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The Janus faces of thyroid carcinoma

João Roberto M. Martins[1]

Thyroid cancer is the most common endocrine neoplasia, and its incidence has increased dramatically in several countries during the last three decades (1). This phenomenon has been attributed to overdiagnosis due to a combination of improvements in new imaging techniques, especially high-resolution ultrasound, and increased access of patients to health care systems (2). Irrespective of controversies regarding whether the growing number of thyroid cancer diagnoses is attributable to overdiagnosis alone (2) or reflects an actual increase in cases (3-5), the majority of tumor recently diagnosed is comprised of small papillary carcinomas (PTCs), (1.5-2.0 cm or less in size). Fortunately, more than 90% of them are curable with total thyroidectomy complemented or not with radioiodine (RAI). Although most PTCs are considered to be low risk, there are cases in which the tumor, even if small, exhibits aggressive behavior, and distant metastases can be present at diagnosis.

In this issue of the *Archives of Endocrinology and Metabolism* (AE&M), these two faces of thyroid cancer were addressed in two well-written papers on patients with PTC in two Latin American countries.

Domínguez and cols. (6), retrospectively reviewed the medical records of 209 patients with papillary thyroid microcarcinoma (PTMC) from 2009 to 2013, with a median follow-up of 4.4 years. Overall, 90% of these cases involved female patients; moreover, despite stratification into a low-risk group, 88% of patients received RAI, a treatment that was provided in accordance with older guidelines (7) and had no impact on tumor recurrence/persistence. As expected, classical PTC was the predominant histology (78%), tumors were frequently unilateral (76.1%), 17.9% of tumors had minimal extrathyroidal extension (ETE), 16.7% of cases involved lymph node metastasis, and there were no distant metastases. According to the American Thyroid Association (ATA) guidelines 2009 risk recurrence stratification (7), 70.8% of tumors were classified as low risk; this percentage increased to 78.5% when the ATA's 2015 risk stratification was used (8). In addition, persistence/recurrence was extremely uncommon: 1.5% of patients exhibited biochemical persistence/recurrence, and 5.5% of patients exhibited structural persistence/recurrence. Patients with persistence/recurrence tended to be younger ($p = 0.08$) and to present with multifocal tumors ($p = 0.07$), but only ETE (univariate analysis, $p = 0.019$) and lymph node involvement (multivariate analysis, $p = 0.001$) were significantly associated with persistence/recurrence. Comparisons of stratification using the 7th and 8th AJCC/TNM systems (9) indicated that when switching from the former system to the latter system, the percentage of patients classified as stage I increased from 89% to 95.2% and the percentage of patients classified as stage II decreased from 10% to 4.8%. Unfortunately, only 33% of patients with recurrent/persistent disease underwent

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preoperative neck ultrasound, which could explain why persistent disease was found during early follow-up.

In general, the data described in that paper resemble those that have previously been reported in the literature (10,11). These data also emphasize the fact that thyroid cancer, especially PTMC, has an excellent prognosis and that correct risk stratification is of great importance in patient management, with the objective of providing more cost-effective patient treatment and avoiding overtreatment. Moreover, these data could encourage local experts to adopt more conservative measures, such as "watchful waiting", in the management of low-risk patients, a measure that has already been suggested by other groups around the world (12-14).

The other face of thyroid cancer refers to distinct outcomes for patients with distant metastases, which are likely to be found in the lungs and bones. Fortunately, this condition is uncommon, occurring in less than 10% of patients with differentiated thyroid carcinoma (DTC); however, such metastases are the main cause of thyroid cancer-related deaths (15).

Another article in this issue of AE&M describes a well-executed retrospective study by Califano and cols. (16), who assessed patients with DTC and bone metastases (BMs) followed at 10 referral endocrinology centers in Argentina to evaluate epidemiology, clinical presentation, treatments, and outcomes. Out of 3,810 patients with DTC, 52 patients (1.3%) had BMs, which were diagnosed less than 6 months after diagnosis of the primary tumor in 46.2% of these patients and later during follow-up in the remaining 53.8% of patients. In contrast to low-risk microcarcinomas, for DTC in this series, PTC accounted for 57.6% of cases, and the follicular variant of thyroid cancer was more frequent. As expected, the majority of patients were classified as having a high risk of recurrence (70.2%) according to the ATA's 2009 guidelines (7). With respect to the extent of disease, BMs were isolated in < 25% of cases, whereas locoregional or other metastatic sites, mostly in the lung (94.4%), were present in the remaining cases. In order of frequency, metastatic sites for BM included the spine, pelvis, ribs, limbs, skull, clavicle and sternum. BMs were frequently symptomatic (65.4%), and pain was the most frequent clinical presentation (73.5%). The treatment modalities were as described by others (15) and included RAI, bisphosphonates, external beam radiotherapy and other therapies (such as tyrosine kinase inhibitor, doxorubicin, thalidomide and radiofrequency ablation). More than 50% of patients died of DTC-associated causes, most of which were related to Hurthle cell variants and fracture at presentation. However, less than one-third of deaths were directly related to BMs; the remaining deaths were caused by other complications, which were mostly related to respiratory events. The authors found a relatively high frequency of negative RAI uptake at the metastatic site (42.3%); as described by Durante and cols. (15), patients who exhibited this characteristic more frequently died of DTC.

Califano and cols. also discussed the fact that when used as a solitary therapy, RAI therapy is rarely curative in patients with distant metastasis; only one patient achieved remission after this treatment. This statement is likely true for cases involving BMs. However, we recall that lung metastases were previously reported to be relatively responsive to RAI (15); thus, in a hypothetical condition in which RAI is associated with complete resection of isolated BM, this therapy is expected to improve overall survival. Certainly, as was well discussed by the authors, complete surgical resection can rarely be achieved if there are multiple BM sites. In such cases, palliative resection might be indicated more to improve quality of life than to impact survival. All of the patients in the examined series who were alive at the end of follow-up (36%) exhibited an incomplete structural response; this phenomenon is likely to be a consequence of broad bone involvement (isolated or combined) in this series. As stated by the authors, the correct management of patients with distant metastasis from DTC requires a multidisciplinary approach and currently remains a challenge; moreover, widespread research continues to be focused on searching for prognostic factors for cancer-specific mortality.

The aforementioned papers describe the two opposing faces of DTC. On one hand, the majority of patients with recently detected DTC have PTMC, which is associated with excellent prognosis and, in general, no mortality. For these diseases, appropriate risk stratification to determine which cancers require specific treatment and ongoing continuous re-stratification are essential to avoid overtreatment (17). For a specifically selected subgroup of low-risk patients, simple surveillance, with no specific treatment, has been proposed and stimulated (12-14). On the other hand, in rare cases involving metastatic DTC, this condition is frequently fatal or associated with persistent/recurrent disease, with great impact on the patient's quality

of life and on healthcare systems worldwide. It has become increasingly challenging to treat this type of tumor. With respect to treatment, particularly for DTC refractory to RAI, molecular targeting therapies against most of the more common pathways involved in thyroid carcinogenesis have been proposed (18). Other targets include promoting immunological intervention, such as by increasing numbers of tumor-associated macrophages (TAMs) or by inhibiting factors associated with evading immunosurveillance (for example, in one study, an inhibitor of PD-1), two strategies that have shown promise (18).

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(Epi) Genetics and the complexity of diabetes mellitus

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Both type 1 (T1D) and type 2 (T2D) diabetes mellitus are complex diseases to which environmental and genetic factors contribute (1). Recently, epigenetic factors have also been recognized as important not only in the etiopathogenesis of these conditions, but also in the development of their chronic complications (2). As if the interaction among environment, genetics and epigenetics is not complex enough, each of these factors has their own complexity. For instance, regarding the genetic setting, both T1D and T2D are polygenic diseases for which several susceptibility genes contribute, each with a relatively small participation (1). In addition, susceptibility genes may vary among different populations, reason why genetic studies should be reproduced in the population of interest.

In this issue of *Archives of Endocrinology and Metabolism* (AE&M), two manuscripts address, respectively, genetic and epigenetic factors associated with diabetes. Pirozzi and cols. (3) evaluated in a population of Brazilian obese patients from the Southeast region, the association of T2D with two polymorphisms, rs1799752 in the gene encoding angiotensin I converting enzyme (*ACE*) and rs1801133 in the gene encoding methylenetetrahydrofolate reductase (*MTHRF*). MTHRF is the enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate whose deficiency increases plasmatic homocysteine concentrations. Both polymorphisms had been previously studied for the association to T2D with conflicting results in different ethnic groups (4,5). No association of these polymorphisms with T2D was found by Pirozzi and cols., in agreement with a previous study performed in Brazilian patients from the South region, which also did not find association of T2D with rs1799752 in the *ACE* gene (6). Given the admixture which characterizes the Brazilian population and the distinct genetic background even among the different Brazilian regions (7), it is important that such genetic studies are carried out, preferably in larger series of patients.

Micro RNAs (miRNAs) are one of the epigenetic mechanisms; they are small non-coding RNAs that repress gene expression at the post transcriptional level (8) and because they regulate several cellular processes, they have been implicated in human diseases (9). miRNA expression profiles have been explored as a potential tool to classify disease states and, in certain situations, to diagnostic applications (10). In the second manuscript, García-Días and cols. evaluated the expression of three miRNAs in peripheral blood mononuclear cells (PBMC) from T1D patients as compared to control (non-diabetes) subjects. A higher expression of miR-155 and a lower expression of miR-326 and miR-146a were observed in T1D patients in comparison to non-diabetes subjects. Interestingly, miR-155 expression was associated with autoimmunity and with the inflammatory status (11). It had been previously demonstrated that different inflammatory mediators, such as tumor necrosis factor

(TNF), are able to induce miR-155 expression in macrophages (12). Because hyperglycemia increases the production of TNF and other cytokines, it is possible that increased miR-155 expression in PBMC from T1D patients reflects a sustained inflammatory state associated with the suboptimal metabolic control (11). Studies like this one exploring the association between clinical and biochemical variables and aberrant expression of miRNAs can improve the understanding of the epigenetic mechanisms triggered by long-standing hyperglycemia, which underlie the concept of Metabolic memory.

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Papillary thyroid microcarcinoma: characteristics at presentation, and evaluation of clinical and histological features associated with a worse prognosis in a Latin American cohort

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ABSTRACT

Objective: We aimed to describe the presentation of papillary microcarcinoma (PTMC) and identify the clinical and histological features associated with persistence/recurrence in a Latin American cohort. **Subjects and methods:** Retrospective study of PTMC patients who underwent total thyroidectomy, with or without radioactive iodine (RAI), and who were followed for at least 2 years. Risk of recurrence was estimated with ATA 2009 and 2015 classifications, and risk of mortality with 7th and 8th AJCC/TNM systems. Clinical data obtained during follow-up were used to detect structural and biochemical persistence/recurrence. **Results:** We included 209 patients, predominantly female (90%), 44.5 ± 12.6 years old, 183 (88%) received RAI (90.4 ± 44.2 mCi), followed-up for a median of 4.4 years (range 2.0–7.8). The 7th and 8th AJCC/TNM system classified 89% and 95.2% of the patients as stage I, respectively. ATA 2009 and ATA 2015 classified 70.8% and 78.5% of the patients as low risk, respectively. Fifteen (7%) patients had persistence/recurrence during follow-up. In multivariate analysis, only lymph node metastasis was associated with persistence/recurrence (coefficient beta 4.0, p = 0.016; 95% CI 1.3-12.9). There were no PTMC related deaths. **Conclusions:** Our series found no mortality and low rate of persistence/recurrence associated with PTMC. Lymph node metastasis was the only feature associated with recurrence in multivariate analysis. The updated ATA 2015 and 8th AJCC/TNM systems classified more PTMCs than previous classifications as low risk of recurrence and mortality, respectively.** Arch Endocrinol Metab. 2018;62(1):6-13

Keywords
Thyroid cancer; head and neck; endocrine

INTRODUCTION

Papillary thyroid microcarcinoma (PTMC) is defined as a papillary thyroid cancer (PTC) whith a diameter 1 cm or less, and its incidence has increased during the last decades (1,2). PTMC comprises nearly 50% of PTC diagnosed worldwide and is associated with a favorable prognosis, which varies depending on the extension of the disease. Intrathyroidal incidental PTMC has the best prognosis, with recurrence and mortality rates as low as 0.5% and 0%, respectively (3-6). On the other hand, the 1-2.8% of patients with PTMC who have distant metastases at diagnosis have a worse prognosis, with high rates of persistence/recurrence (7). Previous studies have associated multifocality, bilateral disease, lymph node (LN) metastasis, tumor diameter of > 0.5 cm, a sum of multifocal tumor diameters greater than 2 cm, and minimal extra thyroidal extension (ETE) with a higher risk of recurrence (8-10).

