).

Overweight/obese subjects have higher hepatic sensitivity to GH. This fact explains the maintenance of IGF-1 concentrations within the normal range despite the reduced secretion of GH observed in these individuals (22). However, there is no elevation

Table 1. Results of the last evaluation of patients with microadenoma on MRI

Patient	Sex	Age (years)	IGF-1 (x ULN)	IGFBP-3	Nadir GH (µg/L)	MRI	Clinical scenario
1	M	52	1.5	Normal	0.2	Microadenoma	Osteoarthritis, hypertension, dyslipidemia, GI, hyperhidrosis
2	F	50	1.42	Normal	0.3	Microadenoma	Headache, paresthesias, hypertension, GI

ULN: upper limit of normal range; MRI: magnetic resonance imaging; GI: glucose intolerance.

of serum IGF-1 (22). It has also been suggested that genotype d3 of the GH receptor (d3-GHR) increases sensitivity to this hormone (23). This fact may explain, at least in part, the higher concentrations of IGF-1 in some patients with acromegaly (for a given concentration of GH), or the greater increase in IGF-1 seen in some patients during treatment with GH (23). However, to our knowledge, there is no study reporting an association between the presence of d3-GHR and elevated IGF-1 in individuals without acromegaly and not treated with GH.

Despite the suggestive clinical scenario and careful definition of "elevated IGF-1" used in this study (see above), after 5 years none of the patient exhibited phenotypic features or changes in physiognomy and 1/3 had spontaneous normalization of IGF-1. All of the patients with persistently elevated IGF-1 continued to present GH suppression and 93% had no apparent tumor on MRI.

Although we cannot rule out acromegaly in the two patients of this series with adenoma on MRI, we believe it is highly unlikely. In addition to persistent GH suppression, reassessment after 5 years (without any intervention) corroborates this conclusion. Considering the interval between the onset of manifestations and the diagnosis in the presence of a typical phenotype (24,25), the absence of the latter and of changes in physiognomy after several years makes the disease unlikely. The lack of an increase in IGF-1 after this period also weakens the diagnosis. We therefore believe that the combination of these findings (persistent suppression of GH, absence of the occurrence of phenotypic features or changes in physiognomy and of an increase in IGF-1 after 5 years) renders acromegaly highly unlikely in these two cases.

In a previous study, acromegaly was not diagnosed in any of the adult patients without a clinical suspicion of the disease and with slightly elevated IGF-1 (up to 1.2 x ULN), but this increase was confirmed in only 15% of the patients (a second measurement was unavailable or normal in the remaining patients) (26). Our results now show that even in patients with a suggestive clinical scenario (1-5) and elevated IGF-1 (> 1.2 x ULN in some), confirmed in a second measurement and without apparent cause, acromegaly is very unlikely in the case of GH suppression in the OGTT. Consequently, the indication of pituitary MRI is questionable in this situation. As discussed earlier, known causes of IGF-1 elevation were excluded and analytical interfering agents do not explain the persistently elevated IGF-1 seen in these patients. We do not know whether these individuals correspond to the portion of the "normal" population that exhibits concentrations outside the reference range, are more sensitive to endogenous GH, or have GH hypersecretion, although not tumoral and suppressive in the OGTT. Further studies on this topic are necessary. Additionally, we do not know whether these persistently elevated concentrations of IGF-1 increase the risk of comorbidities despite the absence of acromegaly, remembering that all of these patients had a combination of two or more of these comorbidities.

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Agreement between BMI and body fat obesity definitions in a physically active population

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ABSTRACT

Objectives: Body mass index (BMI) is a widely used proxy of body composition (BC). Concerns exist regarding possible BMI misclassification among active populations. We compared the prevalence of obesity as categorized by BMI or by skinfold estimates of body fat percentage (BF%) in a physically active population. **Subjects and methods:** 3,822 military firefighters underwent a physical fitness evaluation including cardiorespiratory fitness (CRF) by the 12 min-Cooper test, abdominal strength by sit-up test (SUT) and body composition (BC) by BF% (as the reference), as well as BMI. Obesity was defined by BF% > 25% and BMI ≥ 30 kg/m^2. Agreement was evaluated by sensitivity and specificity of BMI, positive and negative predictive values (PPV/NPV), positive and negative likelihood (LR+/LR-), receiver operating characteristic (ROC) curves and also across age, CRF and SUT subgroups. **Results:** The prevalence of obesity estimated by BMI (13.3%) was similar to BF% (15.9%). Overall agreement was high (85.8%) and varied in different subgroups (75.3-94.5%). BMI underestimated the prevalence of obesity in all categories with high specificity (≥ 81.2%) and low sensitivity (≤ 67.0). All indices were affected by CRF, age and SUT, with better sensitivity, NPV and LR- in the less fit and older groups; and higher specificity, PPV and LR+ among the fittest and youngest groups. ROC curves showed high area under the curve (≥ 0.77) except for subjects with CRF ≥ 14 METs (= 0.46). **Conclusion:** Both measures yielded similar obesity prevalences, with high agreement. BMI did not overestimate obesity prevalence. BMI ≥ 30 was highly specific to exclude obesity. Because of systematic under estimation, a lower BMI cut-off point might be considered in this population. Arch Endocrinol Metab. 2016;60(6):515-25

Keywords

Body composition; firefighters; cardiorespiratory fitness; sensitivity; specificity; BMI; body fat

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INTRODUCTION

Obesity is an epidemic condition that has grown dramatically over the past 50 years, although some data suggest the rate of growth is now slowing (1-3). Nonetheless, recent studies have shown an increase in obesity prevalence in developing countries similar to what was firstly experienced by developed countries (4). The prevalence of obesity has almost doubled between 1980 and 2008 worldwide and increases have occurred in every world region evaluated by the World Health Organization (5). Considering this pandemic scenario and that obesity is a condition associated with other major comorbidities and public health problems, like diabetes (6) and cardiovascular diseases (7), its identification or diagnosis must be based on reliable, simple, and low-cost tools. Several methods and/or techniques have been used to evaluate body composition and to categorize obesity, either for research or for clinical purposes (8). Skinfold thickness and bioelectrical impedance are commonly used for estimating and monitoring body composition in athletics and occupational screenings. The most accurate technique, although more expensive, is the analysis of body composition by the dual-energy X-ray

absorptiometry (DEXA), which is considered to be the gold-standard (9,10). The precision, convenience and cost of these methods vary, so that the test of choice depends on the goal of measurement, time available and resources.

Among this variety of body composition determination methods, body mass index (BMI) is the most widely used and recommended by scientific associations (10,11). One of the best known longitudinal studies that had used BMI to continuously assess obesity prevalence, health-related risks and nutritional status of American adults and children, apart from others outcomes, is the National Health and Nutrition Examination Survey (NHANES) (12). In Brazil, since 2006, the Ministry of Health is conducting similar survey (VIGITEL) with the aim to identify health-related risk factors by phone interviews (13). Like NHANES, VIGITEL also characterizes obesity based on a BMI cut point of ≥ 30 kg/m^2.

Although some possibilities of misclassification of muscle mass as body fat exist (14), BMI has been widely accepted as an appropriate method to estimate obesity prevalence in the public health and health risks contexts. For the most part, misclassification is a significant concern when body composition is evaluated, as in athletic or in physical performance conditions, where the prevalence of fit people with additional weight due to muscle mass is potentially higher than in the general population. In it this context, is plausible to consider that BMI may overestimate overweight and obesity among athletes and among some working populations that are supposedly more active, such as firefighters and other public safety professions. In order to test this hypothesis, we compared the prevalence of obesity as categorized by BMI (≥ 30 kg/m^2) or by body fat percentage (> 25%) in a large cohort of military firefighters.

SUBJECTS AND METHODS

Experimental approach

We performed a cross-sectional study with data collected from standardized records of the 2009 Brasilia Military Firefighters' annual physical fitness evaluations. All participants were military career firefighters who worked for the Federal District (Brasilia) Military Firefighter Brigade (CBMDF – Portuguese acronym). The CBMDF includes all fire departments in the state of the Brazilian Federal District, where the capital Brasília is located. All data were originally collected for occupational purposes as part of the mandatory annual physical evaluation for all CBMDF firefighter under 50 years old. Firefighters above 50 years old were excluded from this study because they perform a different physical evaluation. All physical fitness evaluations were completed during May 2009. This study is part of a project focused in Brasilia firefighter's physical fitness and occupational health: The Brasilia Firefighters Study – BFS.

Subjects

Among the 4216 available physical fitness evaluation records in the database, 394 (9.3%) were excluded: 212 (5.0%) were women and, additionally, 182 (4.3%) men because of incomplete or data containing biologically implausible values. Therefore, all analyses were performed on the cohort of 3,822 men from the CBMDF who had complete data for all variables under analysis.

The use of the recorded physical evaluation data for research purposes was approved by the University of Brasília Faculty of Health Sciences Ethics Committee on Human Research and an authorization from the CBMDF was properly obtained for this study.

Physical activity evaluation

The CBMDF physical evaluation includes components of health-related physical fitness (HRPF). For this study we focused on the body composition, cardiorespiratory fitness (CRF) and abdominal muscle endurance components in order to evaluate the overall agreement between BMI and BF% for defining obesity and its variance across different HRPF groups and age categories.

Body composition was assessed by the body fat percentage (BF%), as the reference method, using the Guedes 3-skinfolds formula, that is based on Brazilian population data (15), as well as BMI according to international guidelines. BMI was calculated using the formula: BMI = (weight in kilograms)/(height in meters)^2. Guedes 3-skinfolds formula is a body fat percentage estimation validated from a Brazilian population that uses the tricipital (TR), suprailiac (SI) and abdominal (AB) skinfolds in a body density formula: D = 1,17136 - 0,06706 log (TR + SI + AB), where "D" is the body density. With the "D" value

calculated, we used the SIRI equation: BF% = [(4.95 / D) - 4.50] x 100, to obtain the BF% (15).

Cardiorespiratory fitness (CRF) was estimated by the 12 min-Cooper test, which is widely accepted as an indirect method for estimating the maximum oxygen consumption (VO_2Max in $mL \cdot kg^{-1} \cdot min^{-1}$). The Cooper test is a running test in which the objective is to run as far as possible within 12 minutes. The distance reached is converted into oxygen consumption (VO_2) using a validated formula (16). All tests were performed on the same athletic running track to improve the test's precision and standardization among participants. For comparisons with other fire service studies, CRF was converted to metabolic equivalents (METs) by dividing VO_2Max values by 3.5 (17).

Abdominal muscular endurance was assessed by the Sit-Up test that is a timed test in which the firefighter had to perform as many sit-up repetitions as possible in one minute, as suggested by the American College of Sports Medicine (ACSM) (18). Apart from CRF and body composition, the abdominal muscle evaluation (Sit-Up test) was included since muscular fitness is a health-related physical fitness component that should be trained to improve health (19) and because it was shown that core strength training could reduce injuries within firefighters (20).

Obesity criteria and analysis

Obesity was defined using standard cut-off points for both indices: BF% > 25% and BMI ≥ 30 kg/m² (21). Comparisons between the two measures were determined using BF%-defined obesity as the reference measure. Agreement analysis was done by the following epidemiological indexes: 1) total agreement (TA), or accuracy, as the sum of the percentage of true positive (TP) and true negative (TN) values (TA = TP + TN); 2) BMI-sensitivity (sensitivity = [TP / (TP + FN)] x 100%), where FN is false negative; 3) BMI-specificity (specificity = [TN / (TN + FP)] x 100), where FP is false positive; 4); 4) Positive predictive value (PPV = [TP / (TP + FP) X 100]); 5) Negative predictive value (NPV = [TN / (TN + FN) X 100]); 6) Positive likelihood ratio (LR+ = [TP / (TP + FN)] / [FP / (FP + TN)]); Negative likelihood ratio (LR- = [FN / (TP + FN)] / [TN / (FP + TN)]) (22). All epidemiological indexes were calculated as their point value and 95% interval of confidence (95%IC).

In order to evaluate if there was any better BMI cut-off point different from the standardized one, we also employed the receiver operating characteristic (ROC) curve approach to analyze agreement and to explore different BMI cut-off points for this specific population.

Considering the possible influences of age and physical fitness on BMI, all agreement analyses were also performed stratifying participants by age, by CRF, using the CRF categories proposed by Baur and cols. (23), and by sit-up performance.

Because many variables were found to be non-normally distributed by skewness and kurtosis test for normality, we used the Cuzick nonparametric test for trend (24) and the area under the ROC curves were compared using the method proposed by Hanley and McNeil (25), with a Bonferoni *post-hoc* test. Pearson correlation coefficient between BMI and BF% was also calculated. Statistical significance was set as a two-tailed p value < 0.05. We used the IBM SPSS Statistics® v17 (IBM Corporation, USA) and Stata 12.1 SE (Stata Corporation, College Station, TX) software packages for processing, analysis, and graphic design of the data.

RESULTS

Mean age ± standard deviation (sd) of the participants was 37.4 ± 4.8 years old, varying from 24 to 49 years. The mean ± sd BMI was 26.5 ± 3.2 kg/m², ranging from 17.6 to 41.4 kg/m².

The overall characteristics of the study population in terms of age, CRF and sit-ups distribution, categorized by BMI subgroups, are shown on Table 1. There was a clear trend for a reduced physical fitness and an increase in age while BMI increases from its normal values (< 25.0 kg/m²) to the overweight and obese categories.

The overall prevalence of obesity estimated by BMI (13.3%) were similar to that obtained by BF% (15.9%), although BMI underestimated the prevalence of obesity in all analyzed categories. The overall difference between these estimates was -2.6%, varying across the CRF, sit-ups and age subgroups, but always smaller than -5.6%. However, the average relative differences were higher (-22.5%) and showed wide variation among subgroups (-2.9% // -65.9%) (Table 2).

We observed a high total agreement: 85.8% (95%CI: 84.7 – 86.9), with good specificity and poor sensitivity: 93.1% (95%CI: 92.2 – 93.9) and 47.4% (95%CI: 43.4 – 51.5), respectively. Furthermore, the agreement varied significantly with the variance in obesity prevalence in different age and physical fitness subgroups (Tables 3-6).

Table 1. Characteristics of the study population. Brazil, 3,822 male firefighters, 2009

	Body mass index			*P* trend[a]
	≤ 25 kg/m²	25–29 kg/m²	≥ 30 kg/m²	
N (row %)	1,274 (33.3)	2,038 (53.3)	510 (13.3)	
Age (yrs), mean (SD)	36.8 (5.0)	37.5 (4.7)	38.6 (4.6)	< 0.001
CRF (MET), mean (SD)	12.7 (1.7)	12.0 (1.7)	10.4 (1.5)	< 0.001
Sit-ups (rep), mean (SD)	27.8 (4.7)	27.4 (5.3)	25.3 (5.7)	< 0.001

CRF: cardiorespiratory fitness; BMI: body mass index; rep: repetitions; [a] nonparametric test for trend (Cuzick).

Table 2. Relative prevalence of obesity by BF% and BMI in Brazil, 3,822 male firefighters, 2009

	Overall	CRF (MET)				Sit-ups (n)				Age (years)		
n	3,822	546	1,259	1,508	509	315	891	2,019	597	329	2,521	972
n %	100	14.3	32.9	39.5	13.3	8.2	23.3	52.8	15.6	8.6	66.0	25.4
		< 10	**10 - 12**	**> 12 - 14**	**> 14**	**≤ 30**	**30 - 40**	**41 - 50**	**> 50**	**20 - 30**	**31 - 40**	**41 - 50**
BF% (%)	15.9	41.6	19.9	7.2	4.1	36.8	21.5	12.9	6.5	9.1	16.1	17.5
BMI (%)	13.3	38.8	17.2	4.9	1.4	31.4	18.1	10.9	4.9	5.8	12.9	17.0
Δ Absolute	-2.6	-2.8	-2.7	-2.3	-2.7	-5.4	-3.4	-2.0	-1.6	-3.3	-3.2	-0.5
Δ Relative	-16.4	-6.7	-13.6	-31.9	-65.9	-14.7	-15.8	-15.5	-24.6	-36.3	-19.9	-2.9

CRF: cardiorespiratory fitness; BF%: body fat percentage; BMI: body mass index; Δ: variation.

Table 3. Agreement between BMI and body fat percentage for defining obesity in Brazil, 3,822 male firefighters, 2009

			Body fat % > 25%		
			Yes	No	Total
			N (%)	N (%)	N
BMI ≥ 30 kg/m²	Yes	N (%)	288 (7.5%)	222 (5.8%)	510
	No	N (%)	319 (8.3%)	2,993 (78.3%)	3,312
	Total	*N*	607	3,215	3,822

Agreement: 85.8% (95%CI 84.7–86.9)
Sensitivity: 47.4% (95%CI 43.4–51.5)
Specificity: 93.1% (95%CI 92.2–93.9)
Positive likelihood ratio: 6.87 (95%CI 5.90–8.00)
Negative likelihood ratio: 0.56 (95%CI 0.52–0.61)
Positive predictive value: 56.5% (95%CI 52.0–60.8)
Negative predictive value: 90.4% (95%CI 89.3–91.4)

Alternative cut-offs			
Cut-off	27 kg/m²	28 kg/m²	29 kg/m²
Agreement, % (95%CI)	69.9 (68.5–71.4)	77.7 (76.4–79.0)	82.9 (81.7–84.1)
Sensitivity, % (95%CI)	82.4 (79.1–85.3)	72.2 (68.4–75.7)	60.5 (56.4–64.4)
Specificity, % (95%CI)	67.6 (65.9–69.2)	78.8 (77.3–80.2)	87.2 (86.0–88.3)
Positive likelihood ratio, (95%CI)	2.54 (2.39–2.70)	3.40 (3.13–3.70)	4.72 (4.22–5.27)
Negative likelihood ratio, (95%CI)	0.26 (0.22–0.31)	0.35 (0.31–0.40)	0.45 (0.41–0.50)
Positive predictive value, % (95%CI)	32.4 (30.1–34.8)	39.1 (36.2–42.0)	47.1 (43.6–50.7)
Negative predictive value, % (95%CI)	95.3 (94.4–96.1)	93.7 (92.8–94.6)	92.1 (91.1–93.0)

The ROC curves for BMI to detect BF%-defined obesity showed an overall area under the curve (AUC) equal to 0.83. When stratified by age, all age-category AUC were similar (p = 0.65) and above 0.82. The same profile was observed for the sit-up stratification, in which the sit-up-categories AUC ranged from 0.77 to 0.85, with no differences among categories (p = 0.14). After stratifying by CRF, the ROC curves analysis showed a low AUC (0.46) for those with the highest CRF (> 14 MET) that was statistically different from all others CRF categories (AUC ≥ 0.78; p < 0.05) but similar within them (p > 0.05) (Figure 1). Further information of the BMI diagnostic performance, using the standardized and alternative cut-off points are shown on Tables 2-6.

A significant correlation between BMI and BF% values was found (r = 0.65; p < 0.001), as shown on Figure 2 in which BMI misclassification is represented by the darker dots.

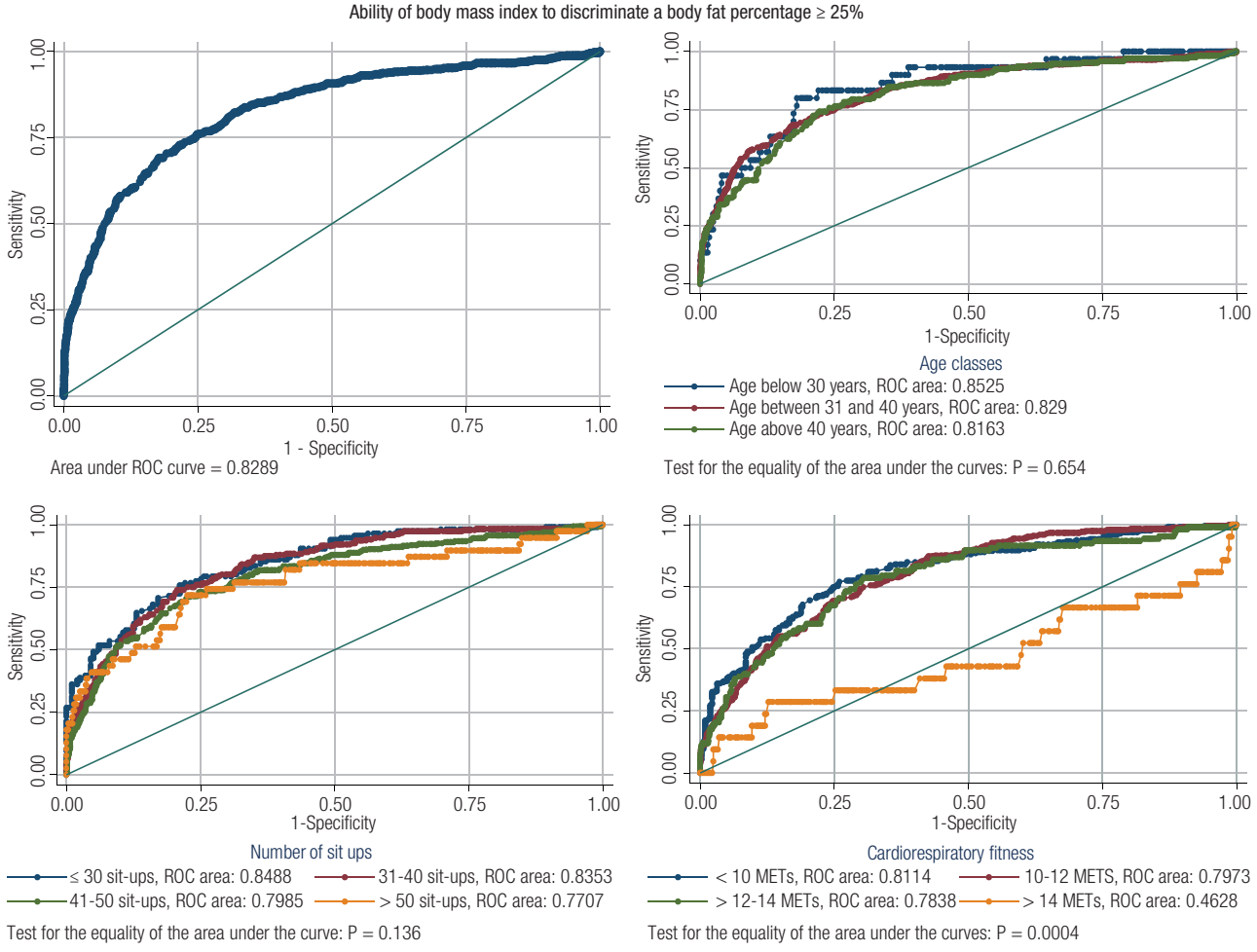

Figure 1. Non-parametric ROC curves showing the ability of BMI to identity obesity (≥ 30 kg/m²) as compared to BF% (> 25%) within 3,822 male military firefighters (upper left panel) and stratified by age (upper right), by the performance on sit up test (bottom left) and by CRF.

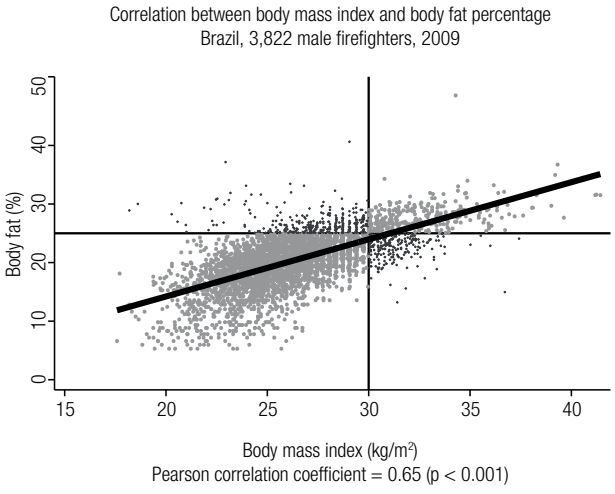

Figure 2. Correlation between BMI and BF% values in 3,822 military male firefighters. The chart shows quadrants defined by the standard cut-off points (BMI ≥ 30 kg/m² and BF% > 25%).

Table 4. Diagnosis of obesity (body fat percentage ≥ 25) based on body mass index. Analysis stratified by cardiorespiratory fitness (n = 3,822 male firefighters)

	Cardiorespiratory fitness (METs)			
	< 10 ***N* = 546**	**10-12** ***N* = 1,259**	**> 12–14** ***N* = 1,508**	**> 14** ***N* = 509**
BMI ≥ 27 kg/m²				
Agreement, % (95%CI)	61.9 (57.7–66.0)	63.8 (61.1–66.4)	73.2 (70.9–75.4)	84.1 (80.6–87.2)
Sensitivity, % (95%CI)	89.9 (85.2–93.5)	85.3 (80.3–89.4)	70.4 (60.8–78.8)	28.6 (11.3–52.2)
Specificity, % (95%CI)	42.0 (36.5–47.6)	58.4 (55.3–61.5)	73.4 (71.0–75.7)	86.5 (83.1–89.4)
Positive likelihood ratio, (95%CI)	1.55 (1.40–1.72)	2.05 (1.88–2.24)	2.65 (2.28–3.08)	2.11 (1.04–4.31)
Negative likelihood ratio, (95%CI)	0.24 (0.16–0.36)	0.25 (0.19–0.34)	0.40 (0.30–0.54)	0.83 (0.63–1.09)
Positive predictive value, % (95%CI)	52.4 (47.3–57.5)	33.8 (30.1–37.6)	17.0 (13.6–20.8)	8.3 (3.1–17.3)
Negative predictive value, % (95%CI)	85.4 (78.8–90.5)	94.1 (91.9–95.8)	97.0 (95.8–97.9)	96.6 (94.4–98.1)
BMI ≥ 28 kg/m²				
Agreement, % (95%CI)	69.0 (65.0–72.9)	70.9 (68.3–73.4)	82.5 (80.5–84.4)	89.8 (86.8–92.3)
Sensitivity, % (95%CI)	85.5 (80.2–89.8)	72.5 (66.5–77.9)	54.6 (44.8–64.2)	14.3 (3.0–36.3)
Specificity, % (95%CI)	57.4 (51.7–62.9)	70.5 (67.6–73.3)	84.6 (82.6–86.5)	93.0 (90.4–95.1)
Positive likelihood ratio, (95%CI)	2.00 (1.75–2.30)	2.46 (2.18–2.78)	3.56 (2.88–4.39)	2.05 (0.68–6.14)
Negative likelihood ratio, (95%CI)	0.25 (0.18–0.35)	0.39 (0.32–0.48)	0.54 (0.43–0.66)	0.92 (0.77–1.10)
Positive predictive value, % (95%CI)	58.8 (53.3–64.2)	38.0 (33.6–42.5)	21.5 (16.8–26.9)	8.1 (1.7–21.9)
Negative predictive value, % (95%CI)	84.7 (79.2–89.2)	91.2 (88.9–93.1)	96.0 (94.8–97.0)	96.2 (94.0–97.7)
BMI ≥ 29 kg/m²				
Agreement, % (95%CI)	74.2 (70.3–77.8)	76.5 (74.0–78.8)	87.9 (86.1–89.5)	93.7 (91.2–95.7)
Sensitivity, % (95%CI)	77.5 (71.5–82.8)	58.2 (51.8–64.3)	39.8 (30.5–49.7)	9.5 (1.2–30.4)
Specificity, % (95%CI)	71.8 (66.5–76.7)	81.1 (78.5–83.4)	91.6 (90.0–93.0)	97.3 (95.5–98.6)
Positive likelihood ratio, (95%CI)	2.75 (2.28–3.32)	3.07 (2.60–3.62)	4.72 (3.54–6.31)	3.58 (0.86–14.8)
Negative likelihood ratio, (95%CI)	0.31 (0.24–0.40)	0.52 (0.44–0.60)	0.66 (0.56–0.77)	0.93 (0.81–1.07)
Positive predictive value, % (95%CI)	66.2 (60.1–71.8)	43.3 (38.0–48.8)	26.7 (20.1–34.2)	13.3 (1.7–40.5)
Negative predictive value, % (95%CI)	81.8 (76.8–86.1)	88.6 (86.4–90.6)	95.2 (93.9–96.3)	96.2 (94.1–97.7)
BMI ≥ 30 kg/m²				
Agreement, % (95%CI)	75.3 (71.4–78.8)	80.6 (78.3–82.8)	91.1 (89.6–92.5)	94.5 (92.1–96.3)
Sensitivity, % (95%CI)	67.0 (60.4–73.0)	44.6 (38.4–51.0)	22.2 (14.8–31.2)	0.0 (0.0–16.1)
Specificity, % (95%CI)	81.2 (76.5–85.3)	89.6 (87.5–91.4)	96.4 (95.3–97.3)	98.6 (97.1–99.4)
Positive likelihood ratio, (95%CI)	3.56 (2.78–4.55)	4.28 (3.41–5.38)	6.22 (3.98–9.72)	1.48[a] (0.09–25.1)
Negative likelihood ratio, (95%CI)	0.41 (0.34–0.49)	0.62 (0.55–0.69)	0.81 (0.73–0.89)	0.99[a] (0.93–1.06)
Positive predictive value, % (95%CI)	71.7 (65.1–77.7)	51.6 (44.8–58.4)	32.4 (22.0–44.3)	0.0 (0.0–41.0)
Negative predictive value, % (95%CI)	77.5 (72.7–81.9)	86.7 (84.4–88.7)	94.1 (92.8–95.3)	95.8 (93.7–97.4)

[a] Values estimated using the substitution formula (0.5 added to all cell frequencies).

Table 5. Diagnosis of obesity (body fat percentage ≥ 25) based on body mass index. Analysis stratified by number of sit-ups (n = 3,822 male firefighters)

	Completed sit-ups (n)			
	≤ 30 *N* = 315	31–40 *N* = 891	41–50 *N* = 2,019	> 50 *N* = 597
BMI ≥ 27 kg/m²				
Agreement, % (95%CI)	68.3 (62.8–73.4)	69.0 (65.9–72.0)	68.5 (66.4–70.5)	77.1 (73.5–80.4)
Sensitivity, % (95%CI)	87.9 (80.6–93.2)	87.0 (81.4–91.4)	78.5 (73.0–83.3)	69.2 (52.4–83.0)
Specificity, % (95%CI)	56.8 (49.6–63.8)	64.1 (60.4–67.7)	67.0 (64.8–69.2)	77.6 (73.9–81.0)
Positive likelihood ratio, (95%CI)	2.03 (1.71–2.42)	2.42 (2.16–2.71)	2.38 (2.17–2.61)	3.09 (2.38–4.01)
Negative likelihood ratio, (95%CI)	0.21 (0.13–0.35)	0.20 (0.14–0.29)	0.32 (0.25–0.41)	0.40 (0.25–0.64)
Positive predictive value, % (95%CI)	54.3 (46.8–61.5)	40.0 (35.2–44.8)	26.0 (23.0–29.2)	17.8 (12.0–24.8)
Negative predictive value, % (95%CI)	89.0 (82.2–93.8)	94.7 (92.3–96.6)	95.5 (94.2–96.6)	97.3 (95.3–98.6)
BMI ≥ 28 kg/m²				
Agreement, % (95%CI)	72.7 (67.4–77.5)	75.3 (72.3–78.1)	77.5 (75.6–79.3)	84.8 (81.6–87.5)
Sensitivity, % (95%CI)	80.2 (71.7–87.0)	76.0 (69.4–81.9)	68.8 (62.8–74.4)	51.3 (34.8–67.6)
Specificity, % (95%CI)	68.3 (61.4–74.7)	75.1 (71.7–78.3)	78.8 (76.8–80.7)	87.1 (84.0–89.8)
Positive likelihood ratio, (95%CI)	2.53 (2.03–3.17)	3.05 (2.63–3.55)	3.25 (2.87–3.67)	3.97 (2.73–5.78)
Negative likelihood ratio, (95%CI)	0.29 (0.20–0.42)	0.32 (0.25–0.41)	0.39 (0.33–0.47)	0.56 (0.40–0.77)
Positive predictive value, % (95%CI)	59.6 (51.5–67.4)	45.6 (40.1–51.3)	32.4 (28.5–36.5)	21.7 (13.8–31.6)
Negative predictive value, % (95%CI)	85.5 (79.1–90.6)	91.9 (89.4–94.0)	94.5 (93.2–95.6)	96.2 (94.2–97.7)
BMI ≥ 29 kg/m²				
Agreement, % (95%CI)	77.1 (72.1–81.7)	79.9 (77.1–82.5)	83.1 (81.4–84.7)	89.9 (87.3–92.2)
Sensitivity, % (95%CI)	74.1 (65.2–81.8)	64.1 (56.8–70.8)	54.6 (48.3–60.8)	41.0 (25.6–57.9)
Specificity, % (95%CI)	78.9 (72.6–84.3)	84.3 (81.3–86.9)	87.3 (85.7–88.8)	93.4 (91.0–95.3)
Positive likelihood ratio, (95%CI)	3.51 (2.63–4.69)	4.07 (3.33–4.98)	4.31 (3.65–5.08)	6.19 (3.80–10.1)
Negative likelihood ratio, (95%CI)	0.33 (0.24–0.45)	0.43 (0.35–0.52)	0.52 (0.45–0.60)	0.63 (0.49–0.82)
Positive predictive value, % (95%CI)	67.2 (58.3–75.2)	52.8 (46.2–59.3)	38.9 (33.9–44.1)	30.2 (18.3–44.3)
Negative predictive value, % (95%CI)	84.0 (77.9–88.9)	89.5 (86.9–91.7)	92.9 (91.5–94.1)	95.8 (93.7–97.3)
BMI ≥ 30 kg/m²				
Agreement, % (95%CI)	78.1 (73.1–82.5)	82.2 (79.5–84.6)	86.6 (85.0–88.0)	93.0 (90.6–94.9)
Sensitivity, % (95%CI)	62.9 (53.5–71.7)	50.5 (43.2–57.8)	40.4 (34.4–46.6)	33.3 (19.1–50.2)
Specificity, % (95%CI)	86.9 (81.4–91.3)	90.8 (88.5–92.9)	93.4 (92.1–94.5)	97.1 (95.4–98.4)
Positive likelihood ratio, (95%CI)	4.82 (3.28–7.08)	5.52 (4.20–7.24)	6.12 (4.87–7.70)	11.6 (6.03–22.4)
Negative likelihood ratio, (95%CI)	0.43 (0.33–0.54)	0.71 (0.67–0.74)	0.64 (0.58–0.71)	0.69 (0.55–0.86)
Positive predictive value, % (95%CI)	73.7 (63.9–82.1)	60.2 (52.2–67.9)	47.5 (40.8–54.3)	44.8 (26.4–64.3)
Negative predictive value, % (95%CI)	80.1 (74.1–85.2)	87.0 (84.3–89.3)	91.4 (90.0–92.6)	95.4 (93.4–97.0)

Table 6. Diagnosis of obesity (body fat percentage ≥ 25) based on body mass index. Analysis stratified by age class (n = 3,822 male firefighters)

	Age (completed years)		
	≤ 30 years *N* = 329	31–40 years *N* = 2,521	> 40 years *N* = 972
BMI ≥ 27 kg/m²			
Agreement, % (95%CI)	81.2 (76.5–85.2)	69.6 (67.8–71.4)	67.0 (63.9–69.9)
Sensitivity, % (95%CI)	80.0 (61.4–92.3)	81.6 (77.5–85.2)	84.7 (78.4–89.8)
Specificity, % (95%CI)	81.3 (76.4–85.5)	67.3 (65.3–69.3)	63.2 (59.8–66.6)
Positive likelihood ratio, (95%CI)	4.27 (3.18–5.74)	2.50 (2.31–2.69)	2.30 (2.06–2.57)
Negative likelihood ratio, (95%CI)	0.25 (0.12–0.50)	0.27 (0.22–0.34)	0.24 (0.17–0.35)
Positive predictive value, % (95%CI)	30.0 (20.3–41.3)	32.5 (29.6–35.4)	32.8 (28.4–37.4)
Negative predictive value, % (95%CI)	97.6 (94.8–99.1)	95.0 (93.8–96.0)	95.1 (92.9–96.8)
BMI ≥ 28 kg/m²			
Agreement, % (95%CI)	85.7 (81.5–89.3)	77.9 (76.2–79.5)	74.6 (71.7–77.3)
Sensitivity, % (95%CI)	53.3 (34.3–71.7)	71.7 (67.1–76.1)	76.5 (69.4–82.6)
Specificity, % (95%CI)	89.0 (84.9–92.3)	79.1 (77.3–80.8)	74.2 (71.0–77.2)
Positive likelihood ratio, (95%CI)	4.83 (3.04–7.69)	3.43 (3.10–3.80)	2.96 (2.57–3.42)
Negative likelihood ratio, (95%CI)	0.52 (0.36–0.77)	0.36 (0.31–0.42)	0.32 (0.24–0.42)
Positive predictive value, % (95%CI)	32.7 (19.9–47.5)	39.8 (36.2–43.4)	38.6 (33.4–44.0)
Negative predictive value, % (95%CI)	95.0 (91.8–97.2)	93.6 (92.3–94.7)	93.7 (91.5–95.5)
BMI ≥ 29 kg/m²			
Agreement, % (95%CI)	89.4 (85.5–92.5)	83.5 (82.0–84.9)	79.4 (76.7–81.9)
Sensitivity, % (95%CI)	46.7 (28.3–65.7)	59.5 (54.5–64.3)	65.3 (57.6–72.4)
Specificity, % (95%CI)	93.6 (90.3–96.1)	88.1 (86.6–89.4)	82.4 (79.6–85.0)
Positive likelihood ratio, (95%CI)	7.34 (4.11–13.1)	4.99 (4.33–5.74)	3.71 (3.08–4.47)
Negative likelihood ratio, (95%CI)	0.57 (0.41–0.80)	0.46 (0.41–0.52)	0.42 (0.34–0.52)
Positive predictive value, % (95%CI)	42.4 (25.5–60.8)	49.0 (44.5–53.5)	44.0 (37.8–50.4)
Negative predictive value, % (95%CI)	94.6 (91.4–96.9)	91.9 (90.6–93.0)	91.8 (89.6–93.7)
BMI ≥ 30 kg/m²			
Agreement, % (95%CI)	91.2 (87.6–94.0)	86.6 (85.2–87.9)	82.2 (79.6–84.6)
Sensitivity, % (95%CI)	33.3 (17.3–52.8)	48.4 (43.5–53.4)	47.6 (39.9–55.4)
Specificity, % (95%CI)	97.0 (94.4–98.6)	93.9 (92.8–94.9)	89.5 (87.2–91.6)
Positive likelihood ratio, (95%CI)	11.1 (4.88–25.1)	7.93 (6.53–9.64)	4.55 (3.52–5.88)
Negative likelihood ratio, (95%CI)	0.69 (0.56–0.74)	0.55 (0.50–0.60)	0.58 (0.51–0.68)
Positive predictive value, % (95%CI)	52.6 (28.9–75.6)	60.4 (54.9–65.8)	49.1 (41.2–57.0)
Negative predictive value, % (95%CI)	93.5 (90.2–96.0)	90.4 (89.1–91.6)	89.0 (86.6–91.0)

DISCUSSION

In this large military firefighter cohort we showed that both BMI and BF% measures yielded similar overall obesity prevalences. Similar results were observed in each age and physical fitness subgroup as well. Also important is the high total agreement between both measures in all groups, ranging from 75.2% to 94.8%.

Contrary to common concerns, BMI did not overestimate obesity prevalence. In fact, BMI actually underestimated obesity in all groups. This BMI-defined obesity underestimation trend has been also reported on several studies with different populations pooled on a meta-analysis published in 2010 (26). This tendency has been an interesting finding among firefighters, since they are supposedly more fit than the general population (27,28).

Poston and cols. also observed a BMI-defined obesity underestimation trend among US firefighters (29). It should be noted that even with the same underestimation tendency, the obesity prevalence reported by Poston and cols. in US firefighters was very high (25.3% to 35.6%) (29,30), as compared to the one observed in this Brazilian cohort. A great distinction between the Brazilian and the US Fire Departments is that the former one is a military institution, with specific annual physical training demands and requirements (31) that may explain part of these differences, whereas the latter is a civil organization with variable training regimens, if any, and rarely applying strict physical fitness requirements.