Due to its good prognosis, some experts have proposed renaming PTMC as "micropapillary tumor" thus avoiding the word "cancer" and its implications regarding overtreatment and the deterioration of patient´s quality of life (11,12). Additionally, the favorable prognosis of PTMC has suggested the need for a less intense treatment with regard to surgery extension, radioactive iodine (RAI) ablation and TSH suppression, without increasing recurrence and mortality (13). Furthermore, active surveillance of selected PTMC patients has shown promising results, with a low risk of enlargement and LN metastases after 10 years of follow-up (14).

These less intense strategies for PTMC treatment are consistent with the updated versions of the 2015 American Thyroid Association (ATA) guidelines and the 8th AJCC/TNM system, which suggest changes in the stratification of the risks of recurrence and mortality, respectively (8,13,15). The most important changes include the following: i) the impact of LN metastases is now said to depend on both their number and size but not on their sole presence; ii) the role of minimal ETE has been reduced; and; iii) the age that determines a higher risk of mortality has been increased from 45 to 55 years (15,16).

Although PTMC represents nearly 50% of PTC patients, there is scarce information on this subject in relation to the Latin American population (5,17-19). Our aim was to describe the characteristics of PTMC at diagnosis and to identify the clinical and histological features associated with persistence/recurrence in a Latin American cohort. We hypothesized that our patients would have low rates of recurrence and mortality. We also expected that the updated ATA 2015 classification and the 8th AJCC/TNM system would induce significant changes in both recurrence and mortality risk stratification, respectively.

SUBJECTS AND METHODS

After receiving the approval of the Ethics Committee, we retrospectively reviewed the electronic medical records of 673 consecutive patients with PTC who were 18 years old or older, who had undergone total thyroidectomy between 2009 and 2013, and who had been followed-up for at least 2 years. We included every patient with PTMC. LN dissection was performed in patients whose preoperative ultrasound, fine needle aspiration biopsy, or intra-operative findings, suggested LN metastases. We used fixed RAI activities based on the extent of the initial disease, and the dose administered was decided by the attending physician, following ATA 2009 and institutional guidelines (8,20). A post-therapy whole body scan (WBS) was performed after RAI. Considering the results of preoperative ultrasound, intra-operative findings, pathology and WBS result, the patients were risk-stratified using the 7th and 8th edition of the AJCC/UICC staging system and the ATA 2009 and ATA 2015 risk of recurrence stratification systems (8,13). Of the 673 potentially eligible patients, a total of 209 patients satisfied the inclusion criteria.

After initial therapy, all patients received levothyroxine to keep TSH between 0.5 and 1.0 uUI/mL, and had at least two neck ultrasounds and two serum thyroglobulin (Tg) and anti-Tg antibodies (TgAb) determinations, either suppressed or stimulated by levothyroxine withdrawal.

Patients were routinely followed every 6 months during the first year, and at 6–12 month intervals thereafter at the discretion of the attending physician, based on the risk of the individual patient and the clinical course of the disease.

Structural neck persistent/recurrent PTC was defined as suspicious LN in neck ultrasound, accompanied by histocytological or Tg in an aspirate study that proved the presence of PTC. Extra-cervical structural persistent/recurrent PTC was defined as the presence of suspicious images on cross-sectional studies (computed tomography scan, or magnetic resonance imaging) or functional imaging (diagnostic WBS or ^{18}FDG PET-scan), requested according to the attending physician criteria. Biochemical persistent/recurrent PTC was defined as the presence of suppressed 1st generation Tg ≥ 0.9 ng/mL or stimulated Tg ≥ 2.0 ng/mL, without structural disease. At final follow-up, the absence of abnormal images in radiological studies accompanied by stimulated Tg < 2.0 ng/mL or suppressed 1st generation Tg < 0.9 ng/mL, were considered no clinical evidence of disease (NED).

Tg was measured using a chemiluminescent assay (Immulite 2000, Siemens, Los Angeles, CA, USA) with a functional sensitivity of 0.9 ng/mL, normalized to CRM 457. TgAb were measured using a chemiluminescent assay (Architect i1000, Laboratories, Abbott Park, IL, USA) (reference value of up to 4.11 IU/mL, analytical sensitivity 1.0 IU/mL). Since May 12th, 2014, second generation Tg was measured using a chemiluminescent assay (Elecsys II, Roche Diagnostics, Rotkreuz, Switzerland) with a functional sensitivity of 0.1 ng/mL, normalized to CRM 457.

Categorical variables are expressed as number and frequencies; continuous variables are expressed as mean ± SD, or median and ranges, as appropriate. Proportions were compared using a Fisher's exact chi2 test. Continuous variables were compared using parametric or non-parametric tests, as appropriate. Analysis was performed using SPSS software (version 15.0.0: SPSS, Inc., Chicago, IL). P-values < 0.05 were considered to be statistically significant.

RESULTS

Patients were predominantly female (90%) and 44.5 ± 12.6 years old: A total of 183 (88%) received RAI (90.4 ± 44.2 mCi) and were followed-up for a median of 4.4 years (range 2.0–7.8 years). Most patients (78%) had classical variant PTC, 61.2% had unifocal, 76.1% had unilateral disease, 17.9% had minimal ETE (microscopic or involving strap muscles), 16.7% had LN metastasis and none had distant metastases at diagnosis (Table 1).

According to the 7th AJCC/TNM system, 89%, 10% and 1% of the patients were classified as stage I, stage III and stage IVA disease, respectively. Using the updated 8th AJCC/TNM system, 95.2% and 4.8% of the patients were classified as stage I and stage II

Table 1. Characteristics of the patients at baseline

	Total Cohort (N = 209)	Persistence/Recurrence		P-value
		No (n = 194)	Yes (n = 15)	
Age at diagnosis (years)	44.5 ± 12.6	44.7 ± 12.8	40.8 ± 9.4	0.08
Female gender	188 (90%)	173 (89.1%)	15 (100%)	0,18
Histological subtype of PTC				0.6
Classical variant	163 (78%)	150 (77.3%)	13 (87.0%)	
Follicular variant	36 (17.2%)	34 (17.7%)	2 (13.0%)	
Other variants	10 (4.8%)	10 (5.130%)	0 (0%)	
Tumor diameter (cm)	0.62 ± 0.25	0,62 ± 0.26	0.65 ± 0.25	0.75
Sum of diameters (cm)	0.83 ± 0.54	0.83 ± 0.55	0.9 ± 0.48	0.9
Sum of all foci diameters > 2 cm	10 (4.8%)	10 (5.1%)	0 (0%)	0.36
Unifocal disease	128 (61.2%)	121 (62.4%)	7 (47.0%)	0.3.
Three or more foci	35 (16.7%)	30 (15.5%)	5 (33.3%)	0.07
Bilateral disease	50 (23.9%)	46 (23.7%)	4 (26.7%)	0.80
Minimal extra thyroidal extension	37 (17.7%)	31 (16.0%)	6 (40.0%)	0.019
Lymphocytic thyroiditis	88 (42.1%)	83 (42.8%)	5 (33.3%)	0.37
T				0.33
1a	172 (82.3%)	154 (82.8%)	8 (61.5%)	
3	37 (17.7%)	32 (17.2%)	5 (38.5%)	
N*				0.001
0	174 (83.3%)	166 (86.0%)	8 (53.3%)	
1	35 (16.7%)	28 (14.0%)	7 (46.7%)	
RAI ablation	183 (88.0%)	168 (86.6%)	15 (100%)	0.40
RAI dose (mCi)	90.4 ± 44.2	88.2 ± 44.2	116.7 ± 36.2	0.69
Length of follow-up (years)	4.4 (2.0-7.8)	4.4 (2.0 – 7.8)	4.2 (2.1 – 6.3)	0.37
AJCC 7th				
I	186 (89%)	173 (89.2%)	13 (86.6%)	0.06
III	21 (10%)	20 (10.3%)	1 (6.7%)	
IVA	2 (1.0%)	1 (0.5%)	1 (6.7%)	
AJCC 8th				0.34
I	199 (95.2%)	184 (95.0%)	15 (100%)	
II	10 (4.8%)	10 (5.0%)	0 (0%)	
ATA 2009				0.006
Low	148 (70.8%)	142 (73.2%)	6 (40%)	
Intermediate	61 (29.2%)	52 (26.8%)	9 (60%)	
ATA 2015				0.002
Low	164 (78.5%)	157 (81%)	7 (46.7%)	
Intermediate	45 (21.5%)	37 (19%)	8 (53.3%)	

* N0 includes patients subjected to lymph node dissection whose biopsy found no tumor, and in whom physical exam, preoperative ultrasound and intraoperative evaluation found no evidence of lymph node metastases.

disease, respectively, with no patients classified as stage III or stage IVA. Regarding risk of recurrence, ATA 2009 classified 70.8% and 29.2% of the patients as low and intermediate risk, respectively, while ATA 2015 categorized 78.5% and 21.5% of the patients as low and intermediate risk, respectively (Table 1).

Of the whole cohort, 194 (93%) patients had no persistence/recurrence, while 3 (1.5%) and 12 (5.5%) had biochemical and structural persistence/recurrence, respectively. There were no PTMC related deaths and the characteristics of patients who recurred are shown in Tables 1 and 2.

In univariate analysis, minimal ETE (OR 3.5 (95% CI 1.2-10.6)) and cervical LN metastasis (OR 5.2 (95% CI 1.7-15.4)) were associated with persistence/recurrence (Table 1). In multivariate analysis, only LN metastasis kept its significance (beta coefficient 4.0 ($p = 0.016$; 95% CI 1.3-12.9)).

The characteristics of the 15 patients who had persistence/recurrence are detailed in Table 2. Preoperative neck ultrasound was performed in only 5 (33%) of them. The median time between initial treatment and persistence/recurrence was 1.2 years (range 0.8-5.8) and 9 cases (60%) were diagnosed earlier than 1.5 years after initial treatment. Among the 12 patients with structural persistence/recurrence, 11 had cervical LN metastases: 5 underwent surgery and RAI, 3 underwent surgery alone, 1 received RAI alone and 2 underwent active surveillance: at the time of analysis, none of the patients showed progression of structural disease, while 7 accomplished a 2nd generation Tg ≤ 0.2 ng/dL, consistent with an excellent response to treatment.

The additional patient with structural disease was a 43-year old woman who had been treated initially with total thyroidectomy and RAI ablation (50 mCi) for a low risk PTMC. She achieved an excellent response (negative neck ultrasound and serum Tg of 0.1 ng/dL), but a 1.7 cm pulmonary metastasis was incidentally diagnosed on a chest X-ray 5.8 years after initial treatment. The lung metastasis was resected, an additional 100 mCi dose of RAI was given and the patient currently has NED.

Of the 3 patients with biochemical persistence/recurrence, all three underwent active surveillance. At the end of the follow-up, none had evidence of structural disease and 2 had achieved Tg ≤ 0.2 ng/dL, demonstrating an excellent response.

Of 194 patients with no persistence/recurrence, 2nd generation Tg was obtained in 146: 143 (98%) had Tg ≤ 0.2 ng/mL, while 3 patients had values between 0.3 and 0.9 ng/mL. None of them developed structural disease.

We found no significant differences for gender, histological subtypes, rate of multifocal disease, ETE, TNM/AJCC system, or risk of recurrence classifications among patients who had received and those who had not received RAI.

DISCUSSION

In this study, in which we evaluated a cohort of 209 Latin American patients, PTMC was associated with good prognosis: there were no PTMC related deaths, 7% of patients had persistence/recurrence, and 204 (98%) had NED at the end of the follow-up. These results are consistent with previously published series, which report mortality and recurrence rates of between 0-3.0% and 0.5-7.9%, respectively (9).