As regard to the possible BMI-misclassification, we should consider that the BMI inherent incapacity to distinguish lean mass from body fat composition is probably higher on intermediate BMI values (from 25.0 to 30.0 kg/m^2), as pointed out elsewhere either for the general population (32) and for specific groups as coronary heart disease patients (33). It should also be considered that the BMI-misclassification occurred not only on the high values of BMI, as usually supposed. Figure 2 shows that there were some volunteers with low BMI values (< 30 kg/m^2) but with a high (> 25%) body fat. Again, most of these cases occurred on intermediate BMI values, specifically between 27.0 to 30.0 kg/m^2. Our data reinforce this hypothesis once the BMI-sensitivity increases while its specificity decreases when the cut-off point is changed from 30.0 to 27.0 kg/m^2, which means that with lower cut-off points some subject with BF% < 25% would be considered to be obese.

Furthermore, almost all indices were affected by CRF, sit-ups and age, with better sensitivity in the less fit (CRF and sit-ups) and in the older groups. On the other hand, specificity was highest among the fittest and youngest groups (Table 2). This trend was observed in all analyzed cut-off points (Tables 3-5). The same trend was also observed for the likelihood ratios, where LR+ tends to increase while cut-off point increases from 27.0 kg/m^2 to the standardized 30.0 kg/m^2 and among better physical fitness and younger groups as compared to those with lower physical fitness and higher age. As regard to the LR- we observed exactly the opposite. LR- values were worse (closer to one) on lower cut-off points and among younger and less fit firefighters while it tended to zero (better values) when the sensitivity tended to be higher, i.e., in lower cut-off points and among the older and less fit groups.

Besides, we observed very low true positive percentages and very high true negative percentages. Despite a very good total agreement, BMI capacity to correctly identify obesity as compared to BF% was very low. On the other hand, BMI was a very good tool to exclude BF%-defined obesity.

Another important finding is that even with the good accuracy observed in all analyzed subgroups, ROC curve stratified by CRF (Figure 1) showed that BMI was not good enough to identify firefighters with BF% > 25% within those with CRF > 14 MET. The reduced AUC (0.46) observed among firefighters with CRF > 14 METs means that BMI was not a helpful tool to identify obesity in this specific subgroup (22). These results reinforce the idea that BMI cut-off points should consider some subgroup specificities, as the CRF itself or the ethnicity, as already proposed by the World Health Organization for the Asian populations (34).

In our analysis, a better agreement was seen when the standardized cut-off point was used. However, it should be highlighted that the high agreement was almost dependent on the high specificity, once the percentage of true positive was very low. When a good sensitivity was obtained (cut-off = 27.0 kg/m^2), the total agreement drops 10% or more, either for the whole population or for any subgroup (Tables 2-5).

While this study has been done with a large firefighter cohort, there are some limitations that must be considered. First, we compared BMI against a reference method (BF% by skinfold thickness) that is not considered the gold-standard one for body composition assessment. However, we aimed to

compare BMI performance against a method that is largely employed worldwide and more suitable for large sample studies than the DEXA, apart from the fact that the estimation of BF% by skinfold thickness has long been recognized as a feasible, valid and low cost method (18,35). Besides the limitations of considering BF% as a reference method, it should be taken into account that BF% estimated by skinfold thickness overcomes the inability of BMI to distinguish lean mass from body fat, which is the BMI most important limitation. Furthermore, Okorodudu and cols. study shows that when BMI obesity diagnostic performance was analyzed considering only the most precise techniques to evaluate body fat, their agreement analysis didn't change significantly (26).

Finally, it is also important to consider that some of the volunteers could have performed bellow their maximum capacity since they knew in advance the minimum threshold that they would need to reach in order to achieve success on the annual physical evaluation. The very high consistency of our data shows that this possibility has probably not affected the results.

In conclusion, this cross-sectional study conducted among a large physically active population showed that BMI and body fat percentage yielded similar obesity prevalence in the whole sample and in each subgroup stratified per age, sit-up and CRF performance. We also observed a very high index of total agreement. Contrary to common concerns, BMI did not overestimate obesity prevalence, even among the fittest subgroup. However, ROC curve analysis demonstrated that BMI standardized cut-off point was not useful to identify obesity in the group with CRF > 14 METs. BMI ≥ 30 km/m^2 showed to be highly specific as a screen to exclude obesity in this large firefighter sample, but it resulted on low sensitivity. Because of systematic underestimation, a lower BMI cut point might be considered in this and other physically active populations

Practical applications

Considering its ease of measurement, low cost and the high total agreement of standardized BMI cut-off point as compared to BF%-obesity definitions, BMI is an excellent screening tool to estimate obesity prevalence in this physically active population. Standardized BMI cut-off point was less useful to identify obesity in the fittest group (CRF >14 METs). Because of systematic obesity underestimation, a lower BMI cut-off point (27.0 kg/m^2) might be considered in this and other physically active populations, especially for obesity prevention programs, when BMI-sensitivity must be emphasized. Our results reinforce the idea that BMI cut-off points should consider some specific characteristics, as age and physical fitness. Our findings are likely generalizable to other similar active populations as such, law enforcement and armed forces professionals.

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Utility of body mass index, waist circumference and waist-to-height ratio as screening tools for hyperglycemia in young people

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ABSTRACT

Objectives: To evaluate the ability of BMI, WC and WHtR to discriminate hyperglycemia in young people, and to determine whether there is an increase in the accuracy with the addition of WC and/or WHtR to BMI. **Subjects and methods:** A cross-sectional study was conducted on 1,139 schoolchildren aged 6 to 17 years from Northeastern Brazil. Body weight, height, WC and fasting glucose levels were measured, and the BMI and WHtR were calculated. The presence of hyperglycemia was defined as a fasting glucose level ≥ 100 mg/dL. **Results:** The prevalence of hyperglycemia was 6.6%. Strong correlations were observed between the anthropometric indicators studied (BMI vs. WC = 0.87; BMI vs. WHtR = 0.87; WC vs. WHtR = 0.90). Hyperglycemia was more likely to be present in young people with excess weight (PR = 1.70), high WC (PR = 1.85), and high WHtR (PR = 1.91). The accuracies to discriminate hyperglycemia were significant, but low, for the individual (BMI = 0.56; WC = 0.53; WHtR = 0.55) and combined indicators (BMI + WC = 0.55; BMI + WHtR = 0.55). **Conclusion:** Our findings do not support the use of BMI, WC or WHtR as screening tools for hyperglycemia in children and adolescents.** Arch Endocrinol Metab. 2016;60(6):526-31

Keywords
Pediatric obesity; anthropometry; pediatrics; hyperglycemia; diabetes mellitus

INTRODUCTION

The worldwide prevalence of overweight and obesity among children and adolescents has increased by about 47% in the last three decades (1). In parallel, a substantial increase has been observed in the prevalence of type 2 diabetes among young people (2). A growing body of evidence indicates that obese children and adolescents are more likely to present different cardiometabolic disorders (3), and that the maintenance of obesity from childhood to adulthood significantly increases the risk of type 2 diabetes (4).

Overweight and obesity are commonly evaluated in children and adolescents using the body mass index (BMI) because of its easy measurement and low cost. Additionally, reference values are available for comparison and the parameter permits continuous evaluation in adults (5). However, the relationship of this parameter with cardiometabolic alterations in young people can be questioned since the BMI estimates total body weight and not the quantity and distribution of fat. On the other hand, waist circumference (WC) and the waist-to-height ratio (WHtR) have gained ground in the evaluation of cardiometabolic risk factors in young people because these indicators propose to estimate central body fat (6,7). A systematic review including 61 studies found that the deposition of central body fat in children and adolescents increases the cardiometabolic risk, irrespective of the definition of abdominal obesity and anthropometric method adopted (3).

Although anthropometric indicators are attractive tools for the evaluation of cardiometabolic risk, it is not clear whether BMI, WC and WHtR are able to discriminate hyperglycemia in children and adolescents, or whether WC and/or WHtR confer additional utility to BMI. Therefore, the objective of this study was two-fold: 1) to evaluate the ability of BMI, WC and WHtR to discriminate hyperglycemia in young people, and 2) to determine whether the inclusion of WC and/or

WHtR increases the accuracy of BMI to discriminate hyperglycemia in young people.

SUBJECTS AND METHODS

Population and sample

The present study is part of a school-based epidemiological study conducted in a city of Northeastern Brazil. The estimated population in 2012 was 34,845 inhabitants, with a human development index of 0.625. The population consisted of school-age children and adolescents of both sexes ranging in age from 6 to 17 years (6.0 to 17.99 years). The students were enrolled in the 1st to 9th grades of elementary school and in the 1st to 3rd year of high school in public and private schools of the city. According to data from the Municipal Education Department, 7,708 students were enrolled in 42 schools in 2011, including 40 public schools [13 urban (N = 5,207) and 27 rural (N = 1,853)] and two private schools (N = 648). The municipality studied comprises an area of 435,932 km^2. For this reason, the Municipal Education Department divided the area of the municipality into six educational nuclei (one urban and five rural nuclei). Among the rural nuclei, the smallest possessed two schools and the largest seven schools in 2011. In the urban area, all schools were located in the same nucleus.

The representative sample size of the larger study was calculated using an estimated prevalence of 50% (for different outcomes), a 95% confidence interval, and a precision of 3 percent points according to Luiz and Magnanini (8). The estimated sample size was 971 children and adolescent; 20% of this number (n = 194) was added to account for possible incomplete data of the subjects or refusal to participate in the data collection.

The sample was selected in two stages, in which the "school" was the primary sampling unit and the "student" the secondary sampling unit. In the first stage, cluster sampling of the schools was used, with proportional stratification by type of school (urban public, rural public, and private) and by educational nucleus for schools in the rural area (in order to guarantee the geographic distribution of the sample in the rural area). Five urban public schools, five rural public schools (one per nucleus studied), and one private school were selected by drawing lots. The estimated sample size per extract was proportional to that observed in the study population (urban public: n = 787; rural public: n = 280; private: n = 98). In the second stage, the students were selected by simple drawing lots considering the number of individuals necessary per school to compose the sample, so that the number would be proportional to the number of students enrolled in each school. The study protocol was approved by the Ethics Committee of Faculdade Maria Milza (Permit No. 126/2011). Only students who voluntarily accepted to participate and whose parents or legal guardian had signed the free informed consent form were included in the study.

Instruments and procedures

The data were collected between August 2011 and May 2012. The sociodemographic variables were obtained by self-report and included age, sex, and socioeconomic class. The last was estimated using the Brazilian Criterion of Economic Classification (9).

Body weight was measured with a Plenna digital scale (capacity of 150 kg) to the nearest 100 g. Height was measured with a Seca portable stadiometer (0 to 220 cm) fixed to the wall, to the nearest 0.1 cm. The two variables were measured using standard techniques (10) and were used to calculate the BMI. Overweight and obesity were defined using the cut-off values proposed by Cole and cols. (11). WC was measured with a non-elastic measurement tape to the nearest 0.1 cm according to procedures recommended by the World Health Organization (12), and was classified as normal or elevated (13). Height and WC were used to calculate the WHtR. A WHtR ≥ 0.5 was classified as high (14). Anthropometric assessment was performed in the morning by two examiners of the same sex as the students to avoid any embarrassment. The two examiners presented intra- and interobserver errors of less than 1% and 1.5% for all measures, which are acceptable according to the literature (15).

Venous blood samples (10 mL) for the measurement of blood glucose levels were collected at the schools in the morning after a 12-hour fast and a normal diet on the previous day. The samples were transported under refrigeration to the Nilson Lomanto Municipal Laboratory of Amargosa, Bahia, for analysis. Glucose levels were determined with an automatic biochemical analyzer (Biosystems®, model A15) by an enzymatic method based on the analysis of plasma fluoride. The presence of hyperglycemia was defined as a fasting

glucose level ≥ 100 mg/dL as proposed by the American Diabetes Association (16).

Statistical analysis

The data were analyzed using the SPSS 15.0 and MedCalc programs. Descriptive analysis consisted of the calculation of mean, median, standard deviation, percentile, and frequency. Differences in glycemic profile, BMI, WC and WHtR between sexes and age groups (children: 6 to 9 years; adolescents: 10 to 17 years) were tested by the Mann-Whitney test ($p < 0.05$). The partial correlation between the anthropometric indicators adjusted for sex and age was evaluated ($p < 0.05$). The prevalence ratio (PR), estimated by Poisson regression with robust variance, was used to verify the association of excess weight (overweight and obesity), elevated WC and high WHtR with hyperglycemia adjusted for sex, age and socioeconomic class. The Wald test was adopted to evaluate statistical significance ($p < 0.05$). The sensitivity, specificity and positive predictive value of excess weight (overweight and obesity) and high WC and WHtR were calculated. The power of the individual (BMI, WC, and WHtR) and combined indicators (BMI with WC and BMI with WHtR) to predict hyperglycemia was evaluated by constructing receiver operating characteristics (ROC) curves. The 95% confidence intervals (95% CI) were calculated and significance was attributed to areas under the ROC curve that showed a lower limit of the respective confidence intervals of 0.50 or higher. The difference in accuracy between the anthropometric indicators, alone or in combination, associated with hyperglycemia was calculated according to Hanley and McNeil (17).

RESULTS

The number of students evaluated was 1,139, with 2.2% of losses due to refusal or absence on the day of data collection. The mean age was 11.51 years (3.33). Table 1 shows the differences in glycemia and anthropometric indicators according to sex and age group. Glycemia was higher in boys, and BMI, WC and WHtR were higher in girls. With respect to differences between children and adolescents, glycemia, BMI and WC were higher in adolescents, while WHtR was higher in children. The prevalence of hyperglycemia was 6.6% (95% CI 5.3-8.3). Young people with hyperglycemia had median (25^{th}, 75^{th} percentiles) glycemia, BMI, WC and WHtR of 103 mg/dL (101, 106), 18.2 kg/m^2 (16.38, 20.28), 65.5 cm (60.0, 74.2) and 0.46 (0.42, 0.51), respectively. The prevalence of overweight was 12.7% (95% CI 10.9-14.8) and the prevalence of obesity was 3.2% (95% CI 2.3-4.3). High WC and WHtR were observed in 17.8% (95% CI 15.7-20.2) and 19.7% (95% CI 17.5-22.1) of the students, respectively.

Strong correlations adjusted for age and sex were observed between all anthropometric indicators (BMI vs. WC = 0.87; BMI vs. WHtR = 0.87; WC vs. WHtR = 0.90). In multivariate logistic regression analysis adjusted for sex, age and socioeconomic condition, hyperglycemia was more likely to be present in young people with excess weight (PR = 1.70, 95% CI 1.03-2.85, p = 0.04), elevated WC (PR = 1.85, 95% CI 1.13-3.03, p = 0.01), and high WHtR (PR = 1.91, 95% CI 1.17-3.12, p = 0.01).

The sensitivity, specificity and positive predictive value were 24.3%, 84.5% and 10.0% for excess weight, 28.4%, 82.7% and 10.4% for WC, and 31.1%, 80.8%

Table 1. Glycemic profile and anthropometric indicators in the children and adolescents studied according to sex and age group. Northeastern Brazil, 2011-2012

	n	Glycemia (mg/dL)[a]	BMI (kg/m^2)[a]	WC (cm)[a]	WHtR[a]
Sex					
Male	506	90 (85, 94)	17.00 (15.60, 19.40)	63.0 (56.5, 70.0)	0.44 (0.42, 0.46)
Female	633	88 (83, 93)	18.10 (15.90, 20.80)	66.9 (59.5, 73.6)	0.46 (0.43, 0.49)
p[b]		0.003	0.001	0.001	0.001
Age group					
Children (6 to 9 years)	363	88 (83, 93)	15.60 (14.60, 17.10)	56.4 (53.0, 60.4)	0.45 (0.43, 0.48)
Adolescents (10 to 17 years)	776	89 (85, 94)	18.80 (16.74, 21.20)	69.0 (63.4, 74.8)	0.45 (0.42, 0.48)
p[b]		0.033	0.001	0.001	0.041
Total	1.139	89 (84, 94)	17.50 (15.70, 20.10)	65.5 (58.0, 72.3)	0.45 (0.42, 0.48)

BMI: body mass index; WC: waist circumference; WHtR: waist-to-height ratio.
[a] Median (25^{th}, 75^{th} percentiles).
[b] Significance level for glycemia, BMI, WC, and WHtR (Mann-Whitney test).

and 10.3% for WHtR, respectively. The accuracies to discriminate hyperglycemia were significant, but low, for all indicators studied. No significant differences were observed between individual and combined anthropometric indicators (Table 2).

Table 2. Accuracy of the anthropometric indicators for hyperglycemia screening in the children and adolescents studied. Northeastern Brazil, 2011-2012

Anthropometric indicator	AUC	95% CI
BMI (kg/m^2)	0.56	0.53-0.59
WC (cm)	0.53	0.50-0.56
WHtR	0.55	0.52-0.58
BMI (kg/m^2) and WC (cm)	0.55	0.52-0.58
BMI (kg/m^2) and WHtR	0.55	0.52-0.58

BMI: body mass index; WC: waist circumference; WHtR: waist-to-height ratio; AUC: area under the curve; 95% CI: 95% confidence interval.

DISCUSSION

The use of simple, easily obtainable and inexpensive measures such as body weight, height and WC for the screening of cardiometabolic risk factors in young people is a promising strategy for the prevention and early diagnosis of diseases such as type 2 diabetes. In this respect, the objective of this study was to evaluate the ability of BMI, WC and WHtR to discriminate hyperglycemia in the pediatric population, and to determine whether the inclusion of WC and/or WHtR increases the accuracy of BMI to discriminate hyperglycemia. Our results showed that the anthropometric indicators evaluated were associated with hyperglycemia. However, BMI, WC and WHtR were not efficient in screening for hyperglycemia in young people because their accuracy was poor. Furthermore, the addition of the indicators of abdominal obesity to BMI did not increase the ability of this index to discriminate young people with and without hyperglycemia.

In recent years, some authors have suggested the use of WC and WHtR for the evaluation of obesity and associated health problems in children and adolescents (6,7). However, others argue that there is not sufficient evidence to use these indicators instead of BMI (18,19). In fact, the average increase in the BMI of children and adolescents has been accompanied by an even more marked increase in WC (20). There might be additional advantages when this measure is corrected for height, such as the absence of a measurement unit, the lack of need for a specific reference population, and the possibility to use a single cut-off to discriminate excess fat in both young people and adults (WHtR = 0.5). However, evidence indicates that measuring height in addition to WC has no additional benefit for predicting cardiometabolic risk (21), and it may not be sufficient to adjust WC for height during the growth periods in childhood and adolescence because of the considerable residual correlation between height and WHtR (ranging from 0.29 to 0.36) (22). Furthermore, at least four different anatomical sites are commonly used to measure WC (23,24), a fact that can produce different prevalences of abdominal obesity (23) and impair the comparison between studies. Moreover, the magnitude of the association between WC and cardiometabolic risk factors in young people seems to be influenced by the site of measurement of this parameter (24).

With respect to the evaluation of general obesity, BMI has been the most used anthropometric indicator for decades. This index has wide applicability and is associated with less inter- and intraobserver error than demonstrated for other measures of obesity in young people (25). The calculation of BMI, which can be considered an obstacle to the use of this index by the population, can nowadays be performed easily using calculators available in electronic devices used in everyday life (*e.g.*, mobile phones). Calculating BMI with a calculator can even be faster than to obtain other anthropometric measures of obesity. On the other hand, although children and adolescents with a high BMI also tend to have high levels of body fat, the BMI does not differentiate lean mass from fat mass and may therefore be considered an inaccurate indicator of body fat, especially among young people with normal or relatively low levels of body fat (26).

Previous studies analyzing anthropometric measures as predictors of hyperglycemia in children and adolescents also observed poor accuracies, ranging from 0.44 to 0.51 for BMI, from 0.44 to 0.52 for WC, and from 0.50 to 0.57 for WHtR (27-30). It is well known that excess body fat is associated with different physiological and pathological states, including alterations in glucose metabolism (31). In this respect, both the amount of total body fat and fat distribution exert moderate effects on glucose metabolism, with a greater effect being observed for abdominal visceral fat (31). Although attractive because they are noninvasive methods to estimate obesity, anthropometric indicators are not exact measures of total body fat and fat distribution (especially abdominal visceral

fat) in children and adolescents (19). This limitation of the anthropometric method, in conjunction with the latency period of hyperglycemia, may explain at least in part the poor accuracies of BMI, WC and WHtR to discriminate hyperglycemia in young people. The phenomenon of "metabolically healthy obesity" observed among adults as well as children and adolescents (32) may also be a confounding factor in the relationship between anthropometric indicators and glycemia. Furthermore, factors not evaluated in the present study, such as the duration of obesity and the rate of recent weight gain/loss, may play an important role in altered glucose metabolism, irrespective of the current weight status (29).

Although the relationship between obesity and hyperglycemia/type 2 diabetes is well established in adults (33), in the pediatric population this subject requires further research. The PR obtained in this study indicated that overweight and obese young people were more likely to have hyperglycemia, irrespective of the anthropometric method used. Some studies also observed this association (7,34), while others did not demonstrate it (27,29). These divergences between the results of studies might be related to the characteristics of the samples such as age group, socioeconomic condition and lifestyle of the subjects studied, as well as methodological aspects, especially the criterion used to define hyperglycemia. In the present study, although a higher PR was observed for WHtR, the magnitude of the associations was similar (WHtR = 1.91, WC = 1.85 and BMI = 1.70). It should be mentioned that PR is not a statistical analysis that distinguishes between the presence and absence of disease; it only suggests an association between exposure and outcome and should therefore not be used to define whether a method is adequate to screen for a certain disease.

Strong correlations were observed in the present study between the three anthropometric indicators, in agreement with the findings of previous studies (18,19). Katzmarzyk and Bouchard (19) analyzed the correlation of total and visceral body fat with BMI and WC in children aged 5 to 18 years and found strong correlations between total and visceral body fat and the two anthropometric indicators. Accordingly, it can be expected that little additional information be obtained when adding WC and/or WHtR to BMI, as confirmed by our accuracy analysis. Chiolero and cols. (18) also observed that the addition of WHtR to BMI did not confer additional discriminatory power for elevated blood pressure in children. Taken together, the results suggest caution to state that WC or WHtR is a better substitute of BMI for the evaluation of body fat, or that the addition of these indicators to BMI improves the screening capacity for cardiometabolic risk in the pediatric population.

A strength of our study is the evaluation of the association of three simple anthropometric indicators with glycemia in a school-based probability sample consisting of children and adolescents of both genders from a developing country. Additionally, to our knowledge, there are no other studies investigating the addition of WC and WHtR to BMI in an attempt to improve the prediction of hyperglycemia in the pediatric population. However, this study has some limitation that need to be considered. A single measurement of fasting glucose levels, although suitable for populational studies, does not reflect the initial alterations in glucose homeostasis nor does it differentiate young people with and without impaired glucose tolerance/type 2 diabetes until significant deterioration in glucose metabolism has occurred (35). The cross-sectional design did not permit to establish cause-effect relationships since the exposure and outcome variables were collected simultaneously. Therefore, studies monitoring changes in body fat and glucose metabolism in young people over time are needed to gain further insight into this topic.

In conclusion, the anthropometric indicators studied were not useful to screen for hyperglycemia; however, obese children and adolescents were more likely to have hyperglycemia. In this respect and taking into consideration that obesity theoretically precedes hyperglycemia, BMI, WC and WHtR should continue to be used for the evaluation of overweight and obesity and consequent monitoring of metabolic diseases at early ages.

Author contribution: TMBQ, and APG designed the study, directed implementation and data collection, analyzed the data, and drafted the manuscript. LRS, and JM edited the manuscript for intellectual content and provided critical comments on the manuscript.

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Hypoparathyroidism and pseudohypoparathyroidism: etiology, laboratory features and complications

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ABSTRACT

Objectives: To identify a clinical profile and laboratory findings of a cohort of hypoparathyroidism patients and determine the prevalence and predictors for renal abnormalities. **Materials and methods:** Data from medical records of five different visits were obtained, focusing on therapeutic doses of calcium and vitamin D, on laboratory tests and renal ultrasonography (USG). **Results:** Fifty-five patients were identified, 42 females and 13 males; mean age of 44.5 and average time of the disease of 11.2 years. The most frequent etiology was post-surgical. Levels of serum calcium and creatinine increased between the first and last visits ($p < 0.001$ and $p < 0.05$, respectively); and serum levels of phosphate decreased during the same period ($p < 0.001$). Out of the 55 patients, 40 had USG, and 10 (25%) presented with kidney calcifications. There was no significant difference in the amount of calcium and vitamin D doses among patients with kidney calcifications and others. No correlation between serum and urinary levels of calcium and the presence of calcification was found. Urinary calcium excretion in 24h was significantly higher in patients with kidney calcification (3.3 mg/kg/d) than in those without calcification (1.8 mg/kg/d) ($p < 0.05$). **Conclusions:** The reduction of hypocalcemia and hyperphosphatemia suggest an effectiveness of the treatment, and the increase in serum creatinine demonstrates an impairment of renal function during follow-up. Kidney calcifications were prevalent in this cohort, and higher urinary calcium excretion, even if still within the normal range, was associated with development of calcification. These findings suggest that lower rates of urinary calcium excretion should be aimed for in the management of hypoparathyroidism. Arch Endocrinol Metab. 2016;60(6):532-6

Keywords
Hypoparathyroidism; renal complication; renal calcification; hypocalcemia; pseudohypoparathyroidism

INTRODUCTION

Hypoparathyroidism (HP) is a metabolic disorder caused either by deficient or absent production of the parathyroid hormone (PTH) by the parathyroid glands or by resistance to PTH in its target tissues (a condition called pseudo-HP) (1,2). HP is a rare disease, with an estimated prevalence of 37 cases per 100,000 inhabitants in the USA (3). In Brazil, studies about the disease itself and data about its epidemiology are lacking. The lower PTH production by the parathyroids may be induced by damage to the glands during surgical procedures of the anterior cervical region (as total thyroidectomy or parathyroidectomy), then being called post-surgical HP; or by autoimmune, infiltrative, genetic or irradiative causes. Physiologically, PTH acts in order to maintain serum levels of calcium and phosphate within the normal range (8.8 to 10.4 mg/dL and 2.5 to 4.5 mg/dL, respectively). Its mechanism of action includes stimulation of bone remodeling with release of calcium and phosphate from the skeleton, and stimulation of the 1α-renal hydroxylase – an essential enzyme for production of calcitriol, which is the active form of vitamin D and therefore important for calcium absorption and bone resorption. In addition, PTH stimulates calcium reabsorption and phosphate secretion in renal tubular cells (1,3,4).

HP is clinically characterized by hypocalcemia and hyperphosphatemia (5,6). The main manifestations of HP include fatigue, paresthesia, tetany, Trousseau and Chvostek signs, cramps, convulsion, hyperreflexia, and cardiac disturbances such as an enlargement of the QT interval on the ECG. All of these are consequences

of the muscle hyperexcitability caused by extracellular hypocalcemia (2,5). Other described manifestations are soft tissue calcification, as in basal ganglia; psychiatric symptoms such as depression; cataract; and alopecia, among others (7,8).

HP is one of the few endocrine hyposecretory diseases whose treatment, in most countries, does not consist of administration of the defective hormone. Rather, it consists of supplementation with high doses of calcium and vitamin D analogues, such as calcitriol and cholecalciferol (9-11). Recently, renal complications have been linked to HP (9). In fact, as the activity of PTH in the renal tubules favoring calcium reabsorption is not repaired, the supplemented calcium is rapidly excreted in the urine. Furthermore, the doses of calcium and vitamin D are usually very high and hypercalciuria is often seen in these patients. Chronic hypercalciuria may lead to nephrocalcinosis, nephrolithiasis, and renal failure (12).

The aims of this study are to identify a clinical profile and laboratorial findings of patients with hypoparathyroidism and to determine the prevalence as well as the predictors for renal abnormalities.

MATERIALS AND METHODS

Selection of patients

This is a retrospective, analytical, and observational study. Data have come from patients of the Bone Clinic of the Division of Endocrinology at Serviço de Endocrinologia e Metabologia da Universidade Federal do Paraná (SEMPR). This study has the approval of the local Ethics Committee in Research on Human Beings.

An active search for patients with HP was performed through the hospital database. The exclusion criterions were disease duration of less than 6 months, which is defined as transient HP (14). Patients whom the cause of the parathyroid surgery were secondary hyperparathyroidism due long-standing renal failure were also excluded from this analyses.

Data collection

Clinical features such as age, gender, HP etiology, age at diagnosis, duration of disease, and doses of treatment (calcium, calcitriol, and/or cholecalciferol) were collected and analyzed. Renal ultrasonography (USG) and cranial tomography (CT), when available, were analyzed to identify the presence of such calcifications as nephrolitiasis and nephrocalcinosis in the kidneys and basal ganglia in the brain. The mean and standard deviation (SD) of serum total calcium, phosphate, creatinine, and PTH as well as 24-hour urinary calcium excretion were collected based on the first and last visit of each patient, as well as on three intermediary visits (5 time points). For the measurement of total serum calcium, phosphorus and creatinine standard kits were used, and analysis was performed using automated spectrophotometric equipment (ADVIA 1650, Bayer, Leverkusen, Germany). Levels of iPTH were determined by chemiluminescence (DPC, Immulite 2000, Los Angeles, CA, USA). The mean doses along with the range (minimum and maximum) of calcium carbonate, calcitriol, and cholecalciferol used by the patients at the five time points were also calculated.

Cockroft-Gault formula (15) was used to calculate the estimated glomerular filtration rate (eGFR) of those patients with weight and creatinine available for the last visit.

Patients who had renal USG results available were divided into two groups – with and without renal calcification – and were compared with each other regarding their clinical and laboratory features as well as doses of calcium and vitamin D.

Statistical analysis

Data were described as frequency and percentage or by mean and SD for qualitative and quantitative variables, respectively. The results of the first and last visit were analyzed. Student's t-test for paired samples or nonparametric Wilcoxon test and/or binomial test was performed as appropriate for quantitative and qualitative variables. Spearman's correlation coefficients were estimated to test the association of disease duration with the laboratory results. For the analysis between the groups with and without renal calcification, Student's t-test for independent samples or nonparametric Mann-Whitney test was used for the quantitative variables, and Fisher's exact test for the qualitative variables. Results with values of $p < 0.05$ have been considered as statistically significant. The data were analyzed using the computational software IBM SPSS Statistics v.20.0.

RESULTS

Main clinical characteristics of the patients are presented in Table 1. Fifty-five patients were identified: 42 (76.4%) females and 13 (23.6%) males, with a mean age of 44.5

± 19.3 years. Post-surgical HP was the etiology in 41 (74.5%) patients, while 5 (9.1%) had pseudo-HP and 9 (16.4%) had autoimmune HP. The age of the patients at diagnosis varied from 5 to 76 years, being on average 32.9 ± 19 years. The average age at diagnosis varied according to the etiologies: 40.4 ± 16.6 years for post-surgical HP, 15 ± 5.2 years for pseudo-HP, and 12.1 ± 7.4 years for those with other causes-HP. The mean duration of disease was 11.2 ± 7.5 years. Ninety-two percent of the patients were taking calcium supplements with a mean dose of 1,232 mg (range 500-2,150), 80% were receiving daily calcitriol in a mean dose of 0.67 ucg (range: 0.25-2), and 75% were taking cholecalciferol in a mean weekly dose of 35,000 UI (range: 7,000-70,000).

Out of the 55 patients, 40 (72.7%) had been submitted to renal USG, whereas 30 (75%) had a normal USG and 10 (25%) had abnormalities such as nephrolithiasis and nephrocalcinosis. No correlation between serum and urinary levels of calcium and the presence of calcification was found. However, weight-adjusted urinary calcium excretion in 24 h was higher in patients with renal calcification than in those without calcification (3.3 vs 1.8 mg/kg/d, $p < 0.05$). Mean values of serum calcium, phosphate and creatinine, and 24-h urinary calcium in each of the five moments are shown in Figure 1, according to the USG results. The values of serum calcium rose significantly from the first to the last visit (6.87 ± 1.65 to 8.62 ± 1.3; $p < 0.001$) in the same way that serum phosphate fell significantly (6.14 ± 2.1 to 4.89 ± 1.0; $p < 0,001$). An inverse correlation was observed between serum calcium and time of disease across different patients, along with a positive correlation with the serum phosphate ($R = -0.32$, $p = 0.033$ and $R = +0.38$, $p = 0.013$, respectively). No correlation was found between disease duration and levels of creatinine, PTH, or 24-h urinary calcium.

Considering all patients, creatinine values increased during this period (0.81 ± 0.17 – 1.1 ± 0.45), also with statistical significance ($p = 0.04$). The mean of PTH for patients with pseudo-HP was 130 ± 146 pg/mL; and for the others, PTH was 7.8 ± 11 pg/mL.

Hypercalciuria was found in 15 patients (urinary calcium > 250 mg/24h for females and > 300 mg/24h for males) (16). The glomerular filtration rate (eGFR) varied from 13.8 to 223 mL/min/1.73 m², and its mean

Table 1. Demographics of patient cohort

	N = 55
Age (years)	44.5 ± 19.3
Gender	
Female	42 (76.4%)
Male	13 (23.6%)
Age of the onset of hypoparathyroidism	32.9 ± 19.0 (5-76)
Duration of hypoparathyroidism	11.2 ± 7.5 (1-32)
Etiology	
Postsurgical	41 (74.5%)
Autoimune	9 (16.4%)
Pseudohypoparathyroidism	5 (9.1%)

Data are presented as mean ± SD (range) for age at onset and duration of hypoparathyiroidism. Data are presented as N (percent) for the subgroups.

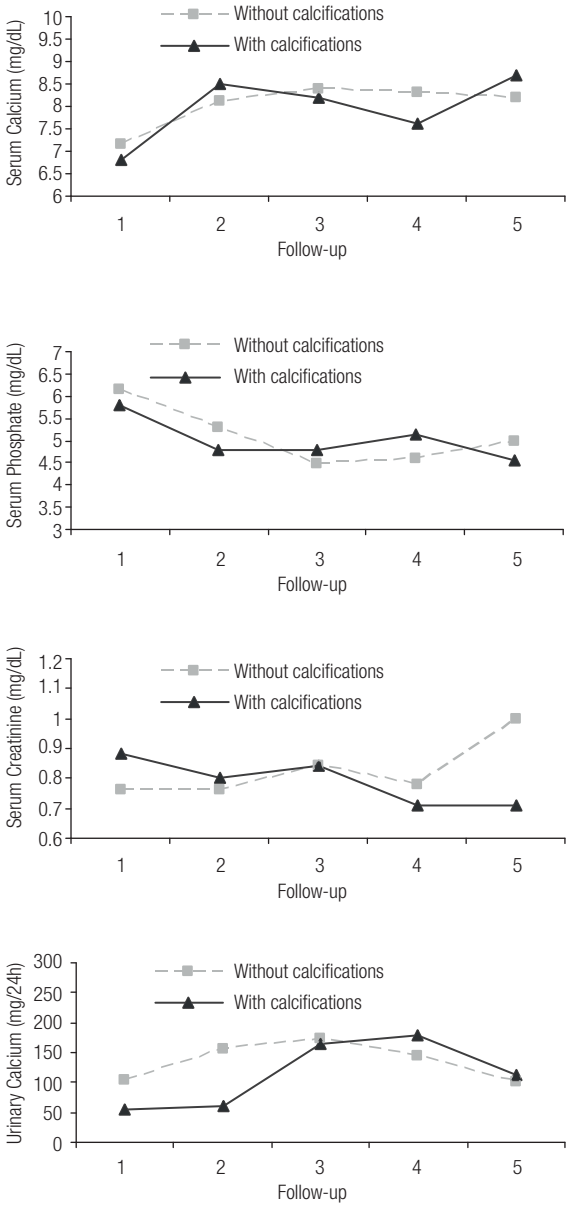
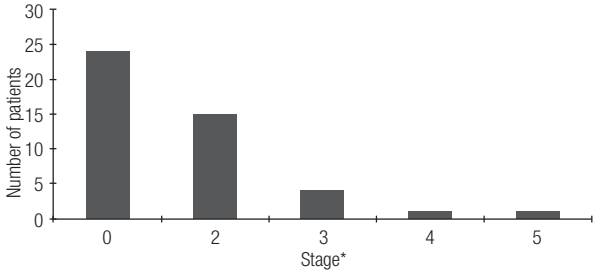


Figure 1. Follow-up of laboratorial findings according to the presence of renal calcification.

was 92.9 ± 36.2 mL/min/1.73 m^2. Consequently, the renal function was in 15 (33.3%) patients in stage II of chronic kidney disease as stated in Kidney Disease Outcomes Quality Initiative (KDIGO) (17), 4 (8.9%) in stage III, 1 (2.2%) in stage IV, and 1 (2.2%) in stage V (Figure 2). Hence, 21 (46.7%) showed an eGFR lower than 90 mL/min/1.73 m^2 and would present a chronic kidney disease at least at stage II.

Eleven patients (20%) had a cranial CT evaluation in their records, while 5 (45%) had basal ganglia calcification (BGC). From these patients with BCG, 2 had post-surgical HP, 1 had pseudo-HP, and 2 had HP caused by other etiologies.



* Stage 1: glomerular filtration rate (GFR) > 90 mL/min/1.73 m^2; Stage 2: GFR 60-80; Stage 3: GFR 30-59; Stage 4: 15-29; Stage 5: < 15 or dialysis.

Figure 2. Distribution of patients according their renal failure stage.

DISCUSSION

This is a pioneer study in our country on hypoparathyroidism (HP) patients followed at a reference center describing the etiologies, laboratory findings, doses of calcium and vitamin D, and complications. In this cohort there was a wide range of age at diagnosis and a high proportion of females, with the post-surgical being the most frequent etiology of the disease (12,18), agreeing with the literature (5,19,20). The most significant clinical feature was the presence of renal complication in this cohort of patients. Kidney calcification and renal function impairment were present in a great number of patients; however, our patients did not have hypercalciuria and the calcium level was within the target range. Kidney calcification and renal function impairment were present in a great number of patients even though mean serum and urinary calcium levels were within the target range. However, higher levels of urinary calcium excretion were seen in patients with renal calcification, suggesting that a upper limit of 4.0 mg/kg/d of urinary calcium (normal range) might not be appropriate for HP patients. Indeed, lower levels of urinary calcium should be the goal for these patients in order to prevent renal complications. Mitchell and cols. reported these findings previously in a cohort of 120 patients with hypoparathyroidism whereas the prevalence of renal calcification was 31% (9). In contrast to our study, they demonstrated a correlation between the duration of the disease and the presence of renal impairment (9). Levy and cols. reported a prevalence of 38% of nephrocalcinosis in children with HP, with the most significant predictors being the degree of relative hypercalcemia and hyperphosphatemia (21). In the present study, no predictor of renal complication was identified such as the doses of calcium and vitamin D supplementation and laboratory abnormalities. This could demonstrate a higher susceptibility some patients may have to developing these calcifications, even when receiving similar doses of treatment as others, or could be the result of the great variability in the serum calcium and phosphorus during the treatment. However, our patients went through a significant worsening of kidney function during the follow-up, despite their younger age. Similarly, Mitchell and cols. found a higher prevalence of eGFR impairment, even though their patients kept serum calcium within the recommended ranges for 86% of the time (9).

In order to avoid these complications, maintenance of serum calcium around the lower limit and serum phosphate around the upper limit of normal ranges is recommended (1,5). In addition, the annual measurement of 24-hour urine calcium is encouraging for ruling out hypercalciuria, as well as renal ultrasound to diagnose nephrolitiases and nephrocalcinosis (5,13). However, it is a challenge to achieve these therapeutic goals, maintaining patients without symptoms, since HP is a chronic disease, and requires long life treatment. Recently, PTH 1-84 has been used in HP treatment in some countries (11,22). This new treatment may lead to control of calcemia without the exposure to high loads of calcium, and therefore may reduce urinary calcium excretion and consequently decrease the incidence of renal complications. Furthermore, this treatment may improve quality of life, an issue that has been discussed in the literature (22,23).

Basal ganglia calcification (BGC) has been established as a possible outcome of HP. Its prevalence demonstrated in the HP cohorts varied significantly from 12% up to 74%. However, it should be noted that the estimated prevalence of BGC in the general population may achieve 12.5% (24). Some studies

have shown BGC to be associated more specifically with the persistent high phosphate levels (9,12,24). Contrasting with kidney calcifications, BGC is not a silent outcome of the disease and could be seen at the diagnosis, and thus the cranial CTs may have been performed in patients who already presented neurological symptomatology, leading to the high percentage of our patients with BGC (45%) among those with CT exams. Mitchell and cols., in the same aforementioned study, also found a small percentage (26%) of patients with cranial CT, along with a high percentage (52%) with the presence of BGC (9). Although post-surgical HP is described as rarely causing this complication, because the diagnosis is made early in post-operative care (7), this was the etiology responsible for HP in two out of our five patients with BGC.

In summary, this study obtained data of HP patients followed up in a tertiary care hospital of Curitiba, in the South of Brazil. In this casuistic, it was observed that the treatment led to laboratorial control of calcium and phosphate serum levels. However, the treatment did not avoid an increase in serum creatinine levels and the presence of kidney calcifications. Higher urinary calcium excretion, even if still within the normal range, was associated with development of calcification. Since HP is most commonly a life-long disease, a complete kidney function evaluation, and the search for calcifications is highly recommended. In addition, lower rates of urinary calcium excretion should be aimed for in these patients.

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Evaluation of preoperative ultrasonographic and biochemical features of patients with aggressive parathyroid disease: is there a reliable predictive marker?

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ABSTRACT

Objective: Parathyroid cancer (PC) represents < 1% of cases of PHPT. Tumors demonstrating atypical histopathologic features and don't fulfill criteria for carcinoma are classified as atypical adenomas (APA). The purpose of this study was to determine a biochemical or ultrasonographic feature that can predict aggressive disease requiring more extensive surgery and closer follow-up. **Subjects and methods:** Twenty eight patients operated for PHPT and diagnosed with atypical adenoma (23 patients) or carcinoma (5 patients) were enrolled in this study. The control group consisted of 102 patients operated between the same dates and diagnosed with classical PA. Classical adenomas, atypical adenomas, and carcinomas were compared according to their biochemical and ultrasonographic parameters. **Results:** Serum Ca levels were significantly higher in the PC group compared with the APA and classical PA groups. Serum median PTH, Serum ALP and UCa was significantly higher in the APA and carcinoma groups compared to the classical PA group. ROC analysis was made to determine the best cut off values for predicting aggressive disease were 12.45 mg/dL, 265.05 pg/mL, 154.5 IU/l, 348.5 mg/day and 21.5 mm for Ca, PTH, ALP, UCa and the adenoma diameter, respectively. Multivariate analysis showed that serum Ca, ALP and isoechoic/cystic appearance were independent predictors for aggressive disease. **Conclusion:** Preoperatively high PTH, ALP, and UCa levels and large lesions with isoechoic or cystic appearances may be predictive of atypical adenoma or carcinoma in patients being evaluated for PHPT. In such cases, surgeons may prefer en bloc parathyroidectomy to minimally invasive surgery. Arch Endocrinol Metab. 2016;60(6):537-44

Keywords

Atypical parathyroid adenoma; parathyroid carcinoma; biochemical markers; ultrasound

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INTRODUCTION

Primary hyperparathyroidism (PHPT) is one of the most common endocrine diseases. It is usually caused by a single lesion; however, occasionally, there may be multiple tumors in the parathyroid glands. Cancer is rarely seen and represents 0.005% of all cancers and < 1% of cases of PHPT in the United States. It affects men and women equally, and its management and outcome are variable (1,2).

Although parathyroid carcinoma (PC) has a characteristic intraoperative gross appearance, including invasion to neighboring anatomical structures and being densely firm, the final diagnosis is made upon histopathological examination, in which an invasive growth pattern or metastasis must be demonstrated. The World Health Organization (WHO) morphological criteria are currently used for diagnosis. Extensive invasion of adjacent tissues and metastatic spread represent the two absolute diagnostic criteria (3). The other criteria include focal coagulation necrosis, irregular dense fibrosis, and capsular, vascular, or neural invasion. The presence of certain cytological and architectural features such as adherence to adjacent organs, a solid growth pattern, broad bands of fibrosis, cytological atypia, and an irregular growth contour do not indicate malignancy but are recognized as atypical features encountered more commonly in malignant than benign tumors. Tumors that demonstrate these atypical features and do not fulfill criteria for carcinoma

can be classified as atypical parathyroid adenomas (APA) (4,5). It is postulated that atypical adenomas may be a preceding stage of carcinoma development, and this entity is expected to include tumors previously referred to as equivocal carcinomas (6). The clinical importance and long-term outcomes as well as appropriate operative management and surveillance are not well defined for APA probably due to the overall low prevalence as well as the lack of a standard definition of APA. For aggressive parathyroid tumors, early diagnosis and a radical therapy approach are paramount if clinical suspicion is made prior to surgery.

There is no single biochemical parameter or imaging property that can distinguish aggressive parathyroid disease (APA and carcinoma) from classic adenomas, and scarce data exist in the literature concerning any suspicious ultrasonographic features that can predict it (7).

Herein, we aimed to evaluate the preoperative imaging and biochemical features of patients histopathologically diagnosed with APA or carcinoma. The purpose of this study was to determine a biochemical or ultrasonographic feature that can predict aggressive disease requiring more extensive surgery and closer follow-up to prevent any lifelong complications.

SUBJECTS AND METHODS

A hundred and sixty two patients were operated for PHPT from January 2011 to September 2015 in our center. Thirty two patients were excluded from the study. Exclusion criteria were; having chronic renal failure (4 patients), secondary or tertiary hyperparathyroidism (2 patients), familial syndromes (1 with MEN1 and 2 with MEN2A) and incomplete patient records (23 patients). Finally twenty eight patients diagnosed with atypical adenoma or carcinoma (23 patients with APA and 5 with PC) and a hundred and two patients operated between the same dates and diagnosed with classical PA with complete histopathology and preoperative patient records were enrolled in this study.

The study was approved by the local ethics committee of the University Hospital. Tumors were classified as either classical parathyroid adenoma (PA) or aggressive parathyroid tumors (APA or PC). APA was defined by the presence of two of the following characteristics: clinical/intraoperative adherence, bands of fibrosis, pronounced trabecular growth, and mitotic rates of > 1/10 high-power fields (hpfs) (4,5). APA also lacked any of the indisputable criteria for malignancy, including invasion of vascular or perineural soft tissue, or surrounding structures (including the thyroid, recurrent laryngeal nerve, trachea, and esophagus) or documented metastatic disease. Insufficient clinical data or missing histopathological reports were defined as exclusion criteria. All patients' demographic, clinical, and biochemical characteristics were reviewed, with an emphasis on collection of parathyroid disease-specific characteristics. Biochemical data included preoperative serum calcium (Ca, mg/dL), phosphorus (P, mg/dL), parathyroid hormone (PTH, pg/mL), and alkaline phosphatase (ALP, IU/L) levels, and 24 h urinary calcium excretion (UCa, mg/day) and they were collected from the records immediately prior to surgery in all patients. Reference ranges for Ca, P, PTH, ALP, and 24 h UCa were 8.5–10.5 mg/dL, 2.5–4.5 mg/dL, 15-60 pg/mL, 36–113 IU/L, and 25–300 mg/day, respectively.

Bone mineral density (BMD) was measured by DXA (QDR-4500, Hologic Inc, Waltham, MA) at the lumbar spine in posterior-anterior projection (L1-L4) (LS BMD) and femoral sites [femoral neck (FN-BMD)]. One-third distal non dominant forearm measurement was not available in most of the patients. BMD was expressed as T-score or Z-score. T-scores was taken into consideration in postmenopausal women and males older than 50 years of age while Z scores were evaluated in premenopausal women and young males.

Imaging data included reports of preoperative ultrasound (US) and sestamibi scans. US was performed by experienced endocrine specialists. All lesions were localized by US in the APA and PC groups whereas all except 4 lesions were localized in the classical PA group. Lesions were recorded as isoechoic, hypoechoic, or cystic according to their ultrasonographic appearance (Figure 1). Sizes and the localization of the lesions were recorded. Classical adenomas, atypical adenomas, and carcinomas were compared according to their biochemical and ultrasonographic parameters.

Statistical analysis

Data analysis was performed using the SPSS version 17.0 software (IBM Corporation, Armonk, NY, USA). For all data, the assumption of normal distribution was verified using a Kolmogorov-Smirnov test. Levene's test was used for the evaluation of homogeneity of variances. Continuous data are shown as the mean ± standard deviation (SD) or the median and interquartile range, and categorical variables are shown as the number of individuals and percentages.

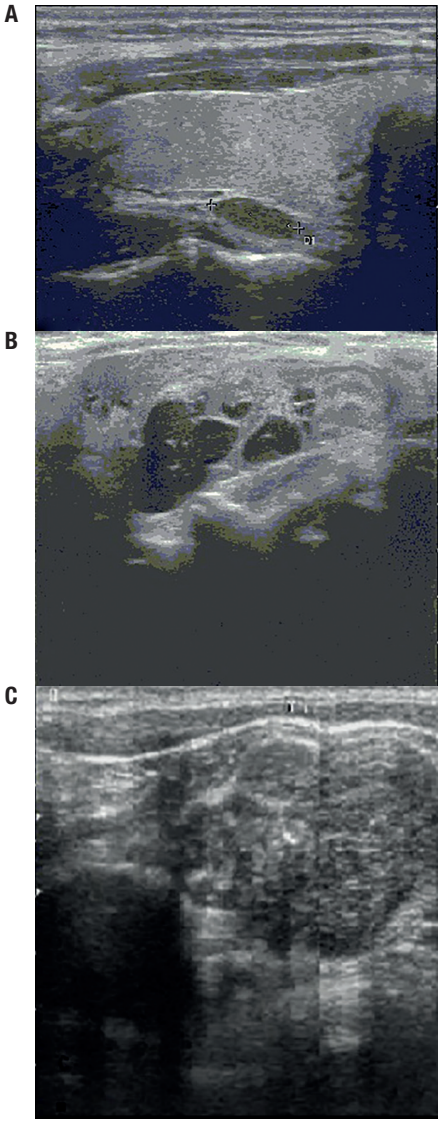

Figure 1. Hypoechoic, cystic and isoechoic appearances of the lesions belonging to a classical adenoma, carcinoma and atypical adenoma, respectively.

The mean differences among groups were analyzed by one-way analysis of variance (ANOVA). The Kruskal Wallis test was used for comparisons of medians, and the Conover's multiple comparison test was used for *post hoc* comparisons. Categorical data were evaluated using the chi-square test or Fisher's exact test, where appropriate.

The area under the receiver operating characteristic (ROC) curve (AUC) and 95% confidence intervals (CI) for both laboratory and adenoma diameter measurements were calculated. When the AUC was statistically significant, the optimal cut off point of each predictor was determined as the value giving the maximum sum of sensitivity and specificity. The diagnostic performances of laboratory and adenoma diameter measurements were evaluated including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

Multiple binary logistic regression with the backward method was used to determine the best predictor(s) that affected diagnosis. Any variable whose univariate test had a p value < 0.05 was included in the multivariate model. Odds ratios, 95% CIs, and Wald statistics were also calculated for each variable.

All variables were considered significant at a value of $p < 0.05$. The Bonferroni correction was applied to multiple comparisons to control for type I errors.

RESULTS

After exclusion of 32 patients (4 with CKD, 23 with incomplete patient records, 2 with tertiary hyperparathyroidism, 1 MEN1 with parathyroid hyperplasia and 2 MEN2A), data from 130 patients were analyzed. There were no significant differences between the groups with regard to age ($p = 0.991$). The sex distribution was significantly different between the classical PA and the APA groups; there were significantly more females in the classical PA group, whereas there were significantly more males in the APA group ($p < 0.05$). Serum Ca levels were significantly higher in the PC group compared with the APA and classical PA groups ($p < 0.001$ and $p = 0.010$, respectively). Serum P levels did not differ between the groups ($p = 0.370$) while PTH levels were significantly higher in the APA and PC groups compared with the classical PA group ($p < 0.05$). Serum ALP and 24 h UCa excretion were significantly higher in the APA and PC groups compared with the classical adenoma group ($p < 0.001$). 25-hydroxy vitamin D levels were not significantly different between the groups. Serum creatinine levels were not significantly different between the groups. FN-BMD expressed as T/Z scores were significantly lower in APA and PC groups compared with the classical PA group whereas LS-BMD scores did not differ significantly.

The largest adenoma diameter measured by preoperative US was higher in the APA and PC groups than in the classical adenoma group ($p < 0.001$), and there were also significant differences between the three groups with regard to the ultrasonographic appearance of the adenomas. Classical PAs had a mostly hypoechoic appearance whereas APA and carcinomas had a more isoechoic or cystic appearance. There were no differences between the groups with regard to

positive involvement in nuclear imaging (p = 0.158). As expected, disease recurrence/persistence and need for an additional surgical intervention were more prevalent in the PC group compared with the other two groups (p = 0.011). Demographic, biochemical, and imaging data are shown in Table 1.

Results of the ROC analysis investigating whether serum Ca, ALP, UCa, and PTH levels had statistically significant diagnostic value in differentiating classical adenomas from aggressive disease (APA or cancer) are shown in Table 2 and Figure 2. The AUC for serum Ca was statistically significant (AUC = 0.636, 95% CI: 0.509–0.762, p = 0.029) (Figure 2A). The best cut-off value for Ca for predicting aggressive disease was 12.45 mg/dL (sensitivity: 42.9%, specificity: 83.0%, PPV: 41.4%, NPV: 83.8%). The AUC for serum P was not statistically significant (AUC = 0.587, 95% CI: 0.450–0.724, p = 0.160), while that for PTH was significant (AUC = 0.787, 95% CI: 0.675–0.898, p < 0.001) and the best cut-off value for PTH was 265.05 pg/mL (sensitivity: 71.4%, specificity: 77%, PPV: 46.5%, NPV: 90.6%) (Table 2, Figure 2B). For serum ALP, the AUC was statistically significant (AUC = 0.843, 95% CI: 0.750–0.936, p < 0.001) and the best cut-off value was 154.5 IU/l for distinguishing aggressive disease from classical adenoma (sensitivity: 74.1%, specificity: 84.6%, PPV: 58.8%, NPV: 91.7%) (Table 2, Figure 2C). The AUC for 24 h UCa was statistically significant and the best cut-off value was 348.5 mg/day (sensitivity: 88.5%, specificity 47.8%, PPV: 32.4%, NPV: 93.6%) (Table 2, Figure 2D).

Table 1. Comparison of the demographic, biochemical and imaging data of the patients in three histological subgroups

	Classic (n = 102)	Atypical (n = 23)	Carcinoma (n = 5)	p-value
Mean age (years)	51.1 ± 14.0	51.3 ± 13.7	50.4 ± 13.7	0.991†
Male/Female	20/80 (20.0-80%)[a]	11/12 (47.8-52.2%)[a]	3/2 (60.0-40%)	**0.006‡**
Median Ca (mg/dL)	11.3 (9.2-14.7)[b]	11.6 (10-16)[c]	16.0 (11.6-16.5)[b,c]	**0.005¶**
Median P (mg/dL)	2.5 (1.1-4.5)	2.3 (2.5-2.9)	2.1 (1.4-3.5)	0.370¶
Median PTH (pg/mL)	167.5 (60-900)[a,b]	448.0 (139-735)[a]	520.0 (163-1077)[b]	**< 0.001¶**
Median ALP (IU/L)	101.0 (27-244)[a,b]	190.0 (66-718)[a]	589.0 (119-1880)[b]	**< 0.001¶**
Median urine Ca (mg/day)	370.5 (68-948)[a,b]	500.0 (362.0)[a]	500.0 (483-900)[b]	**0.002¶**
Mean 25-OH-Vit D (ng/mL)	13.47 ± 0.39	12.84 ± 0.47	11.57 ± 0.34	0.072
Median serum creatinine mg/dL	0.6 (0.4-1.2)	0.8 (0.5-1.8)	1.0 (0.6-1.9)	0.087
Median femur neck T score*	-1.06 (-3. 9-1.1)[a,b]	-1.90 (-3.2- 0.2)[a]	-2.8 (-3.7-0.1)[b]	**0.006**
Median lumbar spine T score*	-1.63 (-5.3-1.1)	-2.38 (-5.5-0.3)	-2.45 (-5.8-0.8)	0.13
Median lesion diameter (mm)	14.5 (5-43)[a,b]	22.0 (5-58)[a]	32.0 (15-44[b]	**< 0.001¶**
USG				**< 0.001‡**
Hypoechoic	95 (95.0%)[a,b]	15 (65.2%)[a]	1 (20.0%)[b]	
Isoechoic	3 (3.0%)[a,b]	5 (21.7%)[a]	3 (60.0%)[b]	
Cystic	2 (2.0%)[a]	3 (13.0%)[a]	1 (20.0%)	
Positive MIBI	63 (65.6%)	18 (78.3%)	5 (100.0%)	0.158‡
Persistent/Recurrent disease	3 (3.0%)[b]	1 (4.3%)[c]	3 (60.0%)[b,c]	**< 0,001‡**

† One-Way ANOVA; ‡ Chi-square test; ¶ Kruskal Wallis test; a: Classic vs Atypical (p < 0.05); b: Classic vs carcinoma (p < 0.05); c: atypical vs carcinoma (p = 0.010). * Z score was used for premenouposal females and males younger than 50 years of age.

Table 2. Areas under curve for biochemical and ultrasonographic parameters for distinguishing classical adenomas from aggressive disease, best cut off points and diagnostic performances

	AUC	95 % CI	p-value	Cut-off	Sensitivity	Specificity	PPV	NPV
Ca	0.636	0.509-0.762	**0.029**	> 12.45	0.429	0.830	0.414	0.838
PTH	0.787	0.675-0.898	**< 0.001**	> 265.05	0.714	0.770	0.465	0.906
ALP	0.843	0.750-0.936	**< 0.001**	> 154.5	0.741	0.846	0.588	0.917
Urinary Ca	0.716	0.604-0.829	**< 0.001**	> 348.5	0.885	0.478	0.324	0.936
Lesion diameter	0.715	0.590-0.839	**< 0.001**	> 21.5	0.571	0.811	0.471	0.865

AUC: area under the curve; CI: confidence interval; PPV: positive predictive value, NPV: negative predictive value.

Among the ultrasonographic parameters, the AUC was statistically significant for the longest lesion diameter (AUC = 0.715, 95% CI: 0.590–0.839, p < 0.001) and the best cut-off value was 21.5 mm (sensitivity: 57.1%, specificity 81.1%, PPV: 47.1%, NPV: 86.5%) (Table 2, Figure 2E).

We performed a univariate analysis to predict risk factors for aggressive disease; variables with a p-value < 0.05 were subsequently included in a multiple logistic regression analysis. When the groups were controlled for all other possible risk factors, the most valuable parameters that predicted aggressive disease were serum Ca, ALP, and ultrasonographic appearance. The risk for aggressive disease increased 10.885-fold with values of ALP > 154.5 IU/L (95% CI: 3.116–37.810, p < 0.001) and 3.8-fold with values of Ca > 12.45 mg/dL (95% CI: 1.061–14.100, p = 0.040). Moreover, the risk for aggressive disease was 37.158-fold higher if the lesion had an isoechoic appearance rather than a hypoechoic appearance (95% CI: 3.027–456.126, p = 0.005). In addition, a cystic appearance also increased aggressive disease risk 11.644-fold compared with a classical hypoechoic appearance (95% CI: 1.023–132.504, p = 0.048). The results of the multivariate analysis are summarized in Table 3.

Table 3. Multivariate analysis of variables that can independently predict presence of aggressive disease

	Odds ratio	95% Confidence interval		Wald	*p*-value
		Lower	Upper		
Ca > 12.45 mg/dL	3.867	1.061	14.100	4.200	**0.040**
ALP > 154.5 IU/L	10.855	3.116	37.810	14.025	**< 0.001**
Hypoechoic	1.000	-	-	-	**-**
Isoechoic	37.158	3.027	456.126	7.984	**0.005**
Cystic	11.644	1.023	132.504	3.914	**0.048**

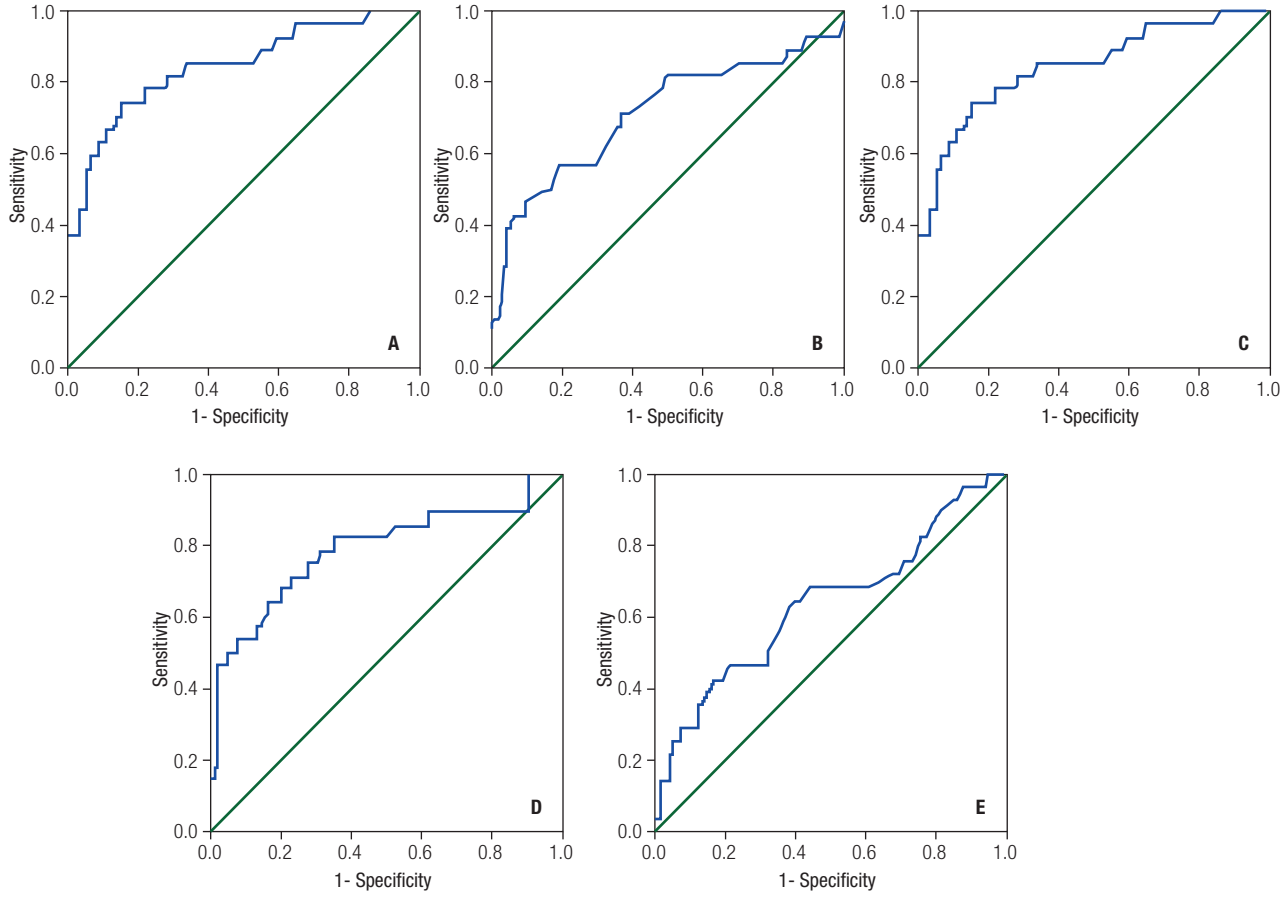

Figure 2. (**A**) ROC analysis for serum Ca (12.3 mg/dL cut-off had 42.9 sensitivity, 83% specificity, (**B**) ROC analysis for serum PTH (265.05 pg/mL cut-off had 71% sensitivity and 77% specificity), (**C**) ROC analysis for serum ALP (154.5 IU/L cut had 74.1% sensitivity and 84.6 specificity), (**D**) ROC analysis for Uca (348.5 mg/day cut off had 88.5% sensitivity and 47.8% specificity, (**E**) lesion size (21.5 mm had 57.1% sensitivity and 81.1% specificity).

The mean follow-up period was 3.2 years and all PC patients had recurrence or metastasis during the follow-up. There was no recurrent disease in the APA or classical PA groups, although three patients in the APA group and one patient in the classical PA group had persistent disease (sustained high levels of serum Ca and PTH within six months of surgery) and required an additional surgery.

DISCUSSION

In this report, we aimed to identify any preoperative biochemical and ultrasonographic variables that could differentiate classical adenomas from more aggressive parathyroid diseases, including APA and carcinomas.

The patient age did not differ significantly among patients with APA, classical PA, and PC in accordance to a previous report (8). However in many other series parathyroid carcinoma patients were younger than ones with benign adenomas (9). The sex distribution was different between the classical adenoma and APA groups, with a significant predominance of males in the APA group. The male-to-female ratio was also higher in the PC group, but was not significantly different from the classical adenoma group, which is most likely due to the low number of patients. Benign adenomas are most frequently seen in females. APA and carcinomas are similar in that the genetic bias that predisposes women to benign adenomas is not as dominant in APA and PC. This is supported by previously described sex-dependent patterns of chromosomal loss in men and women with PC (10).

PHPT is often associated with borderline or mild hypercalcemia (serum Ca < 11 mg/dL); values above 13 mg/dL are unusual in PHPT, although they do occur, and are more common in patients with parathyroid carcinoma-associated hypercalcemia (11). In our study, the median serum Ca level was significantly higher in the carcinoma group than in the APA and classical PA groups, similar to results of a previous report (12). All but one patient with PC had severe hypercalcemia, defined as serum Ca > 14 mg/dL. In a previous study with PC patients, serum Ca was identified as the most promising variable for the differentiation of patients with or without PC (13), whereas in another study, it was not determined to be an independent marker of carcinoma in a multivariate analysis (14). When we considered APA and PC patients together in the aggressive disease group, best cut-off value for Ca for predicting aggressive disease was 12.45 mg/dL. Thus, it was found to be an independent predictive marker for aggressive disease. To our knowledge, our study is the first to detect a cut-off value for distinguishing aggressive disease including APA and carcinomas.

PTH levels were significantly higher in the APA and PC groups compared to the classical PA group, but were not found to be an independent predictor for aggressive disease in the multivariate analysis, supporting the results of a previous report (13). The median PTH levels in the APA and carcinoma groups were similar, which supports a study by Fernandez-Ranvier and cols., which demonstrated no significant differences between the two groups with regard to PTH, although levels were slightly higher in the carcinoma group (15). However, McCoy and cols. demonstrated that PTH levels were significantly lower in patients with benign adenoma compared with PC, whereas they were similar between patients with benign adenoma and APA (16).

Serum ALP was significantly higher in the APA and PC groups than in the classical adenoma group; levels were not significantly different between the APA and PC groups. ALP was an independent predictive marker for aggressive disease, similar to a previous study in which serum ALP was significantly higher in carcinoma patients than in those with benign disease after all variables were adjusted (14). In our study, the best cut off value for ALP for distinguishing aggressive disease from classical adenoma was 154.5 IU/L (sensitivity: 74.1%, specificity: 84.6%). In the aforementioned study, the cut-off value of serum ALP was 285 IU/L and the sensitivity and specificity were 83.3% and 97.0%, respectively (14). Our cut-off value was lower, which was likely to be due to the inclusion of patients with atypical adenomas in the aggressive disease group, who had lower levels of ALP compared to carcinoma patients. We also detected that 24 h UCa excretion was significantly higher in the APA and PC groups than in the classical adenoma group, but it was not an independent predictor for aggressive disease. While interpreting that result it should be kept in mind that UCa is susceptible to the clinical condition and vitamin D status of the patient (17).

In our study most of the patients were vitamin D deficient. Epidemiological studies suggests that the prevalence of hypovitaminosis D (including deficiency and insufficiency) is more prevalent in patients with PHPT than in the general population regardless of sex, age and the season (18). The reason for that co-

existence is not fully understood but there are several explanations in the literature such as increased conversion of 25 OH D to 1, 25 OHD by increased PTH (19). It is well established that hypovitaminosis D might result in increased PTH values and may mask a more significant evaluations in serum Ca, because of reduced Ca absorbtion. Increased PTH levels indeed aggravate bone catabolism, turnover and bone loss (20). In addition to that there is an increased risk of osteoporotic fractures in patients with PHPT in case of low vitamin D levels (21). There are controversies about the effect of vitamin D repletion on BMD at lumbar spine and femur neck. Some authors suggest that BMD is increased with repletion in both sites whereas others claim that 25 OHD has no independent effect on BMD (22,23). Besides the negative effects on BMD, low vitamin D levels may contribute to profound and lengthened hypocalcaemia (hungry bone syndrome) after parathyroidectomy (20). Therefore it has generally been recommended to supplement vitamin D to normalize 25(OH) vitamin D levels, although there are so far no available data to support the premise that this would contribute to the prevention of hypocalcaemia. In our center, we correct vitamin D deficiency aiming at serum levels > 20 ng/mL unless the patient has severe hypercalcaemia in the light of the current studies. There is no consensus about the optimum replacement dose and interval in the literature. In our unit, vitamin D repletion is undertaken using cholecalciferol 50.000 IU/ week for 1 month and vitamin D is checked monthly in order to adjust the maintenance dosage (1500-2000 IU/day).

In our study, femur neck T scores were significantly lower in APA and PC group compared with the classical PA group where as vertebra T scores didn't differ significantly between the groups. It is known that in PHPT patients may have decreased BMD, in particular at more cortical sites (forearm and hip) as compared with more trabecular sites and the degree of bone loss is directly related with disease severity.

Ultrasonography is a convenient and inexpensive imaging modality for the evaluation of the parathyroid gland with a sensitivity and specificity of 98% and 88%, respectively (24). The classical gray-scale imaging features include oval or lobulated extra-thyroidal hypoechoic lesions with a well-defined margin. In the present study, classical PAs had mostly hypoechoic appearances whereas APA and carcinomas had more isoechoic or cystic appearances. In addition, cystic change was an independent predictor for aggressive disease. There are scarce data in the literature concerning the prevalence and relevance of atypical ultrasound features for the evaluation of parathyroid lesions. Our study supports a previous report, which suggested that parathyroid lesions with atypical imaging features are associated with a higher incidence of malignancy (25). Moreover, in our study, the AUC for the longest adenoma diameter measured by ultrasonography was statistically significant, and the best cut-off value for predicting aggressive disease was 21.5 mm. In a previous report, the cut-off value for PC was 3 cm (26), while in another study the median tumor size at the time of diagnosis in 286 cases of PC treated in the USA from 1985 to 1995 was approximately 3.3 cm (27).

In our study the incidence of PC was higher than reported in the literature. Although parathyroid carcinoma (PC) is an uncommon finding, accounting for only 1-2% of patients with primary hyperparathyroidism (PHPT); a relatively higher incidence has been reported in Italy and Japan, in consistent with our study (28,29). Another possible etiology that can explain the higher incidence may be the fact that our hospital is a tertiary center receiving more complicated cases who are candidates for surgery. Moreover, the possible effects of radiation exposure after the Chernobyl disaster might have contributed to the higher incidence of cancers. Case reports and retrospective identification of several cases of parathyroid carcinoma in patients exposed to radiation have appeared in the literature in the last three decades supporting our hypothesis (30).

In this study we found that certain laboratory and clinical parameters were predictive for presence of aggressive disease. However we also observed that even mild clinical and laboratory disease may be associated with parathyroid carcinoma and benign disease could be found in large lesions with severe clinical picture. Similarly, Agarwal and cols. showed severe clinical features such as palpable parathyroid tumor, advanced skeletal and renal manifestations, and very high serum calcium and parathyroid hormone levels are common in Indian PHPT patients although only few have PC (31). Moreover Sulaiman and cols. showed that most of larger parathyroid lesions were benign PAs associated with specific genomic features (32).

A limitation of our study was that histopathology was not interpreted by one pathologist only, and ultrasonography was performed by two different endocrine specialists. Moreover, the number of patients diagnosed with carcinoma was small.

In conclusion, the clinical distinction between classical adenoma and aggressive parathyroid disease is of critical importance to determine the appropriate extent of resection and follow-up. Preoperatively high PTH, ALP, and UCa levels and large lesions with isoechoic or cystic appearances may be predictive of atypical adenoma or carcinoma in patients being evaluated for PHPT. In such cases, surgeons may prefer en bloc parathyroidectomy to minimally invasive surgery. It should also be kept in mind that those results are specific for our population, need to be verified in different populations and meanwhile the results should be interpreted with caution.

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Metabolic syndrome and sexual function in postmenopausal women

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ABSTRACT

Objective: The purpose of this study was to evaluate whether female sexual dysfunction (FSD) is associated with metabolic syndrome (MS) and to identify factors that contribute to FSD in postmenopausal women. **Subjects and methods:** This was a cross-sectional study in 111 sexually active women aged 45-65 years. We applied the Female Sexual Function Index (FSFI) to evaluate the participant's sexual function and a structured questionnaire to collect demographic, socioeconomic, clinical, anthropometric, and laboratory data. **Results:** The prevalences of MS and FSD were 68.5% and 70.3%, respectively. After logistic regression analysis, we identified the following variables associated with FSD: married status (prevalence ratio [PR] 1.69, 95% confidence interval [95% CI] 1.16-2.47, $p < 0.01$), 6-10 years elapsed since menopause (PR 1.60, 95% CI 1.22-2.09, $p < 0.01$), occurrence of climacteric symptoms (PR 1.01, 95% CI 1.00-1.02, $p = 0.03$), and history of sexual abuse (PR 1.40, 95% CI 1.12-1.73, $p < 0.01$). **Conclusion:** We found a high prevalence of MS and FSD, but no association between both. Married status, time elapsed since menopause, climacteric symptoms, and history of sexual abuse emerged as factors associated with FSD on multivariate analysis. Arch Endocrinol Metab. 2016;60(6):545-53

Keywords
Metabolic syndrome; female sexual function; menopause; postmenopausal

INTRODUCTION

A woman's sexual health is associated with several psychological and interpersonal factors, and may be affected by aging and metabolic changes. In this context, female sexual dysfunction (FSD) is a multifactorial and multidimensional biological problem (1). The concept of FSD includes the occurrence of difficulty in any phase of the sexual response cycle or pain associated with intercourse (2).

Menopause is a transitional life phase in which a woman undergoes hormonal, physical, psychological, and social adjustments. Previous studies have demonstrated that menopause may negatively influence sexuality, leading to an important dysfunctional condition and affecting quality of life (3,4).

The decrease in estrogen levels associated with menopause may lead to changes in lipid profile, increase in body weight, and accumulation of abdominal fat. A recent report addressed in the Princeton III Consensus suggests an association between female sexual function with cardiovascular and metabolic diseases (5).

During the climacteric phase, a woman's cardiovascular risk profile undergoes changes characterized by the onset or worsening of some risk factors such as central obesity, hypertension, and dyslipidemia. These factors, in addition to hyperglycemia and insulin resistance, constitute the concept of metabolic syndrome (MS) (6).

Obesity, hypertension, dyslipidemia, and type 2 diabetes, which are conditions frequently present in individuals with MS, are considered risk factors for atherosclerosis and endothelial dysfunction. In FSD, endothelial dysfunction impairs tissue oxygenation and causes subsequent functional and structural damage to the female genital tract (7). A decrease in pelvic blood flow secondary to atherosclerotic disease leads to fibrosis of the vaginal wall and clitoral smooth muscle, eventually resulting in vaginal dryness and dyspareunia (8).

A study has shown the occurrence of low sexual function scores in premenopausal women evaluated with the Female Sexual Function Index (FSFI). Decreases in FSFI total scores were proportional to an increase in the number of MS components (9). Another study in postmenopausal women also found an association between MS and low sexual function scores (10). In contrast, Kim and cols., while studying middle-aged women, found no association between MS and most of

the components of sexual function (11). Following the same line, a study in climacteric women by Politano and cols. found no association between MS and decreased sexual function, except for increased age which was associated with a decreased sexual function (12).

Among the various factors involved with female sexual function, MS seems to be an emergent concern, due in part to its elevated global prevalence. However, it is yet not known whether the effects of MS are significant enough to lead to FSD (11); in fact, data from the literature are still conflicting regarding the association between MS and FSD.

Both conditions are multifactorial, and their diagnoses depend on the characteristics of the studied population and the criteria adopted for diagnosis. The duration and severity of the MS also seem to have an effect on a woman's sexuality; however, it is still unclear how severe the MS must be to exert this effect (11,12).

The purpose of the present study was to evaluate whether FSD is associated with MS and identify the factors that contribute to FSD in postmenopausal women.

SUBJECTS AND METHODS

The study population in this observational, cross-sectional study comprised postmenopausal women attending the outpatient clinic of the Department of General Gynecology at the Fernandes Figueira National Institute for Women, Children, and Youth Health/IFF (Rio de Janeiro, Brazil) between August and December 2013.

In order to calculate the sample size, we used information from the study by Martelli and cols. (10) who evaluated the occurrence of FSD in postmenopausal women using the FSFI questionnaire. These authors found a prevalence of FSD of 68% in patients with MS, compared with 41% in those without MS. Based on that, we estimated in our study a sample size of 200 women. The sample size necessary to detect a difference in FSD prevalence was calculated at a power of 80% and a significance level (α) of 0.05. Since the sample size obtained for each FSFI domain was smaller than that calculated for all FSFI domains as a whole, we calculated the sample sizes at a lower power (56.89%) for the domain lubrication and at a higher power (95.81%) for the domain arousal.

The inclusion criteria comprised women aged between 45 and 65 years, with amenorrhea for longer than 12 months, serum follicle-stimulating hormone (FSH) level ≥ 40 mIU/mL (13), and reporting sexual activity within the last 4 weeks from the date of the interview. The exclusion criteria were the use of hormone replacement therapy (HRT) in the past year, past or current chemotherapy or radiation therapy due to cancer, pelvic surgery for bilateral oophorectomy, premature menopause, neurological disease, type 1 diabetes, thyroid disease, hyperprolactinemia, and homosexual relationship.

During the study period, a total of 416 postmenopausal women aged between 45 and 65 years visited the outpatient clinic and were invited to participate in the study. Of all women, 40 refused to participate, 46 had a history of cancer, 53 were on HRT, 24 had reached menopause prematurely, 37 had undergone bilateral oophorectomy, and two had neurological diseases. Of the remaining 214 women, two were excluded for failing to draw blood and 13 for presenting an FSH value ≤ 40 mIU/mL. Among the remaining 199 women, only 111 reported sexual activity within the prior 4 weeks from the study and were included in the analysis.

The project was approved by the Research Ethics Committee (REC) at the Fernandes Figueira Institute under protocol number CAAE 03498812.7.0000.5269 and REC certificate of approval number 359.174. All participants signed an informed consent form.

A structured questionnaire with closed questions was applied to each participant to identify demographic, socioeconomic, and clinical variables. The participants' schooling level was evaluated by the number of years of complete study. The household *per capita* income was considered as the sum of all monthly earnings of the family members, and the result was categorized in numbers of minimum wages in Brazilian currency (Brazilian real [R$]).

We used the International Consultation on Incontinence Questionnaire – Short Form (ICIQ-SF) to evaluate the occurrence of urinary incontinence (UI). The ICIQ-SF is composed of four questions that evaluate the frequency, severity, and impact of UI. The scores of the total result of questions that evaluate severity range from 0 to 21 points (14). Women with scores ≥ 8 were considered symptomatic for UI (15).