Previous reports have shown that non-incidental PTMC has higher risk of recurrence than incidental PTMC (6). The patients included in our series have a similar profile as those with non-incidental PTMC according to the previous series: predominantly female, 44.5 ± 12.6 years old, tumor diameter 0.62 ± 0.25 cm, multifocality (38.8%), bilaterality (23.9%), minimal ETE (17.7%), and LN metastasis (16.7%) (6). This similarity to non-incidental PTMC may explain why the recurrence rate was close to the highest reported.

Published data reported rates of LN metastases between 10-50%, with the highest rates found when prophylactic central LN dissection is performed (21). In our series, only 16.7% of PTMC had LN metastases, which is probably due to our policy of performing therapeutic neck dissection exclusively in patients with clinical evidence of N1 disease. This might also explain the impact of LN metastases in persistence/recurrence after multivariate analysis. It is currently known that the impact of LN metastases on recurrence depends on the number and size of the metastases, and on the presence of extra-nodal extension (8). Although it was controversial in the past, retrospective studies have not found lower rates of recurrence in PTC patients subjected to prophylactic central neck dissection, but may be associated with higher risks of complications (22,23).

Table 2. Characteristics of 15 patients with persistent or recurrent disease

Patient number	ATA 2015	Type of persistence/ recurrence	Time between initial treatment and to persistence/ recurrence (years)	Preoperative neck ultrasound done	Serum Tg (ng/mL)		Follow-up neck US	FNA	Therapy	2nd Gen Tg (ng/mL)	Length of follow-up (years)	Status at end of follow-up
					Suppressed	Stimulated						
1837	Low	Structural	0.8	No	< 0.9	NA	(+)	(+)	Sx	< 0.1	6.8	NED
1897	Low	Structural	2.2	No	3.6	NA	(+)	(+)	Sx + RAI	< 0.1	6.7	NED
2015	Intermediate	Structural	1.4	No	10.6	NA	(+)	NA	RAI	0.7	3.9	Biochemical disease
1952	Low	Structural	0.9	No	3.4	NA	(+)	(+)	Sx + RAI	< 0.1	6.1	NED
1997	Low	Structural	5.81	No	0.1	3.2	(-)	Chest CT and Dx scan compatible with 1.7 cm pulmonary metastasis	Sx + RAI	0.1	6.5	NED
1511	Low	Structural	1.2	No	5.5	NA	(+)	(+)	Sx + RAI	< 0.1	7.5	NED
1732	Intermediate	Structural	1.0	No	1.2	33	(+)	(+)	Sx + RAI	6.5	0.2	NED
2172	Intermediate	Structural	0.6	No	5.0	NA	(+)	(+)	Sx	4.8	<0.1	NED
2174	Intermediate	Structural	0.7	No	3.8	21	(+)	(+)	Sx	5.0	0.2	NED
2315	Low	Structural	1.96	Negative	< 0.9	NA	(+)	(+)	AS	0.2	4.5	Neck US non-specific nodule (granuloma) + negative chest CT
2232	Intermediate	Structural	4.0	No	< 0.9	NA	1 cm suspicious lateral cervical lymph node	Not done	AS	< 0.1	4.9	Stable neck US
2296	Intermediate	Structural	1.8	Cervical lymph nodes (+)	1.0	1.8	(+)	(+)	Sx + RAI	< 0.1	4.3	NED
1506	Low	Biochemical	0.9	Negative	2.4	27.1	(-)	(-)	AS	1.0	6.9	Biochemical disease (negative neck US and ^{18}FDG PET- CT)
1520	Intermediate	Biochemical	2.4	Cervical lymph nodes (+)	< 0.9	2.8	(-)	NA	AS	< 0.1	7.5	NED
2195	Intermediate	Biochemical	1.1	Negative	< 0.9	2.7	(-)	NA	AS	0.2	4.8	NED Neck US + Chest CT negative

Tg: thyroglobulin; NA: not applicable; Sx: surgery; RAI: radioactive iodine; NED: not evidence of disease; CT: computed tomography; Dx scan: ^{131}I diagnostic scan; AS: active surveillance; US: ultrasound.

In our series, minimal ETE had no impact on recurrence/persistence. Although it may be controversial and ATA 2015 guidelines still consider its presence to be criterion with which to classify patients as being at intermediate risk of recurrence, our findings are consistent with other studies that found no association with recurrence (10,24). Furthermore, the updated 8th TNM/AJCC system discouraged its role as a predictor of high risk of mortality in PTC, and recent data has shown poor agreement among expert pathologists in terms of defining its presence (15,25).

As patients were treated following older guidelines, 183 (93%) patients received RAI: they had similar characteristics at presentation to those who were not ablated, and RAI had no impact on recurrence/persistence, which is consistent with ATA 2015 recommendations and other series (13,26). We also recognize that currently a significant number of these patients would not undergo total thyroidectomy, as PTMC prognosis is similar in patients treated with partial thyroidectomy (13,27).

In our series, most patients with persistence/recurrence disease, 11 of 15, had structural regional disease. When analyzing their characteristics, only one third of them had preoperative neck ultrasound, and in two thirds persistence/recurrence was diagnosed within 1.5 years of initial treatment. These two facts suggest that the incomplete preoperative stratification is an explanation for the increased rate of structural persistent disease found early on in the follow-up. They also emphasize that the need for a comprehensive preoperative ultrasound staging in patients with PTMC is as important as it is in non-PTMC patients (28). From 2012 onwards, preoperative ultrasound staging was established as part of our routine protocol for every patient with diagnosis of thyroid cancer.

Of the 11 patients with cervical persistent/recurrence, 9 were actively treated with surgery, RAI, or a combination of both. The remaining 2 patients underwent active surveillance: one had NED and the other had stable findings at the end of the follow-up. This finding supports a less aggressive management of low volume cervical LN disease in properly selected patients, which has been previously associated with 5-10% risks of local progression, without or without very low risk of distant metastases (29,30).

Only one patient developed a distant metastasis during follow-up, which was incidentally discovered in a chest X-ray 5.81 years after initial treatment with total thyroidectomy and RAI. The patient had had a long period of excellent response, making recurrence very unlikely. After a lung metastasis resection and an additional RAI dose, the patient currently has NED.

An additional interesting finding is the impact of the updated risk of mortality and recurrence classifications. Regarding recurrence, in our series the rate of low risk patients rose from 70.8% to 78.5% according to ATA 2009 and 2015 classifications, respectively, which is consistent with the low rates of recurrence found in PTMC (8,13). Based on the recently published 8th AJCC/TNM system, the rate of stage I disease increased to 95%, and none of the patients were classified as stage III (8,13,15). These two changes are consistent with the very low risk of death related to PTMC in our and others series (6,16,31). These improvements in the stratification of PTMC patients should allow physicians to better adjust the intensity of therapy and avoid overtreatment in most patients with PTMC, which has been associated with side effects and deterioration of quality of life (9). Even more, in properly selected patients, active surveillance has shown to be feasible, with low risk of progression of disease and lower rates of side effects related to therapy (14,32).

A limitation of this study is the short time of follow-up, a median of 4.4 years (range 2.0-7.8). However, although PTC may recur a long time after initial treatment, nearly 50% and up to 80% of the recurrences occur during the first 3 and 5 years after initial treatment, respectively (33). In addition, it is important to note that among the 194 patients who did not have persistence/recurrence, 2nd generation Tg was available in 146: 143 had Tg ≤ 0.2 ng/mL, which is consistent with an excellent response to treatment and a very low risk of recurrence (13,34,35). The remaining 3 patients had negative imaging studies and stable serum Tg of between 0.3 and 0.9ng/mL, which is consistent with an indeterminate response, a category that still has good prognosis, with less than 1% risk of death and nearly 15% risk of structural progression (13).

In conclusion, we found a low rate of recurrence and no mortality associated with PTMC in Latin American patients. LN metastasis was the only feature associated with higher risk of recurrence, which emphasizes the importance of a comprehensive preoperative neck ultrasound evaluation. Updated ATA 2015 and 8th AJCC/TNM systems had a significant impact on patient stratification by increasing the rate of patients

classified as at low risk of recurrence and mortality, respectively.

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Outcomes of patients with bone metastases from differentiated thyroid cancer

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ABSTRACT

Objective: Bone metastases (BM) from differentiated thyroid cancer (DTC) are associated with poor survival rates. Due to the low frequency of this entity, we performed a multicentric retrospective study that aimed to evaluate the presentation, outcome and causes of death in this population. **Subjects and methods:** We reviewed file records from 10 databases. BM were diagnosed by: i) biopsy and/or ii) radioiodine (RAI) bone uptake + elevated thyroglobulin (Tg) levels and/or c) bone uptake of 18-FDG in the PET-CT scan + elevatedTg levels. **Results:** Fifty-two patients with DTC were included (44% male, mean age 54 years); 58% had papillary histology. BM were synchronous with DTC diagnosis in 46% of the participating cases. BM were symptomatic in 65% of the cases. Multiple BM were present in 65% of patients, while simultaneous metastatic disease in additional sites was found in 69%. Ninety-eight percent of patients received treatment for the BM, which included RAI therapy in 42 patients; 30 of them received cumulative RAI doses that were larger than 600 mCi ^{131}I. The mean follow-up after a BM diagnosis was 34 months. The 2- and 5-year survival rates after diagnosis of the first BM were 64% and 38%, respectively. The status on the last evaluation was DTC-related death in 52% of the patients; 26% of them died from direct complications of BM or their treatments. **Conclusion:** BM are usually radioiodine-refractory and are associated with a short overall survival, although most of the patients died of causes not directly related to the BM. Arch Endocrinol Metab. 2018;62(1):14-20

Keywords
Thyroid cancer; distant metastases; bone metastases

INTRODUCTION

In most published series, patients with differentiated thyroid cancer (DTC) have a 10-year overall survival rate of 85-93% (1). However, when distant metastases occur, the overall survival may decrease to 50% at 5 years of follow-up (1-4).

Among cases, the most frequent localizations of DTC metastases are the lungs in 50%, bones in 25 to 30% and both sites in 20% (5-8).

The treatment of distant metastases usually involves the use of radioactive iodine (RAI), which may be administered several times during follow-up. Nevertheless, once DTC is no longer amenable to RAI therapy or surgery, the expected survival declines rapidly, and death from thyroid carcinoma within 3 years is common (8,9).

Bone metastases (BM) may severely reduce the quality of life (QoL) in patients with disseminated thyroid cancer, causing pain, fractures and spinal cord compression, among other complications (10). BM is a difficult clinical problem that requires a multidisciplinary approach.

Cancer is generally incurable once it has metastasized to the bone. It has been suggested that bone marrow can serve as reservoir for dormant tumor cells, thereby rendering them resistant to RAI and conventional chemotherapeutic agents (11).

However, the causes of death in patients with DTC and BM have not been widely evaluated, probably due to the low frequency of this entity. Therefore, we decided to perform a retrospective review of patients with DTC and BM that were followed up in 10 referral

endocrinology units in Argentina in order to evaluate the epidemiology, forms of presentation, treatments modalities and outcome, including overall survival and causes of death.

SUBJECTS AND METHODS

We reviewed the file records of patients with DTC whom were treated at 10 hospitals. Since this was a retrospective study, written consent was deemed unnecessary. Confidentiality of patients' data was maintained in accordance with each institution's standards. We included patients who were treated with total thyroidectomy (either with or without lymph node dissection) and remnant ablation. The patients were classified according to the TNM staging system (AJCC/UICC; 7 ed) (12) and to the risk of recurrence classification from the American Thyroid Association (13). The baseline characteristics of the patients who were included are shown in Table 1.

Table 1. The baseline characteristics of 52 patients with bone metastases and differentiated thyroid cancer who were included in the study

Characteristics	Number of patients (%)
Age at diagnosis of thyroid cancer, median (years)	54.5
Range	15-54
> 45 years	42 (80.7)
Male/female	23/29 (44.3/55.7)
Pathological diagnosis	
Papillary thyroid cancer	30 (57.6)
Classic	17
Follicular variant	9
Tall cell	3
Oncocytic	1
Follicular thyroid cancer	19 (36.5)
Hürthle cell carcinoma	3 (5.7)
Stage (AJCC/UICC7ªed)	
I	4 (8)
II	6 (12.5)
III	7 (15)
IVa	7 (15)
IVb	1 (1.5)
Ivc	23 (48)
Risk of recurrence (ATA 2009) (n = 47)	
Intermediate	14 (29.7)
High	33 (70.2)

BM were diagnosed by: i) biopsy and/or ii) after ^{131}I bone uptake associated with elevated thyroglobulin (Tg) levels and/or iii) abnormal bone uptake of ^{18}fluordeoxyglucose in the positron emission tomography (PET/CT) scan associated with elevated Tg levels.

The following clinical-pathological features of the BM were considered for the analysis: methodology for the diagnosis of the BM, the number and localization of the BM foci, the symptoms generated by the BM, the sites of RAI radioiodine uptake after the administration of the ^{131}I dose and the presence of loco-regional disease and/or other non-skeletal metastatic sites.

BM were classified as synchronous if they were detected within the first 6 months after the diagnosis of the DTC and metachronous if they were found later in the follow-up.

Clinical management during follow-up

After the initial approach, the patients were followed by assessing their clinical status in response to initial therapy by using thyroid hormone withdrawal (THW)-stimulated Tg, along with diagnostic or post-treatment whole body scans (WBS) and neck ultrasonography. All patients underwent morphological imaging, including computed tomography (CT) and/or PET/CT. The response to treatment was defined as follows: i) remission: undetectable stimulated Tg levels with negative post-dose WBS associated to the absence of structural images in CT and or PET/CT); ii) biochemical persistence: detectable Tg levels under thyroid hormone suppressive therapy or after THW, or persistent/increasing levels of TgAb with negative post dose WBS associated to the lack of structural images in CT and/or PET/CT); and iii) incomplete structural response.

Patients with BM received RAI treatments (mean cumulative activity: 391 mCi ^{131}I, median 200 mCi, range 100-1500 mCi) until i) adverse events related to RAI appeared or ii) no response to treatment was observed due to progressive disease. Different modalities for the treatment of BM were also evaluated.

We considered the causes of death due to DTC and classified them as secondary to BM when they could be attributed to a direct complication of BM (such as pulmonary thromboembolism caused by immobilization, decubitus ulcer infections in bedridden patients or complications of their treatment, pathological fracture or due to disseminated DTC).

Statistical analyses were performed using SPSS software (version 21: SPSS Inc., Chicago Il). Quantitative variables are expressed as means ± SD; qualitative data are expressed in percentages. Continuous variables were

compared using Fisher's exact test and the x^2 test was used to compare categorical variables. Kaplan-Meier curves are used to present survival times. Values were considered statistically significant at $p < 0.05$.

RESULTS

Out of a total of 3810 patients with DTC, 52 (1.3%) were found to have BM and were included in the study (Table 1). BM were diagnosed synchronously (at the moment of the diagnosis of the DTC) in 46.2% of patients (n = 24) and metachronously in 53.8% (n = 28). Metachronous metastases were diagnosed 7-240 months after the initial diagnosis (median: 72 months).

BM were symptomatic in 65.4% of the cases (n = 34). Pain was the most frequent clinical presentation (73.5%, n = 25), while fractures and neurological symptoms were each reported in 8.8% of the cases (n = 3). The characteristics of BM can be observed in Table 2. Either as a presenting symptom or as a later event, spinal cord compression was noted in 6 cases (11,5%).

Simultaneous metastatic disease in sites other than bone was found in 69.2% of the patients (n = 36). The lung was the most frequently affected site in 94.4% of the cases (n = 34), followed by the brain, retroperitoneum, adrenal glands and skin (6.6%).

Table 2. Radioiodine uptake, localization and extent of disease in 52 patients with bone metastases from differentiated thyroid cancer

Characteristics	Number of patients (%)
Radioiodine uptake at metastatic site	
Positive	30 (57.6)
Negative	22 (42.3)
Number of BM	
Solitary	18 (34.6)
Multiple	34 (65.3)
Metastatic site	
Spine	38 (29.2)
Pelvis	25 (19.2)
Ribs	24 (18.4)
Limbs	19 (14.6)
Skull	13 (10)
Clavicle	8 (6.1)
Sternum	3 (2.3)
Extent of disease at the time of BM diagnosis	
Isolated BM	12 (23)
BM+ other metastatic sites	22 (42.3)
BM+ locoregional disease	4 (7.6)
BM+ locoregional + other metastases	14 (26.9)

The blood calcium levels were normal in all cases.

Stimulated Tg levels at the time of diagnosis of BM (excluding two patients with positive TgAb) were available in 43 cases. They were < 100 ng/mL in 16% of patients, between 100-1000 ng/mL in 39.5% and > 1000 ng/mL in the remaining 46.5%.

Only one patient was not treated due to advanced disease and poor performance status. The remaining patients received treatment for their BM.

The treatment modalities included RAI therapy in 80.7% (n = 42; 30 of them received cumulative RAI doses larger than 600 mCi ^{131}I), intravenous monthly bisphosphonates in 57.6 % (n = 30) and 19 of them received pamidronate, while the remaining 11 were treated with zoledronate, surgery in 50% (n = 26), external beam radiotherapy 50% (n = 26), other therapies in 13.4% (sorafenib n = 4, doxorubicin n = 2, thalidomide and radiofrequency ablation, one patient each).

The mean follow-up after diagnosis of first BM was 34.4 ± 33.7 months (median: 24 months, range: 1-67 months). More than 50% of patients died of causes related to the DTC. Hürthle cell carcinoma (30 vs 0%, p 0,005) and fracture as a presenting symptom (20 vs 2.4%, p 0,007) were more frequent in patients who died of DTC-related causes. Radioiodine uptake was found less frequently in patients who died from DTC, although it did not reach statistical significance (40 vs. 61%, p ns). The status on last evaluation can be observed in Figure 1. All of the patients who were alive with persisting disease at the end of the follow-up period had an incomplete structural response.

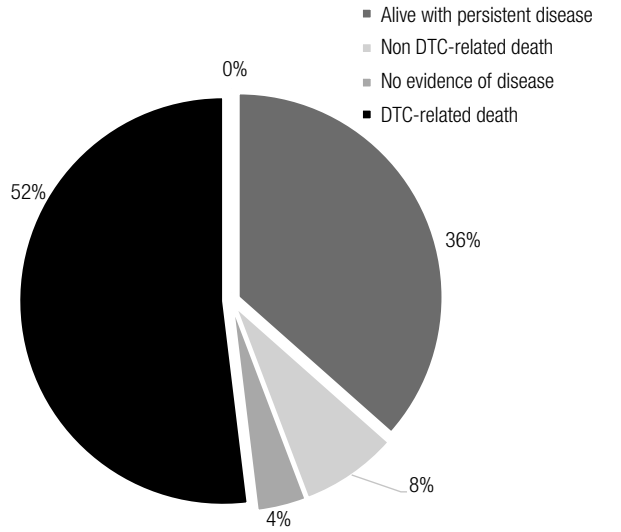


Figure 1. Status of patients with bone metastases from differentiated thyroid cancer at the end of follow-up.

When DTC-related causes of death (n = 27) were analyzed, 25.9% of the patients (n = 7) died of direct complications of BM or their treatment. The remaining 20 died of other complications related to other localizations of DTC (Table 3).

Table 3. Causes of death in 27 patients with bone metastases from differentiated thyroid cancer

Cause of death	Number of patients (%)
Non-BM related	20 (74.1)
Respiratory insufficiency	11
Airway obstruction	2
Central nervous system progression	2
Sepsis	2
Treatment complications	1
Other	4
Secondary to complications of BM	7 (25.9)
Pulmonary thromboembolism	3
Sepsis	1
Treatment complications	1
Other	2

The 2- and 5-year survival rates after the diagnosis of the first BM were 63.5% and 38%, respectively; the overall survival is depicted by the Kaplan-Meier curve in Figure 2.

The median overall survival was 24 months (range 1-120 months).

When we analyzed mortality related to BM (Table 4), the presence of papillary thyroid cancer and painful BM were more frequent in patients who died from causes not related to the BM.

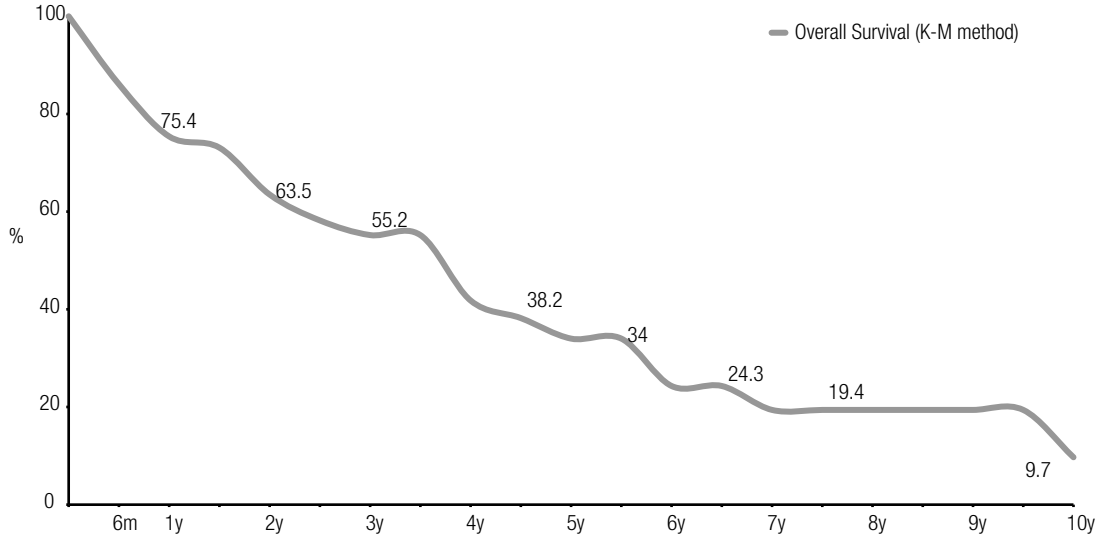
Table 4. Analysis of variables related to mortality in patients who died from bone metastasis compared to those who died from other causes

	Death related to BM (n = 10)	Death related to other causes (n = 19)	P
< 45 years old	2 (20%)	4 (21.1%)	NS
≥ 45 years old	8 (80%)	15 (78.9%)	
Female	4 (40%)	9 (47.4%)	NS
Male	6 (60%)	10 (52.6%)	
Histology			
Papillary	3 (30%)	15 (78.9%)	0.02
Follicular	4 (40%)	4 (21.1%)	NS
Hürthle	3 (30%)	-	0.003
Synchronous	5 (50%)	6 (31.6%)	NS
Metachronous	4 (40%)	13 (68.4%)	
Asymptomatic	4 (40%)	7 (36.8%)	NS
Pain	1 (10%)	10 (52.6%)	0.03
Fracture	2 (20%)	1 (5.3%)	NS
Tumor	2 (20%)	-	
Other	1 (10%)	1 (5.3%)	NS
Positive RAI uptake	4 (40%)	14 (73.7%)	0.08
Solitary	4 (40%)	4 (21.1%)	NS
Multiple	6 (60%)	15 (78.9%)	

RAI: radioiodine; NS: non-significant.

DISCUSSION

Local or distant metastases occur in nearly 10% of patients with DTC (14). In this setting, therapeutic options may include the use of RAI, surgery, and/or the use of external beam radiotherapy (EBRT), among others (15). Nevertheless, between one-third to two-thirds of patients with metastatic DTC will become RAI



y: year; K-M: Kaplan-Meier.

Figure 2. Overall survival after the development of the first bone metastasis in patients with differentiated thyroid cancer (n = 52).

refractory, and this situation is often seen in patients with BM (8). As we observed in our study, most patients fulfill the current criteria for RAI refractoriness (16).

This subgroup of patients generally has a poor overall prognosis, with 10-year survival rates of only 10% and median survival from the discovery of metastases of only 3 to 5 years (8).