Symptoms related to the climacteric phase were evaluated with the Blatt-Kupperman Menopausal Index (BKMI), which is a global quantitative evaluation of the occurrence of such symptoms. The symptomatology is evaluated with 11 questions regarding hot flashes,

arrhythmias, dizziness, headache, paresthesia, tingling sensation, arthralgia and myalgia (classified as somatic symptoms), as well as fatigue, nervousness, and sadness (these last three symptoms are categorized as psychological symptoms). The higher the score, the more symptomatic the women were (16). For the present analysis, we used the mean total score of the questionnaire.

The participants' sexual function was evaluated with the FSFI, a validated questionnaire that assesses the woman's sexual response and quality of life over the previous 4 weeks (2,17). This questionnaire is constituted by 19 questions about the domains of sexual response: desire and subjective arousal, lubrication, orgasm, satisfaction, and pain or discomfort. Individual scores are obtained by the sum of the items that constitute each domain and are multiplied by the factor of this domain, resulting in the weighted score. The final score (which may range from a minimum of two to a maximum of 36) is obtained by the sum of the weighted scores in each domain. The cutoff value for the score that determines the occurrence of FSD is 26.5, with higher scores indicating better sexuality (17).

Two trained investigators applied the questionnaires and collected anthropometric measurements in a private room.

To establish the diagnosis of MS, we used the criteria defined by the Joint Scientific Statement for Harmonizing the Metabolic Syndrome issued by the International Diabetes Federation (IDF); American Heart Association; National Heart, Lung, and Blood Institute; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. We considered as having MS those participants who presented three of the following criteria: waist circumference ≥ 88 cm (as established for Brazilian women) (18), serum triglyceride level ≥ 150 mg/dL, serum high-density lipoprotein cholesterol (HDL-C) level ≤ 50 mg/dL, systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, and fasting blood glucose level ≥ 100 mg/dL. Use of medications and treatment for high blood pressure, diabetes, hypertriglyceridemia, and low HDL-C values were also considered in the diagnosis of MS since they are also part of the syndrome's criteria (6).

Blood pressure was evaluated in the participant's non-dominant arm using a mercury sphygmomanometer accurate to 2 mmHg while the participant remained seated after resting for at least 10 minutes. The participants' weights were measured with a digital scale (Leader, Model LD1050) and represented in kilograms, and their heights were measured using a Wiso® stadiometer 200 cm long and accurate to 0.05 cm.

The participants' body mass index (BMI) was calculated by the weight divided by the square of the height and was represented in kg/m^2. The BMI results were categorized according to the classification determined by the World Health Organization for adults (19). The patients were categorized as eutrophic when having a BMI between 18.5-24.9 kg/m^2, overweight when between 25.0-29.9 kg/m^2, and obese when ≥ 30.0 kg/m^2.

Blood samples were collected after fasting for determination of levels of glucose, HDL-C, triglycerides, C-reactive protein (CRP), free thyroxine, thyroid-stimulating hormone, FSH, estradiol, and prolactin. Blood tests were evaluated by enzyme-linked fluorescence (ELFA) and immunofluorometric assays using the kit VIDAS® (bioMérieux). An immunonephelometry assay was used to evaluate the serum concentrations of CRP using the CardioPhase® hsCRP reagent and a Siemens BN II equipment. According to the results of high-sensitive CRP (expressed in mg/dL), patients were classified as low risk (when < 0.1 mg/dL), intermediate risk (when between 0.1 and 0.3 mg/dL), and high risk (when > 0.3 mg/dL) (20).

Statistical analysis

The results of the statistical analyses for continuous variables with normal distribution are presented as mean and standard deviation. When normality was not observed, the results are expressed as median and minimum and maximum values. The Shapiro-Wilk test was used to verify the normality of the data. Bivariate analyses were performed to identify factors potentially associated with MS. Pearson's chi-square test measured the statistical significance of the association between MS and categorical variables. Fisher's exact test was applied in cases with at least one expected frequency below five. To compare continuous measures, Student's *t*-test was used for variables with normal distribution and the Mann-Whitney test for those without normal distribution. Poisson regression models with robust variance estimation were employed to determine factors associated with sexual dysfunction.

The variance inflation factor was used to detect possible multicollinearity. Univariate regression analyses were carried out, and variables with a *p* value below 0.20 were included in a multiple regression model. The significance level adopted to define the factors statistically associated with sexual dysfunction in the multiple regression model was 5%. The magnitude of the association between explanatory and outcome variables was quantified as prevalence ratio (PR) and corresponding 95% confidence intervals (95% CI). The software used for the analysis of the data were SPSS, version 20, and R, version 3.0.2.

RESULTS

Among the 111 women evaluated, the average age was 55.9 ± 4.8 years and 78.4% were married. The median duration of school education was 8 years and the mean household *per capita* income was R$ 600. Of all participants, 83.8% were multipara, the mean age at menopause was 48.3 years, 11.7% were smokers, 27.9% consumed alcohol regularly, 56.8% were sedentary, 57.7% used antihypertensive drugs, 23.4% used hypoglycemic drugs, and 16.2% used hypolipidemic drugs. A total of 68.5% of the participants had a diagnosis of MS (Table 1).

Table 1. Sociodemographic, clinical, anthropometric, and metabolic characteristics according to the presence or absence of metabolic syndrome (MS)

Variable	Metabolic syndrome			*P* value
	Total (n = 111)	Absent (n = 35)	Present (n = 76)	
Age (years)	56.0 (45-65)	55.5 (45-64)	57.0 (48-65)	**0.02**
Marital status				
Married	87 (78.4%)	27 (31.0%)	60 (69.0%)	1.00
Not married	24 (21.6%)	8 (33.3%)	16 (66.7%)	
Schooling years	8 (0-17)	8 (2-14)	8 (0-17)	0.22
Number of pregnancies				
Nulliparous	5 (4.5%)	4 (80.0%)	1 (20.0%)	**0.02**
Primiparous	13 (11.7%)	6 (46.2%)	7 (53.8%)	
Multiparous	93 (83.8%)	25 (26.9%)	68 (73.1%)	
Age at menopause	48.3 (±4.54)	47.9 (±3.70)	48.4 (±4.90)	0.60
Duration of menopause (years)				
1 to 5	50 (45.0%)	17 (34.0%)	33 (66.0%)	0.57
6 to 10	28 (25.2%)	10 (35.7%)	18 (64.3%)	
More than 10	33 (29.7%)	8 (24.2%)	25 (75.8%)	
Blatt-Kupperman Menopausal Index	22.7 (±12.83)	22.3 (±12.23)	22.9 (±13.18)	0.81
Smoker				
Yes	13 (11.7%)	4 (30.8%)	9 (69.2%)	0.91
No	61 (55.0%)	18 (29.5%)	43 (70.5%)	
Former	37 (33.3%)	13 (35.1%)	24 (64.9%)	
Alcohol consumption				0.65
Yes	31 (27.9%)	11 (35.5%)	20 (64.5%)	
No	80 (72.1%)	24 (33.8%)	56 (70.0%)	
Physical activity				
Active	48 (43.2%)	15 (31.2%)	33 (68.8%)	1.00
Sedentary	63 (56.8%)	20 (31.7%)	43 (68.3%)	
Body mass index				
Eutrophic	23 (20.7%)	15 (65.2%)	8 (34.8%)	**< 0.01**
Overweight	43 (38.7%)	14 (32.6%)	29 (67.4%)	
Obese	45 (40.5%)	6 (13.3%)	39 (86.7%)	

continuation

Variable	Metabolic syndrome			*P* value
	Total (n = 111)	Absent (n = 35)	Present (n = 76)	
Waist circumference				
Normal	23 (20.7%)	16 (69.6%)	7 (30.4%)	**< 0.01**
Increased (≥ 88 cm)	88 (79.3%)	19 (21.6%)	69 (78.4%)	
Systolic blood pressure				
Normal	64 (57.7%)	28 (43.8%)	36 (56.2%)	**< 0.01**
Elevated (≥ 130 mmHg)	47 (42.3%)	7 (14.9%)	40 (85.1%)	
Diastolic blood pressure				
Normal	64 (57.7%)	29 (45.3%)	35 (54.7%)	**< 0.01**
Elevated (≥ 85 mmHg)	47 (42.3%)	6 (12.8%)	41 (87.2%)	
Blood glucose				
Elevated (≥ 100 mg/dL)	55 (49.5%)	4 (7.3%)	51 (92.7%)	**< 0.01**
Normal	56 (50.5%)	31 (55.4%)	25 (44.6%)	
High-density lipoprotein cholesterol				
Low (< 50 mg/dL)	61 (55.0%)	12 (20.0%)	48 (80.0%)	**< 0.01**
Normal	50 (45.0%)	23 (45.1%)	28 (54.9%)	
Triglycerides				
Increased (≥ 150 mg/dL)	45 (40.5%)	6 (13.3%)	39 (86.7%)	**< 0.01**
Normal	66 (59.5%)	29 (43.9%)	37 (56.1%)	
C-reactive protein				
Low < 0.1 mg/dL	27 (25.0%)	11 (40.7%)	16 (59.3%)	**< 0.01**
Moderate 0.1–0.3 mg/dL	42 (38.9%)	19 (45.2%)	23 (54.8%)	
High > 0.3 mg/dL	39 (36.1%)	5 (12.8%)	34 (87.2%)	

Tests: Student's *t*-test for equality of means, Mann-Whitney U test, Pearson's chi-square test, and Fisher's exact test.

On univariate analysis, the following variables were potentially associated with FDS: married status ($p = 0.02$), *per capita* income ($p = 0.05$), vaginal childbirth ($p = 0.02$), time elapsed since menopause ($p < 0.01$), BKMI value ($p = 0.02$), prior bladder surgery ($p < 0.01$), alcohol consumption ($p < 0.01$), and history of sexual abuse ($p < 0.01$).

Regarding the FSFI, we analyzed the total scores and the scores in each domain of the questionnaire. The median FSFI score in the overall cohort was 23.9, which is below the cutoff score of 26.5 determined for the questionnaire (Table 2). To evaluate the relationship between the components of MS and FSD, we analyzed the total FSFI scores according to the number of MS components, but we found no significant differences. After a multiple regression analysis, we identified the following variables associated with FSD: marital status, time elapsed since menopause, climacteric symptoms, and history of sexual abuse (Table 3).

Table 2. Total score and scores in each domain of the Female Sexual Function Index (FSFI) questionnaire according to the presence and absence of metabolic syndrome

Domain	Metabolic syndrome			*P* value
	Total (n = 111)	Absent (n = 35)	Present (n = 76)	
FSFI	23.9 [8.8-34.9]	22.8 [10.0-32.5]	24.8 [8.8-34.8]	0.25
Desire	3.0 [1.2-6.0]	2.4 [1.2-4.8]	3.0 [1.2-6.0]	0.12
Arousal	3.6 [1.2-6.0]	3.3 [1.2-5.7]	3.9 [1.2-6.0]	0.07
Lubrication	4.2 [1.2-6.0]	3.6 [1.2-6.0]	4.2 [1.2-6.0]	0.62
Orgasm	4.0 [1.2-6.0]	4.0 [1.2-6.0]	4.2 [1.2-6.0]	0.75
Satisfaction	4.8 [0.8-6.0]	4.8 [1.2-6.0]	4.8 [0.8-6.0]	0.63
Pain	4.0 [0.0-6.0]	3.6 [1.2-6.0]	4.4 [0.0-6.0]	0.47

Test: Mann-Whitney U.

Table 3. Multiple regression model of variables associated with risk of sexual dysfunction

Variable	Adjusted prevalence ratio (95% confidence interval)	*P* value
Marital status		
Married	1.69 (1.16-2.47)	**< 0.01**
Not married	1.00	---
Income *per capita*	0.99 (0.99-1.00)	0.07
Number of vaginal births	0.98 (0.89-1.07)	0.61
Number of cesarean births	0.94 (0.80-1.10)	0.41
Duration of menopause		
1 to 5 years	1.00	---
6 to 10 years	1.60 (1.22-2.09)	**< 0.01**
> 10 years	0.94 (0.69-1.28)	0.67
Blatt-Kupperman Menopausal Index	1.01 (1.00-1.02)	**0.03**
Bladder surgery		
Yes	1.02 (0.75-1.37)	0.92
No	1.00	---
Smoker		
Yes	1.04 (0.56-1.97)	0.89
No	1.00	---
Former	1.16 (0.91-1.49)	0.23
Alcohol consumption		
Yes	0.74 (0.50-1.10)	0.14
No	1.00	---
History of sexual abuse		
Yes	1.40 (1.12-1.73)	**< 0.01**
No	1.00	---
C-reactive protein		
Low	1.00	---
Moderate	1.07 (0.82-1.40)	0.60
High	0.89 (0.66-1.20)	0.45
Waist circumference (≥ 88 cm)		
Increased	0.83 (0.65-1.07)	0.14
Normal	1.00	
Blood pressure (≥ 130/≥ 85 mmHg)		
Elevated	1.02 (0.82-1.26)	0.87
Normal	1.00	
Triglycerides (≥ 150 mg/dL)		
Elevated	1.09 (0.86-1.37)	0.47
Normal	1.00	
High-density lipoprotein cholesterol (< 50 mg/dL)		
Low	1.31 (0.97-1.78)	0.08
Normal	1.00	

Poisson's regression model.

DISCUSSION

In the present study, we found no association between MS and FSD. The FSD rate in women with MS was not higher than that in the group of women without MS. Similarly, none of the FSFI domains were associated with MS, showing that the sexual function of the participants was not significantly impaired by the occurrence of MS.

To establish the diagnosis of MS in our study, we used the criteria defined by the Joint Scientific Statement for Harmonizing the Metabolic Syndrome (6) and identified an MS prevalence of 68.5% among postmenopausal women. Another cross-sectional study that has evaluated the sexual function of climacteric women with MS found a prevalence of 62.1% when using the criteria defined by the IDF. In contrast, in the study by Ponholzer and cols., who also adopted the IDF criteria, the prevalence of MS in postmenopausal women was 32.6% (7).

Studies carried out in populations from different countries have revealed a high prevalence of MS, with rates oscillating from 8.0% to 24.0% in men and 7.0% to 46.0% in women (21). In postmenopausal women, MS rates have been reported to vary between 22.0% and 69.0% (22). Overall, the MS rates vary according to the diagnostic criteria adopted in the study and characteristics of the observed population, including sex, age, and ethnicity of the participants and associated morbidities.

We chose the FSFI questionnaire because this instrument is easily administered in women of a broad age group, including postmenopausal women, but also because it is widely used in international research, as reported in several studies (9,11,10,23). The FSFI was developed as a brief self-report instrument to assess the main dimensions of the female sexual function. The questionnaire was designed to evaluate the female sexual response and quality of life in clinical and epidemiological studies (17), and it is in line with new models of female sexuality (2). The current standard cutoff value of the total FSFI score to diagnose sexual dysfunction is 26.5. Therefore, women presenting scores equal to or below 26.5 are considered to have sexual dysfunction (24).

Previous studies have demonstrated a close relationship between MS and male sexual dysfunction, especially related to erectile dysfunction (25,26), and

more recent discussions have attempted to show this association between MS and FSD (9,10).

One of the first studies to find a positive association between MS and FSD was published by Esposito and cols. in the evaluation of premenopausal women. These authors observed a decrease in the FSFI score in women with MS when compared with controls. Although the authors adopted the FSFI questionnaire to evaluate sexual function, they used another scale to determine the occurrence of FSD (9). Another study in postmenopausal women also found a higher prevalence of FSD in women with MS when compared with controls, demonstrating a possible association between FSD and MS. This study also showed that high levels of triglycerides are associated with a higher risk of FSD (10).

Ponholzer and cols., in a cohort study including premenopausal and postmenopausal women, reported an association in premenopausal women of MS and FSD, restricted to the desire component of the sexual function. However, MS had no effect on any of the sexual function components in postmenopausal women when comparing those with and without MS. This result may have differed because these authors used a questionnaire validated for the evaluation of women's sexual health (7).

Kim's and cols., while studying sexual function in middle-aged women, noticed that MS had little influence on their participants' sexuality (11). Another cross-sectional study carried out in climacteric women evaluated the relationship between decreased sexual function and MS and also found no association between FSD and MS, with the exception of older age, which was associated with decreased sexual function (12).

These differences between studies can occur due to several factors such as differences in study design, population ethnicity, diagnostic criteria and cutoff points for both FSD and MS. Another question that must be considered is that FSD can be different according to the severity of the components of the MS (11). In our study, we were unable to evaluate the association between FSD and severity of MS due to the small number of participants.

Based on the pathophysiology of MS, this condition may affect the female sexual function due to its association with vascular inflammation and endothelial dysfunction, which can impair vessel oxygenation and reduce the blood supply to the pelvis (27). However, vascular inflammation and endothelial dysfunction are not frequently observed in the initial phase of MS. Following this hypothesis, the female sexual function would only be impaired in more severe or chronic cases of MS. This contrasts with the observations in men, in whom sexual dysfunction is intimately associated with cardiovascular diseases and MS in the very beginning of the disease (28).

Inflammatory biomarkers are important tools to monitor the progress of endothelial dysfunction. The inflammatory marker CRP is associated with cardiovascular morbidity in the long-term and has a stronger prognostic value regarding cardiovascular events than other biomarkers such as homocysteine and lipoprotein. Moreover, it has already been shown that patients presenting four or five MS components are at increased risk of developing cardiovascular disease (29). Based on this information, we chose to use CRP to verify a possible association between MS and FSD. Esposito and cols. found an inverse relationship between FSFI scores and CRP levels (9). However, the present study found no association between CRP and FSD.

On multivariate analysis, we found that married status was a risk factor for FSD. Another study that has reported similar results demonstrated that married status was a stronger risk factor for FSD and that the partner had an important influence on the woman's sexuality.

According to our findings, women who had reached menopause 6-10 years before the study had a higher risk of FSD. Some studies have demonstrated that the increase in age and time elapsed since menopause may be associated with FSD (30), since women with more time elapsed since menopause may be more susceptible to suffering the consequences of the decline in hormone levels, which increase their chances of having vaginal atrophy, urinary tract infections, UI, and FSD (8).

Although FSD increases with age, it seems that the distress associated with the loss of sexual desire is minimized with age (31). This was observed in a study by Graziottin, who found that the distress associated with FSD is more prevalent in younger women (32).

The biological components of sex and sexuality change substantially with aging in terms of intensity and quality of sexual response. However, some women who are more experienced at this phase of life have fewer conflicts regarding their sexuality. This allows them to seek new ways to exercise their sexuality, motivated by their developed wisdom, better knowledge of their bodies, and maturity (13). This may explain why women who reached menopause more than 10 years

before our study have a lower risk of FSD than those who did so between 6 and 10 years before.

The results of our study show that the occurrence of climacteric symptoms, evaluated by the BKMI had a negative influence on the participants' sexual function. This finding is aligned with that by De Lorenzi and Saciloto (30) in a study evaluating the frequency of sexual activity in postmenopausal women. Another study, also on the influence of climacteric symptoms on sexual function in middle-aged women, has demonstrated that the greater the severity of the symptoms, the higher the chance of the woman of presenting FSD (4).

During menopause, women present higher vasomotor, psychological, and urogenital symptoms associated with hypoestrogenism. The decrease in estrogen levels results in a reduction of pelvic support and lubrication of urogenital tissues, causing pain and difficulty during sexual activity. However, sexuality is not influenced only by hormonal factors; it is also associated with the emotional status of the woman, the quality of her relationship with her partner, and the environment in which she lives (33).

Other important aspects that interfere with the capacity of sexual response are psychological factors. Low self-esteem, anxiety, past traumatic experiences, sexual violence, sexual abuse during childhood, and rape create a negative impact on a woman's sexuality (34). In our study, a history of sexual abuse was associated with the highest risk of FSD. A dysfunctional condition may appear due to organic causes, but it will very often be aggravated by an emotional and psychosocial event (35).

Some limitations of our study must be considered in the interpretation of our results. Considering that the study had a cross-sectional design, we are unable to infer a cause-and-effect relationship or evaluate the onset of FSD and MS. The women who participated in our study may not represent those in the general population, given that they were recruited from an outpatient clinic. Another factor that must be considered is the small number of participants in our study. Although more than 400 women visited the outpatient clinic, only 111 satisfied all inclusion criteria of the study.

Further longitudinal studies are required to evaluate the temporality and causality between FSD and MS and its components, and the factors predisposing to FSD. This should be done taking into account the fact that both FSD and MS are multifactorial conditions; therefore, the impact of confounding factors must be considered in the analysis, including psychological issues and relationship with the partner, among others.

In conclusion, we found no association between MS and FSD, or influence of metabolic risk factors on the domains of female sexual function. The women in our study population had high prevalences of FSD and MS, the latter attributing a high cardiovascular risk profile. Multivariate analysis evidenced that the factors associated with FSD were married status, time elapsed since menopause, climacteric symptoms, and history of sexual abuse.

The fact that FSD has been found to be associated with different factors support the concept that the female sexuality is a very complex and multifactorial subject that requires further studies. Offering monitoring and support for both women and men regarding aging and its complications can help prevent MS and improve sexual function and quality of life.

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Incidence and prevalence of clinically relevant pituitary adenomas: retrospective cohort study in a Health Management Organization in Buenos Aires, Argentina

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ABSTRACT

Objectives: The main purpose of this study was to estimate the incidence rate and prevalence of clinically relevant pituitary adenomas (PAs) within the Hospital Italiano Medical Care Program (HIMCP), a well-defined population of 150,000 members living in the urban and suburban area of the city of Buenos Aires. We defined clinically relevant PAs as those associated with endocrine dysfunction and/or mass effect. **Subjects and methods:** A retrospective open cohort study was conducted, including all members of the HIMCP over 18 years old, with active memberships during the period of the study, from January 1st 2003, to January 1, 2014. The incidence rates (IRs) were standardized (SIR) to the World Health Organization (WHO) 2000 standard population and were expressed per 100,000 members/year. Prevalence was estimated at January 1, 2014, and was expressed per 100,000 persons. The clinical records have been electronically managed since 2001. All lab and imaging studies were done in-house. **Results:** The overall SIR was 7.39/100,000/year (95% CI 4.47-10.31). Female patients had a specific IR significantly higher than male patients (5.85 vs.1.54) and represented 73% of the affected members. Regarding tumor size, 61.4% were microadenomas, and the mean age at diagnosis was 46.4 years. Prolactinomas had the highest SIR (5.41), followed by acromegaly (Acro) and non-functioning adenomas (NFAs) with overlapping 95% CIs (0.44-1.41 and 0.31-0.99, respectively). Microprolactinomas were more frequent in female (72.6%) ($p < 0.01$) and younger members (38 vs.60 years; $p < 0.04$). The overall prevalence rate was 97.76/100,000. Prolactinomas had the highest prevalence (56.29), followed by NFAs (21.48), Acro (14.07) and CD (5.93). **Conclusion:** Our results demonstrate that clinically relevant PAs are more common than usually suspected, especially prolactinomas and growth-hormone secreting PAs. These data highlight the need to increase the awareness of PAs, thereby enabling early diagnosis and treatment. Arch Endocrinol Metab. 2016;60(6):554-61

Keywords
Incidence and prevalence; pituitary adenomas

INTRODUCTION

The prevalence estimates of pituitary adenomas (PAs) are inconsistent. According to epidemiological data derived from cancer registries, the prevalence of PAs is 25 cases per 100,000 inhabitants (1), and according to the most recent report from the Central Brain Tumor Registry of the United States, they account for approximately 15% of all brain tumors (2). On the other hand, postmortem studies have reported a mean prevalence of 11%, with the majority of tumors being microadenomas (3,4). With the widespread use of MRI, PA detection seems to have increased. In fact, in the meta-analysis by Ezzat and cols. (5), PAs were found in up to 22.5% of imaging studies. However, the findings of autopsy and imaging studies are not related to clinically relevant PAs but rather to asymptomatic tumors, yet clinically relevant PAs are associated with increased morbidity and mortality (6).

Recent epidemiological studies have shown that both the incidence (7-9) and prevalence (7,10-12) of PAs may have been previously underestimated.

The main purpose of this study was to estimate the incidence and prevalence rates of clinically relevant PA within the Hospital Italiano Medical Care Program (HIMCP), a well-defined population living in the urban and suburban area of the city of Buenos Aires.

We defined clinically relevant PAs as those associated with endocrine dysfunction and/or mass effect.

SUBJECTS AND METHODS

Study setting

The study population was the members of a prepaid health maintenance organization, HIMCP, managed by a general, tertiary-level university hospital in Argentina (HIBA) that serves a community of over 150,000 members. Health care services are provided by physicians in two main hospitals and 24 peripheral outpatient medical clinics, located mainly in Buenos Aires's inner city.

According to the 2010 Census, a total of 2,890,151 inhabitants live in Buenos Aires's inner city, covering an area of 202 km^2. Approximately 92% of this population is of white South European descent, and there is a minority of mixed native and other ethnicities (2010 Census. INDEC. Dirección General de Estadísticas y Censos. Argentina, http://www.indec.gov.ar) (13). Approximately 5% of this population is affiliated with the HIMCP.

Argentina's health care system is maintained by three major providers: the state, the private sector and social security (the last two covering almost 18.3 million people, distributed among about 300 entities of varying scope and size). Beneficiaries of the private sector can freely choose their health maintenance organization.

The HIBA provides health services to two kinds of patients: patients affiliated with the HIMCP and patients belonging to other health providers sent to our hospital, as a tertiary center for evaluation. Only patients belonging to the HIMCP were included in the prevalence and incidence estimates. The patients are clearly identified by health provider, name, photograph, identification number and date of birth in the electronic database, thus preventing record duplication or misallocation. Before being admitted into the HIMCP, new patients must sign a sworn affidavit stating their pre-existing diseases and health conditions. A general practitioner will then perform a complete medical history and physical exam during the admission process.

A retrospective open cohort study was conducted that included all members of the HIMCP over 18 years old, with active memberships during the whole study period, from January 1, 2003, to January 1, 2014.

All medical care interventions including diagnostic studies – diagnostic laboratory tests and MRI imaging – were performed at HIBA and registered in a centralized electronic database.

Data gathering

Cases of PA were identified by an exhaustive search in the HIMCP's electronic database using the following Systematized Nomenclature of Medicine Clinical Terms (SNOMED-CT): acromegaly (Acro), Cushing's disease (CD), prolactinoma, non-functioning adenoma (NFA) and thyrotropinoma. Related search terms (hyperprolactinemia, pituitary tumor, sellar or intrasellar tumor, pituitary adenoma) were also used. Every case among patients that was diagnosed and followed by endocrinologists, general practitioners, gynecologists, urologists, neurosurgeons and neuro-ophthalmologists was confirmed and classified by three trained staff endocrinologists. The PA subtypes were prolactinomas, NFAs, Acro, CD and thyrotropinoma. To ensure that no preexistent PAs were included as new cases, only those cases of patients with more than twelve months as members of the HIMCP were included to estimate the incidence rate.

A diagnosis of prolactinoma was established when serum prolactin levels were higher than 60 ng/mL in the presence of a pituitary tumor, and the patients showed therapeutic response to dopamine agonists. Patients with hyperprolactinemia without the presence of a pituitary tumor were not included. Acro was defined by levels of insulin-like growth factor type 1 (IGF1) above the reference range for age and gender, and unsuppressed GH in the oral glucose tolerance test, in the presence of a pituitary tumor. The diagnosis of CD was based on biochemical evidence of ACTH-dependent hypercortisolemia with unsuppressed ACTH levels (greater than 20 pg/mL) in the presence of a pituitary adenoma, or bilateral inferior petrosal sinus sampling showing a central: peripheral ratio > 2.0 or a post-desmopressin stimulation ratio > 3.0. NFA was diagnosed by the presence of a pituitary tumor not associated with clinical or biochemical evidence of hormone hypersecretion. Thyrotrophinoma was diagnosed if an inadequately normal or high TSH secretion was demonstrated in the presence of high free thyroxin levels and a pituitary adenoma. In the cases where surgery was performed, definitive diagnosis was based on pathological and immunohistochemical results. Patients were excluded from the analysis

when the available data did not support a definitive diagnosis of PA after surgery or the specific diagnosis of non-adenomatous lesions was established by histopathological study.

Hormone-deficiency syndromes were defined following established criteria: the hypothalamic-pituitary-adrenal axis was considered impaired when the morning serum cortisol level (8-9 hours) was lower than 3 mcg/dL or the response to cosyntropin stimulation was lower than 18 mcg/dL. The pituitary-thyroid axis was deemed deficient when the serum free and/or total thyroxin level was low for the reference range in the presence of normal or low TSH. In patients with normal serum prolactin levels, the hypothalamic-pituitary-gonadal axis was considered affected in men when their baseline circulating testosterone levels were below the reference range, in association with low or normal levels of follicle-stimulating (FSH) and luteinizing (LH) hormones. In women, this diagnosis was established when FSH levels were inadequately low in menopausal women, or when hypogonadotropic amenorrhea was detected in premenopausal women. The growth hormone axis was not explored in any of the patients in the series by means of an insulin hypoglycemia test or any other stimulating test.

Patient characteristics including age, gender and clinical presentation and diagnosis were recorded. Clinical features at presentation were classified as: 1) hormone excess, in cases with symptoms related to confirmed pituitary hormone excess; 2) mass effects, in cases with headaches and/or visual impairment; and 3) hypopituitarism, in cases with symptoms of confirmed pituitary-hormone deficiency. PAs without at least one of these three clinical features were considered clinically irrelevant and excluded.

All of the patients with incidentally found PAs were reviewed. If the patient's medical record revealed any of the clinically relevant features mentioned above, even if previously undetected, the patient was also included as a case.

Regarding size, PAs with a maximal diameter of or greater than 10 mm were considered macroadenomas, while smaller ones were considered microadenomas.

Statistical analysis

The incidence rates (IRs) were age standardized to the World Health Organization's (WHO's) 2000 standard population (SIR) (14) using a direct method. For SIR estimation, those patients with less than one year as members of the HIMPC were excluded to avoid the inclusion of prevalent cases. Unadjusted age-specific IRs were also estimated. All of the reported incidence rates are expressed per 100,000 members/year. We used rate ratios with their 95% confidence interval (95% CI) to compare sex-specific SIRs.

Prevalence was estimated by the Wilson method on January 1, 2014. For this purpose, members with PA fulfilling the inclusion criteria who were alive and belonged to the HIMCP at the time were included. All of the prevalence rates and 95% CIs are per 100,000 members.

The continuous variables are expressed as means and standard deviations (SD). The categorical variables are expressed as percentages of the total cases or groups.

Ethics

This study complies with the tenets of the Helsinki Declaration and was approved by the Ethics Committee of the HIBA.

RESULTS

For the incidence estimates, 101 patients with PA matched our inclusion criteria within a population of 1,286,781.47 member-years at risk. The mean age at diagnosis of PA was 46.39 (18.2) yrs., with a predominance of female patients (n = 74; 73.3%) and the female patients being significantly younger (34.1 [0.9] vs. 53.9 [21.9]; $p < 0.04$). Sixty-one percent of the PAs were microadenomas (n = 62; 61.4%). The most common subtype was prolactinoma (57.43%) followed by NFA (18.81%), Acro (16.83%) and CD (6.93%). No thyrotrophinomas were diagnosed. The overall SIR was 7.39 (4.47-10.31). The female subjects had a significantly higher incidence than their male counterparts (5.85 vs. 1.54, respectively) with a SIR rate ratio of 3.79 (2.44-5.90) (Table 1). The incidence of PA increased with age in males, whereas the peak incidence among females was in the 30-40 age group, as shown in Figure 1A. Regarding clinical features at diagnosis, prolactinoma, Acro and CD mostly presented with signs and symptoms of hormone excess, while most patients with NFAs experienced mass effects and hypopituitarism. Further details regarding the incidence rates and clinical data of all of the PAs and subtypes are shown in Table 1.

For the prevalence estimates, a total of 132 patients with PA were identified within the total population of HIMCP members alive on January 1, 2014 (135,019 adult members; 81,422 women and 53,597 men). The mean age at diagnosis of PA was 44.4 (17.2) yr, with a predominance of female patients (n = 102; 77.3%). Fifty-two percent of the PAs were microadenomas (n = 69, 52.3%). The most common subtype was prolactinoma (57.58%), followed by NFA (21.97%), Acro (14.39%) and CD (6.06%). No thyrotrophinomas were detected. The estimated prevalence rate was 97.76/100,000. A higher prevalence was found in the female patients: 125.27 (103.22-152.04) vs. 55.97 (39.21-79.89) in the male patients. Further details regarding prevalence estimates are shown in Table 2 and Figure 2.

Prolactinomas

Prolactinomas had the highest SIR: 5.41 (2.57-8.25). The mean age at diagnosis was 37.5 (13.5) yr, with a very high proportion of females (81%), most of them having microadenomas (84.5%). Microadenomas were more frequent in female vs. male patients: 53 (72.6%) and 9 (34.6%), respectively ($p < 0.001$). Patients with microadenomas were also younger than those with macroadenomas: 38 (12.8) vs. 60.1 (17.3) yr ($p < 0.05$). The highest IR for females was reached in the 3-40 age group, whereas no significant incidence peak was found among the male patients (Figure 1B).

Prolactinomas had also the highest prevalence rate: 56.29 (44.98-70.44)/100,000; 83% were harbored by women, and 70% were microadenomas (Table 2).

Table 1. Incidence rates and clinical features in the 101 patients included for incidence estimates

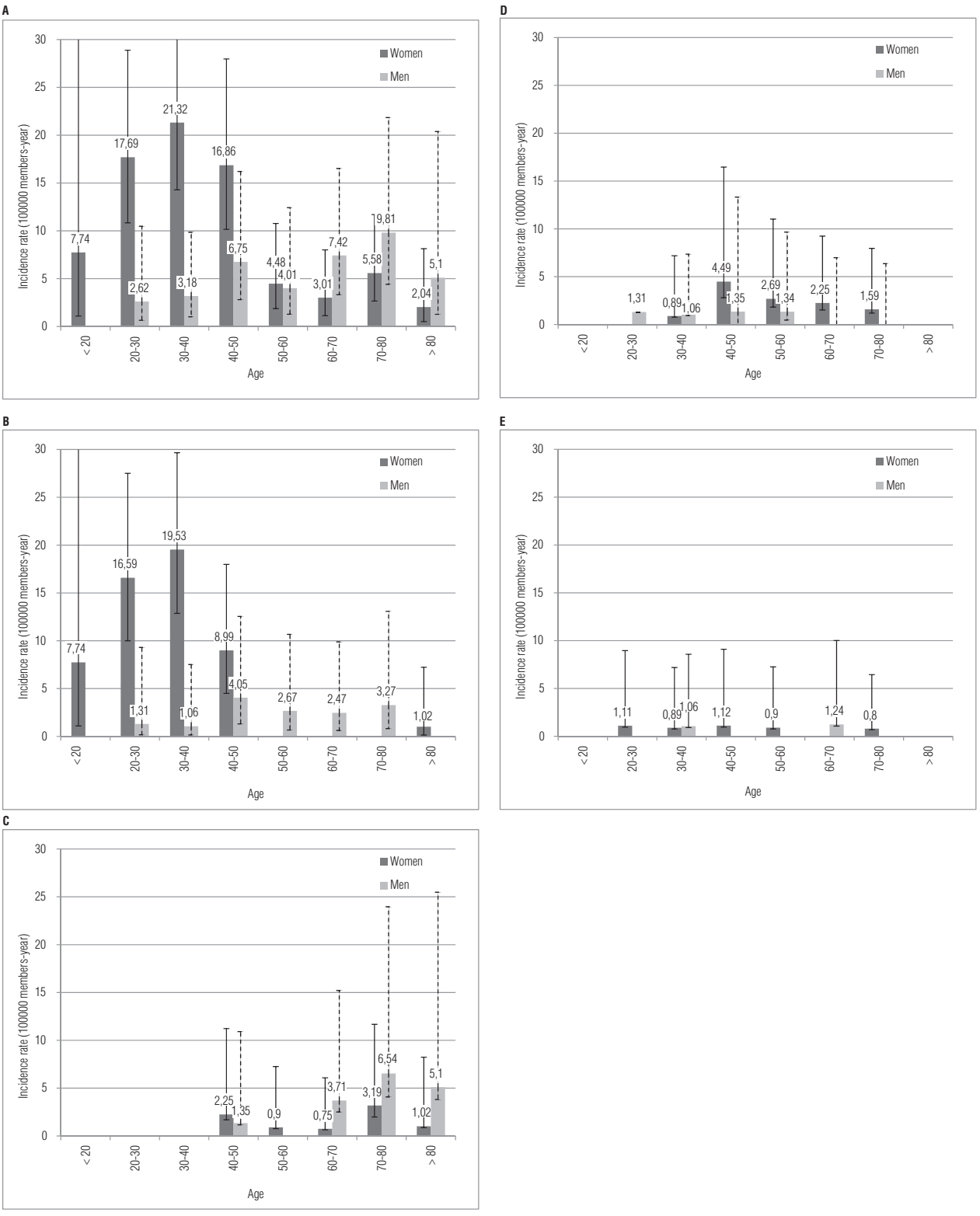
	Total PA	Prolactinomas	NFA	ACRO	CD
Number of patients (%)	101 (100)	58 (57.43)	19 (18.81)	17 (16.83)	7 (6.93)
Mean age at diagnosis (SD)	46.39 (18.2)	37.5 (13.5)	68.7 (13.5)	51.5 (14.1)	47.2 (16.7)
Female patients (n) (%)	74 (73.3)	47 (81)	9 (47.4)	13 (76.5)	5 (71.4)
Microadenomas (n) (%)	62 (61.4)	49 (84.5)	1 (5.3)	6 (40)	6 (85.7)
Clinical features at presentation					
Hormone excess (n) (%)	81 (81)	58 (100)	-	16 (94.1)	7 (100)
Mass effects (n) (%)	23 (23)	4 (6.9)	16 (88.9)	2 (11.8)	1 (14.3)
Hypopituitarism n (%)	9 (9)	1 (1.7)	8 (44.4)	-	-
SIR (95%CI)	7.39 (4.47 – 10.31)	5.41 (2.57 – 8.25)	0.65 (0.31 – 0.99)	0.92 (0.44 – 1.41)	0.4 (0.08 – 0.73)
IR male (95%CI)	1.54 (0.9 – 2.18)	0.72 (0.26 – 1.17)	0.37 (0.12 – 0.62)	0.34 (0 – 0.67)	0,12 (0.05 – 0.29)
IR female (95%CI)	5.85 (3 – 8.69)	4.69 (1.89 – 7.5)	0.28 (0.05 – 0.51)	0.59 (0.23 – 0.94)	0.28 (0.01 – 0.56)
IR rate ratio (female/male) (95%CI)	3.79 (2.44 – 5.9)	6.56 (3.4 – 12.65)	0.75 (0.31 – 1.85)	1.75 (0.57 – 5.37)	2.39 (0.46 – 12.3)

IR and SIRs are expressed per 100,000 members/year. IR: incidence rate; SIR: standardized incidence rate; PA: pituitary adenomas; NFA: non functioning adenomas; Acro: acromegaly; CD: Cushing's disease; SD: standard deviation; %: percentage; CI: confidence interval.