DTC has a high tendency to metastasize to bone compared to other tumors; it was reported to be the third most frequent solid tumor after breast and prostate cancer (17), although the physiopathology of BM from DTC is largely uncharacterized.

Follicular thyroid cancer and papillary thyroid cancer show different patterns of spread. The former has a higher propensity to disseminate to bones (7-28% versus 1.4-7% for with papillary thyroid cancer), probably due to a higher frequency of haematogenous dissemination (10,18). However, in absolute terms, in our study we found a greater number of patients with papillary carcinoma and BM, similar to that reported in other series (19). This finding is probably related to the overall higher incidence of papillary thyroid cancer compared to follicular thyroid cancer that, in most current series, comprises only 4 to 7% of the cases.

Skeletal related events (SRE), such as pathological fractures, spinal cord compression, pain and hypercalcemia, are frequent events that adversely affect the QoL of DTC patients with BM (20). As expected, in the present series, 65% of the patients presented symptoms related to BM.

Most of the BM are located in the spine (21) and, as a consequence, spinal cord compression may occur in 14 to 50% of the cases (22,23). In the present series, it was found in 11% of the cases.

Nearly half of the patients in our study presented with pain as the initial symptom. Osseous pain was more frequent in those patients who died due to causes not related to BM (52.6% vs. 10%). It is possible that pain may have led to an earlier BM diagnosis before severe complications occur.

The incidence of hypercalcemia in BM from DTC is variable and has been reported to be as low as 0-4% (19,20,23) to 25-37% (24). We found no cases of hypercalcemia in the present series. Tumoral hypercalcemia is mainly caused by parathyroid-hormone-related peptide secretion (25). Nevertheless, the physiopathology of hypercalcemia occurring in patients with DTC remains to be elucidated.

Considering the treatment modalities, a combination of multiple strategies is generally recommended for patients with BM from DTC (19). Nearly 90% of our patients received more than one option of treatment. In addition to ^{131}I therapy (81%), surgery (50%), EBRT (50%) and bisphophonates (60%) were frequently used. In selected cases, chemotherapy, sorafenib and radiofrequency ablation were also indicated.

RAI was used in 81% of patients in our series; 60% of them showed uptake of ^{131}I in BM. However, only one patient achieved remission after RAI as the only modality of treatment (cumulative activity of 400 mCi ^{131}I). Sabra and cols. demonstrated the lack of oncological benefit in the majority of patients treated with I-131 for distant metastases (9), highlighting that RAI therapy is rarely curative in these patients when used as a solitary therapy. However, an impact on the survival of patients treated with RAI was shown by some authors (19,22,26). Moreover, the complete surgical resection approach followed by RAI therapy has been related to better overall survival (19,27-29).

The complete resection of BM can rarely be achieved when bone disease comprises multiple sites (as was the case in 63% of our series) and the palliative resection of BM has less of an impact on survival (84.4% vs. 55.3%) (29). It should only be considered to obtain a better QoL (30). The mean 5-year survival in DTC patients with BM ranges from 41% to 87% (20,26,28,31). Accordingly, we found that 38% of patients in our series were alive 5 years after the diagnosis of the first BM.

Prognostic factors for cancer-specific mortality in patients with distant metastases from DTC have been widely studied (8,32-34). Most series consistently report that follicular carcinoma, poorly differentiated histology, a lack of radioiodine uptake, advanced age, widespread disease and BM *per se* are predictors of a worse prognosis and shorter overall survival. Poorly differentiated thyroid cancer patients were excluded from the present study; however, all of the remaining risk factors were found in the majority of our population. In this setting, other metastatic sites (such as the lung) or local recurrences are often the immediate causes of death. In the study by Kitamura and cols. (32), the causes of death were respiratory insufficiency (43%), circulatory failure (15%), hemorrhage (15%) and airway obstruction (13%). Nevertheless, in some cases it was not possible to identify the specific cause, due to the simultaneous compromise of several organs.

In the existing literature, the rates of mortality due exclusively to BM are variable and they are usually low (0 to 24%) (6,35,36). In our study, less than one-third of the deaths were directly related to BM. In the remaining cases, death was caused by other complications of advanced DTC, mainly related to respiratory events.

In conclusion, to our knowledge, this is the first multicentric study performed in Latin America on this topic. BM cause significant morbidity and should be managed with a multidisciplinary approach that aims to improve the QoL. Patient selection is important to tailor therapy to individual needs. Further studies with bone targeted agents are needed to assess the utility of these therapeutic modalities in DTC patients. BM are usually RAI refractory and are associated with a decrease in overall survival, although the causes of death are mainly related to complications of non-BM localizations of DTC.

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The relationship between of *ACE* I/D and the *MTHFR* C677T polymorphisms in the pathophysiology of type 2 diabetes mellitus in a population of Brazilian obese patients

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ABSTRACT

Objectives: This study aimed to evaluate the frequencies of the *angiotensin converting enzyme* (*ACE*) gene insertion/deletion (I/D) and *methylenetetrahydrofolate reductase* (*MTHFR*) gene C677T polymorphisms in obese patients with and without type 2 diabetes mellitus (T2DM). **Subjects and methods:** These polymorphisms were analyzed by polymerase chain reaction in 125 patients with obesity, 47 (T2DM) and 78 (Control Group). **Results:** No significant difference was found on comparing the T2DM and Control Groups in respect to the genotypic frequencies of the polymorphisms - (II: 13.3% vs. 12.0%; ID: 37.8% vs. 37.3; DD: 48.9% vs. 50.7%; CC: 36.2% vs. 39.0%; CT: 46.8% vs. 49.3%; TT: 17.0% vs. 11.7%), and alleles (I: 32.2% vs. 30.7%; D: 67.8% vs. 69.3%; C: 59.6% vs. 63.6%; T: 40.4% vs. 36.4%) and their synergisms in the pathophysiology of T2DM. On analyzing the T2DM Group, there were no significant differences in the presence of complications. In this population of Brazilian obese patients, no correlation was found between the *ACE* and *MTHFR* polymorphisms in the development of T2DM. **Conclusion:** Analyzing only the group with diabetes, there was also no relationship between these polymorphisms and comorbidities. Arch Endocrinol Metab. 2018;62(1):21-6

Keywords

Type 2 diabetes mellitus; obesity; angiotensin-converting enzyme gene; methylenetetrahydrofolate reductase gene; polymorphism

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is considered a serious public health problem (1,2). Currently, there are about 415 million diabetics in the world, with more than 14 million in Brazil (3). In addition to the known environmental risk factors such as obesity, there are several genetic changes that are correlated to the pathophysiology of T2DM and its complications (4,5).

As in hypertension, some studies implicate *angiotensin converting enzyme* (*ACE*) gene polymorphisms in the pathogenesis of T2DM, nephropathy and diabetic retinopathy (6,7). The relationship of *MTHFR* polymorphisms associated with hyperhomocysteinemia and changes in the folate cycle is unclear in respect to the development of T2DM with some studies linking these conditions with microvascular complications (8,9).

Epidemiological studies show the relationship of environmental factors in the development of T2DM in the Brazilian population, but few epidemiological studies show the relationship of this metabolic disease with genetic alterations in the same population (10,11), especially in respect to the polymorphisms of the *ACE* and *MTHFR* genes. This study aimed to evaluate the frequency of the insertion/deletion (I/D) polymorphism of the *ACE* gene and the C677T polymorphism of the *MTHFR* gene in a population of obese individuals with and without T2DM. Additionally, the association between these polymorphisms and the occurrence of T2DM and its complications were investigated.

SUBJECTS AND METHODS

Subjects

This study enrolled 125 obese adults, 47 with T2DM and 78 nondiabetic patients (Control Group) from June 2015 to November 2015. Inclusion criteria were male and female patients aged 18 to 65 with a body mass index (BMI) greater than or equal to 30 kg/m^2. The Control Group was matched with respect to gender, BMI and ethnicity (Caucasian and Afro-Brazilian).

Individuals who had two fasting glucose measurements ≥ 126 mg/dL or glycemia levels ≥ 200 mg/dL two hours after ingesting 75g dextrosol or glycated hemoglobin (HbA1c) ≥ 6.5% were considered diabetics according to the criteria of the American Diabetes Association (12). Moreover, patients who were already taking medication to treat diabetes (metformin, sulfonylureas, inhibitor of α-glucosidase, pioglitazone inhibitor, DDP-4, GLP-1 agonists, SGLT-2 inhibitors and insulin) were included in this group.

Microvascular complications, such as diabetic retinopathy (diagnosis by an ophthalmologist), diabetic nephropathy (microalbuminuria, macroalbuminuria or chronic renal failure) and diabetic neuropathy (according to clinical conditions, and physical examination) were also investigated in diabetic patients. Additionally, this study investigated whether the patients had previous diagnoses of hypertension, dyslipidemia or cardiovascular disease (myocardial infarction, stroke and peripheral arterial disease). This research was approved by the Ethics Committee of the Universidade Estadual Paulista "Julio de Mesquita Filho" (UNESP/IBILCE) in São José do Rio Preto, and followed the ethical principles stated in the Declaration of Helsinki. Informed consent was obtained from all participating individuals.

Sample collection

After fasting for 10 hours, 4 mL of peripheral blood were drawn by venipuncture of the upper limbs and placed into tubes containing 5% EDTA in order to extract DNA from leukocytes (13).

I/D polymorphism of the ACE gene

The investigation of the I/D polymorphism of 278 bp (rs1799752) in the *ACE1* gene (17q23.3) was carried out by polymerase chain reaction (PCR) using two amplifications with different primers. The first amplification used the ACE1 (sense) 5'CTGGAGACCACTCCCATCCTTTCT3' and ACE2 (antisense)5'GATGTGGCCATCACATTCGTCAGAT3' primers; the resulting insertion fragment has 490 bp and the deletion fragment has 190 bp. In this first amplification, there is a preference for the insertion allele to amplify as a deletion allele. Thus, samples homozygous for the deletion allele are submitted to a second amplification to identify heterozygous samples. In this second amplification, the ACE3 (sense) 5'TGGCACGACAGCGCCGCCCACTAC3' and ACE4 (antisense) 5'TCGCCAGCCCTCCATGCCCATAAT3' primers are used. The amplified insertion fragment has 355 bp and the deletion fragment is not amplified (14). Both amplifications were analyzed by 2.0% agarose gel electrophoresis under a constant current of 80V for 30 minutes and visualized under ultraviolet (UV) light after staining with ethidium bromide.

C677T polymorphism of the *MTHFR* gene

The PCR-restriction fragment length polymorphism (RFLP) technique was used to investigate the C677T polymorphism (rs1801133) of the *MTHFR* gene. The MTHFR1 (sense) 5'TGAAGAAGGAGGTGTCTGCGG3' and MTHFR2 (antisense) 5'AGGACGGTGCGGTGAGAGTG3' primers were used for amplification generating a 198 bp fragment which was digested using the *HinfI* enzyme (G↓ANTC). The mutation creates a restriction site for the enzyme. The wild homozygous genotype (CC) gives a 198 bp band, the homozygous TT genotype has two bands – one of 175 bp and the other of 23 bp and the heterozygous genotype (CT) gives all three bands (198 bp, 175 bp and 23 bp) (15). The result was analyzed by 4.0% agarose gel electrophoresis under a constant current of 80V for 40 minutes and visualized under UV light after staining with ethidium bromide.

Statistical analysis

Statistical analyzes were performed using the R program, version 3.2.3 (http://www.r-project.org) and p-values < 0.05 were considered significant. Quantitative variables, tested for normal distribution and homogeneity of variances, are expressed as means ± standard deviation. Mean scores between groups were compared using Student's t test and differences in proportions were evaluated using the Fisher exact test or Pearson chi-square test using the Rcmdr

package (16). Allelic and genotypic frequencies and Hardy-Weinberg deviations were evaluated using the SNPassoc package (17). A binary logistic regression model was constructed to evaluate the association of polymorphisms in patients with T2DM in different genetic models of inheritance. The p-value was adjusted for variables using the same logistic regression model using the SNPassoc package (17).

RESULTS

Table 1 shows the characteristics of the obese, T2DM and nondiabetic Groups (Control Group). The T2DM Group had a mean age of 47.8 ± 8.8 years and the mean age of the Control Group was 41.6 ± 11.5 years (p-value = 0.02). There was no significant differences in relation to the gender or ethnicity.