Table 2. Prevalence rate and clinical features of the 132 patients included for prevalence estimates

	Total PA	Prolactinomas	NFA	ACRO	CD
Number of patients n (%)	132 (100)	76 (57.5)	29 (21.9)	19 (14.5)	8 (6.1)
Mean age at diagnosis (SD)	44.4 (17.2)	37.4 (14.2)	60 (16.7)	47.8 (13.2)	46.6 (15.3)
Female patients n (%)	102 (77.3)	63 (82.9)	19 (65.5)	14 (73.7)	6 (75)
Microadenomas n (%)	69 (52.3)	54 (70)	1 (3.4)	8 (42.1)	6 (75)
Clinical features at presentation					
Mass effects n (%)	34 (26.6)	8 (10.7)	24 (92.3)	2 (10.5)	0
Hormone excess	99 (77.3)	74 (98.7)	0	19 (100)	6 (75)
Hypopituitarism	7 (5.5)	1 (1.3)	6 (23.1)	0	0
Prevalence (95%CI)	97.76 (82.45 – 115.91)	56.29 (44.98 – 70.44)	21.48 (14.96 – 30.85)	14.07 (9.01 – 21.98)	5.93 (3 – 11.69)
Female prevalence (95%CI)	125.27 (103.22 – 152.04)	77.37 (60.49 – 98.97)	23.34 (14.94 – 36.45)	17.19 (10.24 – 28.86)	7.37 (3.38 – 16.08)
Male prevalence (95%IC)	55.97 (39.21 – 79.89)	24.26 (14.18 – 41.5)	18.66 (10.14 – 34.34)	9.33 (3.98 – 21.84)	3.73 (1.02 – 13.61)

All prevalences rates/100,000 members alive at 1st January 2014. PA: pituitary adenomas; NFA: non-functioning adenomas; Acro: acromegaly; CD: Cushing's disease; SD: standard deviation; %: percentage; CI: confidence interval.



Incidence rate (IR) per 100,000 members/year according to 10 year age groups in male and females: < 20 years; ≥ 20 to < 30 years; ≥ 30 to < 40 years; ≥ 40 to < 50 years; ≥ 50 to < 60 years; ≥ 60 to < 70 years, ≥ 70 to < 80 years; ≥ 80 years.

Figure 1. Overall and subtype pituitary adenomas incidence rate.

A: All pituitary adenomas; **B:** Prolactinomas; **C:** Non-functioning adenomas; **D:** Acromegaly; **E:** Cushing disease.

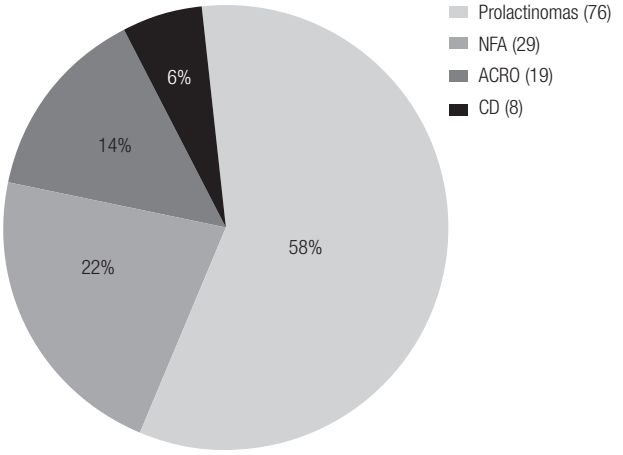


Figure 2. Distribution of pituitary adenoma subtypes for prevalence estimates in percentage (%) and number (n). NFA: non-functioning adenomas; Acro: acromegaly; CD: Cushing's disease.

Non-functioning adenomas

The SIR of NFAs was 0.65 (0.31-0.99). The mean age at diagnosis was 68.7(13.5) yr, which was significantly higher than that for prolactinomas, and 52.6% occurred in males. All of the NFAs but one were macroadenomas (94.7%).

Although the SIR was higher in males, this difference was not statistically significant (Table 1). NFAs showed the peculiar feature of increasing their incidence with age, especially in males, with the highest IRs attained in the 70-80 age group for both genders (Figure 1C).

The prevalence rate for NFAs was 21.48 (14.96-30.85)/100,000; 65.4% were harbored by female patients, and all but one were macroadenomas (Table 2).

Acromegaly

The SIR for Acro was 0.92 (0.44-1.41), the mean age at diagnosis was 51.5 (SD 14.1) yr and 76% of the patients were female. Sixty percent were macroadenomas. The highest IR in women was reached in the 40–50 age group, with no distinctive peak of incidence in males (Figure 1D). Although the SIR was higher in females, this difference was not statistically significant (Table 1).

The prevalence rate was 14.07 (9.01-21.98)/100,000. They occurred mostly in women (73.7%) and were macroadenomas in 57.9% of the cases (Table 2).

Cushing's disease

The SIR for CD was 0.4 (0.08-0.73). The mean age at diagnosis was 47.2 (SD 16.7) yr, and most of them were microadenomas (85.7%). Although females had higher SIRs, this difference was not statistically significant (Table 1).

The prevalence rate was 5.93 (3-11.69)/100,000; 75% were harbored by females, and 76.6% were microadenomas (Table 2).

DISCUSSION

This is the first study to estimate the prevalence and incidence of pituitary adenomas in Latin America. In this retrospective study, we found high incidence and prevalence of clinically relevant PA. Most of them were prolactinomas, predominantly microadenomas in female and younger patients. The incidence of PAs found in our study is higher than those reported by other authors, and the overall prevalence is similar to that published in Belgium, which in turn was the highest population-based prevalence ever published. NFAs were less frequent than in other reports, whereas acromegaly showed a prevalence rate in keeping with other published series but a higher incidence.

Some epidemiological studies of PA have been published in the last few years. In three of them (7-9), the SIR was estimated at about 4/100,000/year, which is significantly higher than previously reported (6). In three other studies (10-12), the reported prevalence of PAs was significantly higher than the one previously estimated from cancer registries (1), in agreement with our results.

To the best of our knowledge, only one study (7) estimates both the incidence and prevalence rates of PAs, like ours. Both epidemiological outcome measures are important for assessing the real burden of these tumors on health care resources because although they are benign, they are an important cause of morbidity and mortality.

The first aspect to discuss regarding our results is the high SIR [7.4 (4.47-10.31)/100.000 members/year], which appears to be higher than previously published results. However, the 95% CI overlaps those of Malta [4.27 (3.7–4.9)] (7) and Finland [3.98 (3.37-4.6)] (8), and are only significantly higher than those reported in Sweden [3.9 (3.6-4.3)] (9).

The prevalence rate of PA in our cohort was 97/100,000, or 1 PA for every 1,030 individuals, which is very similar to that reported by Liege (94/100.000, 1 PA/1,064 individuals) (10), and slightly higher than other reports that estimate the prevalence of PAs between 75 and 80/100,000 (7,11,12).

The overall SIR, as clearly depicted in Figure 2, mainly arises as a result of the high incidence of prolactinomas [5.41 (2.57-8.25)], which accounted for 57% of all of our PAs. Although not significantly higher than that of Finland [2.16 (1.70-2.63)] (8), our incidence of prolactinomas is higher than those of Malta (7) and Sweden (9). This could be attributed to the fact that our study included patients diagnosed and treated not only by endocrinologists and neurosurgeons but also by gynecologists and general practitioners, who usually care for patients with microprolactinomas in our health program. As put forward by the authors of the Swedish series, different inclusion criteria can alter SIR estimates: the relatively low incidence of prolactinomas and high incidence of NFAs in their study has been attributed to the inclusion of PAs from a register that mainly captures cases reviewed by endocrinologists. Our case-finding strategy of using all of the subspecialties that diagnose and care for PAs as a source of incidence data could be revealing a more complete picture, and rendering a more valid estimate of incidence, than endocrinology department-based studies, especially for prolactinomas. Moreover, the prevalence of prolactinomas in our study, 56.3/100,000, is also very similar to that of the Belgian study (66/100,000), which also included cases treated by general practitioners and those from other medical subspecialties besides endocrinologists (10).

Of all of the tumor subtypes, significant female gender predominance was found only in prolactinomas that were clearly more prevalent in women (81%) in our cohort. Prolactinomas also affected younger females, and most were microadenomas, showing the gender differences described by several authors (15-17).

NFAs were the second-most-frequent tumor subtype, at 18% of our incidence cohort; they were associated with either hypopituitarism or mass effects and, in contrast to prolactinomas, were mainly macroadenomas and did not show a gender predominance. NFAs were less frequent in our study, although the SIR [0.65 (0.31-0.99)] was similar to the one reported in Finland [1.02 (0.86-1.19)] (8), and the age of peak incidence was between the fifth and seventh decades. This can be attributed to the fact that when we excluded clinically irrelevant tumors, many were NFA microadenomas. The findings are further supported by the high proportion of macroadenomas, 95%, when compared to other series that quote 64% and 82%, respectively (9-11). Our prevalence of NFAs was 21.48/100.000, which is in agreement with the prevalence reported by other epidemiological studies between 14/100,000 (10) and 26/100,000 (7).

Interestingly, the incidence of Acro in our cohort appears to be high [0.92 (0.44-1.41)] and is only comparable to the one reported in Malta [0.31 (0.19-0.53)] (7). In the same way, the prevalence of Acro in our study (14.07/100,000) was also considerably higher than previously estimated (4 /100,000) (18), but similar to those of Malta (12.5/100,000) (7) and Liege (12.2/100.000) (10). However, it was lower than the one estimated when screening for elevated IGF1 levels in a primary care setting, in a cross-sectional study from Germany (19), and almost half the one reported in a recent study from Belo Horizonte, based on screening with a questionnaire that assessed the enlargement of extremities (20). All of these reports suggest that the prevalence of growth-hormone-secreting pituitary adenomas has been heretofore underestimated. This is important from the perspective of healthcare resource allocation, as most patients with acromegaly harbor macroadenomas and surgery may not result in remission of the disease. Prolonged treatment with somatostatin analogs and/or pegvisomant is costly, and an adequate estimation of the prevalence and incidence of Acros will allow for an accurate assessment of its burden on healthcare resources.

The SIR of CD found in our study, 0.40 (0.08-0.73), is higher but not significantly different from that found in previous studies: 0.07 (0.03-0.21) to 0.18 (0.11-0.25) (7-9). Nonetheless, it is lower than the incidence found in commercially insured patients under 65 years old in the United States (8 cases per million/year) that was recently published (21). CD is difficult to diagnose, so larger cohorts of patients are likely needed to estimate the true prevalence and incidence of this disease.

The HIBA is a tertiary care center. However, it is important to underscore that our results were not influenced by referral bias, since only patients belonging to the HIBA health program, HIMCP, were included, per our stringent inclusion criteria. Observer and selection bias were also precluded by including only members who did not have a PA on admission and did not develop it during the twelve months following their affiliation, further strengthening our results. Furthermore, the similarities of our results to those reported by other investigators, especially those for PA in the well-defined area of Liege, suggest that no significant selection bias occurred in our study.

The main weakness of our study is that it refers to a specific Buenos Aires population belonging to a prepaid medicine program, and not a geographically defined population like Malta or Liege. Nevertheless, the population cared for by our hospital network is numerically larger than those of many other published series; it includes approximately 5% of the population of Buenos Aires and is thus a representative sample. Although patients from outside Buenos Aires and its suburbs may also be included in the health care program, over 150,000 members have valid addresses within the city's limits.

The main strengths of our study are the inclusion of different specialists like gynecologists and general practitioners in the central medical record database, the use of diagnostic criteria in agreement with international standards, access to individual patient data and the confirmation of the diagnoses by one of the three neuroendocrinology specialists in our group. The centralized medical records in our medical center include all lab and imaging studies performed in-house, minimizing data loss, and allocation errors.

In conclusion, our results demonstrate that clinically relevant pituitary adenomas are more common than usually suspected, especially prolactinomas and growth-hormone secreting PAs. These data highlight the need to increase awareness of PA, thereby enabling early diagnosis and treatment of these tumors, which mainly affect the economically active population and are associated with increased morbidity and mortality.

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Treatment of hypothyroidism with levothyroxine plus liothyronine: a randomized, double-blind, crossover study

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ABSTRACT

Objective: To compare the effects of a unique fixed combination levothyroxine/liothyronine (LT4/LT3) therapy in patients with primary hypothyroidism. **Subjects and methods:** This is a randomized, double-blind, crossover study. Adults with primary hypothyroidism (n = 32, age 42.6 ± 13.3, 30 females) on stable doses of LT4 for ≥ 6 months (125 or 150 µg/day) were randomized to continue LT4 treatment (G1) or to start LT4/LT3 therapy (75/15 µg/day; G2). After 8 weeks, participants switched treatments for 8 more weeks. Thyroid function, lipid profile, plasma glucose, body weight, electrocardiogram, vital signs, and quality of life (QoL) were evaluated at weeks 0, 8 and 16. **Results:** Free T4 levels were significantly lower while on LT4/LT3 (G1: 1.07 ± 0.29 vs. 1.65 ± 0.46; G2: 0.97 ± 0.26 vs. 1.63 ± 0.43 ng/dL; P < 0.001). TSH and T3 levels were not affected by type of therapy. More patients on LT4/LT3 had T3 levels above the upper limit (15% vs. 3%). The combination therapy led to an increase in heart rate, with no significant changes in electrocardiogram or arterial blood pressure. Lipid profile, body weight and QoL remained unchanged. **Conclusions:** The combination therapy yielded significantly lower free T4 levels, with no changes in TSH or T3 levels. More patients on LT4/T3 had elevated T3 levels, with no significant alterations in the evaluated outcomes. No clear clinical benefit of the studied formulation could be observed. Future trials need to evaluate different formulations and the impact of the combined therapy in select populations with genetic polymorphisms. Arch Endocrinol Metab. 2016;60(6):562-72

Keywords
Clinical trial; combined modality therapy; cross-over studies; hypothyroidism; levothyroxine; triiodothyronine; liothyronine; quality of life; randomized

INTRODUCTION

Levothyroxine sodium (LT4) is the drug of choice for the treatment of patients with hypothyroidism (1-5). The LT4 formulations available have a half-life of six days and provide fairly stable blood levels of thyroxine (T4) after ingestion of an oral daily dose (6). When treating patients with hypothyroidism, normal blood levels of TSH and free T4 (fT4) are achieved in most patients, with improvements in hypothyroid signs and symptoms.

However, approximately 5-10% of patients continue to report symptoms of hypothyroidism, despite their TSH levels being within the normal reference range (7). Mounting evidence suggests that LT4 monotherapy cannot assure a euthyroid state in the blood and in all tissues simultaneously, and that normal serum thyroid-stimulating hormone (TSH) levels in patients receiving LT4 reflect only pituitary euthyroidism (8). This could be attributed to the fact that the peripheral conversion from T4 to triiodothyronine (T3) is not sufficient to restore normal T3 levels. In animal studies, only the combination levothyroxine/liothyronine (the synthetic form of triiodothyronine; LT4/LT3) ensured euthyroidism in all tissues of thyroidectomized rats (9).

In humans, several clinical trials have evaluated whether LT4/LT3 combination therapy was able to reverse overt hypothyroidism and improve symptoms and quality of life (QoL). The results from the first randomized, double-blind, crossover trial were reported in 1970. These showed no positive results regarding patient preference toward LT4/LT3 therapy and revealed a high incidence of hyperthyroid symptoms (possibly due to excessive doses of LT4/LT3) (10). In 1999, Bunevicius and cols. described an increase in well-being, mood and psychometric functionality in patients treated with LT4/LT3 (11), and similar results

were replicated by another group (12). Those findings could not be further replicated by other studies, and recent meta-analyses of those trials did not show any evidence supporting a superior effect of combination treatment (13,14). However, in the clinical setting, some patients who complain of hypothyroid symptoms (despite having normal TSH and fT4 levels) have mentioned improvements when empirically treated with LT4/LT3 and preferred that type of therapy (15). Therefore, it is still unclear whether the combination therapy LT4/LT3 is superior to LT4 monotherapy in patients with hypothyroidism.

The aim of the present study was to compare the effects of a unique, fixed combination LT4/LT3 therapy (75 μg of LT4 and 15 μg of LT3, once a day) on thyroid hormone levels, body weight, vital signs, metabolic parameters, and QoL of patients with primary hypothyroidism.

SUBJECTS AND METHODS

Study subjects

The study was approved by the Federal University Hospital of Paraná Ethics Committee, and written informed consent was obtained from all study participants, who were recruited from the Clinical Hospital of the Federal University of Paraná, Curitiba, Brazil. Inclusion criteria were participants of any gender, between 15 and 65 years old, with an established diagnosis of primary hypothyroidism, who had received stable doses of LT4 during the previous six months (125 or 150 μg/day). Exclusion criteria were: diabetes mellitus or serious concomitant diseases (such as liver, renal or heart failure); use of drugs or substances that alter the pharmacokinetics and measures of serum TSH and of thyroid hormones; pregnancy; and use of hormonal contraception. Participants with diagnosis of depression were not excluded, provided they had been receiving adequate antidepressant treatment for the previous six months.

Study design

This was a randomized, double-blind, crossover study. One group of participants was randomized to continue receiving their usual dose of LT4 for 8 weeks, followed by use of combination therapy LT4 plus LT3 for 8 more weeks (G1). The participants included in the second group (G2) were randomized to switch their usual therapeutic regimen to the combination therapy LT4/LT3 for 8 weeks and to go back to their usual LT4 dose for another 8 weeks. Participants received capsules containing either their usual dose of LT4 (125 μg or 150 μg; Euthyrox, Merck S/A, Brazil) or LT4/LT3 (75 μg of LT4, plus 15 μg of LT3; Novothyral, Merck KGaA, Germany). Participants were advised to take their medication once daily, half an hour before breakfast. At the end of each eight-week period, participants received another batch of capsules. Drug dispensing was performed by one unblinded investigator (G.A.C.). This individual did not participate in the assessment of the results. Adherence to the therapy was assessed by direct questioning during follow-up visits and pill counting. Adverse events were evaluated by standard anamnesis and physical examination.

Participants were evaluated at baseline and at the end of each 8-week period. At each visit, venous blood samples were collected from all participants after a 12-hour fast in order to measure serum TSH, total T3, fT4, glucose, total cholesterol, HDL cholesterol and triglycerides.

Laboratory methods

TSH was determined in duplicates by Immulite® 2000 chemiluminescence TSH third-generation kit (Diagnostic Products Corporation, CA, USA; reference values [RV] 0.4-4.0 mUI/L; sensitivity 0.002 mUI/L; intra-assay coefficient of variation [CV], 3.8-12.5%; and inter-assay CV, 4.6-12.5%). Free T4 was measured in duplicates by Immulite® 2000 chemiluminescence enzyme-linked immunosorbent assay kit (Diagnostic Products Corporation, CA, USA; RV, 0.8-1.9 ng/dL; sensitivity, 0.15 ng/dL; intra-assay CV, 4.4%-7.5%; and inter-assay CV, 4.8%-9.0%). Total T3 was measured in duplicates by Immulite® 2000 chemiluminescence enzyme-linked immunosorbent assay kit (Diagnostic Products Corporation, CA, USA; RV, 82-179 ng/dL; sensitivity, 19 ng/dL; intra-assay CV, 4.4%-12%; and inter-assay CV, 5.3%-15%). Glucose was measured by enzymatic method (Hexokinase II, Bayer, Germany). Serum total cholesterol, HDL cholesterol and triglycerides were measured by enzymatic colorimetric methods (Sera-Pak Cholesterol Fast Color kit, HDL Advia and Sera-Pak Triglyceride Fast Color kits, Bayer, Germany).

Body weight, resting heart rate and arterial blood pressure were measured at baseline and at the end of each treatment period. Resting heart rate and arterial blood pressure were measured in the sitting position. Twelve-lead electrocardiogram (ECG) was obtained at the end of each treatment period.

Quality of life evaluation

At each visit, QoL was assessed using a disease-specific questionnaire adapted from the Health Related Quality of Life (HRQOL) questionnaire (16), which in turn was elaborated to detect impaired well-being in subjects with hypothyroidism. We selected 29 items from the HRQOL questionnaire and added four items: palpitation, insomnia, irritability and anxiety. Therefore, our questionnaire consisted of 33 items grouped into three categories: physical complaints (12 items), energy and general well-being (11 items), and mood and emotions (10 items). Scoring was based on a six-point scale from zero to five, with zero representing a more favorable state. Participants also completed a visual analog scale (VAS) at baseline, 8 weeks and 16 weeks that assessed overall well-being, interest in sex and ability to engage in physical activities, social interactions and work. In the VAS, scores from -2.5 (the worst possible state) to +2.5 (the best possible state) were given for each of the five questions in 0.25-point increments (overall feeling, ability to engage in physical activity, ability to engage in social interaction, ability to work and interest in sex). Each patient was evaluated by the same blinded examiner during all three sessions (either J.K. or F.Y.M.).

Statistical analysis

Clinical and biochemical data were analyzed by one-way ANOVA, Dunn's multiple comparison test, paired or unpaired (where appropriate) t-test for variables with normal distribution, and Wilcoxon signed-rank test or Mann-Whitney U test for variables without normal distribution. To compare sample proportions, a z-test was employed. Quality of life scores were analyzed by Friedman rank-sums test. Correlations between QoL scores and thyroid hormone levels were calculated using Spearman's or Pearson's correlation coefficients. All variables were tested for normality by Kolmogorov-Smirnov test. Analyses were performed using GraphPad Prism 5.04.

RESULTS

A total of 39 patients were considered for inclusion. Seven patients were excluded before randomization, one because of pregnancy, one because of divorce, two because of onset of diabetes mellitus, and three because of increased TSH levels during the previous six months (Figure 1).

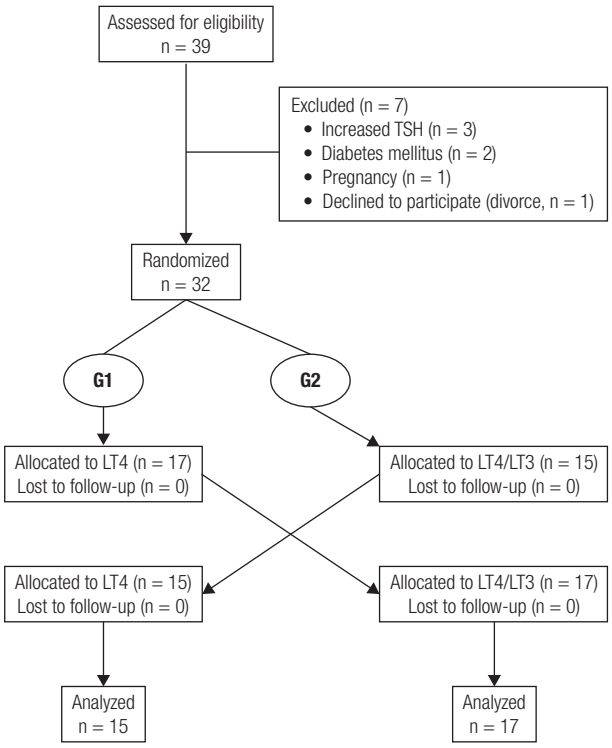

Figure 1. CONSORT diagram.

Thirty-two participants who fulfilled the inclusion and exclusion criteria were recruited. Twenty-three participants (71.9%) had autoimmune or idiopathic hypothyroidism, six (18.7%) had post-surgical hypothyroidism acquired after therapy for differentiated thyroid carcinoma, and three (9.4%) had radioiodine-induced hypothyroidism due to Graves' disease. Mean age was 42.6 ± 13.3 years old, and 94% were female. The median duration of hypothyroidism was 4.5 years (minimum 1, maximum 33 years). The same proportion of participants was being treated with either 125 µg or 150 µg of LT4 per day. Participants were randomized to continue taking their usual LT4 dose (G1; n = 17) or to switch to the LT4/LT3 combination therapy (G2; n = 15). Baseline clinical and biochemical data were similar for both groups (Table 1). Serum basal TSH levels ranged from 0.001 to 4.5 mU/L in G1 and from 0.001 to 8.425 mU/L in G2. Six patients had suppressed TSH as a result of treatment for thyroid carcinoma. On the contrary, some patients had increased TSH levels at the time of randomization. Ideally, serum basal TSH concentrations would be within normal range. However, we decided to keep all patients in the statistical analysis because each crossover patient served as his or her own control.

Table 1. Baseline clinical and biochemical data

	Prior to randomization (n = 32)	Group 1 (G1) (n = 17)	Group 2 (G2) (n = 15)	P (group 1 vs. group 2)
Age (years-old)	42.6 ± 13.3	39.5 ± 14.2	46.1 ± 11.86	0.170
Gender (F:M)	30:2	15:2	15:0	0.143
Participants on LT4 125 µg/day (%)	50	52.9	46.7	0.726
Time of hypothyroidism diagnosis (years)	4.5 (1 – 33)	5 (1 – 33)	4 (1 – 23)	0.595
Autoimmune/idiopathic hypothyroidism (%)	71.9	58.8	86.7	0.080
Post-surgical hypothyroidism (%)	18.7	23.5	13.3	0.460
Radioiodine-induced hypothyroidism (%)	9.4	17.6	0	0.088
Body mass index (kg/m^2)	28.5 ± 5.6	27.3 ± 5.0	29.8 ± 6.0	0.211
Resting heart rate (beats per minute)	76.7 ± 9.8	79.1 ± 9.0	74.1 ± 10.3	0.160
Systolic blood pressure (mmHg)	120.0 (90.0 – 150.0)	120.0 (100.0 – 140.0)	120.0 (90.0 – 150.0)	0.612
Diastolic blood pressure (mmHg)	78.9 ± 11.3	77.1 ± 11.2	81.0 ± 11.5	0.335
TSH (mU/L)	0.309 (0.001 – 8.425)	0.078 (0.001 – 4.500)	0.535 (0.001 – 8.425)	0.117
Total T3 (ng/dL)	93.6 ± 11.6	93.2 ± 11.6	94.0 ± 12.0	0.847
Free T4 (ng/dL)	1.26 ± 0.26	1.26 ± 0.25	1.26 ± 0.28	0.956
Glucose (mg/dL)	99.0 ± 12.5	97.6 ± 10.6	100.5 ± 14.5	0.532
Total cholesterol (mg/dL)	191.1 ± 35.4	188.8 ± 36.9	193.7 ± 34.8	0.706
HDL (mg/dL)	47.2 ± 11.9	47.5 ± 12.4	46.9 ± 11.8	0.891
Triglycerides (mg/dL)	139.7 ± 68.0	144.5 ± 75.0	134.3 ± 61.2	0.679

Note: results are shown as mean ± standard deviation or median (minimum – maximum).

Over the 16-week period, significant changes were observed only for fT4 levels (one-way ANOVA P < 0.001 in both groups), with significantly lower levels observed while taking LT4/LT3 treatment (1.07 ± 0.29 at week 16 vs. 1.65 ± 0.46 ng/dL at week 8 in G1; 0.97 ± 0.26 at week 8 vs. 1.63 ± 0.43 ng/dL at week 16 in G2) (Figure 2A). T3 levels were similar over the study in G1 (one-way ANOVA P = 0.662) and G2 (one-way ANOVA P = 0.247), and median T3 levels were not affected by type of therapy (Figure 2B). Changes in TSH levels were not significant in G1 (one-way ANOVA P = 0.05) or in G2 (one-way ANOVA P = 0.819; Figure 2C).

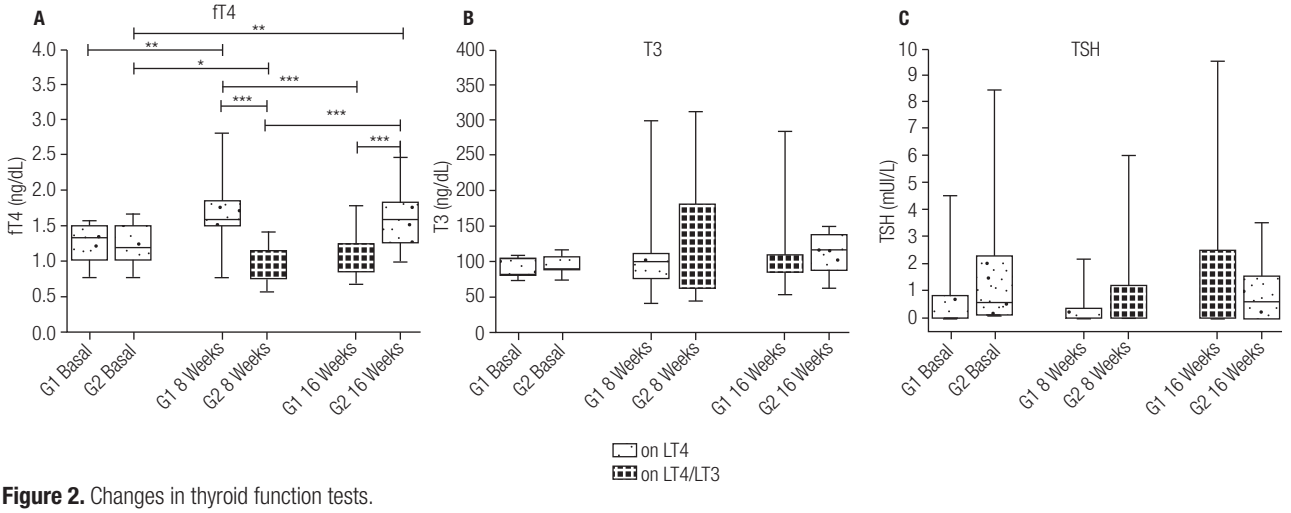

Figure 2. Changes in thyroid function tests.

Only statistically significant comparisons are depicted.

* P < 0.05; ** P < 0.01; *** P < 0.001.

G1: Group 1, on LT4 monotherapy; G2: Group 2, on LT4/LT3 combination therapy.

The analysis of the results according to type of therapy (LT4 or LT4/LT3) showed that fT4 levels were significantly lower and resting heart rate was slightly higher while participants were taking the combination therapy. Other outcomes were similar, regardless of type of therapy (Table 2). Furthermore, five patients had T3 > 180 ng/dL while on LT4/LT3 (mean 239.4 ± 57.1 ng/dL, highest 311.5 ng/dL), whereas only one participant had increased T3 levels (298.5 ng/dL) when on LT4 therapy.

No significant adverse effects were reported, and all subjects completed the study. Although resting heart rate was slightly higher during the combination LT4/LT3 treatment, it remained within the normal range. In addition, no arrhythmias were detected on resting ECG.

All participants responded to the adapted HRQOL questionnaire. Global median scores improved after randomization ($P < 0.001$). Scores categorized into subgroups (physical complaints, energy/general well-being, and mood/emotions) also improved after randomization ($P < 0.05$). However, there was no difference in global scores when comparing the two types of therapy ($P = 0.888$). Furthermore, scores in each subgroup were similar for both treatments (all $P > 0.05$) (Figure 3). There were no correlations between global scores and thyroid hormone levels (fT4 and T3) at baseline or while on LT4 or LT4/LT3 (all $P > 0.05$). However, there was a positive correlation between TSH and global scores while on LT4/LT3 ($r = 0.4164$, $P = 0.018$). Correlation analysis between scores by subgroups and thyroid hormone levels showed correlation only between energy/general well-being scores and TSH levels while on LT4/LT3 ($r = 0.4123$, $P = 0.019$) and between mood/emotions scores and TSH levels, also while on LT4/LT3 ($r = 0.4011$, $P = 0.023$).

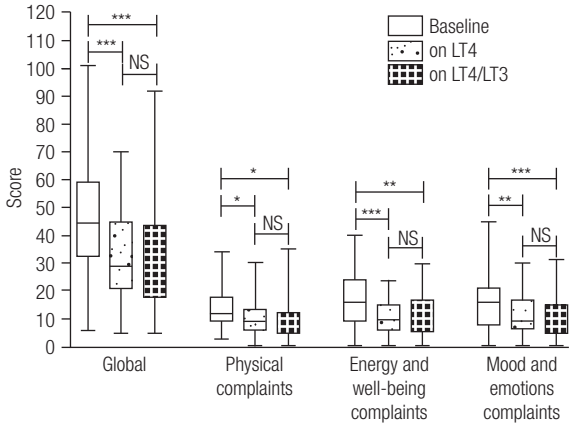

Figure 3. Scores of quality of life, global and by type of complaint. Box-plots depict median and range.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

By analyzing the 33 items of the adapted HRQOL questionnaire individually, we observed that 15 items had significantly different median scores when comparing scores at baseline to scores when on LT4 to scores of LT4/LT3 combination therapy ($P < 0.05$). According to these scores, QoL improved for all 15 of these specific items. When analyzing the effect of each type of therapy within these 15 items, lack of improvement from baseline was observed more frequently when participants were on LT4/LT3 combination therapy. Median scores did not change for 3 items while participants were on LT4/LT3, whereas scores remained the same for only one item while participants were on LT4. Conversely, compared

Table 2. Clinical data, thyroid function tests and biochemical results while on LT4 or LT4/LT3

	On LT4 monotherapy	On LT4/LT3 combination therapy	P
Body mass index (kg/m^2)	28.5 ± 5.7	28.4 ± 5.7	0.212
Resting heart rate	74.6 ± 9.5	77.4 ± 9.2	0.046
Systolic blood pressure (mmHg)	118.1 ± 13.3	117.5 ± 11.1	0.875
Diastolic blood pressure (mmHg)	75.6 ± 9.0	74.1 ± 8.7	0.446
TSH (mU/L)	0.189 (0.004 – 3.495)	0.638 (0.013 – 9.500)	0.540
T3 (ng/dL)	103.8 (40.0 – 298.5)	98.5 (44.7 – 311.5)	0.742
Free T4 (ng/dL)	1.64 ± 0.44	1.03 ± 0.28	< 0.0001
Glucose (mg/dL)	102.9 ± 23.7	99.0 ± 14.1	0.191
Total cholesterol (mg/dL)	188.4 ± 31.3	190.9 ± 34.9	0.493
HDL (mg/dL)	49.1 ± 10.1	48.2 ± 10.4	0.286
Triglycerides (mg/dL)	130.6 ± 53.4	135.7 ± 66.6	0.623

Note: results are shown as mean ± standard deviation or median (minimum – maximum).

to baseline, 14 items had their scores improved while on LT4; on LT4/LT3, lower scores were obtained in 12 items of the adapted HRQOL questionnaire. In a *post hoc* analysis, only two items were significantly different when the two treatments were compared. Median scores concerning brittle nails were lower while on LT4, and scores regarding shortness of breath were lower while on LT4/LT3. Concerning the four items that were added to the original questionnaire (palpitation, insomnia, irritability and anxiety), no significant changes were observed between the two regimens (Table 3).

Table 3. Itemized scores of quality of life

N	Symptom	Prior to randomization	On LT4	On LT4/LT3	P value (baseline vs. LT4 vs. LT4/LT3)	P value (baseline vs. LT4) *Post hoc*	P value (baseline vs. LT4/LT3) *Post hoc*	P value (LT4 vs. LT4/LT3) *Post hoc*
Physical complaints	Feeling cold	0 (0-4)	0 (0-2)	0 (0-4)	0.032	0.018	0.025	0.904
	Dry skin	1.5 (0-5)	1.5 (0-4)	1.5 (0-4)	0.007	0.002	0.031	0.275
	Cold skin	0 (0-4)	0 (0-5)	0 (0-3)	0.640	NA	NA	NA
	Tightening of clothes	1 (0-5)	0 (0-5)	0 (0-5)	0.113	NA	NA	NA
	Dry hair	0.5 (0-5)	0.5 (0-5)	0 (0-5)	0.076	NA	NA	NA
	Puffiness of hands	1 (0-3)	0 (0-3)	0 (0-3)	0.013	0.006	0.013	0.778
	Brittle nails	0 (0-5)	0 (0-3)	1 (0-5)	0.013	0.021	0.557	0.004
	Muscle cramps	0.5 (0-5)	0 (0-5)	0 (0-5)	0.182	NA	NA	NA
	Shortness of breath	1 (0-4)	1.5 (0-3)	1 (0-5)	0.018	0.453	0.006	0.038
	Swollen feet	1 (0-5)	0 (0-4)	0 (0-3)	0.071	NA	NA	NA
	Pins and needles in hands/feet	0 (0-3)	0 (0-3)	0 (0-5)	0.433	NA	NA	NA
	Palpitation	1 (0-5)	1 (0-5)	0.5 (0-5)	0.182	NA	NA	NA
Energy and general well-being	Feeling tired	2.5 (0-5)	2 (0-4)	1 (0-5)	0.002	0.003	0.002	0.879
	Feeling need for more sleep	1.5 (0-5)	1 (0-3)	1 (0-4)	0.587	NA	NA	NA
	Needing nap during day	1 (0-5)	1 (0-3)	1 (0-3)	0.220	NA	NA	NA
	Needing more time to do daily chores	1 (0-5)	0 (0-4)	0 (0-3)	0.001	0.003	0.001	0.758
	Insomnia	0 (0-5)	0 (0-5)	0 (0-5)	0.658	NA	NA	NA
	Slower physically	1 (0-5)	0 (0-3)	0.5 (0-3)	0.013	0.012	0.006	0.797
	Slower mentally	2 (0-5)	1 (0-4)	1 (0-5)	< 0.001	0.002	< 0.001	0.340
	Needing more time for calculations	1.5 (0-5)	1 (0-5)	1 (0-4)	0.044	0.013	0.128	0.307
	Going out less	1 (0-5)	1 (0-4)	1 (0-5)	0.120	NA	NA	NA
	No energy to get through the day	2 (0-5)	1 (0-5)	1 (0-4)	0.001	< 0.001	0.016	0.135
	Slowing of movements	0 (0-5)	0 (0-2)	0 (0-5)	0.072	NA	NA	NA
Mood and emotions	Feeling frustrated	0 (0-5)	0 (0-4)	0 (0 -3)	0.005	< 0.001	0.016	0.135
	Feeling discouraged	2 (0-5)	1 (0-5)	1 (0-5)	0.002	0.001	0.004	0.538
	Difficulty concentrating	1 (0-5)	1 (0-4)	1 (0-4)	0.093	NA	NA	NA
	Deterioration of memory	1 (0-5)	1 (0-4)	1 (0-4)	0.066	NA	NA	NA
	Losing interest in sex	1.5 (0-5)	1 (0-5)	1 (0-5)	0.476	NA	NA	NA
	Losing interest in activities/hobbies	1 (0-5)	1 (0-3)	1 (0-4)	0.495	NA	NA	NA
	Feeling depressed	1 (0-5)	0 (0-5)	0 (0-5)	0.021	0.005	0.118	0.191
	Feeling worthless	0 (0-5)	0 (0-2)	0 (0-3)	0.971	NA	NA	NA
	Irritability	2 (0-5)	1 (0-5)	1 (0-5)	0.011	0.033	0.003	0.359
	Anxiety	2 (0-5)	1 (0-5)	1 (0-5)	0.069	NA	NA	NA

Note: scores are shown as median (minimum – maximum). Scoring was based on a six-point scale from zero to five, with zero representing a more favorable state.

NA: not applicable; G1: Group 1, on LT4 monotherapy; G2: Group 2, on LT4/LT3 combination therapy.

Mean VAS scores were similar at baseline (0.47 ± 0.91), while on LT4 (0.21 ± 0.91), and while on LT4/LT3 (0.44 ± 1.26; one-way ANOVA P = 0.5175).

We performed a sub-analysis according the etiologies of hypothyroidism: autoimmune or idiopathic hypothyroidism, post-surgical hypothyroidism acquired after therapy for differentiated thyroid carcinoma and radioiodine-induced hypothyroidism due to Graves' disease. No significant changes were observed in VAS scores or QoL scores (global and subgroup scores) between the two treatments. We also performed a subanalysis for the group with high T3 levels (T3 > 180 ng/dL), which showed no significant differences regarding QoL scores (global and subgroup scores), VAS scores, body mass index, arterial blood pressure or resting heart rate.

DISCUSSION

Currently, the drug of choice for the treatment of hypothyroidism is levothyroxine sodium (1-5), and LT4/LT3 combination therapy should be considered solely as an experimental treatment modality (7). There is conflicting animal and human data suggesting that the addition of liothyronine to levothyroxine therapy improves outcomes (15). To clarify the role of liothyronine in the treatment of hypothyroidism, we conducted a randomized, double-blind, crossover clinical trial in which participants received LT4 alone for 8 weeks and LT4/LT3 for another 8 weeks. Our results suggest that the combination therapy LT4/LT3 significantly determines lower serum free T4 levels without significantly altering serum TSH, total T3, lipids, or fasting glucose levels. The type of treatment implemented did not affect body mass index and arterial blood pressure. Despite the fact that resting heart rate was slightly higher in participants taking the combination therapy, neither ECG alterations nor significant adverse effects were observed. Furthermore, no differences were observed regarding overall quality of life, and LT4/LT3 therapy was significantly superior to LT4 for only one of the 33 items evaluating quality of life.