There were no statistical differences in the percentages of subjects with Class I, II and III obesity between the T2DM and Control Groups (p-value = 0.416). However, as noted in the general population, both groups had a higher proportion of patients with Class I obesity compared to Class II and III (p-value < 0.01) (18).

There was no significant difference between the two groups in respect to the number of individuals with hypertension and cardiovascular disease. However, the T2DM Group had a higher frequency of dyslipidemia compared to the Control Group. No patients in the Control Group had retinopathy, nephropathy or neuropathy due to other causes, thus the T2DM Group had more patients with these manifestations.

There was no statistically significant differences in the genotypic and allelic frequencies of the polymorphisms of the *ACE* and *MTHFR* genes between the T2DM and Control Groups (Table 2). Additionally, the genotypes of the polymorphisms evaluated showed no predisposition for elevated risk or protection against the development of T2DM.

Different models of gene inheritance were evaluated to check any predisposition for elevated risk or protection against T2DM by comparing the two groups. The results are shown in Table 3, and according to the findings, none of the analyzed inheritance models (codominant, dominant, recessive and additive log) have any influence on the development of T2DM in this obese population.

Table 1. Demographic and clinical data of the study participants

Characteristic	T2DM (n = 47)	Controls (n = 78)	p-value
Age (mean ± SD)	47.8 (8.8)	41.6 (11.5)	0.020[c]
Male [n (%)]	17 (36.2)	19 (24.4)	0.157[b]
Female [n (%)]	30 (63.8)	59 (75.6)	
Caucasian [n (%)]	39 (83.0)	62 (79.5)	0.631[b]
Afro-Brazilian [n (%)]	8 (17.0)	16 (20.5)	
Obesity [n (%)]			
Class I (BMI: 30 – 34.99)	23 (48.9)	43 (55.1)	0.416[b]
Class II (BMI: 35 – 39.99)	13 (27.7)	24 (30.8)	
Class III (BMI: ≥ 40)	11 (23.4)	11 (14.1)	
Retinopathy [n (%)]	3 (6.4)	0	< 0.05[a]
Nephropathy [n (%)]	7 (14.8)	0	< 0.05[a]
Neuropathy [n (%)]	7 (14.8)	0	< 0.05[a]
Hypertension [n (%)]	24 (51.1)	30 (38.5)	0.168[b]
Dyslipidemia [n (%)]	25 (53.2)	12 (15.4)	< 0.05[b]
Cardiovascular disease [n (%)]	3 (6.4)	1 (1.3)	0.148[a]

T2DM: type 2 diabetes mellitus; SD: standard deviation

[a] Fisher exact test; [b] Pearson chi-square test or chi-square goodness-of-fit test; [c] Student's *t*-test.

Table 2. Genotypic and allelic frequencies of *ACE* I/D and *MTHFR* C667T polymorphisms in patients with type 2 diabetes mellitus (T2DM) and controls

Genotype/ Allele	T2DM	Controls	OR (95% CI)	p-value
ACE (rs1799752)	(n = 45)	(n = 75)		
Genotype				
II	6 (13.3)	9 (12.0)	0.87 (0.27-2.77)	
ID	17 (37.8)	28 (37.3)	0.95 (0.43-2.12)	0.97
DD	22 (48.9)	38 (50.7)	1.00	
Allele				
I	29 (32.2)	46 (30.7)	0.93 (0.53-1.63)	0.88
D	61 (67.8)	104 (69.3)		
MTHFR (rs1801133)	(n = 47)	(n = 77)		
Genotype				
CC	17 (36.2)	30 (39.0)	1.0	
CT	22 (46.8)	38 (49.3)	0.98 (0.44-2.16)	0.71
TT	8 (17.0)	9 (11.7)	0.64 (0.21-1.96)	
Allele				
C	56 (59.6)	98 (63.6)	0.84 (0.49-1.42)	0.59
T	38 (40.4)	56 (36.4)		

95% CI: 95% confidence interval.

Different combinations of polymorphisms in the T2DM and Control Groups were evaluated in relation to possible synergism between the polymorphisms to check any predisposition for elevated risk or protection against T2DM. Significant differences between groups were not found with any combination of polymorphisms (Table 4).

The number of individuals with T2DM as well as microvascular complications (retinopathy, nephropathy or diabetic neuropathy) or other diseases related to metabolic syndrome such as hypertension, dyslipidemia, and cardiovascular diseases was small and no statistically significant difference was found in relation to polymorphisms of the *ACE* and *MTHFR* genes.

DISCUSSION

Brazil has one of the highest number of obese and diabetic patients in the world but few epidemiological studies exist, especially related to the association of genetic polymorphisms with this disease and its

Table 3. Analysis of the association of type 2 diabetes mellitus (T2DM) with *ACE* I/D and *MTHFR* C677T gene polymorphisms in different models of inheritance

Model	Genotype	T2DM	Controls	OR (95% IC)	p-value
ACE (rs1799752)		n = 45	n = 75		
Codominant	II	6 (13.3)	9 (12.0)	0.87 (0.27-2.77)	0.97
	ID	17 (37.8)	28 (37.3)	0.95 (0.43-2.12)	
	DD	22 (48.9)	38 (50.7)	1.00	
Dominant	DD	22 (48.9)	38 (50.7)	1.00	0.85
	ID-II	23 (51.1)	37 (49.3)	0.93 (0.44-1.95)	
Recessive	II	6 (13.3)	9 (12.0)	1.00	0.83
	DD-ID	39 (86.7)	66 (88.0)	0.89 (0.29-2.68)	
Additive log	-----	-------	------	0.94 (0.55-1.59)	0.81
MTHFR (rs1801133)		n = 47	n = 77		
Codominant	CC	17 (36.2)	30 (39.0)	1.0	0.71
	CT	22 (46.8)	38 (49.4)	0.98 (0.44-2.16)	
	TT	8 (17.0)	9 (11.7)	0.64 (0.21-1.96)	
Dominant	CC	17 (36.2)	30 (39.0)	1.0	0.76
	CT-TT	30 (63.8)	47 (61.0)	0.89 (0.42-1.88)	
Recessive	TT	8 (17.0)	9 (11.7)	0.65 (0.23-1.81)	0.41
	CC-TT	39 (83.0)	68 (88.3)	1.00	
Additive log	-----	-------	------	0.84 (0.49-1.43)	0.52

95% CI: 95% confidence interval.

Table 4. Association analysis of type 2 diabetes mellitus (T2DM) using combinations of polymorphisms (*ACE* I/D and *MTHFR* C667T)

Combination	T2DM (n = 45)	Controls (n = 74)	OR (95% IC)	p-value
DD/CC	8 (17.8)	15 (20.3)	0.85 (0.33-2.20)	0.74
DD/CT	12 (26.7)	18 (24.3)	1.13 (0.48-2.64)	0.77
DD/TT	2 (4.4)	5 (6.8)	0.64 (0.12–3.45)	0.63
ID/CC	7 (15.6)	9 (12.2)	1.31 (0.45-3.86)	0.59
ID/CT	7 (15.6)	15 (20.3)	0.72 (0.27-1.94)	0.52
ID/TT	3 (8.8)	3 (3.5)	2.46 (0.51-13.81)	0.35
II/CC	1 (2.2)	5 (6.8)	0.31 (0.03-2.77)	0.41
II/CT	2 (4.5)	3 (4.0)	1.14 (0.18-7.12)	0.89
II/TT	3 (36.2)	1 (39.0)	5.2 (0.53-51.74)	0.15

95% CI: 95% confidence interval.

complications (3,10,11,18). Two groups of obese patients, with and without T2DM, were matched with respect to gender, BMI and ethnicity (Caucasian and Afro-Brazilian). The main objective of this study was to evaluate the frequency of polymorphisms (ACE I/D and MTHFR C677T) and their influence on the pathophysiology of T2DM and microvascular complications in individuals with a BMI ≥ 30 kg/m^2.

According to the findings of this study, the *ACE* I/D and *MTHFR* C677T polymorphisms are not involved in the development of T2DM in this population. Studies had previously correlated these polymorphisms, especially the I/D polymorphism of the *ACE* gene, with T2DM (4-9) even in Indian women with gestational diabetes (19). These polymorphisms have also been implicated in the development of microvascular complications linked to diabetes (20-22). However, studies in Caucasian populations show no significant relationship with the polymorphism of the *ACE* gene (23). Although the Brazilian population is multiethnic, most of the participants of this study (80.8%) were Caucasians, which supports this finding.

Recent studies point to a synergism between different polymorphisms as the triggering factor for diseases that have a genetic component in their pathogenesis, such as T2DM. Although some studies point to this effect on the development of diabetes, including synergism between the *ACE* I/D and *MTHFR* C677T polymorphisms (24), our study found no significant difference between the patients and controls. Moreover, in the group of patients with T2DM, we did not find significant differences in the analysis of these polymorphisms in relation to microvascular complications and comorbidities such as hypertension, dyslipidemia and cardiovascular disease.

One study reported a higher frequency of the D allele in patients with T2DM and cardiovascular disease and the DD genotype had higher risk for these individuals to present with a cardiovascular event (25). Another study in patients with coronary artery disease, with and without diabetes, showed that the DD genotype is associated with poor coronary collateral circulation, which implies worse ischemic conditioning during acute myocardial infarction (26). The frequencies of the D allele and the DD genotype of the *ACE* gene were higher in both groups pointing to an increased cardiovascular risk probably due to obesity and not T2DM. However, these results should be interpreted with caution, as the numbers of individuals previously diagnosed with cardiovascular disease was very small in both groups.

One study of a cohort of Brazilian subjects did not find any association between the I/D polymorphism of the *ACE* gene and patients with T2DM and metabolic syndrome according to the criteria of the World Health Organization (27), and there are no studies reporting the risk of individuals with this polymorphism developing diabetes. Although there is a Brazilian study that correlates homocysteine levels in patients with and without T2DM to the polymorphism of the *MTHFR* gene (28), there are no studies showing any association of this gene with the risk of developing diabetes in the same population.

Even with the two matched groups, despite the higher age in the T2DM group and more patients with hypertension (not statistically significant) and dyslipidemia (with statistical significance) compared to the Control Group, the number of participants was small in this study and because only patients with obesity were selected, this evaluation did not include overweight patients with metabolic problems.

In conclusion, no association was found between the polymorphisms of the *ACE* and *MTHFR* genes and the development of T2DM in this population. Additionally, no evidence of synergism between the polymorphisms of these two genes and the emergence of diabetes was found. Our group did not find any relationship of polymorphisms of the *ACE* and *MTHFR* genes with microvascular complications and diseases associated to the metabolic syndrome (hypertension, dyslipidemia and cardiovascular diseases) in the group of T2DM patients. This is the first multiethnic Brazilian population study to evaluate polymorphisms of the *ACE* and *MTHFR* genes in obese individuals but our cohort is too small to confirm the data in view of the heterogeneity of the Brazilian population.

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Expression of miR-155, miR-146a, and miR-326 in T1D patients from Chile: relationship with autoimmunity and inflammatory markers

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ABSTRACT

Objective: The aim of this research was to analyze the expression profile of miR-155, miR-146a, and miR-326 in peripheral blood mononuclear cells (PBMC) of 47 patients with type 1 diabetes mellitus (T1D) and 39 control subjects, as well as the possible association with autoimmune or inflammatory markers. **Subjects and methods:** Expression profile of miRs by means of qPCR using TaqMan probes. Autoantibodies and inflammatory markers by ELISA. Statistical analysis using bivariate correlation. **Results:** The analysis of the results shows an increase in the expression of miR-155 in T1D patients in basal conditions compared to the controls ($p < 0.001$) and a decreased expression level of miR-326 ($p < 0.01$) and miR-146a ($p < 0.05$) compared T1D patients to the controls. miR-155 was the only miRs associated with autoinmmunity (ZnT8) and inflammatory status (vCAM). **Conclusion:** Our data show a possible role of miR-155 related to autoimmunity and inflammation in Chilean patients with T1D.
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Keywords
miRNAs; inflammation; type 1 diabetes; autoimmunity

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease triggered by T cells that destroy pancreatic beta cells. This destruction takes place by means of a complex interaction between active lymphocytes, cytokines, and macrophages (1). During the initial step of the disease, β cells are exposed to high levels of cytokines that cause the activation of the immune system and trigger the insulitis process. This inflammatory environment results in β cell damage, decreased insulin production, and the consequent destruction of β cells through apoptosis (2).

The first indication that miRNAs may be involved in regulating the β cell function was the identification of miRNAs specifically expressed in human pancreatic islets – miR-375 and miR-376 (3). In the last decade, a number of miRNAs have been described that are capable of regulating pancreatic function (4).

The expression of miRNAs may be induced by a variety of stimuli-including cell stress and inflammation, which either induce or suppress its expression in response to different stimuli, which may influence some biological processes and have pro- or anti-inflammatory effects (5) – such as hyperglycemia in patients with T1D – which increases the inflammatory response by increasing cytokines. This effect is associated with increased expression of Toll receptors (6,7), and has been correlated with studies on PBMC cultures stimulated with high glucose concentrations, which showed an increase in the expression levels of TNF-α, IL-1β, and IL-6 (8). It has been shown that stimulation by TNF-α induces the expression of certain miRNAs, including miR-146a and miR-155, which affect the pathogenesis of some diseases such as rheumatoid arthritis (6,9). Studies show the involvement of miR-155 in the activation and maturation of T and B lymphocytes. This is why it has been associated with many autoimmune diseases, such as rheumatoid arthritis; thus, an increase in the expression level of

this miRNA is observed both in fibroblasts and in the PBMC of patients with this disease (10). miR-146a and miR-155 are described to be altered in T lymphocytes of patients with rheumatoid arthritis (9). miR-326 is observed to be altered in PBMC of multiple sclerosis patients (10) and shows higher expression levels in T1D patients from Italy (11,12). The aim of this study was to analyze the expression levels of the miRNAs miR-146a, miR-155, and miR-326 in PBMC from T1D and healthy patients, and to estimate their possible relationships with inflammatory or autoimmunity status in Chilean children with T1D.

SUBJECTS AND METHODS

Subjects

This study involves 47 T1D patients aged 6–11 years from the metropolitan region of Santiago in Chile, recruited from the Institute of Maternal and Child Research (IDIMI) of the San Borja Arriarán Hospital. T1D was diagnosed based on the American Diabetes Association (ADA) criteria. In all cases, a survey was applied to gather the patient's family medical and clinical history. The presence of possible chronic complications in T1D patients was corroborated through a survey and through the hospital clinical history; this included normal renal function (microalbuminuria) and normal eye fundus. In addition, 39 samples from healthy individuals (control group) aged 13–30 years were used. During the blood sample collection, patients and controls who declared the presence of previous febrile state (three days) or some inflammatory process were excluded from the study. The blood samples of T1D patients and controls were collected in the hospital after an informed consent was signed by parents of patients younger than 10 years and/or directly by patients older than 10 years. This study has been approved by the Ethics Committee of IDIMI and Faculty of Medicine, University of Chile.

Extraction and culture of PBMC

The 10 ml of drawn blood was diluted with phosphate buffered saline (PBS) at a ratio of 1:1 to facilitate the handling of the sample. The PBMC was extracted and incubated, as previously described (13).

Extraction of total RNA and miRNAs analysis

Total RNA extraction was performed using the TRIZOL method (Invitrogen) following the manufacturer's instructions. Single-stranded cDNA was synthesized from 300 ng of total RNA taken at dilutions of 2–10 ng of RNA in each sample. To assess the relative expression of miRNAs, stem-loop RT real-time PCR was performed (Applied Biosystems, Foster City, CA, USA) with specific primers for each miRNA. Expression levels were determined using TaqMan MGB probes and TaqMan Universal PCR Master Mix II (2x) in triplicate in an equipment from Agilent Technologies (CA, USA). The expression levels of miRNAs – miR-155, miR-146a, and miR326 – were normalized to a small RNA called RNU48, as an internal control.

Serological analysis

Anti-GAD65, anti-IA2, and anti-ZnT8 antibodies were determined through enzyme immunoassay (ELISA) using the Medizym commercial kits (Berlin, Germany). Antibody detection was carried out semi-quantitatively through reference to the value of 5 IU/mL for GAD65, 10 IU/mL for IA2, and 15 IU/ml for ZnT8. The analysis of inflammatory markers included human ultrasensitive C Reactive Protein (usCRP, BioVendor, Czech Republic) and the measurements of TNFα, IL-6, vCAM, and C-peptide concentrations determined by ELISA (R&D Quantikine Human ELISA Assay, UK). HbA1c levels were measured using a commercially available automatic system (DCA 2000, Bayer Diagnostics, Tarrytown, NY, USA).

Statistical analyses

We used the REST© (Relative Expression Software Tool) program, designed especially for analyzing the results of qPCR using the Pfaffl equation. Afterwards, tests were performed to evaluate the statistical significance or non-significance of the results, regarding the variations in expression observed between patients and controls. All subsequent calculations were performed using the Graph Pad Prism 6 (Graph Pad Software, Inc. San Diego CA, USA). The Shapiro-Wilk normality test was used and the effect of glucose was studied in GraphPad using the Kruskal-Wallis test. To determine the relationship between gene expression and clinical records, the bivariate correlation test was used. A p value of < 0.05 was considered as statistically significant.

RESULTS

Table 1 describes the clinical, immunological, and inflammatory characteristics of all individuals included

in this study. T1D patients showed a high pattern of autoimmunity and a pattern of 28% inflammation by mean of usCRP over 3 mg/dL. This was not observed in the control group. All controls subjects tested negative to autoantibodies profile. TNFα, usPCR, IL-6, and vCAM were significantly elevated in T1D patients compared to control subjects.

Overall, miR-155 expression was significantly higher in T1D patients than in controls (Figure 1A). On the contrary, miR-326 and miR-146a expressions were lower in T1D subjects (Figures 1B and 1C). In order to find relationships in miRNAs expression regarding autoantibody and inflammatory profile in T1D patients, 2x2 ANOVA was performed (Figures 2, 3 and 4). Regarding miR-155, only a significant interaction between Znt8 low or high titer and VCAM low or high expression was observed ($p < 0.01$), presenting the Znt8 H/VCAM H (the higher) and the Znt8 L/VCAM L (the lower) miR-155 expression (Figure 2A). On the other hand, miR-326 presents a significant interaction when contrasted in the presence of at least two positive autoantibodies in serum with either low/high IL6 or VCAM presence (Figures 3B and C), although these interactions seem to have come from a different pattern of expression between factors (lower expression at IL-6 H and higher at VCAM H in the two positive autoantibody conditions, although no significant differences between groups were found). Finally, miR-146a expression only showed a tendency toward a higher expression induced by higher IL-6 presence (Figures 4B and D). This is especially significant when the sample is dichotomized in presence of three positive autoantibodies (Figure 4D).

Table 1. Clinical, immunological and inflammatory parameters in T1D patients and controls

	T1D patients (n = 47)	Healthy controls (n = 39)	p-value
Age (years)	15.5 ± 3.9	19.5 ± 7.7	NS
BMI (kg/m^2)	23.8 ± 3.3	25.6 ± 3.2	NS
Glycemia at debut (mmol/L)	31.3 (17.7 – 58.3)	-	-
HbA1c (%)	8.6 (6.7 – 15.5)	-	-
C-peptide (pmol/L)	94 ± 29	762 ± 314	0.01
Disease duration (years)	3.4 ± 1.9	-	-
Chronic complications*	Negative	-	-
Positive anti-ZnT8 (%)	67	Negative	-
Positive anti-GAD65 (%)	76	Negative	-
Positive anti-IA2 (%)	81	Negative	-
TNF-α (pg/mL)	4.2 ± 1.6	2.4 ± 1,3	0.01
usCRP (ng/mL)	1.71 (0.19 – 14.1)	1.28 (0.4 – 2.7)	0.03
IL-6 (pg/mL)	2.16 (0.93 – 5.61)	0.87 (0.72 – 1.44)	0.05
vCAM (ng/mL)	276.5 (101.6 – 567.9)	139.4 (91.7 – 349.2)	0.01

* Renal function (normal microalbuminuria); diabetic retinophaty: eye fundus examination.

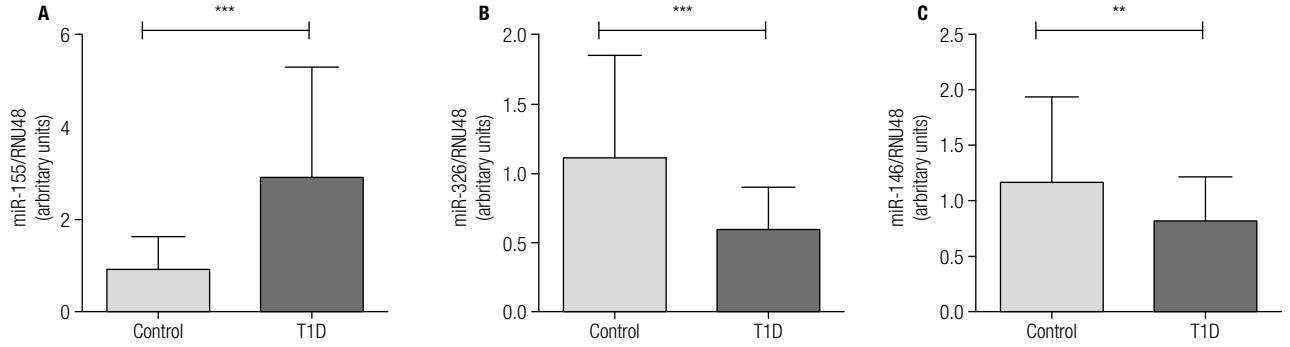

Figure 1. Expression of miR-146a (a); miR-55 (b) and miR-326 in control subjects (n = 37) and T1D patients (n = 47) in baseline conditions. Kruskal Wallis, Dunn post hoc test. ** $p < 0.01$; *** $p < 0.001$.

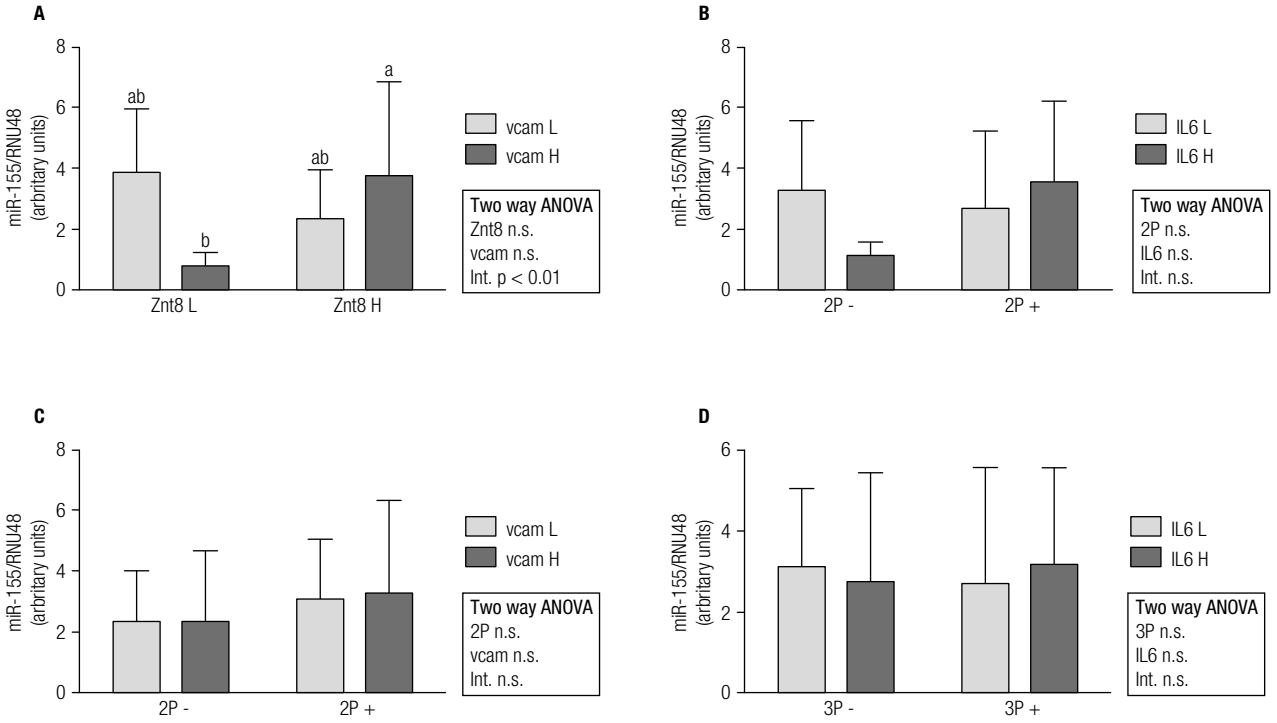

Figure 2. miR-155 gene expression and relationship with autoimmune and inflammatory status in T1D patients (L = low; H = high; 2P- = two negative autoantibodies; 2P+ = two positive autoantibodies; 3P- = three negative autoantibodies; 3P+ = three positive autoantibodies).