Autoimmune thyroid disease is present in about 70% of hypothyroid individuals, and patients with autoimmune thyroid disease are at high risk of developing other autoimmune diseases. The development of these associated conditions might go unnoticed, and some patients have persistent nonspecific symptoms despite apparently adequate levothyroxine. In addition, there is the possibility that thyroid autoimmunity itself might give rise to nonspecific symptoms, independent of thyroid function (8).

The adult thyroid gland secretes all the circulating T4 and 20% of T3 present in the blood. The remaining 80% of the circulating T3 derives from peripheral 5'-deiodination of the secreted T4 (17). Evidence from thyroidectomized animal and human studies suggests that thyroid hormone replacement therapy in the form of LT4 is not sufficient to lead to euthyroidism or to replenish 100% of the circulating and tissular T3, even assuming that peripheral conversion from T4 to T3 is normal (18,19). In fact, a true euthyroid state (in plasma and tissue) may be attained only when LT4/LT3 combination therapy is employed (9). This piece of evidence, along with the observation that many hypothyroid patients do not have their symptoms improved with LT4 therapy alone despite normal thyroid function tests, led to the development of human trials assessing the efficacy of LT4/LT3 in the treatment of hypothyroidism.

To date, 15 clinical trials have evaluated the combination therapy of LT4 plus synthetic liothyronine in patients with hypothyroidism [reviewed by Escobar-Morreale and cols. (15)], and one study evaluated desiccated thyroid extract containing both T3 and T4 (20). Clear benefits of the combination therapy were only observed among two groups from Lithuania and Denmark, which reported improvements in mood, well-being and psychometric functionality (11,21), along with better scores of QoL, depression and anxiety (12). In the first trial showing positive results, the significant benefit of the combination therapy was evident only in a group of athyreotic subjects who had previously had thyroid cancer. Thus, it is not clear whether the results were related to the cause of hypothyroidism (21). In the other positive study, treatment dose was titrated to aim for stable serum TSH levels. Significant improvements in QoL, depression and anxiety scales were observed with a considerably lower T4:T3 ratio of 2.5:1 (12). The study with the largest sample size and longest follow-up was conducted by Saravanan and cols. In that study, 697 patients with primary hypothyroidism were initially randomized and treated in a non-crossover, double-blind design at a T4:T3 weight ratio of 5:1. That study reported a slight improvement in mood, QoL and anxiety after 3 months of the combination treatment, which was not confirmed subsequently after 1 year (22). Furthermore, a meta-analysis evaluating

11 randomized controlled trials with a total of 1,216 patients concluded that there is no evidence supporting the superiority of LT4/LT3 combination therapy regarding improvements in bodily pain, mood, fatigue, QoL, cognition, body weight or blood lipids (13). Not even patients with depression (11), high dissatisfaction with LT4 therapy (23), psychiatric symptoms (24), or fatigue (25) benefited from the combination therapy.

In our study, we evaluated 32 participants with stable, treated primary hypothyroidism, most of them of autoimmune or idiopathic etiology. However, some patients were athyreotic following treatment for thyroid cancer or had hypothyroidism because of the administration of radioactive iodine therapy for Graves' disease. Also, both genders were included in the study; therefore, this heterogeneity may be a confounding factor, as previously observed (21). Instead of using a substitution approach to calculate the dose of LT4/LT3, we recruited participants who were within a very limited LT4 daily dose range (125 or 150 µg/day) and used a fixed combination approach. We administered LT4/LT3 at a fixed dose of 75 µg of LT4 plus 15 µg of LT3, regardless of their initial LT4 dose, in the same 5:1 weight ratio as previously evaluated in other studies (12,22-24). Doses were not adjusted during the study period, as was done by Nygaard and cols. (12). To our knowledge, no other studies have employed such strict recruitment criteria, and the fixed dose of 75 µg of LT4 plus 15 µg of T3 has been evaluated only by one study (24). That study recruited participants taking any dose of LT4, and a substitution approach was employed to calculate the LT4/LT3 dose for a subgroup of patients (usual LT4 dose minus 25 µg of LT4 plus 15 µg of LT3). Within this subgroup of patients that was probably overtreated, half of the participants underwent TSH suppression and experienced increases in heart rate and significant weight loss. Nygaard and cols.'s study recruited patients on different daily doses of LT4; a few participants whose dose prior to randomization was 125 µg/day were treated with a dose that was close but not equal to ours (75 µg of LT4 + 20 µg of T3). In the study by Clyde and cols., a few participants who were initially on LT4 125 µg/day received the same combination dose as the one employed in our study. However, the LT3 dose was administered twice daily (26).

We submitted participants to both types of therapy for 8 weeks each, which led to significantly lower plasma levels of fT4 while on LT4/LT3 therapy. This is an expected finding that was reported in a previous meta-analysis, which also reported no effect of the combination therapy on plasma TSH, total T3 or lipids (13). In our study, comparisons of results observed at baseline to those observed at 8 weeks and at 16 weeks also showed that fT4 levels were lower when participants were under LT4/LT3 therapy. Also, there were no changes in TSH and T3 levels over the 16-week period. Although we could not identify increases in T3 levels caused by the combination therapy, it is possible that these results are falsely low due to the short half-life of liothyronine, as well as the fact that blood samples were collected 24 hours after administration of the LT4/LT3 tablets.

Although the T4:T3 ratio of 5:1 used in our study is similar to those used in other studies [but different than the 13:1-20:1 weight ratio recommended by the European Thyroid Association (7)], we have not assessed whether that dose led to both blood and tissular euthyroidism. Excessive doses of LT4/LT3 may contribute to increases in resting heart rate (27), bone remodeling markers (24,28-30) and serum aminotransferases (30), leading to clinical manifestations of hyperthyroidism (31). In our study, we observed a borderline significant increase in heart rate by 3 beats/minute, which did not lead to adverse events. Even though atrial arrhythmias have been reported in two studies in which patients were overtreated during the combination treatment (10,32), we did not detect arrhythmias on resting 12-lead ECG. Furthermore, no significant changes were detected in arterial blood pressure, in concordance with previous clinical trials (10-12,22-24,32).

We also evaluated body weight (as this can be affected by slight variations in thyroid hormone levels), body composition and resting energy expenditure (33). In one previous study, significant decreases in body weight after the combination therapy were observed. However, the combination therapy led to overtreatment in many patients, obtaining a median TSH of 0.07 mU/L (24). Nevertheless, similar to our results, other clinical trials did not reveal significant changes in body weight after LT4/LT3 therapy (11,12,22,23).

Overt hypothyroidism is classically associated with negative effects on lipid metabolism (34), which improves significantly with LT4 therapy (35,36). However, very few trials showed a benefit of the LT4/LT3 combination treatment on blood lipids (37). In our study, we did not observe significant changes in

total cholesterol, LDL-cholesterol, HDL-cholesterol or triglycerides, in concordance with previous studies (11,22). Additionally, the combination therapy did not affect fasting glucose levels. Since we did not measure fasting insulin levels, we could not assess the impact of LT4/LT3 therapy on insulin sensitivity.

Hypothyroidism may induce affective and cognitive dysfunction (38,39). Most studies have assessed changes in mood, cognition function and QoL during combination therapy with LT4/LT3 (11,12,21,23-26,28,30-32,40,41). Two meta-analyses including 10 (14) and 11 (13) of these studies showed no improvement in bodily pain, well-being, mood, fatigue, QoL or cognition. It should be noted that the evaluation of QoL, well-being, mood and thyroid symptoms is not standardized across studies, and different instruments (such as the SF-36, Hamilton Scales of Depression and Anxiety, and Thyroid Symptom Questionnaire) have been employed. In our study, we adapted and translated to Portuguese the questionnaire developed and employed by Jaeschke and cols. (16) and Clyde and cols. in their clinical trials (26). The analysis of our adapted HRQOL questionnaire revealed no significant changes in global scores or in scores within each category (physical complaints, energy and well-being, and mood and emotions). Interestingly, in 15 items of the questionnaire, we observed improvement in the scores throughout the study period when compared with baseline scores. This could be explained by the Hawthorne effect, or the tendency of patients to describe improvement in health simply due to participation in a trial (6). However, when excluding the baseline scores and performing the *post hoc* analysis between the two treatments, only two out of the 33 evaluated items had significantly different scores. Participants on LT4 reported improvement in the "brittle nails" complaint, whereas those on LT4/LT3 reported reduced shortness of breath. In hypothyroid patients, breathing complaints are unspecific and their clinical importance is unclear. By contrast, fragile nails are the most prevalent symptom in patients with nail involvement in hypothyroidism (42). Nevertheless, it is unclear why this symptom was improved by LT4 therapy. When correlating scores with TSH, fT4 and T3 levels, we could identify only moderate correlation between TSH and global scores, between TSH and energy/general well-being scores, and between TSH levels and mood/emotions scores (all while on LT4/LT3). The lack of effect on QoL was also evident in the analysis of the scores obtained from the VAS.

Whereas a previous study showed that LT4 monotherapy leads to no change in serum free T3 in the first 4 hours post-dose, LT4/LT3 therapy can determine a marked rise by 42% in serum free T3 within the first 4 hours after ingestion of LT4/LT3 (27). This acute rise can cause hyperthyroid symptoms that were not detected by our adapted HRQOL questionnaire. We added four items to that questionnaire (palpitation, insomnia, anxiety and irritability) because we postulated that LT4/LT3 therapy could induce some complaints that would have been missed by the original HRQOL questionnaire. Among those four items, only irritability scores improved from baseline with both therapies. However, those scores were not significantly different from each other.

We performed a 16-week crossover trial to eliminate any carryover effect. By doing so, we observed that TSH levels while on LT4 or LT4/LT3 were similar, and TSH levels remained fairly constant throughout the study. Moreover, we used a commercially available presentation of LT4/LT3 that can be applied in clinics, thereby avoiding dosing errors when compounding liothyronine. However, we did not assess patient preference, which might be higher when taking LT4/LT3 and unrelated to improvements (or lack thereof) in quantitative scores (20). Furthermore, we did not employ a washout period between the LT4 treatment and the combined therapy, to avoid submitting our participants to unnecessary clinical hypothyroidism. In addition, oral liothyronine has a shorter half-life, and significant results could have been observed if we instead had administered a constant, steady supply of T3 by means of enteric, transdermal or intramuscular sustained-release preparation or twice daily (8). Different results could have been observed in a more homogeneous, larger sample, but several similar trials evaluated ≤ 40 participants, with adequate statistical power (11,21,25,28,30,32,37,41). Similarly, the duration of our study may not have been long enough to detect changes in peripheral parameters. However, combination therapy has been evaluated for ≤ 8 weeks in other similar trials (10,11,21,28). Finally, despite being validated in English, our HRQOL questionnaire did not undergo validation in Portuguese.

Despite the fact that our study and previous meta-analyses failed to find clear benefits in the treatment of hypothyroid individuals with combination LT4/LT3, it is possible that a subgroup of patients with deiodinase 2 (*DIO2*) polymorphisms can benefit from combination

therapy (43). One such polymorphism in the D2 gene (Th92Ala) is associated with reduced T4 to T3 activation in skeletal muscle and thyroid, as well as alterations in thyroid–pituitary feedback. A suggestive indication to the presence of this polymorphism could be a higher-than-normal ratio of free T4 to free T3 (6,44). Other polymorphisms in the phosphodiesterase 8B (*PDE8B)* may also alter the carrier's genetically determined TSH set-point, leading to sustained hypothyroid symptoms despite normal TSH levels (7). To date, it is still unclear whether polymorphisms play a role in response to therapy in patients with hypothyroidism (45-47).

Even though animal studies support combined levothyroxine plus liothyronine therapy, clinical trials in humans have not shown the advantages of that approach over administration of levothyroxine alone. The preference of some patients for combined therapy (found in some trials) may have a genetic background. Hence, this should be balanced against the possibility of adverse events resulting from the addition of liothyronine to levothyroxine. Currently available oral liothyronine preparations have an inadequate pharmacokinetic profile. Similarly, commercial preparations that contain levothyroxine and liothyronine contain an excess of the latter and do not mimic the proportion of levothyroxine to triiodothyronine present in normal human thyroidal secretion. Future studies need to evaluate the effect of long-acting, slow-release forms of T3, which mimic normal physiological endogenous T3 production. Furthermore, prospective and longer trials are required to further evaluate the response to the combination LT4/LT3 treatment in patients with the polymorphisms in genes affecting thyroid economy.

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Circulating early biomarkers of atherogenesis in participants of the Longitudinal Study of Adult Health (ELSA-Brasil) without diabetes or cardiovascular disease

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ABSTRACT

Objective: Our aim was to describe the distribution of selected biomarkers according to age and sex, adjusted for HOMA-IR and adiposity, in a subset of middle-aged individuals of Brazilian Longitudinal Study of Adult Health-ELSA without diabetes mellitus or CVD. **Subjects and methods:** This cross-sectional study was conducted in 998 participants of the ELSA-Brasil without diabetes and/or cardiovascular disease. In addition to the traditional risk factors, several biomarkers concentrations were compared according to sex, age groups (35-44; 45-54 yrs) and HOMA-IR tertiles. Linear regression was used to examine independent associations of sex and age with selected novel biomarkers, adjusted for body adiposity and HOMA-IR. **Results:** Fifty-five percent were women. Men had higher mean values of body mass index, waist circumference, blood pressure, plasma glucose, HOMA-IR, worse lipid profile and higher E-selectin and lower leptin concentrations than women; while women had higher levels of HDL-cholesterol and leptin than men. Mean values of waist circumference, systolic BP, plasma glucose and apolipoprotein B (Apo B) increased with age in both sexes. Leptin and E-selectin concentrations increased across HOMA-IR tertiles. Independent associations of Apo B with age were found only in male sex, while of leptin with body mass index and HOMA-IR, and of E-selectin with HOMA-IR in both sexes. **Conclusions:** In conclusion, our data indicate age, sex, adiposity and, consequently, insulin resistance, influence circulating levels of Apo B, leptin and E-selectin, suggesting that those aspects should be taken into consideration when assessing these parameters for research or clinical purposes in individuals at relatively low cardiometabolic risk. Arch Endocrinol Metab. 2016;60(6):573-81

Keywords
Biomarkers; atherogenesis; adiposity; age; sex; insulin resistance

INTRODUCTION

Cardiovascular diseases (CVD) are major causes of death in both sexes (1). Mortalities due to CVDs are declining, but they are still the leading causes of death in several developed and developing countries (2-5). In Brazil, population aging has resulted in an increased chronic disease burden, but age-standardized mortalities from CVDs have declined (6). The decline has mostly been attributed to the control of traditional cardiovascular risk factors such as smoking, elevated blood pressure levels and lipids concentrations (7-9). However, obesity is increasing worldwide, and diabetes mellitus is considered one of the major epidemics of the century (10).

Until their fifties, women have lower rates of CVDs than men of the same age (11). This apparent protection from CVDs has been attributed to adequate levels of endogenous estrogens rather than to ageing. Several years after menopause, cardiovascular mortality rates in women show a steep increase (12). Despite disparities in the detection of CVDs, treatment and prognosis between sexes, basic differences in physiology and physiopathology definitely contribute to different cardiovascular morbidity and mortality rates in men and women. Gender differences in the impact of traditional risk factors are recognized. Female smokers are significantly more likely to suffer an ischemic heart disease than men (13). The impact of diabetes on

the risk of coronary death is significantly greater for diabetic women than diabetic men (14). More recently, studies investigated whether novel biomarkers could particularly influence cardiovascular risk in women (15). Nowadays, distinct prediction models of cardiovascular events have been proposed for each sex (15-18).

Numerous early markers of atherogenesis, mainly inflammatory and hemostatic markers, have been described during the last decade. Markers of inflammation, insulin resistance and endothelium dysfunction are increased in obesity (19) and are considered underlying mechanisms of the atherosclerotic disease (20). Attempts to improve risk prediction by adding inflammatory markers, such as the C-reactive protein, to existing risk scores have been reported (15,18). Also, studies have investigated the role of other inflammatory and endothelial dysfunction markers in predicting diabetes and CVDs. However, limited data are reported concerning the distribution of these circulating biomarkers in individuals with lower cardiometabolic risk and how those values vary according to age and sex (21,22).

In Brazil, a large multicenter cohort study, the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil), was conducted to evaluate risk factors associated with CVDs and diabetes mellitus in civil servants from six sites (23). The availability of traditional cardiovascular risk factors and a broad spectrum of circulating biomarkers of atherogenesis in the ELSA-Brasil baseline data represent an opportunity to describe these biomarkers in a large sample of middle-aged adults.

Our aim was to describe the distribution of selected biomarkers according to age and sex, adjusted for homeostasis model assessment (HOMA-IR) and adiposity, in a subset of middle-aged participants of ELSA-Brasil without diabetes mellitus or a CVD.

SUBJECTS AND METHODS

This is a cross-sectional study that was developed based on baseline data from the multicenter cohort study ELSA-Brasil. A subsample of the participants of an ELSA-Brasil research center, located in the University of São Paulo, was included. The main objective of ELSA-Brasil is to investigate the incidence and progression of diabetes and CVDs and their biological, behavioral, environmental, occupational, psychological and social factors. Details on objective and methodological aspects were previously reported (23). Briefly, all active or retired employees of six Brazilian universities, aged 35-74 years, were eligible for the cohort study. The first examinations of 15,105 individuals (54% women) were carried out from August 2008 through December 2010. A random sample of 1,000 individuals of the research center from the University of São Paulo without diabetes and CVDs aged between 35 and 54 years was drawn from all 5,061 participants of ELSA-Brasil in São Paulo. Two individuals were excluded from the final sample because insufficient aliquots were frozen for the analysis of novel biomarkers. The institutional ethics committee approved the study, and written consent was obtained from all individuals.

Participants had an initial interview at the job site and were then scheduled for clinical exams and laboratory tests in the research center. Body weight and height were measured using calibrated electronic scales and a fixed rigid stadiometer, and participants wore light clothing and no shoes. Body mass index (BMI) was calculated as weight (kilograms) divided by squared height (meters). Waist circumference was measured with an inextensible tape according to the World Health Organization technique (24). Blood pressure (BP) was taken three times after a 5-minute rest in the sitting position. The mean of the second and third measurements was used in the analyses (25).

After overnight fasting, participants underwent a 2-hour, 75-gram oral glucose tolerance test. The blood samples were taken for several determinations at the University Hospital of the University of Sao Paulo (USP). For all the laboratory procedures, the ELSA-Brasil study had rigorous standards that are well described in a paper about pre-analytical aspects, including choosing the collection place, identifying and preparing participants, identifying collection site(s), applying and timing tourniquets, administering venipuncture techniques, ordering the collection of tubes, collecting by volume, handling tubes and processing biological samples and centrifuging, freezing and transporting biological material (26,27).

Plasma glucose was measured using the hexokinase method (ADVIA Chemistry; Siemens, Deerfield, Illinois, USA). Total cholesterol was assessed using the cholesterol oxidase method (enzymatic colorimetric). HDL-cholesterol was assessed using the homogeneous colorimetric method without precipitation. Triglycerides were assessed using the glycerol-phosphate peroxidase method according to Trinder

(enzymatic colorimetric) assays (ADVIA Chemistry; Siemens, Deerfield, Illinois, USA). The low-density lipoprotein cholesterol concentrations were calculated with the Friedewald equation. The American Diabetes Association's diagnostic criteria were used to diagnose categories of glucose tolerance (28). The HOMA-IR was used to assess insulin resistance (29). Aliquots were frozen at -80ºC for further determinations of hormone apolipoproteins, markers of inflammation and endothelium adhesion molecules (30).

Insulin was determined using enzyme-linked immunoenzymatic assay (ELISA) (Siemens, Tarrytown, USA) and high-sensitivity C-reactive protein through immunochemistry (Dade Behring, Siemens, Marburg, Germany). ELISA kits were also used for the determination of adiponectin and leptin (Enzo Life Sciences, Farmingdale, NY, USA), apolipoprotein B (Apo B), E-selectin and transforming growth factor β1 (TGF-β1) (Abnova Corp, Taipei, Taiwan), lipoprotein (a) (Lp(a)) (ALPCO Diagnostics, Salem, NH, USA), asymmetric dimethylarginine (ADMA) and fibrinogen (Affinity Biologicals Inc., Hamilton, ON, Canada). Interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) were determined using the Bio-Plex® Pro Human Cytokine 4-plex assay panel (Biorad, São Paulo, SP, Brazil). Intra-assay coefficients of variation ranged from 1.8 to 7.2, except for those of TGF-β1, which reached 10%. Inter-assay coefficients varied from 0.9 to 9.1 except for TGF-β1 (12.7%) and E-selectin (14.4%).

Statistical analysis

Descriptive data were either expressed as means and standard deviations (SDs) or as medians (interquartile range). Distributions of the biomarker concentrations were skewed and thus log-transformed before analysis to achieve normality. Biomarker means by sex and age group (35-44 and 45-54 years) were compared using an unpaired student t-test when normally distributed. Frequencies were compared using a chi-squared test. Participants were categorized into tertiles of HOMA-IR, and data were compared via ANOVA. A Pearson (r) or Spearman (rho) coefficient was used to test correlations between each biomarker and measures of obesity (BMI and waist circumference) according to sex. Whenever a significant correlation was found, a sex-specific multivariate analysis using multiple linear regression was used to examine independent associations of age, adiposity and HOMA-IR with the selected novel biomarkers.

Sensitivity analyses, excluding participants who used medications (antihypertensive and/or lipid reducing agents) or smoked, were conducted to avoid their influence on the associations found. Considering that conditions related to clinical inflammation could interfere in measurements of these biomarkers, some procedures were performed. For the blood sample collection, individuals who self-reported infectious diseases were invited to return to the clinic after they recovered. However, chronic inflammatory disorders were not exclusion criteria in our sample. Participants reported rheumatologic diseases (n = 13), rheumatoid arthritis (n = 12), lupus (n = 2), arthrosis (n = 55) and arthritis (n = 24). In order to control for these potential confounding factors, sensitivity analyses considering these conditions were performed and nonsteroidal anti-inflammatory drugs (n = 1), acetylsalicylic acid (n = 7), hormonal anti-inflammatory drugs (n = 1) and CRP levels > 9.9 mg/L (n = 28) were used. Less than 5% of women in the sample were using hormone replacement therapy for menopause, which had a negligible influence on results. Pre-diabetes status was identified in 661 individuals who had similar profiles to the whole group [mean age of 46.3 (SD 4.8) years; 53% of men, mean BMI was 26.9 (SD 4.2) kg/m^2]. The results of sensitivity analyses did not differ when pre-diabetic individuals or those who used anti-hypertensive drugs (n = 120) were excluded. Therefore, results excluding these participants were not shown. All statistical analyses were performed using the Statistical Package for Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

The mean age of the 998 individuals studied was 45.8 (SD 4.9) years; 55% were women and 19% had a BMI ≥ 30 kg/m^2. Of them, 18.4% were current smokers, being 22.6% of men and 15.0% of women (p < 0.01).

Men had higher mean values of waist circumference, BP levels, fasting plasma glucose levels and HOMA-IR and worse lipid profiles than women (Table 1). Mean values of waist circumference, systolic BP, fasting plasma glucose and Apo B increased in the older group (45-54 years) of both sexes. In women alone, total cholesterol,

LDL-cholesterol and triglyceride values were higher in the older group. Men had higher E-selectin while women had higher leptin levels. In women, HOMA-IR, Il-6 and TNF-α values were higher in the older group, while in men, leptin and CRP values were higher in the same age group. No other biomarker differed between the age groups of both sexes.

Biomarkers were compared across tertiles of HOMA-IR (Figure 1). E-selectin and leptin concentrations gradually increased across tertiles in men (ANOVA, $p < 0.001$ for E-selectin and leptin) and women (ANOVA, $p = 0.007$ for E-selectin and $p < 0.001$ for leptin). In all categories of HOMA-IR, higher E-selectin and lower leptin concentrations were observed in men. Despite men's higher Apo B values in each category of HOMA-IR compared with women, no trend across tertiles was verified. No statistical difference between the other biomarkers was found according to HOMA-IR tertiles (data not shown).

Correlations of interest regarding anthropometric measurements and plasma glucose with biomarkers were assessed for the total sample before variables were entered into the regression models. Correlations between waist circumference and Apo B (rho = 0.034, $p = 0.490$ and rho = 0.093, $p = 0.037$) and E-selectin (rho = 0.178, $p < 0.001$ and rho = 0.124, $p = 0.004$) were evaluated among men and women, respectively. BMI was directly correlated to leptin concentrations in men (rho = 0.363, $p < 0.001$) and women (rho = 0.425, $p < 0.001$). As expected, both adiposity parameters, BMI and waist circumference, were correlated to lipid and plasma glucose levels (data not shown). E-selectin was also correlated to plasma glucose in women (rho = 0.090, $p = 0.038$) but not men (rho = 0.059, $p = 0.213$).

Table 1. Mean (SD) values of cardiovascular risk factors and biomarkers according to sex and age group in a subset participants of ELSA-Brasil

	Women			Men		
	Total	35 - 44 yrs	45 - 54 yrs	Total	35 - 44 yrs	45 - 54 yrs
BMI (kg/m^2)	26.3 (4.2)	25.8 (4.2)	26.6 (4.2)$^{£}$	26.6 (4.0)	26.3 (4.2)	26.8 (4.0)
Waist circumference (cm)	83.3 (10.7)	81.2 (10.1)	84.5 (10.9)$^{£}$	91.3 (10.7)$^{¤}$	89.8 (11.1)	92.2 (10.4)$^{£}$
Systolic BP (mmHg)	112 (13)	109 (12)	113 (13)$^{£}$	122 (14)$^{¤}$	119 (12)	124 (15)$^{£}$
Diastolic BP (mmHg)	72 (10)	71 (10)	73 (10)	78 (10)$^{¤}$	76 (9)	80 (11)$^{£}$
Plasma glucose (mg/dL)	100 (7)	98 (7)	102 (8)$^{£}$	105 (8)$^{¤}$	104 (7)	106 (9)$^{£}$
HOMA-IR$^{\#}$	1.7 (1.2)	1.5 (1.1)	1.8 (1.3)$^{£}$	2.1 (1.8)$^{¤}$	1.9 (1.7)	2.2 (1.8)
Total cholesterol (mg/dL)	208 (36)	197 (34)	214 (36)$^{£}$	212 (37)	207 (34)	214 (38)
LDL-cholesterol (mg/dL)	126 (31)	118 (29)	131 (31)$^{£}$	132 (33)$^{¤}$	129 (30)	134 (34)
HDL-cholesterol (mg/dL)	60 (13)	59 (13)	60 (13)	50 (11)$^{¤}$	48 (8)	51 (13)$^{£}$
Triglycerides (mg/dL)	110 (61)	101 (61)	115 (61)$^{£}$	150 (86)$^{¤}$	153 (39)	148 (82)
Apolipoprotein B (mg/dL)$^{\#}$	187 (171)	154 (132)	206 (188)$^{£}$	205 (196)$^{¤}$	159 (114)	232 (228)$^{£}$
Lipoprotein (a) (mg/dL)$^{\#}$	17.1 (25.8)	15.6 (11.2)	18.0 (31.5)	16.2 (14.1)	17.5 (16.8)	15.4 (12.1)
Fasting Insulin (μUI/mL)$^{\#}$	6.8 (4.9)	6.3 (4.4)	7.0 (5.2)	7.9 (6.7)	7.6 (6.5)	8.2 (6.8)
Leptin (ng/mL)$^{\#}$	25.3 (38.4)	25.7 (28.4)	25.0 (43.5)	10.5 (9.8)$^{¤}$	9.2 (8.1)	11.3 (10.6)$^{£}$
Adiponectin (mcg/mL)$^{\#}$	13.6 (18.1)	14.6 (26.3)	13.0 (10.4)	12.1 (14.8)	11.8 (13.0)	12.3 (15.8)
C-reactive protein (mg/L)$^{\#}$	2.7 (3.5)	2.6 (3.0)	2.8 (3.8)	2.2 (4.2)$^{¤}$	1.7 (2.4)	2.5 (5.0)$^{£}$
Interleukin-6 (pg/mL)$^{\#}$	19.3 (36.8)	14.5 (10.7)	22.2 (45.6)$^{£}$	18.7 (41.2)	18.0 (28.0)	19.2 (47.4)
Interleukin-10 (pg/mL)$^{\#}$	10.6 (60.3)	17.2 (95.5)	6.8 (20.2)	10.5 (59.3)	9.3 (58.6)	11.1 (59.7)
TNF-α (pg/mL)$^{\#}$	24.2 (84.4)	14.9 (23.2)	29.8 (104.9)$^{£}$	28.1 (147.2)	20.3 (52.5)	32.7 (181.4)
MCP-1 (pg/mL)$^{\#}$	42.4 (33.5)	42.1 (34.1)	42.5 (33.2)	40.6 (29.8)	41.4 (31.7)	40.3 (28.6)
TGF-β1 (pg/mL)$^{\#}$	19.6 (19.0)	17.9 (16.1)	20.6 (20.6)	22.3 (9.4)	18.3 (14.6)	24.6 (118.5)
E-selectin (ng/mL)$^{\#}$	80.9 (54.5)	79.5 (51.9)	81.8 (56.1)	93.7 (61.9)$^{¤}$	91.4 (59.7)	95.0 (63.2)
ADMA (μmol/L)$^{\#}$	0.20 (0.12)	0.21 (0.12)	0.19 (0.12)	0.20 (0.10)	0.19 (0.12)	0.20 (0.09)
Fibrinogen (g/L)	1.6 (0.4)	1.6 (0.3)	1.6 (0.4)	1.6 (0.4)	1.6 (0.3)	1.6 (0.4)

BP: blood pressure; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein-1; TGF-β1: transforming growth factor β1; ADMA: asymmetric dimethylarginine. # log-transformed variables for analyses. Student t test used to compare sex and age groups. $^{¤}$ $P < 0.05$ for men versus women $^{£}$ $P < 0.05$ for 35-44 versus 45-54 years.

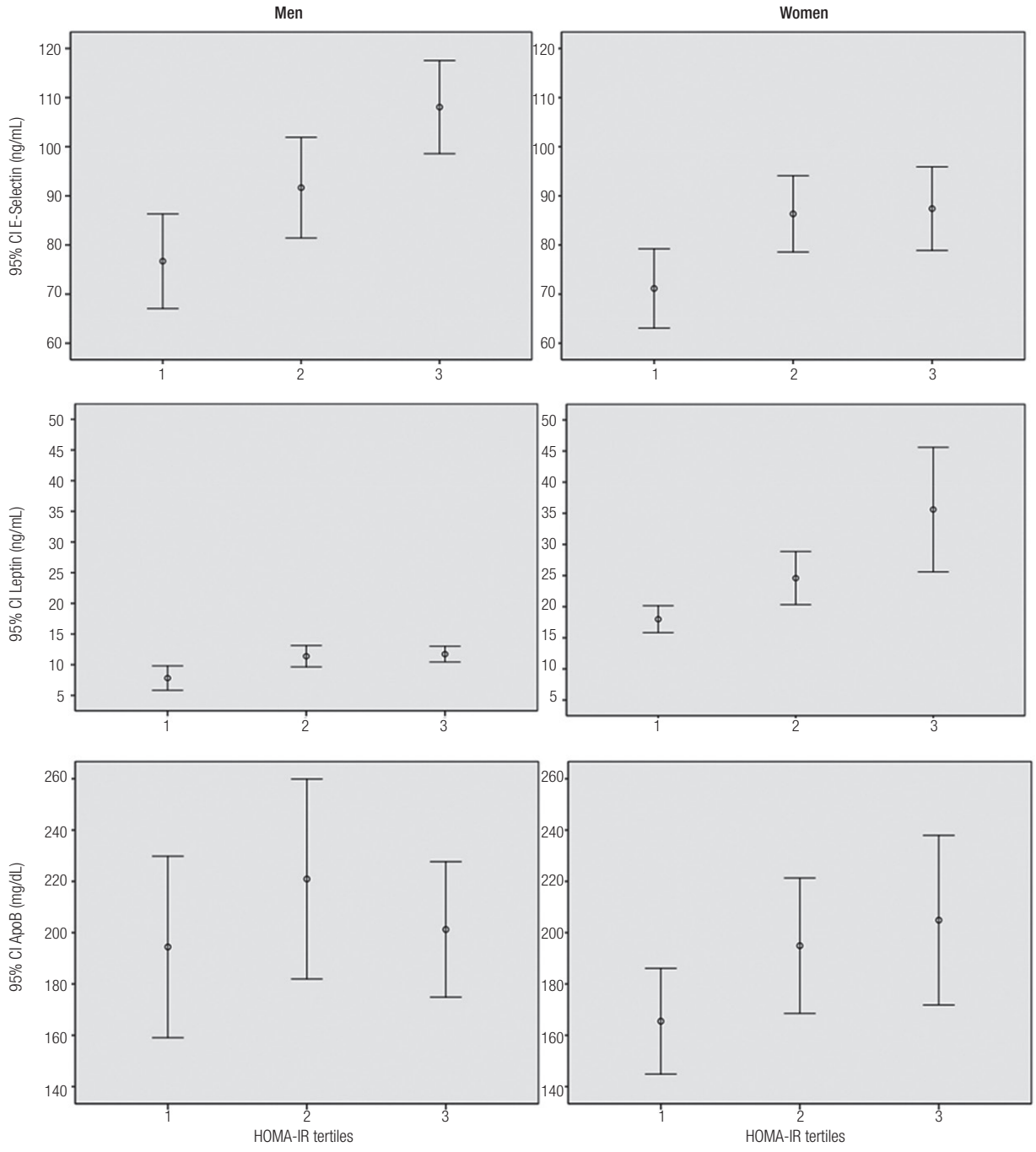

Figure 1. Means and 95% confidence intervals of E-selectin, leptin and apolipoprotein B across tertiles of HOMA-IR according to sex.

Multiple regression analysis models were run separately for men and women using Apo B, leptin and E-selectin as dependent variables (Table 2) since these biomarkers differed between sexes in Table 1. All models included age, adiposity (waist circumference in apolipoprotein B and E-selectin models and BMI in the leptin model) and HOMA-IR. Among men, Apo B was associated with age independent of adiposity and the insulin resistance index. For both sexes, leptin was strongly associated with BMI and HOMA-IR independent of age. E-selectin was associated with waist circumference in the age-adjusted model but lost significance when HOMA-IR was included in the final model for both sexes.

Table 2. Linear regression models for apolipoprotein B, leptin or E-selectin (dependent variables) for men and women

	Men											
	Apolipoprotein B				Leptin				E-selectin			
	r^2	β	95% CI	p	r^2	β	95% CI	p	r^2	β	95% CI	p
Age	0.016	0.030	0.009–0.051	0.006	0.134	0.013	-0.004–0.029	0.127	0.008	0.004	-0.010–0.019	0.559
Adiposity*	0.018	-0.011	-0.023–0.001	0.065	0.184	0.068	0.044–0.092	0.000	0.011	0.005	-0.003–0.013	0.242
HOMA-IR	0.025	0.047	-0.023–0.117	0.189	0.150	0.067	0.013–0.120	0.015	0.046	0.076	0.028–0.124	0.002
	Women											
	Apolipoprotein B				Leptin				E-selectin			
	r^2	β	95% CI	p	r^2	β	95% CI	p	r^2	β	95% CI	p
Age	0.002	0.008	-0.012–0.028	0.430	0.156	-0.012	-0.026–0.004	0.142	0.008	-0.006	-0.021– 0.09	0.427
Adiposity*	0.002	0.001	-0.007–0.010	0.823	0.118	0.030	0.053–0.093	0.000	0.002	0.008	-0.005–0.010	0.542
HOMA-IR	0.020	-0.001	-0.086–0.085	0.988	0.165	0.093	0.028–0.158	0.005	0.028	0.097	0.033–0.160	0.003

CI: confidence interval.

All models included age and adiposity. * Waist circumference was used in apolipoprotein B and E-selectin models and BMI in the leptin model.

DISCUSSION

Attempts to improve the prediction of cardiovascular events have motivated investigations into biomarkers involved in the early atherosclerotic process. Ageing is a major risk factor for atherosclerosis, and its manifestations in women are closely related to menopause. In the present study, a broad spectrum of circulating biomarkers of inflammation and endothelial dysfunction as well as traditional risk factors were examined in association with age and sex in a large sample of middle-aged adults of both sexes. As many of these markers track body adiposity, our study also calls attention to the influence of anthropometric measurements and the insulin resistance index. Our findings add new knowledge to these determinations: associations of Apo B with age and sex and of leptin and E-selectin with sex, adiposity and insulin resistance suggest that these demographic and metabolic characteristics should be taken into consideration when assessing these biomarkers for research or clinical purposes.

Atherogenesis starts early in life and accelerates with ageing. Our study reinforces that, in parallel, blood pressure levels, plasma glucose and lipids (except HDL-cholesterol) tend to increase in both sexes. In our female sample, the 45- to 54-year-old group showed higher BMIs and waist circumferences. The waist circumference value was higher in the oldest age group, confirming previous observations of central fat accumulating with age in men and women (31). Certainly, cytokines secreted by the adipose tissue contribute to disturbances of glucose and lipid metabolism and BP elevation with ageing (32). The deterioration of these traditional risk factors has common underlying mechanisms, such as inflammation and insulin resistance. Our data on increased IL-6 and TNF-α in older women and increased CRP in older men in our sample are in agreement with this pathophysiological pathway. Actually, inflammatory markers were shown to be correlated to Apo B, which is present in most atherogenic lipid particles in circulation. It is interesting that these results were found when the traditional risk factors were almost within the normal range (Table 1).

It was postulated that adhesion molecules, like selectins and CAMs, play a role in the development of the arterial plaque (33). We found that the greater concentrations of a soluble molecule that promotes leukocyte adhesion to endothelia in men is compatible with higher cardiovascular risk in this sex, particularly in the 35 to 54 age group. The association with sex was previously described in a sample of young individuals (21,22). Since the production of adhesion molecules is stimulated by metabolic disturbances (33), it was important to test the association of E-selectin with an insulin resistance index. Even after adjusting for adiposity and age, the correlation of E-selectin to HOMA-IR persisted. Other investigators had similar findings that showed that adhesion molecule concentrations were influenced by sex, age and HOMA-IR in individuals with low cardiometabolic risk (21,22). Apart from the limitations of the present study's design, our findings

could suggest that visceral adipose tissue might be influencing E-selectin synthesis, maybe through adipose tissue derived cytokines that induce insulin resistance. In animals, adipocyte was shown to express an adhesion molecule named ACAM (34). It was previously demonstrated that this endothelial, cell-specific adhesion glycoprotein is partially stimulated by inflammatory cytokines — like TNF-α and interleukins — produced by adipocytes, leukocytes and other cells (35). Transcription of the E-selectin gene is exclusively expressed on the endothelial cell surface and is mainly dependent on TNF-α-induced activation of the nuclear factor kappa B (35). For our knowledge, prospective studies have yet to prove whether circulating levels of E-selectin could be useful for identifying early abnormalities in the atherosclerotic process and whether different values should be considered for each sex.

Unadjusted analysis showed higher leptin concentrations in women, which was expected because it is an adipose tissue derived hormone. It is recognized that, at similar BMI levels, women have more fat mass than men (36). Leptin concentration is known to be associated with fat mass in women from puberty. Interestingly, higher mean values of leptin in the older group were only observed for men, which could be explained by their tendency to increase visceral adipose tissue with ageing. Interestingly, the association of leptin with body adiposity persisted after additional adjustment for insulin resistance index. This finding may indicate that such an association is highly dependent on the production of this hormone by the adipocyte and, to a lesser extent, related to the condition of insulin resistance.

On average, participants of the present study were overweight and 19% were obese. Being overweight is a recognized condition of insulin resistance (37) commonly accompanied by resistance to leptin (38). Insulin and leptin resistance are seen as the interface between inflammation and metabolism in obesity-related CVD. It would be reasonable to suppose that an increase in body adiposity with ageing might contribute to a deteriorating cardiovascular risk profile. However, this cross-sectional design precludes investigating a cause and effect relationship.

The findings of higher central adiposity, HOMA-IR and concentration of Apo B and E-selectin in men are compatible with the fact that the cardiovascular risk profile is worse in men than in pre-menopausal women (39,40). Our sample included a large number of middle-aged adults (younger than 55 years old), most of them women (55%), who did not have any overt CVD. Another advantage was the very low rate of hormone replacement therapy. Studies with these sample characteristics are relatively uncommon in the literature.

Our study has limitations. One major limitation is related to the high inter-individual, and possibly intra-individual, variability. Ideally, biomarkers should be determined on several occasions to obtain variability rates. Looking at the r^2 values, concentrations of these biomarkers are weakly explained by the variables entered in the regression models. However, it is well known that age, sex and adiposity influence cardiovascular risk assessments. Our results suggest that these variables should also have an impact on some of the novel biomarkers. Therefore, they might be taken into account when their values are examined. The study design does not allow the establishment of a cause and effect relationship. Further investigation is needed to determine whether some novel cardiovascular biomarkers could contribute to earlier risk prediction needs.

In conclusion, our data indicate that age, sex, adiposity and, consequently, insulin resistance influence circulating levels of Apo B, leptin and E-selectin, thereby suggesting that those factors should be taken into consideration when assessing these parameters for research or clinical purposes in individuals with relatively low cardiometabolic risk.

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Authors' contributions: BAP participated in the study design, organization of the data, analysis of novel biomarkers, statistical analysis, interpretation of the results, draft the article. FFRF participated in the review of the statistical analysis and of the

article. SB participated in the design of the study, interpretation of the results and review of the article. BBD conceived of the ELSA-Brasil study, participated in the design interpretation of the results and review of the article. MIS conceived of the ELSA-Brasil study, participated in interpretation of the results and review of the article. IMB conceived of the ELSA-Brasil study, participated in interpretation of the results and review of the article. PAL conceived of the ELSA-Brasil study, participated in interpretation of the results and review of the article. SRGF conceived of the actual study, design of the study, participated in interpretation of the results and review of the article. All authors read and approved the final manuscript.

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Thyroxine increases *Serca2* and *Ryr2* gene expression in heart failure rats with euthyroid sick syndrome

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ABSTRACT

Objective: The current study was aimed at analyzing sarcoplasmic reticulum Ca^{2+} ATPase (*Serca2*) and ryanodine receptor type 2 (*Ryr2*) gene expression in rats subjected to surgery that induced HF and were subsequently treated with T4 using physiological doses. **Materials and methods:** HF was induced in 18 male Wistar rats by clipping the ascending thoracic aorta to generate aortic stenosis (HFS group), while the control group (9-sham) underwent thoracotomy. After 21 weeks, the HFS group was subdivided into two subgroups. One group (9 Wistar rats) with HF received 1.0 µg of T4/100 g of body weight for five consecutive days (HFS/T4); the other group (9 Wistar rats) received isotonic saline solution (HFS/S). The animals were sacrificed after this treatment and examined for signs of HF. Samples from the left ventricles of these animals were analyzed by RT-qPCR for the expression of *Serca2* and *Ryr2* genes. **Results:** Rats with HF developed euthyroid sick syndrome (ESS) and treatment with T4 restored the T3 values to the Sham level and increased *Serca2* and *Ryr2* gene expression, thereby demonstrating a possible benefit of T4 treatment for heart function in ESS associated with HF. **Conclusion:** The T4 treatment can potentially normalize the levels of T3 as well elevated *Serca2* and *Ryr2* gene expression in the myocardium in heart failure rats with euthyroid sick syndrome. Arch Endocrinol Metab. 2016;60(6):582-6

Keywords
Reverse triiodothyronine; heart failure; therapeutic use; calcium channels; triiodothyronine

INTRODUCTION

Euthyroid sick syndrome (ESS) is defined as a drop in the levels of triiodothyronine (T3) and increase in the levels of reverse T3 (rT3), with or without decrease in thyroxine (T4) or thyroid-stimulating hormone (TSH) (1,2) in critically infirm patients (3,4). Both hyperfunction and hypofunction of thyroid have multiple effects on the cardiovascular system; therefore, normal endocrine function is essential for cardiovascular health (5).

ESS occurs in various clinical conditions, such as malnutrition, liver or kidney failure, systemic disease, human immunodeficiency virus (HIV) infection, trauma and post-operative infection (6). Its appearance in patients with heart failure is an indicator of the severity of the disease (7). In addition, it is unclear whether the appearance of ESS is only a prognostic marker of HF or that ESS contributes to the worsening of HF (8). However, it is known that normal thyroid hormone levels are restored when the HF is resolved.

A drop in T3 levels is the most common abnormality in decompensated HF. T3 levels reduce rapidly within the first 24 hours of escalation of the disease, while rT3 levels increase and TSH and total and free T4 levels remain normal (2).

The activity of various channels and pumps located in the sarcolemma and in the sarcoplasmic reticulum (SR) regulates the transit of intracellular Ca^{2+}, modulating the contraction and relaxation of the myocardium. It is also known that a reduction in T3 promotes a reduction in the expression of calcium transport proteins; sarcoplasmic Ca^{2+}ATPase (*Serca2*) and ryanodine receptor type 2 (*Ryr2*) (9,10).

It is not clear whether treatment with thyroid hormone in patients with HF and ESS results in favorable hemodynamic changes and improves heart

function (11). While some experimental work has shown favorable results after treatment (principally with T4 analogues) (10-12), other studies have shown either no difference in relation to the placebo, or an increase in the number of collateral effects while providing no benefit in relation to the placebo (13,14).

Hence, the present study assessed the effect produced by treatment of HF-induced ESS with physiological doses of T4 on *Serca2* and *Ryr2* mRNA expression in rats. We found that HF-induced ESS resulted in decreased T3 levels and a decrease in *Serca2* and *Ryr2* gene expression. T4 treatment after HF increased *Serca2* and *Ryr2* gene expression.

MATERIALS AND METHODS

Animal models and experimental protocol

All procedures outlined in this study were approved by the Ethics Committee of Botucatu Medical School (Unesp, SP, Brazil) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council in the 1996 (15).

A total of 50 male Wistar rats (aged 60 days and weighing approx. 392 g) were used for this study. The animals were initially divided into two groups: sham (10 rats submitted to simulated surgery, thoracotomy without the insertion of a clip) and HFS group (40 rats submitted to thoracotomy with the insertion of a clip). They were anesthetized posteriorly and subjected to trichotomy of the anterior median region of the thorax. After that, the animals were placed in the supine position and manual ventilation was provided by positive pressure with oxygen at 100%. A median sternotomy was carried out and a clip was placed in the case of the HFS group; the thoracic cavity was closed with 5.0 mononylon thread.

After surgery, the animals were assessed daily in order to detect signs of HF such as tachypnea, cyanosis, hair standing on end, edema, ascites, pleural-pericardial effusion, atrial thrombus, and hepatomegaly; the animals that did not present signs of HF were excluded from the study. About 50% of the animals developed HF after surgery. At the end of 21 weeks, eighteen rats developed aortic stenosis (HFS group), which is a characteristic of HF. The HFS group was subdivided into two groups, as shown in Figure 1. The HFS/T4 group (9 rats) was administered T4 (L-T4 Sigma) at a physiological dose of 1.0 μg/100 g (16,17) of body weight (BW) subcutaneously, twice a day (7 am and 7 pm) for 5 days. This period is sufficient for the pharmacokinetics and pharmacodynamics of thyroxine (18,19). The HFS/S group (9 rats) was administered saline solution injections (0.9% NaCl, without T4) instead of T4. At the end of the treatment, all the animals were sacrificed.

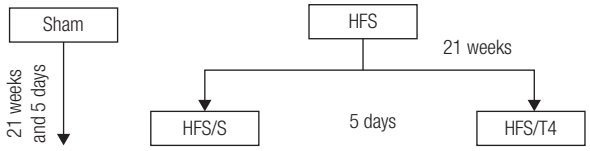

Figure 1. Experimental protocol.

Sham (n = 9), heart failure syndrome (HFS, n = 18), HFS/S administered saline 0.9% NaCl without T4 (n = 9), and HFS/T4 administered T4 at 1.0 μg/100 g BW (HFS/T4, n = 9).

Serca2 and *Ryr2* gene expression

Total RNA was extracted from cardiac tissue (left ventricle) using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1000 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Real-time PCR (RT-qPCR) was used to quantitatively measure the messenger RNA (mRNA) levels of *Serca2a* (Rn00568762_m1) and *Ryr2* (Rn01470303_m1) using TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA) and the Applied Biosystems StepOne Plus detection system. Each sample was run in triplicate for RT-qPCR. Cycling conditions were as follows: enzyme activation at 50°C for 2 min; denaturation at 95°C for 10 min; cDNA amplification for 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Cyclophilin (Rn00690933_m1) was used as the internal control and gene expression was quantified in relation to the values for the control groups (C15, C30, and C45, respectively) with the $2^{-\Delta\Delta Ct}$ method (20).

Serum analysis

Blood was collected in dry tubes and then centrifuged at 1008 g (Eppendorf Centrifuge 5804R) for 10 min for separating the serum from whole blood. Serum concentrations of free T3, free T4, and TSH were measured by Genese Laboratory-Botucatu-SP with the Luminex Corporation's xMAP™ Tecnology, by immunofluorescence reaction.

Statistical analysis

For biometric statistical analysis, the Student's t-test (21) was used. Gene expression and hormone data were analyzed using analysis of variance (ANOVA) complemented by Bonferroni's test. The data are expressed as mean ± standard deviation and a significance level of 5% was adopted.

RESULTS

Biometric and tissue wet/dry weight ratio data

The animals subjected to aortic stenosis surgery developed HF, established by the following findings: tachypnea, ascites, pleural effusion, atrial thrombus, and liver congestion (22-25). None of the nine sham group exhibited any of these clinical or pathological features. Biometric and tissue wet/dry weight ratio data for HFS and sham group are presented in Table 1. BW was greater in the sham group as compared to the HFS group. Normalized LV and RV (right ventricle) weights were greater in the HFS group than in the sham group. Lung wet/dry ratio was greater in the sham than the HFS group. No significant difference in liver wet/dry ratio was observed among the groups. The comparison of the groups HFS/S and HFS/T4 not showed significant statistical difference (data not shown) for biometric and tissue wet/dry weight ratio data.

Table 1. General characteristics of groups: Sham and HFS

	Sham (n = 9)	HFS (n = 18)
BW	452.62 ± 18.85[b]	348.89 ± 34.98[a]
LVW/BW	1.78 ± 0.13[a]	3.10 ± 0.30[b]
RVW/BW	0.51 ± 0.06[a]	0.95 ± 0.18[b]
Liver W/Dg/g	3.46 ± 0.79[a]	3.07 ± 0.58[a]
Lung W/Dg/g	4.54 ± 0.07[a]	5.24 ± 0.40[b]

Values are mean ± standard deviation n: number of rats; Sham: thoracotomy without the insertion of clip; HFS: rats with the insertion of a clip (aortic stenosis); BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight. The averages followed by different alphabets ($p < 0.01$) show significance according to the Student's *t*-test.

Reduction in T3 levels after HF

The levels of thyroid hormones in the serum of the animals are presented in Table 2. Statistically significant reduction in the levels of T3, and no change in T4 and TSH levels was observed in the HFS/S group in comparison to the sham group, but exogenous administration of T4 led to a significant increase in T3 levels in the HFS/T4 group to the levels of the sham group.

Table 2. Hormonal measurement of free T3, T4, and TSH

Variable	Groups		
	Sham	HFS/S	HFS/T4
Free T3 (ug/mL)	12.13 ± 1.09[b]	9.44 ± 1.44[a]	12.05 ± 1.40[b]
Free T4 (ug/mL)	646.02 ± 27.53[a]	671.3 ± 49.25[a]	652.0 ± 131.50[a]
TSH (ug/mL)	5.16 ± 2.34[a]	4.98 ± 2.36[a]	5.68 ± 2.17[a]

TSH: thyroid stimulating hormone; T3: triiodothyronine; T4: thyroxine; Sham = thoracotomy without the insertion of a clip, HFS/S = aortic stenosis without T4 (saline, 0.9% NaCl), HFS/T4 = aortic stenosis with 1.0 µg T4/100 g BW. Data expressed as mean ± standard deviation. ANOVA complemented by Bonferroni's test was used. Use of same alphabets represents $p > 0.05$; different alphabets represent $p < 0.05$.

HF decreased *Serca2* and *Ryr2* levels, but T4 administration elevated them

RT-qPCR data provided a quantitative assessment of *Serca2* and *Ryr2* gene expression, illustrated in Figure 2. *Serca2* and *Ryr2* expression was reduced in the HFS group as compared to the sham group, while an increase in *Serca2* and *Ryr2* expression was observed in HFS/T4 group as compared to the HFS/S group.

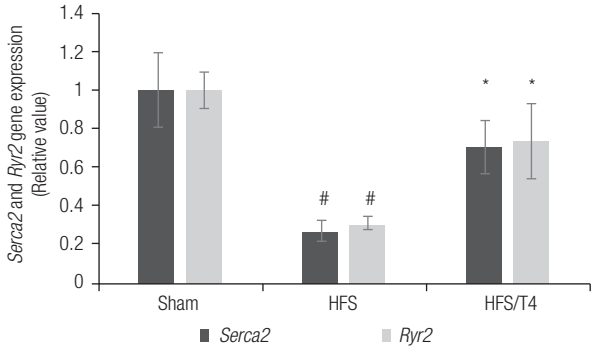

Figure 2. Gene expression of *Serca2* and *Ryr2* decreases in HF, while an increase is seen upon administration of T4.

Sham = thoracotomy without the insertion of a clip, HFS = aortic stenosis without T4, HFS/T4 = aortic stenosis with 1.0 µg T4/100 g BW. Data expressed as mean ± standard deviation. ANOVA was used, complemented by Bonferroni's test. The # symbol represents the Sham group vs HFS ($p < 0.001$); the * symbol represents the HFS group vs HFS/T4 ($p < 0.001$).

DISCUSSION

The role of thyroid hormone therapy is unclear in patients with HF and low serum T3 levels; this therapy, however, results in improvement in ventricular function. Some studies suggest the use of thyroid

hormone analogs such as diiodothyropropionic acid and gene therapy along with T3 and/or T4 hormone replacement to modify thyroid hormone receptor or deiodinase expression and activity (26).

Using an experimental model as a base for studying ESS in HF, we were able to successfully induce ESS in animals with HF and at the same time demonstrate a reduction in the serum level of T3 in these animals. The decrease in T3 observed in our model is probably due D1, involved in the production of T3, as demonstrated by previous studies from our group. This study analyzed the conversion of T4 to T3 or rT3 in the liver and kidneys and found that animals with HF had a higher concentration of rT3 while no T3 was detected (27). The other possibility is the increase of D3 activity in the cardiomyocyties (28).

Our group has previously demonstrated that clipping surgery of the ascending thoracic aorta is an effective technique to induce HF and ESS in rats (27). These results are similar to those of Pimentel and cols. (29) and Hamilton (7), which demonstrated a correlation between the degree of HF and ESS; hence, the presence of ESS in HF patients is a prognostic factor in HF.

In the presence of ESS (reduced T3 levels), the gene expression of the intracellular calcium pumps is reduced. This results in altered intracellular calcium movement, which affects the contraction and excitation of the heart muscles, thus compromising the function of the heart (9,10).

Therefore, animals from the HFS group presented with ESS and showed decreasing *Serca2* and *Ryr2* gene expression. We showed the effect of treatment of HF with T4 (HFS/T4), in a physiological dose and verified the subsequent increase in expression of *Serca2* and *Ryr2* in the HFS/T4 group (Figure 1). This could be a result of conversion of administered T4 into T3; T3 is known to be essential for gene transcription of these pumps (9,10).

Some studies have suggested administration of T4 as a treatment for ESS that develops in acute kidney injury, in newborns with lung disease, and with the use of supraphysiological doses (12,13). The current study is significant primarily since we investigated the role of T4 in the treatment of ESS in HF, which is an understudied topic.

Treatment with T4 normalized T3 levels in the HFS/T4 group (Table 2) compared to those seen for HFS/S, thereby demonstrating that treatment with T4 can potentially reverse hormonal changes caused by ESS in HF. Previously four randomized controlled trials (RCTs) were conducted using either T4 or T3 for treatment of ESS. Two of these RCTs used T3 and the other two used T4 as the hormone for treatment of ESS. Three out of the four studies used these hormones in pharmacologic doses. All four studies showed that hormonal treatment further suppressed plasma TSH, and none of trials showed a therapeutic benefit to patients (30). In contrast, the present study demonstrated that T4 administration at a physiologic dose after HF normalizes T3 levels (HFS/T4) and does not suppress TSH secretion (Table 2). The normalization of serum T3 in HF rats, following T4 administration, resulted in a significant increase in *Serca2* and *Ryr2* mRNA expression, thereby demonstrating the utility of this hormone to normalize molecular and hormonal changes produced by HF-induced ESS.

In conclusion, the T4 treatment can potentially normalize the levels of T3 as well elevated *Serca2* and *Ryr2* gene expression in the myocardium in heart failure rats with euthyroid sick syndrome.

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Interactions between prolactin and kisspeptin to control reproduction

Jose Donato Jr.[1], Renata Frazão[2]

ABSTRACT

Prolactin is best known for its effects of stimulating mammary gland development and lactogenesis. However, prolactin is a pleiotropic hormone that is able to affect several physiological functions, including fertility. Prolactin receptors (PRLRs) are widely expressed in several tissues, including several brain regions and reproductive tract organs. Upon activation, PRLRs may exert prolactin's functions through several signaling pathways, although the recruitment of the signal transducer and activator of transcription 5 causes most of the known effects of prolactin. Pathological hyperprolactinemia is mainly due to the presence of a prolactinoma or pharmacological effects induced by drugs that interact with the dopamine system. Notably, hyperprolactinemia is a frequent cause of reproductive dysfunction and may lead to infertility in males and females. Recently, several studies have indicated that prolactin may modulate the reproductive axis by acting on specific populations of hypothalamic neurons that express the *Kiss1* gene. The *Kiss1* gene encodes neuropeptides known as kisspeptins, which are powerful activators of gonadotropin-releasing hormone neurons. In the present review, we will summarize the current knowledge about prolactin's actions on reproduction. Among other aspects, we will discuss whether the interaction between prolactin and the *Kiss1*-expressing neurons can affect reproduction and how kisspeptins may become a novel therapeutic approach to treat prolactin-induced infertility. Arch Endocrinol Metab. 2016;60(6):587-95

Keywords

Prolactin receptor; pSTAT5; hypothalamus; *Kiss1*; infertility

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INTRODUCTION

Prolactin is a protein hormone produced and secreted by the anterior pituitary gland. Sparse evidence, at least in rodents, suggests that prolactin may also be locally produced in some brain areas, but the physiological importance of this production is unknown (1). Prolactin secretion is controlled by hypothalamic endocrine neurons, especially the tuberoinfundibular dopamine (TIDA) neurons located in the arcuate nucleus of the hypothalamus (ARH). These neurons secrete dopamine into the hypophyseal portal system, which leads to the activation of dopamine D2 receptors in pituitary lactotrophs, causing suppression of prolactin gene expression and prolactin secretion (1). Prolactin is transported by the circulatory system and acts on target cells via specific receptors located on the plasma membrane (1,2). Serum prolactin crosses the blood-brain barrier by a PRLRs-independent mechanism (3). The function of PRLRs in TIDA neurons is to allow these cells to sense circulating prolactin levels and consequently regulate pituitary prolactin secretion through negative feedback mechanisms. Several other cell populations also express PRLRs, including different brain regions, as well as the bone, adipose tissue, gut, skin, immune system and reproductive tract (2,4-9).

Clinical evidence indicates that hyperprolactinemia is a frequent cause of reproductive dysfunction and may lead to infertility in males and females (10-13). Pathological hyperprolactinemia is mainly caused by the presence of a prolactinoma or is due to pharmacological effects induced by drugs that interact with the dopamine system. Loss-of-function mutations in the gene that encodes the PRLRs can also be a rare cause of hyperprolactinemia (11). The objective of the present review is to summarize and discuss recent advances in the discovery of possible mechanisms linking prolactin signaling and the control of reproduction, especially regarding the role of kisspeptins as novel potential targets to treat prolactin-induced infertility.

PROLACTIN SIGNALING

Multiple isoforms of membrane-bound PRLRs have been identified, differing in the length and composition of their cytoplasmic tail. In rats, for example, the

following three major PRLRs isoforms were identified: short (291 amino acids), intermediate (393 amino acids), and long (591 amino acids). In mice, one long and three short isoforms of the PRLRs have been described (2). After PRLRs activation, different intracellular signaling pathways can be recruited to induce prolactin biological effects. The receptor activation results in a rapid phosphorylation of Janus kinase 2 (JAK2), which is constitutively associated with the intracellular domain of PRLRs. Activation of JAK2 leads to the phosphorylation of tyrosine residues. Phosphotyrosines serve as binding sites for transducer molecules, such as the signal transducer and activator of transcription (STAT) protein family. Three members of this family are recognized as transducer molecules of PRLRs, including STAT1, STAT3 and STAT5a/b. STAT5, earlier known as mammary gland factor, is recognized as the most important transducer of the PRLRs. STAT proteins become phosphorylated by the PRLR/JAK2 complex and form hetero- or homodimers through its phosphotyrosine residues with another phosphorylated STAT molecule. STAT dimers then translocate to the nucleus, where genomic effects on target genes can occur. The phosphotyrosine residues of the activated PRLR may also serve as a docking site for others adapter proteins which can lead to the activation of different signaling pathways, such as the mitogen-activated protein kinase (MAPK) cascade or the phosphatidylinositol 3-kinase (PI3K) cascade (1,2). In addition, it has been demonstrated that PRLRs activation is also involved in rapid acute effects that lead to changes in membrane excitability. For example, prolactin acutely induces rapid effects on the membrane excitability of neurons (14-18). Such effects occur because PRLRs activation can activate fast-acting signaling mechanisms, such as the PI3K pathway, tyrosine kinase-dependent K^+ channels or the production of intracellular messengers that open voltage-independent Ca^{2+} channels, which in turn allows for ionic changes across the cell membrane (1,15). One of the final mechanisms induced by PRLRs activation is protein synthesis that can in turn desensitize the receptor itself. The JAK/STAT complex can be inhibited by suppressors of cytokine signaling (SOCS) proteins, which inhibit JAK kinases and compete with STAT for docking sites on PRLRs. These proteins include SOCS1, SOCS3 and cytokine-inducible SH2-containing protein (CIS) (1).

PROLACTIN-MEDIATED REPRODUCTIVE FUNCTIONS IN PERIPHERAL ORGANS

Prolactin is best known for its role in mammopoiesis and lactogenesis. Mammary gland development includes the formation of a branched ductal system that is decorated with terminal and lateral lobules in wild-type virgin adult mice. Following pregnancy and in response to prolactin production, the alveolar development of the mammary gland is greatly amplified. Several studies have provided consistent evidence that such progression is directly modulated by the recruitment of STAT5 proteins upon PRLRs activation. In $PRL^{-/-}$ or $PRLR^{-/-}$ female mice, terminal end buds form during puberty and the ductal tree grows normally. However, in adult $PRL^{-/-}$ mice, the mammary gland ductal system grows into an extended branching network that is devoid of both terminal and lateral lobulations (19). The differentiation of ductal elements also occurs in the global STAT5a, STAT5b and double knockout STAT5a/b female mice; however, development of terminal buds occurs to a lesser extent as compared to wild-type mice (20,21). Deficiencies in mammary gland development were even observed in non-lactating heterozygous PRLRs knockout female mice, indicating that such development is dependent upon prolactin signaling (22). Of note, mammary gland development upon pregnancy could not be observed in $PRLR^{-/-}$ female mice, as well as in global double STAT5a/b knockout females due to their infertility. The total body of research has led to the determination that STAT5 is the principal transcription factor mediating mammopoiesis and lactogenesis. Global STAT5a knockout mice fail to lactate due to incomplete mammary gland development, even after maximal stimulation of prolactin secretion induced by suckling. Conversely, mammary gland development occurs in a relatively normal manner in STAT5b knockout mice (20,21). In addition, the specific deletion of the *Stat5* locus only from the mammary epithelium, using *Cre-loxp*-mediated recombination, determined that this protein is essential not only for pregnancy-mediated cell proliferation/differentiation but also for the survival of mammary epithelium and maintenance of differentiation (23).

The effects of prolactin on fertility have been well-characterized using knockout mouse models, indicating that reproduction is clearly dependent upon the signaling of this hormone, at least in rodents. Both short and long isoforms of PRLRs have been

described to be expressed in the granulosa, interstitial and luteal cells of the ovaries, and the endometrium, myometrium and decidua in the uterus (24-26). Estradiol is the main ovarian hormone that stimulates prolactin secretion. Estradiol acts at the pituitary level to modulate prolactin gene expression and at the hypothalamus to modulate the activity of neurons known to be prolactin responsive (1,9). Depending on the hormonal milieu, PRLRs expression in several tissues (*e.g.*, the ovaries, uterus and hypothalamus) may change along the estrous cycle or during pregnancy and lactation (24,25). In the ovaries, prolactin acts in concert with gonadotropins to stimulate progesterone production by luteal cells and to induce the increase in progesterone receptor expression in the uterus. Progesterone produced by the ovaries is essential for implantation of the fertilized ovum, maintenance of pregnancy and the inhibition of ovulation (2). Prolactin signaling disruption leads to reproductive deficits in mice. For example, $PRL^{-/-}$ or $PRLR^{-/-}$ female mice are infertile (19,22). Adult $PRL^{-/-}$ female mice have irregular estrous cycles, with multiple days of proestrus or estrus. These mutants fail to become pregnant, despite no obvious defects in ovarian histology (19). Similarly, $PRLR^{-/-}$ female mice are infertile, despite regularly mating every 3-4 days, in comparison to wild-type animals that mated every 12 days. The study of preimplantation development of embryos demonstrated that most of the fertilized eggs failed to develop correctly in $PRLR^{-/-}$ mothers, although the embryos that failed to develop were capable of normal development when transferred to wild-type mothers. In addition, it was found that the uterus of a $PRLR^{-/-}$ female was not able to accept the implantation of wild-type blastocysts, indicating that the lack of PRLRs made the uterus refractory to implantation (22). Despite the reproductive defects exhibited by mutant females, $PRL^{-/-}$ male mice are fully fertile, and most $PRLR^{-/-}$ males are fertile, which demonstrates that the infertility in knockout females is caused by the lack of prolactin's luteinizing effects (19,22). However, it is worth mentioning that prolactin has this particular function only in rats and mice, but not in all mammals. Double STAT5a/b knockout female mice, but not solely STAT5a or STAT5b knockouts, are infertile as well (20,21). STAT5a/b knockout mice ovaries exhibit either few or no corpora lutea, which was determined to be the main cause of their infertility (21).

THE HYPOTHALAMUS AS A TARGET OF PROLACTIN TO MODULATE SEVERAL BIOLOGICAL FUNCTIONS

Prolactin-responsive cells are densely distributed in the central nervous system, especially in the hypothalamus (9,27,28). Several biological functions are regulated by prolactin through its action on defined hypothalamic neuronal populations, including the regulation of prolactin secretion through negative feedback, the expression of maternal behaviors and the modulation of energy balance and the reproductive axis (Figure 1). The best known hypothalamic circuitry involving prolactin effects is composed of TIDA neurons that act as a synchronous network to release dopamine and control prolactin secretion (1,15-17). TIDA neurons are directly responsive to prolactin as demonstrated by the induction of STAT5 phosphorylation (pSTAT5) after an acute prolactin stimulus and a direct postsynaptic depolarization of cell membranes, which stimulates dopamine secretion (15-17). However, during lactation, dopamine secretion is suppressed to allow for physiological hyperprolactinemia. Because TIDA neurons remain electrically responsive to prolactin during lactation, the significant decrease in tyrosine hydroxylase phosphorylation is the best-known mechanism so far that is responsible for the suppression of dopamine secretion during this period (17). In addition, prolactin is also known as an important factor

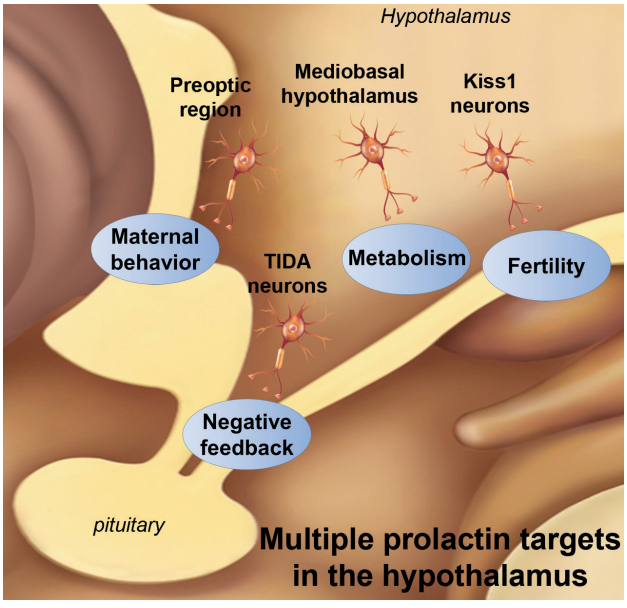

Figure 1. Scheme that summarizes different biological functions regulated by prolactin through its action on defined hypothalamic neuronal populations.

that mediates adaptive responses related to maternal behaviors. In this case, the effects of prolactin on maternal care depend on neurons distributed in the preoptic area (29). In rodents, maternal care can be analyzed by evaluating the latency of the animal to exhibit behaviors, such as retrieving pups to the nest, grouping them and crouching over them for a set amount of time. Previous studies performed bilateral infusions of prolactin into the preoptic region, which resulted in a pronounced stimulation of maternal behavior (30). Animals infused with prolactin directly into the medial preoptic area (MPO) retrieved the pups, crouched over them and displayed full maternal behavior significantly faster than respective controls (30). In contrast, overall full maternal response was impaired in animals given bilateral infusions of a PRLRs antagonist into the MPO (31). Interestingly, while $PRLR^{-/-}$ female mice showed a complete disruption of maternal behavior (22,32), brain-specific STAT5a/b knockout mice showed no postpartum maternal behavior deficits, demonstrating that STAT5 signaling is not required for the expression of maternal care. Consequently, other signaling pathways recruited by PRLRs activation may be necessary to modulate maternal behavior. In fact, we have previously demonstrated that fast STAT5-independent signaling pathways are acutely recruited by prolactin to modulate the membrane excitability of neurons located in the MPO (14).

Prolactin may also regulate food intake and other metabolic aspects. Hyperprolactinemia is frequently associated with metabolic imbalances, such as obesity and diabetes mellitus (33). Accordingly, $PRLR^{-/-}$ mice show a lean phenotype (34). Several studies also indicate that prolactin or placental lactogens contribute to the metabolic changes typically observed during pregnancy, such as the increase in food intake and adiposity. These studies showed that central prolactin infusions induce a leptin resistance state that changes the metabolism towards a positive energy balance (35,36). Indeed, several populations of leptin receptor-expressing neurons in mice are directly responsive to prolactin (37). Furthermore, inactivation of the *Socs3* gene in leptin receptor-expressing cells improves leptin sensitivity in pregnant mice and mitigates major gestational metabolic adaptions (38). Altogether, these findings suggest that changes in prolactin levels during pregnancy lead to leptin resistance, which, in turn, is responsible for orchestrating many metabolic adaptations commonly observed in pregnant animals. Thus, these studies indicate that prolactin may centrally modulate energy balance in specific situations.

INTERACTION BETWEEN PROLACTIN AND THE KISSPEPTIN SYSTEM

Hyperprolactinemia frequently causes disruption of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion, and may lead to hypogonadism and infertility in humans and animal models (12,39-41). The most common symptoms of hyperprolactinemia in women are galactorrhea, amenorrhea and infertility. In men, the most frequent symptoms are typically secondary to a sellar mass effect, such as headaches and visual field defects due to the pituitary enlargement (40,41). A small percentage of male patients present with symptoms such galactorrhea, loss of libido, erectile dysfunction, changes in sperm quality and infertility (12,40). To better understand how hyperprolactinemia affects reproduction, several studies investigated possible prolactin-target neurons that may modulate the reproductive axis. Although GnRH neurons were thought to be potential candidates to be directly regulated by prolactin, it has been demonstrated that only a very small percentage of GnRH neurons express the PRLRs or prolactin-induced pSTAT5. In addition, membrane excitability of GnRH neurons is not acutely modulated by prolactin (16,42), suggesting that other neuronal populations are probably responsible for the prolactin-mediated effects on gonadotropin secretion. Recently, studies provided new evidence that prolactin may modulate the reproductive axis by acting on a specific population of hypothalamic neurons that express the *Kiss1* gene (4-8,43). The *Kiss1* gene encodes neuropeptides, known as kisspeptins, that are critically involved in reproduction. Loss-of-function mutations in the genes encoding kisspeptins or the kisspeptin receptor (*KISS1R*, also known as *GPR54*) leads to the disruption of puberty and infertility in both humans and animal models (44-46). Conversely, a *KISSR*-activating mutation leads to precocious puberty in humans (47). *Kiss1*-expressing neurons exhibit a very defined distribution in the brain. These neurons are mainly located in the anteroventral periventricular nucleus (AVPV), the rostral periventricular nucleus (PeN) and the ARH of the hypothalamus in rodents (48). Of note, some research groups denominate AVPV and PeN neurons together as the rostral periventricular

area of the third ventricle (RP3V) in rodents (4,42). The confirmation that *Kiss1*-expressing neurons are directly modulated by prolactin levels was provided by the demonstration that most of *Kiss1*-expressing neurons co-express the PRLRs (6,7). Additionally, these receptors are functional because an acute prolactin stimulus can induce pSTAT5 in *Kiss1*-expressing neurons (detailed information can be found on Table 1) (4,5,8). Similar results were obtained by our group using a transgenic mouse model that allows the visualization of *Kiss1*-expressing neurons through a reporter protein. Approximately 80% of *Kiss1*-expressing neurons in the ARH express pSTAT5 after acute intraperitoneal (i.p.) prolactin administration in female mice in diestrus (Figure 2). The expression of PRLRs and the induction of pSTAT5 by prolactin together indicate that prolactin may regulate the activity of *Kiss1*-expressing neurons and kisspeptin secretion. More evidence that the interaction between prolactin and kisspeptin system causes significant impact to the hypothalamic-pituitary-gonad axis was provided by studies that evaluated the consequences of prolactin infusion on *Kiss1* mRNA expression in animal models (Table 1). Systemic or intracerebroventricular (icv) prolactin infusion suppresses hypothalamic *Kiss1* expression leading to a reduction in plasma LH levels (4,5,43).

Table 1. Summary of the studies that investigated the interaction between prolactin and kisspeptin

Reference	Species	Comments
Presence of prolactin receptors in *Kiss1*-expressing neurons		
Kokay and cols., 2011	Rat	86% of *Kiss1*-expressing neurons in the AVPV co-express PRLRs mRNA in OVX+E2 treated females 79% and 45% of *Kiss1*-expressing neurons in the ARH of OVX or OVX+E treated animals, respectively, co-express PRLRs mRNA
Li and cols., 2011	Sheep	60% of ARH kisspeptin immunoreactive neurons co-express PRLRs in OVX females
	Human	Not described
Evidence of responsiveness to prolactin		
Brown and cols., 2014	Mouse	65% of kisspeptin immunoreactive neurons in the AVPV and 35% of kisspeptin neurons in the PeN express prolactin-induced pSTAT5 in mice in diestrus
Araujo-Lopes and cols., 2014	Rat	70-80% of ARH kisspeptin immunoreactive neurons express pSTAT5 after prolactin icv infusion (0.5 μg/2 μL) in virgin OVX or lactating animals
Sjoeholm and cols., 2011	Rat	65-75% of ARH kisspeptin neurons of primiparous rats co-express pSTAT5 after icv prolactin infusion (2.5 or 100 ng/rat)
Li and cols., 2011	Sheep	No co-expression between *Kiss1* mRNA and prolactin-induced pSTAT5 (icv, 20 μg/hr/1 week) in the ARH of OVX+E2 females
	Human	Not described
Effects of prolactin on *Kiss1* expression		
Sonigo and cols., 2012	Mouse	Chronic infusion of prolactin (7 μg/24 hr/28 days) significantly decreases hypothalamic *Kiss1* mRNA and kisspeptin immunoreactivity in the AVPV and ARH
Brown and cols., 2014	Mouse	3 sc doses of prolactin (100 μg/200 μg) cause a suppression of *Kiss1* mRNA in the ARH of OVX mice
Araujo-Lopes and cols., 2014	Rat	Icv (4 μg/μL) or sc (0.5 mg/0.2 mL) prolactin injection causes a significant reduction of *Kiss1* mRNA in the ARH of OVX rats
Li and cols., 2011	Sheep	Icv prolactin infusion (20 μg/hr/1 week) does not significantly change *Kiss1* mRNA expression in the ARH of OVX+E2 females
	Human	Not described
Effects of kisspeptin on prolactin secretion		
Szawka and cols., 2010	Rat/cell culture	Kisspeptin-10 icv infusion (3 nmol) increases serum prolactin release in OVX+E2 and proestrus females, but had no effect in OVX females, diestrus females or males Kisspeptin-10 does not alter prolactin secretion in anterior pituitary cell culture
Hashizume and cols., 2010	Goat	Intravenous administration of kisspeptin-10 (5 mg/kg) does not alter basal serum prolactin levels
Kadokawa and cols., 2008	Bovine/ cell culture	Kisspeptin-10 (1 μM or 10 μM) increases media prolactin concentration of anterior pituitary cells, extracted from 8-month-old castrated male calves
Ramaswamy and cols., 2009	Monkey	Intravenous infusion of kisspeptin-10 (10 or 30 μg) induces no change in prolactin serum concentration in male *Macaca mulatta*
Jayasena and cols., 2014	Human	Acute or twice-daily for 1 week sc administration of kisspeptin-54 (6.4 nmol/kg) induced no effect on serum prolactin levels in healthy women

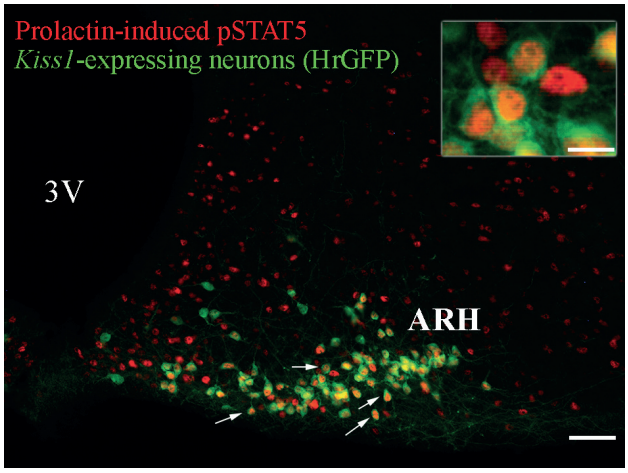

Figure 2. Prolactin-responsive *Kiss1*-expressing neurons in the mouse arcuate nucleus. Fluorescent photomicrographs of hypothalamic sections showing the expression of humanized Renilla green fluorescent protein (hrGFP) which is expressed under the transcriptional control of the *Kiss1* gene and prolactin-induced pSTAT5 immunoreactivity of a female mouse in diestrus. Details about this mouse model can be found in previous publications (Cravo and cols., 2013). Mice received a single i.p. injection of ovine prolactin (10 µg/g) before perfusion. Arrows illustrate examples of prolactin-responsive *Kiss1*-expressing neurons. The *inset* represents a higher magnification of dual-labeled neurons in the ARH. Abbreviations: 3V, third ventricle. Scale bar: photomicrograph = 100 µm; inset = 23 µm.

Interestingly, not only may prolactin affect the activity of *Kiss1*-expressing neurons, but kisspeptins possibly control prolactin secretion as well. Administration of kisspeptin-10 to culture media prepared with anterior pituitary cells extracted from 8-month-old castrated male calves increased prolactin secretion (49). However, several other studies were unable to demonstrate such effect (Table 1) (50-53). In humans, for example, acute or a week of twice-daily subcutaneous (sc) administrations of kisspeptin-54, at a dose known to stimulate gonadotropin secretion, induced no effect on serum prolactin levels in healthy women (53). In fact, the ability of kisspeptin to induce prolactin secretion seems to be directly related to circulating estrogen levels, as demonstrated in rodents. Icv infusion of kisspeptin-10 increases serum prolactin levels only in ovariectomized E2-primed (OVX+E2) or proestrus rats, but had no effect in ovariectomized (OVX) females, diestrus females or male rats (52). Additionally, Ribeiro and cols. (54) demonstrated that kisspeptins increase prolactin secretion through inhibition of TIDA neurons in an estrogen-dependent manner, not only in female rats but also in males. The demonstration, at least in rodents, that kisspeptin administration may induce prolactin secretion through TIDA neurons inhibition suggests that *Kiss1*-expressing neurons may also be part of the feedback circuitry responsible for modulating prolactin secretion.

MAY KISSPEPTIN BE USED AS A NOVEL THERAPEUTIC APPROACH TO TREAT PROLACTIN-INDUCED INFERTILITY?

Because kisspeptins are known as the most important activators of GnRH neurons (55) and they are able to cause a powerful stimulation in LH and FSH secretion in both humans and animal models (48,56-58), kisspeptin administration may have potential therapeutic effects to treat infertility. The first evidence of this effect was provided by a clinical trial in which women with functional hypothalamic amenorrhea were subjected to a protocol of twice-daily sc kisspeptin-54 administration for 2 weeks. After the first kisspeptin-54 injection, women with functional hypothalamic amenorrhea showed a rapid and marked increase in plasma gonadotropins and estradiol levels, in comparison to the vehicle group. However, 2 weeks of kisspeptin-54 treatment led to receptor desensitization and no further significant effect on gonadotropins secretion was observed (58). In fact, the treatment of women with hypothalamic amenorrhea was more effective when kisspeptin-54 was administered sc twice-weekly over a prolonged period. This protocol elevated the levels of reproductive hormones even after 8 weeks of treatment (59). Additionally, the effects of kisspeptins administration on egg maturation in women undergoing in vitro fertilization therapy have also been tested. A single sc injection of kisspeptin-54 was able to trigger egg maturation sufficiently to result in fertilization, embryo implantation, and successful live birth in women with subfertility. Of note, the efficacy rate of kisspeptin treatment was similar to that obtained by conventional therapy (60).

Kisspeptin administration can also restore gonadotropin secretion and ovarian cyclicity in a prolactin-induced infertility model in mice (43). Hyperprolactinemia was induced by a chronic sc infusion of prolactin. While control animals displayed regular estrous cycles, hyperprolactinemic female mice showed disruption of their cycles. Remarkably, daily i.p. injections of kisspeptin-10 recovered estrous cyclicity and ovulation rate even in hyperprolactinemic mice (43). Additionally, Sonigo and cols., (43) tested whether the suppression of GnRH release induced by

hyperprolactinemia could be reversed by kisspeptin treatment. To demonstrate the effects of kisspeptins on GnRH secretion, medial basal hypothalamus explants obtained from female mice were treated in a culture medium. As expected, GnRH release into the medium was significantly inhibited after exposure to prolactin. Notably, co-treatment with kisspeptin-10 was able to restore GnRH secretion, demonstrating that prolactin inhibitory action on gonadotropin secretion is mediated by changes in kisspeptin secretion (43). Therefore, the reduction of *Kiss1* gene expression is now believed to be the primary cause of the suppression of gonadotropin secretion during hyperprolactinemia. Nevertheless, whether kisspeptin may be used as a novel therapeutic approach to treat prolactin-induced infertility in humans still requires further investigation.

In conclusion, recent evidence indicates that *Kiss1*-expressing neurons are important mediators of prolactin's effects on reproduction. Prolactin acts directly on *Kiss1*-expressing neurons and induces suppression of *Kiss1* mRNA expression and kisspeptin secretion, leading to a lower activation of GnRH and gonadotropins secretion. Therefore, hyperprolactinemia-induced infertility can possibly be treated with kisspeptin replacement. Furthermore, kisspeptins seem to contribute to the control of prolactin secretion, which highlights a putative bidirectional interaction between prolactin and the kisspeptin system.

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A boy with Prader-Willi syndrome: unmasking precocious puberty during growth hormone replacement therapy

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SUMMARY

Prader-Willi syndrome (PWS) is a genetic disorder frequently characterized by obesity, growth hormone deficiency, genital abnormalities, and hypogonadotropic hypogonadism. Incomplete or delayed pubertal development as well as premature adrenarche are usually found in PWS, whereas central precocious puberty (CPP) is very rare. This study aimed to report the clinical and biochemical follow-up of a PWS boy with CPP and to discuss the management of pubertal growth. By the age of 6, he had obesity, short stature, and many clinical criteria of PWS diagnosis, which was confirmed by DNA methylation test. Therapy with recombinant human growth hormone (rhGH) replacement (0.15 IU/kg/day) was started. Later, he presented psychomotor agitation, aggressive behavior, and increased testicular volume. Laboratory analyses were consistent with the diagnosis of CPP (gonadorelin-stimulated LH peak 15.8 IU/L, testosterone 54.7 ng/dL). The patient was then treated with gonadotropin-releasing hormone analog (GnRHa). Hypothalamic dysfunctions have been implicated in hormonal disturbances related to pubertal development, but no morphologic abnormalities were detected in the present case. Additional methylation analysis (MS-MLPA) of the chromosome 15q11 locus confirmed PWS diagnosis. We presented the fifth case of CPP in a genetically-confirmed PWS male. Combined therapy with GnRHa and rhGH may be beneficial in this rare condition of precocious pubertal development in PWS. Arch Endocrinol Metab. 2016;60(6):596-600

INTRODUCTION

Prader-Willi syndrome (PWS), also known as Prader-Labhart-Willi syndrome, is a complex neurogenetic disorder characterized by neonatal hypotonia, psychomotor delay, early-onset hyperphagia, short stature, hypogonadism, sleep disturbance, learning disabilities, and behavioral and psychiatric disorders; it also represents the most common form of genetic obesity. Its incidence is estimated between 1:10,000 and 1:30,000 living births with the prevalence of 1:50,000. The expression of paternally active genes located on chromosome 15q11-q13 is lost in PWS, which may occur due to a deletion in this chromosomal segment (65-70%), maternal uniparental dissomy (25-30%), or imprinting defects (1-2%), even though rare gene mutation (< 0.1%) and balanced translocation (0.1%) can also be found (1,2). Diagnosis of PWS is made according to the Holm and Cassidy criteria (3), but it must be confirmed by genetic analysis.

Patients with PWS have multiple characteristics associated with hypothalamic dysfunctions, such as hyperphagia, growth hormone (GH) deficiency, and abnormal pubertal development (1). Usually these patients have hypogonadism manifested as genital hypoplasia and unilateral or bilateral cryptorchidism, incomplete pubertal development, and infertility, which are attributed to both hypothalamic dysfunction and/or to primary gonadal defect (4). In fact, hypogonadism is one of the eight major clinical diagnostic signs of PWS, whereas isolated premature adrenarche is frequently observed and is accepted as a minor criterion (3).

Despite the common clinical picture of delayed or incomplete puberty, rare cases with true precocious puberty have been described in this syndrome (5). Here, we report a male patient with PWS caused by a classical hypermethylation of the *locus* 15q11 who experienced growth hormone deficiency and central precocious puberty (CPP). To the best of our knowledge, this is the first case of CPP reported in boys with PWS during GH replacement. Informed consent was obtained from the patient's legal guardian for publishing this case report.

CASE REPORT

The patient was born at term by operative delivery at 39 gestational weeks (2.46 kg, 47 cm). His parents were healthy, non-consanguineous, and from Caucasian descent. Hypotonia and bilateral cryptorchidism were present at birth as well as poor sucking and low weight gain during the first month of life. Failure to thrive was diagnosed in the next months; no brain abnormalities were detected on the computed tomography scan. At the age of 2, he began to develop hyperphagia, followed by rapid weight gain and signs of mild psychomotor deficiencies; the clinical diagnosis of PWS was then first suspected. At the age of 3, he was submitted to surgical orchiopexy. At the age of 6, he was referred to our pediatric endocrinology unit for evaluation of obesity and short stature. His height was below the 3rd percentile, weight between the 25-50th percentile range, body mass index between the 90-95th percentile range with normal bone density and 37.9% total body fat. At presentation, he had no signs of sexual development (Tanner stage I): no pubic hair, nonpalpable left testis (1.05 cm^3), normal right testis (1.53 cm^3) (both measured by ultrasonography), and stretched penile length of 1.5 cm. Otherwise, his examination was normal, except for low ears implantation, relatively small hands and feet, valgus knee, and mild learning disabilities. The Holm and Cassidy criteria score was 9; the genetic test demonstrated only the methylated maternal allele, thus confirming the clinical diagnosis of PWS by the methylation-specific PCR (MS-PCR) performed ten years ago. Figure 1 presents anthropometric measurements plotted on current standardized growth curves for PWS subjects (6).

Initial laboratory analyses resulted in prepubertal hormone levels (Table 1). The peak growth hormone response to clonidine testing was 1.75 μg/L (normal > 7 μg/L), suggesting the diagnosis of GH deficiency. Considering that the patient presented an inadequate growth rate but had no nutritional deficiency or hypothyroidism, therapy with recombinant human growth hormone (rhGH) replacement (0.15 IU/kg/day) was started at the age of 7 (Figure 1). In the beginning of the rhGH treatment, his insulin-like growth factor 1 (IGF-1) level recovered to the middle-to-upper normal range (Table 1), and his growth velocity was 6 cm/10 months.

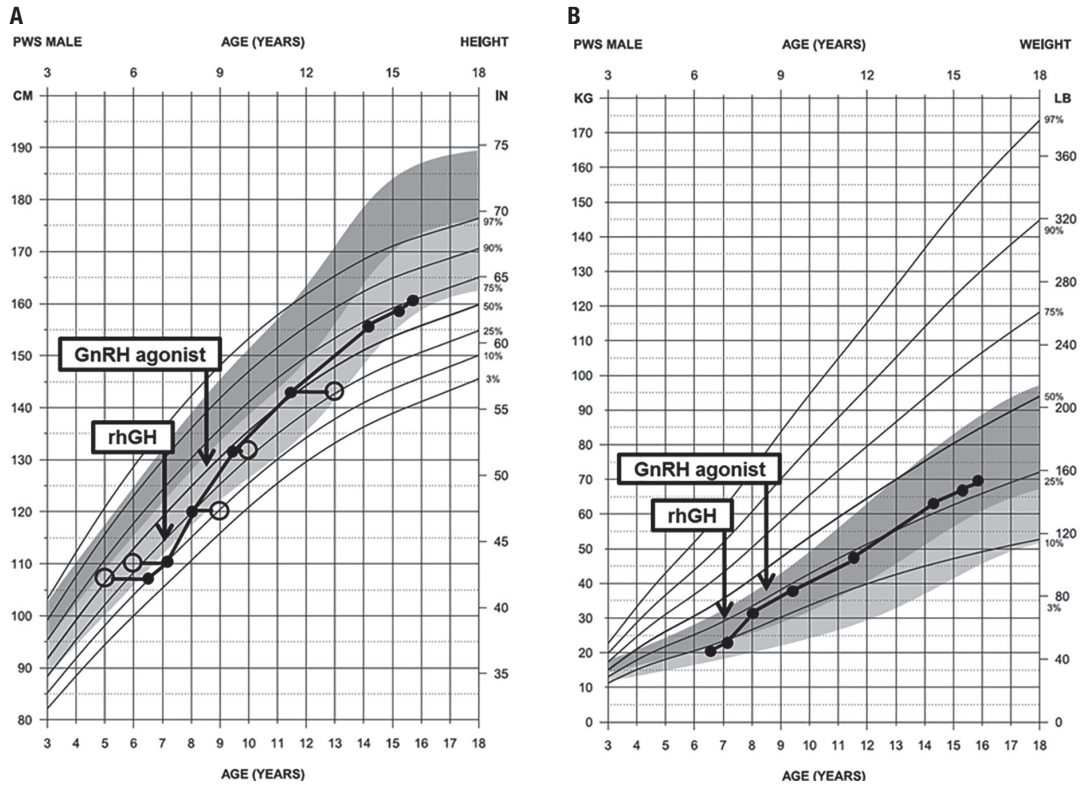

Figure 1. Growth and obesity status of the reported patient: a boy with Prader-Willi syndrome. Stature (**A**) and weight (**B**) are plotted against age as solid circles in syndrome-specific growth standards. Bone ages are represented as open circles (**A**). Arrows indicate the beginning of the growth hormone replacement (rhGH, somatropin) and the depot leuprorelin acetate therapy (GnRHa) for central precocious puberty. The growth curves were constructed using data from the PWS-specific growth chart (6).

Table 1. Nine-year follow-up of anthropometric evaluation, clinical examination, and laboratory findings of a Prader-Willi syndrome boy with central precocious puberty

Anthropometrical data										
Age (years)	6.60	7.10	8.00	8.50	8.80	9.40	10.80	11.50	14.30	15.20
Height (cm) [z-score][a]	107 [-2.32]	110 [-2.37]	120.50 [-1.20]	123 [-1.19]	126 [-0.82]	132 [-0.45]	138 [-0.63]	143 [-0.43]	156 [-1.20]	159 [-1.38]
Weight (kg)	20.9	23.2	30.9	32.9	33.4	38.3	45	48.8	62.3	66
BMI (kg/m^2) [centile][a]	18.25 [95]	19.17 [97]	21.30 [99]	21.6 [99]	21.04 [98]	21.90 [98]	23.60 [99]	23.80 [98]	25.50 [97]	26.10 [96]
Growth therapy follow-up										
IGF-1[b] (µg/L)	75.40		339	518		346		466	218	
[age-specific reference ranges]	[52-297]		[57-316]	[64-345]		[74-388]		[111-551]	[220-972]	
rhGH (U/kg/day)	Somatropin (0.15)						Somatropin (0.1)			
Puberty blocking follow-up										
Bone age[c] (years)	5	6	9			10		13	14	
Genitalia stage (Tanner)	I	I	II	II	II	II	II-III	III	III	IV
Right testis volume[d] (mL)		3	4	4				3	3	6
Left testis volume[d] (mL)	np	np	2 (r)	2.5				np	np	5
Pubic hair (Tanner)	I	I	II	II-III		III-IV	III-IV	III	III	IV
LH (IU/L)	< 0.50		0.50	0.47		1.66	0.94		< 0.07	
FSH (IU/L)	2.88		5.60[e]	8.80[e]		8.70	5.85		0.15	
Testosterone (ng/dL)	< 0.1		13[e]	55[e]		19	< 20		14.45	
GnRHa (mg/month)	Leuprorelin (3.75)									

[a] STAT GrowthCharts™ (WHO, Geneva, 2006). [b] IGF-I measured by an automated chemiluminescence immunoassay (Nichols Institute Diagnostics, San Clemente, CA, USA). [c] According to the Greulich and Pyle method. [d] Measured using Prader orchidometer. [e] Hormone levels above the normal range.
BMI: body mass index; FSH: follicle-stimulating hormone; GnRHa: gonadotropin-releasing hormone analog; IGF-1: insulin-like growth factor 1; LH: luteinizing hormone; np: nonpalpable; r: retractile; rhGH: recombinant human growth hormone.

At the age of 7.6, his parents reported he was presenting psychomotor agitation, aggressive behavior, and also frequent penile erections. Physical examination revealed signs of puberty, including asymmetric enlargement of testes (1.5 and 4 cm^3, left and right respectively) and penis length (4.5 cm). Biochemical and imaging evaluation for peripheral precocious puberty revealed no evidence of congenital adrenal hyperplasia, human chorionic gonadotropin (hCG), or androgen-secreting tumors. At the age of 8 (Table 1), he presented Tanner stage II, bone age of a 9-year old, and laboratory analyses confirmed the diagnosis of CPP: precocious pubertal level of total testosterone (54.7 ng/dL) and luteinizing hormone (LH) peak 15.8 IU/L after gonadotropin-releasing hormone (GnRH) stimulation test; magnetic resonance imaging (MRI) of hypothalamic-pituitary region was normal.

Treatment for precocious puberty was started at the age of 8.8 with GnRH analog (GnRHa) (depot leuprorelin acetate 3.75 mg i.m. every 28 days), and the rhGH dose was adjusted to 0.1 IU/kg/day. Patient's response to treatment was satisfactory, as evaluated under pubertal blockade by GnRHa (Table 1) at the age of 9.4: total testosterone was 18.7 ng/dL (normal for age 9.8 – 19.6 ng/dL), LH two hours after leuprorelin 3.75 mg i.m. was 1.66 IU/mL, and IGF-1 was 346 µg/L (normal for age 74-388 µg/L). He was treated with leuprorelin 11.25 mg i.m. every three months until the age of 13. Bone age deviation and pubertal Tanner stage III had no progression during GnRHa therapy, with spontaneous resolution of testicular size discrepancy, but a second left orchiopexy was needed. At the last endocrine visit, Tanner stage was V, growth velocity was adequate for age, and his treatment was rhGH 0.08 IU/kg/day and topiramate 50 mg twice a day. We have recently performed a more precise molecular diagnosis test, the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) of the locus 15q11 performed by SALSA MS-MLPA (Kit ME028, MRC Holland, Amsterdam, Netherlands). MS-MLPA revealed a hypermethylation pattern of the SNPRN probes, suggesting that the two

chromosome 15s came from the mother and none from the father (maternal uniparental disomy) or that a maternal-only DNA-methylation pattern occurred despite the biparental inheritance (imprinting defect). No abnormality in copy number was detected in this region, excluding a deletion.

DISCUSSION

Here, we described the case of a boy presenting common features of PWS including obesity, short stature, hypotonia, and hypogonadism (characterized by cryptorchidism and small penis); after the onset of rhGH therapy, he surprisingly presented early signs of pubertal development. According to a large database of PWS children, growth hormone deficiency was present in 80% of patients, and 86.7% were treated; hypogonadism was present in 49% of patients (7). Gonadal axis function, however, is not homogeneous; normal gonadotropin response to GnRH test is quite common. but hypergonadotropic hypogonadism has also been described (4). Moreover, many children present premature adrenarche that is manifested with slightly advanced bone maturation related to obesity; usually, puberty fails to progress beyond this stage. Nevertheless, CPP has been rarely reported in PWS (5). Our patient fulfilled all diagnostic criteria for CPP (i.e., age, advanced skeletal age, testicular volume, pubertal LH response to GnRH test, and androgen levels). According to our literature review, this is the fifth case of CPP in a genetically-confirmed PWS male (5,8-10). GnRH blockade has been reported in two cases (5,9). Abnormal imaging of the hypothalamic-pituitary region was observed in two cases: empty sella (8) and pituitary microadenoma (9).

The locus 15q11 contains a domain of imprinted genes in which loss of expression of the paternally-inherited allele of genes including *SNRPN*, *NDN*, *MAGEL2*, and *MKRN3* is associated with PWS. Mapping of microdeletions associated with PWS has identified a 4.3 kb region of the *SNPRN* gene that appears to be required for establishment and/or maintenance of the paternal epigenotype across the imprinting center domain (11). Therefore, it has been speculated that deleterious defects such as paternal deletion, maternal uniparental disomy, and methylation abnormalities of the SNPRN probes in the MS-MLPA could lead to the loss of paternal PWS-imprinting center and presumably lose expression of the distal genes, including the makorin ring finger protein 3 (*MKRN3*) (12). In this study, we demonstrated a classical hypermethylation pattern of 4 SNPRN probes, indicating a PWS diagnosis (1). Interestingly, several loss-of-functions mutations of *MKRN3* gene, an imprinted gene located on chromosome 15q11, Prader-Willi critical region, were described in non-syndromic patients with familial CPP (13,14). However, the MKRN3 role in the PWS associated with CPP remains unknown. Here, the genetic diagnosis of PWS was first detected using a DNA methylation analysis and then confirmed by a MS-MLPA. The DNA methylation is usually the first line test in the PWS diagnosis and can correctly diagnose PWS in up to 99% of the cases; however, this technique cannot distinguish between deletions, uniparental disomy, or imprinting defects (1). The advantage of MS-MLPA over traditional DNA methylation is that MS-MLPA investigates five distinct differentially methylated sites (4 probes for SNPRN and one probe for NDN methylated sites) rather than just one locus and can assess the deletion status at the same time as the DNA methylation

The natural course of PWS is a significant increase in fat mass over the years that can be worsened by GH deficiency (2). Beyond improving longitudinal growth, the rhGH replacement in our patient also aimed to improve body composition, bone density, physical capacity, basal energy consumption, cognition, and life quality. As we could observe, there was a significant increase in longitudinal growth rate (10 cm/year) in the first year of treatment with rhGH, which is uncommon in patients with PWS, once they usually have low IGF-1 levels compared to exogenous obesity. GH secretion and puberty may be correlated since GH exerts direct effects on gonadal function and may influence reproductive activity by increasing secretion and sensitivity of gonadotropin releasing hormone (15). However, during the rhGH therapy in PWS children, the simultaneous activation of the pituitary-gonadal axis is certainly very unusual (7). Another case of male PWS previously reported with CPP was treated by replacing rhGH, but his pubertal development had preceded rhGH therapy (5).

Despite the fact that precocious puberty has been recognized in few PWS cases, there is controversy whether treatment with GnRHa should be used. Patients with PWS commonly fail to complete puberty, and, as a consequence, there was no final stature loss, in spite of slight bone age advance (4). In the present case,

the decision to treat with GnRHa was made because of the development of aggressive behavior but also the bone age advance. The GnRHa was started at the age of 9 and showed good clinical and laboratory response. After 6 months of treatment, adequate puberty blocking was evidenced by normal bone age progression and stabilization of secondary sexual characteristics.

The rare manifestations of CPP in patients with PWS have been attributed to brain lesions (5,10). Moreover, morphological abnormalities were also associated with GH deficiency in PWS patients, and hypothalamic dysfunction was frequently associated with pituitary hypoplasia (10). Conversely to these observations, our patient's imaging of the hypothalamic-pituitary region was normal. In some conditions, CPP could be associated with rhGH exposure during childhood (15,16). We report the first case of CPP during rhGH replacement in a male patient with PWS without hypothalamic imaging abnormalities. Although it was suggested that one of the causes of precocious puberty or accelerated progression of puberty might be due to GH therapy associated with hypothalamic dysfunction, no clear causal association between the rhGH replacement and the CPP development has been made in this case. In conclusion, we would like to emphasize that CPP may be diagnosed in PWS patients, and the combined therapy (rhGH replacement and GnRHa) may be beneficial, since extreme short stature was avoided in this case, while appropriated pubertal progression was restored. Although the benefits of rhGH therapy in PWS is well established (2), there is no consensus in the management of such a gonadotrophic disorder because few cases of precocious puberty in patients with PWS have been reported in the literature.

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Coexistence of resistance to thyroid hormone and ectopic thyroid: ten-year follow-up

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SUMMARY

Resistance to thyroid hormone (RTH) coexisting with ectopic thyroid is rare. Here we report a case of RTH with ectopic thyroid. A ten-year-old girl had been misdiagnosed as congenital hypothyroidism and treated with levothyroxine since she was born. Ten-year follow-up showed that the elevated thyrotropin was never suppressed by levothyroxine and no signs indicating hyperthyroidism or hypothyroidism despite elevated FT3 and FT4 levels. Therefore the girl developed no defects in physical and cognitive development. Pituitary adenoma was excluded by magnetic resonance imaging. Ultrasonography did not find the thyroid gland in the normal place, while the thyroid scan found a large lingual thyroid gland. The octreotide inhibition test showed a reduction in thyrotropin by 41.98%. No mutation was detected in the thyroid hormone receptor (*THR*) β, *THR*α, thyrotropin receptor (*TSHR*), and *GNAS1* genes. To our knowledge, it is an interesting RTH case coexisting with lingual thyroid. Arch Endocrinol Metab. 2016;60(6):601-4

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INTRODUCTION

Resistance of thyroid hormone (RTH) is a rare genetic disease characterized by reduced tissue sensitivity to thyroid hormone. The hallmark of RTH is elevated circulating thyroid hormones with unsuppressed thyrotropin (TSH) (1). Most of RTH cases are caused by a mutation in the thyroid hormone receptor (*THR*) β gene (2). Recently, several reports described the RTH patients due to heterozygous truncating mutations in *THR*α (3). The clinical presentation of RTH is highly variable including hyperthyroidism, hypothyroidism and asymptomatic. Detection of RTH by a positive neonatal TSH screening test has been described in rare cases (4).

Ectopic thyroid is a rare embryological aberration. Together with thyroid agenesis and hypoplasia, thyroid ectopy is classified as thyroid dysgenesis. Whereas the mutations in the *NKX2-1, PAX8, FOXE1, NKX2-5, TSHR* genes have been reported in a minority of patients with thyroid dysgenesis (5). Lingual thyroid accounts for approximately 90% of ectopic thyroid tissue. However, the incidence is close to approximately 1:100,000. The majority of patients with lingual thyroid are asymptomatic (6,7). Subclinical and overt hypothyroidism can be observed in some patients, while hyperthyroidism is uncommon (8-10). The inactivating mutations in the guanine nucleotide binding subunit 1 gene (*GNAS1*) that encodes G protein α-subunit and causes mild TSH resistance in pseudohypoparathyroidism (PHP) type Ia is significantly rare (11).

The coexistence of RTH and ectopic thyroid is extremely rare and is difficult to distinguish, three cases have been reported (12-14). Here we describe an unusual patient with RTH and ectopic thyroid. We decided to screen the mutations in the *THR*α, *THR*β, *TSHR*, and *GNAS1* genes in the study. The patient in the present study was followed up for ten years.

CASE REPORT

The girl was born at 39 weeks in June, 2005. Newborn screening after birth revealed that the serum TSH level was 36.9 μIU/mL, while the FT4 was 9.01 pmol/mL

(normal range: TSH, 0.5-5.0 μIU/mL; FT4, 8.56-25.6 pmol/mL). She had no signs of hypothyroidism, such as low growth rate, abdominal distention, mottled skin, open posterior fontanelle, prolonged jaundice, and lethargy. Thyroid ultrasound was not performed at that time, and the diagnosis of congenital hypothyroidism (CH) was retained. Levothyroxine (L-T4) replacement was given according to the misdiagnosis. However, the treatment of L-T4 had no effect on TSH levels, and FT4 were always elevated (Figure 1).

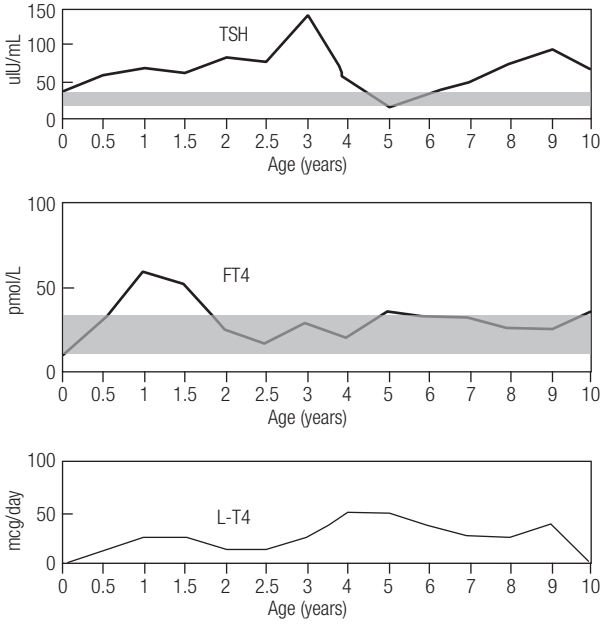

Figure 1. Clinical course of the levels of TSH and FT4 and doses of L-T4 during the 10 year follow-up.

In August 2014, the patient was referred to our endocrine center due to abnormal thyroid function for 10 years. Physical examination showed that the patient had no symptoms of hypothyroidism or hyperthyroidism. Her weight was 25 kg, height was 132 cm, and heart rate was 81 beats/minute (Figure 2). She was slightly thin, but did not exhibit muscle wasting or tremor. We stopped the administration of L-T4 for 2 months and then confirmed the thyroid function of the patient and her family members (Table 1). The patient still showed elevated TSH levels despite high levels of FT3 and FT4, which suggests RTH. Ultrasonography did not find the thyroid gland in the normal place, while the thyroid scan found the enlarged lingual thyroid (Figure 3). Pituitary magnetic resonance imaging (MRI) showed no pituitary tumor in the sella turcica. Her bone age was normal and IQ was 90. Her parents' thyroid function was normal without goiter. The octreotide inhibition test was negative, a decrease of up to 41.98% in TSH level was observed (Table 2). Interestingly, the level of platelet was 318-448 × 10^9/L (normal range: 100-300 × 10^9/L) during the 10 year follow-up.

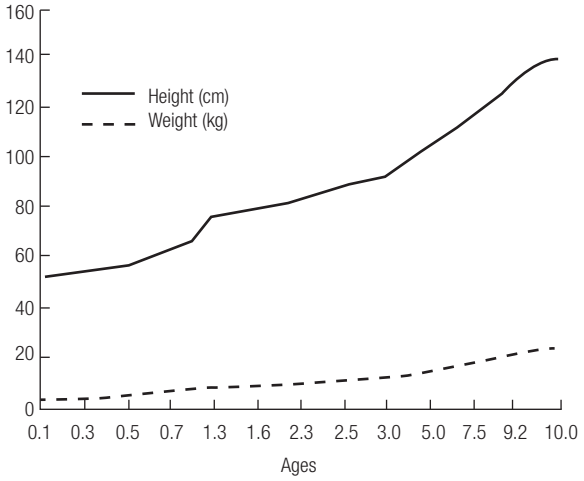

Figure 2. The growth rate (height and weight) of the patient during ten-year follow-up.

Table 1. Detection of thyroid function of family members

Variables	Normal range	Daughter	Mother	Father
Age (years)	/	10	39	43
TSH (μIU/mL)	0.34-5.44	67.23	2.88	4.86
FT4 (pmol/L)	7.91-20.59	35.42	13.08	13.42
FT3 (pmol/L)	2.92-5.93	14.05	4.15	3.57
TT3 (nmol/mL)	1.30-3.10	5.89	1.89	1.52
TT4 (nmol/mL)	66.00-181.00	257.10	124.56	78.64
Tg (ng/ml)	1.15-130.77	329.60	66.74	37.87
TGAb (IU/mL)	0-34.00	< 10.00	-	-
TPOAb (IU/mL)	0-12.00	5.96	-	-
Thyroid volume	/	Enlarged	Normal	Normal

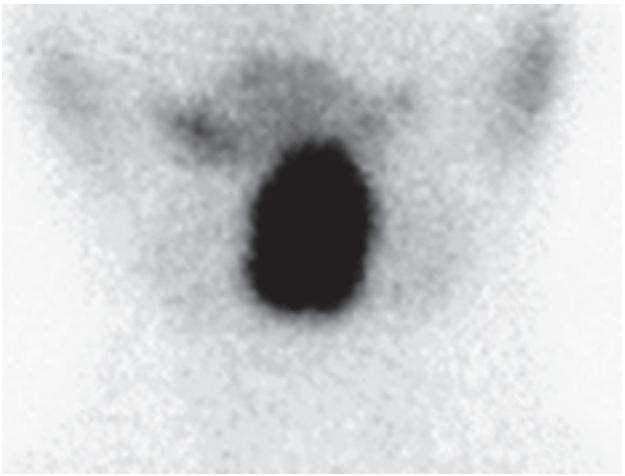

Figure 3 ^{99m}Tc-pertechnetate thyroid scan showing a single enlarged lobe with intense uptake in the neck.

Table 2. Sandostatin inhibition test in the patient

Time	TSH (μIU/ml)	TT3 (nmol/l)	TT4 (nmol/l)	FT3 (pmol/l)	FT4 (pmol/l)	TSH/ basic value (%)
0 h	59.050			12.22	34.14	100.00
2 h	46.330	3.26	188.90	10.86	30.47	78.46
4 h	45.130	3.30	186.20	11.11	31.87	76.42
6 h	28.320	3.29	190.00	12.46	33.00	47.96
8 h	27.700	3.12	180.81	11.73	32.35	46.91
24 h	24.790	3.40	191.04	12.76	34.01	41.98

Genomic DNA was extracted from peripheral blood leukocytes. Primers were designed to target the flanking intron regions of the exons. All exons of the *THR*β (MIM# 190160, GenBank NM_001128177.1), *THR*α (MIM# 199334.3, GenBank NM_190120), *TSHR* (MIM# 603372, GenBank NM_000369.2) and *GNAS1* (MIM# 139320, GenBank NM_080425.3) genes were amplified using PCR. Sequence analysis indicated no mutation in the four genes.

DISCUSSION

Here we describe a ten-year-old patient with RTH and lingual ectopic thyroid gland. Our patient had no mutation in the *THR*β, *THR*α, *TSHR* and *GNAS1* genes. The patient was misdiagnosed as CH due to the elevated level of TSH. Ten-year follow-up showed the serum levels of TSH, FT3, and FT4 were always high but without complications.

RTH is a rare syndrome characterized by decreased tissue responsiveness to thyroid hormone. RTH is mostly caused by mutations in the *THR*β gene, which encode for the thyroid hormone receptor beta unit (2). However, we did not find any mutations in the *THR*β, *THR*α, *TSHR* and *GNAS1* genes. According to previous reports, nearly 10% of the patients with RTH had no mutations in the coding region of *THR*β and 5% of the patients did not have mutations in both the *THR*β and *THR*α genes (3,15). There are multiple factors, including cofactors, transporters, deiodinases, and binding proteins, that may affect the actions of thyroid hormone (16). It is likely that defects in the factors that may affect the actions of thyroid hormone account for this patient's phenotype.

The different degree of hypothyroidism can be observed in some patients with ectopic thyroid. The patient had the evaluated TSH but normal FT4 at birth. In our opinion, normal FT4 at birth could be related to ectopic thyroid. A similar situation can be observed in the previous literature (14).

Thyrotropin releasing hormone (TRH) stimulating and octreotide inhibition tests could be used in the diagnosis of RTH (17). However, TRH is no longer commercially available in China. The TSH value in our patient remained 41.98% after 24 hours of injection of somatostatin. In general, suppression of serum TSH in patients with TSH-secreting adenomas was significantly higher than patients with RTH. The elevated level of platelet was not reported previously, and the clinical significance needs more research.

LT-4 supplementation was necessary to the RTH patients with hypothyroidism (18-20). While other patients with RTH rarely require treatment, treatment is clearly necessary in the patient due to the high concentrations of TSH, which may cause further expansion of the lingual thyroid tissue (21). Bromocriptine (Brc), a dopamine agonist, has also been reported to suppress inappropriate TSH secretion in RTH, and it can be used alone or in combination with 3,5,3'-triiodothyroacetic acid (TRIAC) (22). However, the patient's parents rejected L-T4 treatment for their child.

In conclusion, we describe the patient who met strict clinical and biochemical criteria for RTH but had no mutations in the considered genes. This patient was even more unusual because she had a lingual thyroid without hyperthyroidism or hypothyroidism symptoms.

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Trial registration: ChiCTR-CCS-14004734.

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1x ao dia em combinação fixa[10]

*Quando comparada a metformina IR isolada ou associada a glipizida ou dapagliflozina isolada ou metformina XR isolada.

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XigDuo XR® (dapagliflozina + cloridrato de metformina) comprimidos revestidos de liberação prolongada. **Indicações:** XigDuo XR® é indicado como adjuvante à dieta e exercícios para melhorar o controle glicêmico em adultos com diabetes melittus tipo 2 quando o tratamento com ambos, dapagliflozina e metformina, é apropriado. XigDuo XR® não é indicado para uso em pacientes com diabetes tipo 1. XigDuo XR® não deve ser usado para o tratamento da cetoacidose diabética. **Contraindicações: doença ou disfunção renal moderada a grave** (p.ex.: níveis de creatinina sérica ≥1,5 mg/dL [homens], ≥1,4 mg/dL [mulheres] ou TFGe <60 mL/min/1,73 m2 ou ClCr <60 mL/min pelo Cockcroft-Gault), inclusive secundária a condições como choque, IAM e septicemia; acidose metabólica aguda ou crônica, incluindo cetoacidose diabética, com ou sem coma, que deve ser tratada com insulina; história de reação de hipersensibilidade grave à substância ativa ou a qualquer um dos excipientes; disfunção hepática. **Cuidados e Advertências**: acidose láctica (metformina plasmática > 5 µg/mL - maior risco em idosos, disfunção renal, doença hepática, insuficiência cardíaca congestiva, hipoxemia, desidratação, sepse, ingestão excessiva de álcool e uso de contraste intravascular), disfunção renal, disfunção hepática, ingestão excessiva de álcool, cetoacidose (maior risco em disfunções pancreáticas como DM1, pancreatite, cirurgia pancreática, redução da dose de insulina, redução da ingestão calórica, infecções, cirurgias, doenças concomitante e abuso de álcool), níveis de vitamina B12 (risco de redução em pacientes susceptíveis), procedimentos cirúrgicos, alterações no estado clínico, medicações concomitantes que afetem a função renal ou a hemodinâmica ou a eliminação da metformina, administração de meio de contraste intravascular iodado (aumento do risco de insuficiência renal aguda), estados de hipóxia (choque, ICC, IAM, insuficiência renal pré-renal), mau controle glicêmico secundário a febre, trauma, infecção ou cirurgias, pacientes sob risco de depleção de volume intravascular (idosos, uso de diuréticos), uso concomitante com medicamentos que causam hipoglicemia (insulina e sufoniluréias), sepse urinária e pielonefrite, uso geriátrico, gravidez, lactação, uso pediátrico, câncer de bexiga ativo. **Categoria de risco na gravidez: C. Interações medicamentosas:** *com dapagliflozina* (sem alterações clínicas relevantes, sem necessidade de ajuste de dose): bumetanida, sinvastatina, rifampicina, ácido mefenâmico; *com metformina*: medicamentos catiônicos (**cimetidina**), glibenclamida, furosemida, nifedipino; outros medicamentos hiperglicemiantes (tiazidas e outros diuréticos, corticosteroides, fenotiazinas, produtos da tireoide, estrógenos, contraceptivos orais, fenitoína, ácido nicotínico, simpatomiméticos, medicamentos bloqueadores do canal de cálcio e isoniazida). Interferência com teste do 1,5-anidroglucitol (1,5.AG). **Reações adversas:** infecção genital, infecção do trato urinário, poliúria, dorsalgia, cefaleia, hipoglicemia, desidratação, hipovolemia ou hipotensão, diarreia, náuseas, vômitos, redução dos níveis séricos de vitamina B_{12}, aumento do hematócrito. **Posologia:** deve ser individualizada com base no regime atual do paciente, desde que não exceda a dose máxima recomendada de 10 mg de dapagliflozina e de 2000 mg de cloridrato de metformina de liberação prolongada. XigDuo XR® deve, de modo geral, ser administrado uma vez ao dia com a refeição da noite. **Apresentações:** XigDuo XR® comprimidos revestidos de liberação prolongada de: 5 mg/1000 mg em embalagens com 14 e 60 comprimidos; 10 mg/500 mg em embalagens com 14 comprimidos e 10 mg/1000 mg em embalagens com 14 e 30 comprimidos. **USO ADULTO. USO ORAL. VENDA SOB PRESCRIÇÃO MÉDICA. SE PERSISTIREM OS SINTOMAS, O MÉDICO DEVERÁ SER CONSULTADO.** Para mais informações, consulte a bula completa do produto. www.astrazeneca.com.br. Reg. MS – 1.0180.0407 (XIG005_min).

CONTRAINDICAÇÃO: doença renal ou disfunção renal moderada a grave. **INTERAÇÃO MEDICAMENTOSA:** cimetidina.

Material destinado ao profissional de saúde.

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