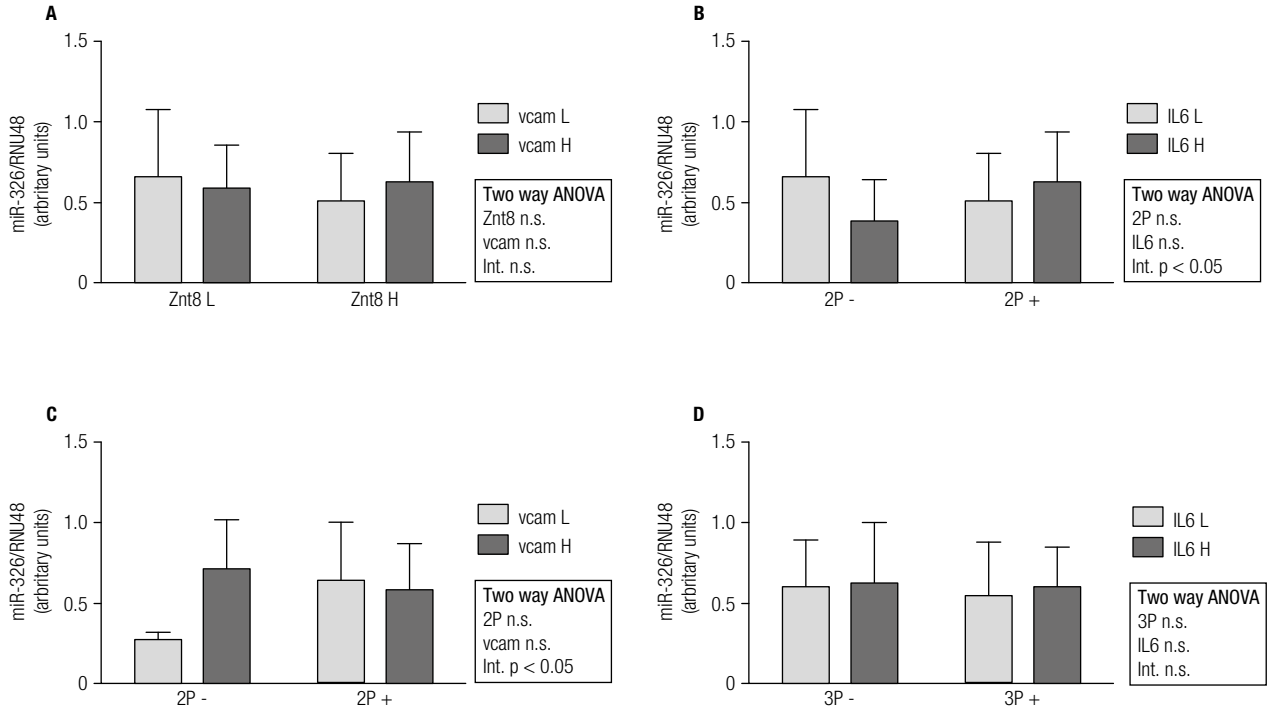

Figure 3. miR-326 gene expression and relationship with autoimmune and inflammatory status in T1D patients (L = low; H = high;2P- = two negative autoantibodies; 2P+ = two positive autoantibodies; 3P- = three negative autoantibodies; 3P+ = three positive autoantibodies).

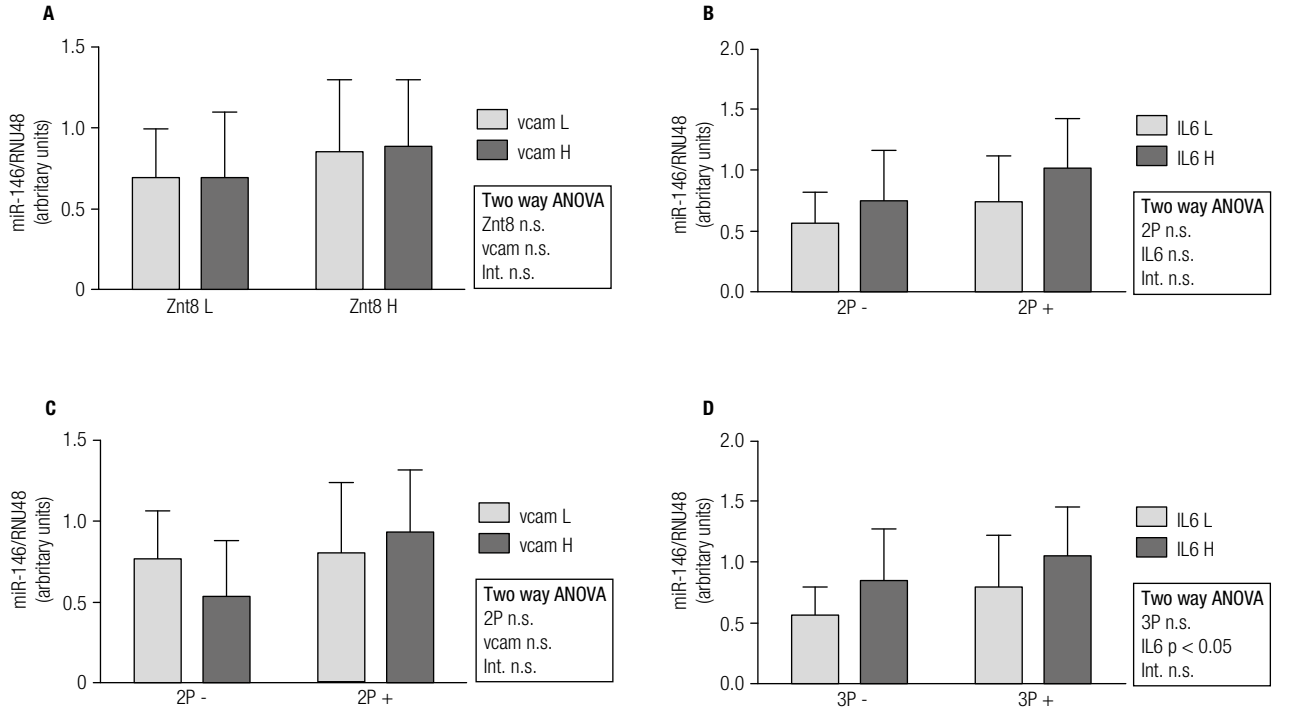

Figure 4. miR-146a gene expression and relationship with autoimmune and inflammatory status in T1D patients (L = low; H = high; 2P- = two negative autoantibodies; 2P+ = two positive autoantibodies; 3P- = three negative autoantibodies; 3P+ = three positive autoantibodies).

DISCUSSION

Despite the massive expansion in miRNAs studies and extensive investigation in several diseases, the role of miRNAs in T1D has only recently been explored. T1D – an eminent autoimmune disease – suggests a possible connection between these miRNAs and the immune system components.

The relationship between miRNAs and the various components of the immune system has been addressed in different autoimmune pathologies previously. miR-155 is related with the immune response of macrophages to different types of inflammatory mediators, such as TNF-α, which can induce the expression of miR-155 in macrophages and monocytes (14,15). miR-146a is associated with innate immunity and inflammation (16). In mice, these miRNAs have shown a deficiency in accordance with cytokine production after LPS stimulation (17,18). miR-146a acts by stimulating TLR4 toll-like receptors that activate TRAF6 and IRAK1 and genes that control cytokine production, thus suggesting that miR-146a participates in regulating cytokines release (19). miR-146a expression has been described as decreased in patients with T1D and is associated with high levels of GADA (20). In our previous studies, we analyzed several miRNAs in T1D and control subjects. We obtained a different expression profile in PBMC submitted to increase concentrations of glucose (13,21). The above point opens up the possibility of searching miRNAs that are capable of sensing subtle changes in glucose profiles.

It is known that hyperglycemia increases the production of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, which act by way of NF-kB (22). The medium in which T cells are found in T1D patients is a hyperglycemic environment leading to a sustained inflammatory state. This altered environment in which T cells are found could change the expression of some miRNAs (12,14). miR-155 is a microRNA that has been previously studied in rheumatoid arthritis, where there has been an increase in the expression levels (14), as well as in the PBMC of patients with systemic lupus erythematosus (SLE) (23). miR-155 is also a miRNA that is linked to inflammation and acts through the signaling of Toll receptors, which activate TAB 2, Ik-B and whose final objective is NF-kB, which is responsible for producing pro-inflammatory cytokines such as TNF-α and IL-1β (13). TNF-α is a potent inflammatory mediator produced by T cells; it has been linked to T1D and it is over-expressed in the inflammatory phenomena (insulitis). This inflammatory phase is characterized by an infiltrate composed mainly

of CD4 and CD8 T lymphocytes β cells (24). In our study, the basal expression of miR-155 is elevated in T1D patients compared than control subjects. Our results are similar to what has been reported in viral myocarditis in myocardial cells, where the increased expression of miR-155 has been described (25). The low expression of miR-146a observed in T1D patients could be associated with an overproduction of inflammatory cytokines, as observed in studies with mice, which show that the decrease in the expression levels of miR-146a is associated with an excessive increase in proinflammatory cytokines (TNF-α and |IL-6) in response to LPS or cytokines, as in the case of sepsis and asthma (26). Our observation is consistent with the effect that occurs in vesicular stomatitis, where the low expression of miR-146a induces the production of pro-inflammatory cytokines such as TNF-α, |IL-1β, and IL-8. All these antecedents make us presume that miR-146a could regulate inflammation through a negative feedback through NF-kB to maintain a controlled immune response (15).

Regarding miR-326, in 2011, Sebastiani and cols. (12) reported a high positive correlation between this miRNA and autoimmunity. However, this analysis was carried out only for a group of T1D patients, without comparing their miRNA levels with that of healthy subjects, as was done in the work of Du and cols. (27) on multiple sclerosis. Our study shows differences between miR-326 expression between T1D patients and controls, and no relationship based on the number of positive autoantibodies. Finally, our study shows a tendency for possible relationships between the expression of miR-22 and ZnT8 antibodies among a group of patients who tested positive for this autoantibody, which could indicate an association effect. A recent study reports that 32 miRNAs located in the same genomic region (Chromosome 14q32) could act on the mRNA of several T1D autoantigens; 12 of these miRNAs were sensitive to changes in glucose. This study shows no data on ZnT8 (28). The relationship between miRNAs and the environmental factors (virus, diet) – related with the T1D and the immune system regulation – is a field of research that is currently being explored (29).

Our study describes an increase in the expression of miR-155 and a decrease in the expression of miR-146a and miR-326 in T1D patients, compared to control subjects. A possible interaction was observed between miR-155 and ZnT8 autoantibody, but no interaction was described to inflammatory status in T1D (related with vCAM and IL-6 levels). Regarding our cell model, PBMC represent a diverse population of cells; as such, each distinct cell type may have a unique miRNA expression profile. Finally, an important aspect to be considered in our study is the age of the disease among patients with T1D. This study includes young patients with a short evolution of their disease. In general, during this period, the patients have a metabolically stable picture. Our findings should be interpreted with caution if we consider advanced stages of the disease. Several microRNAs have been linked to complications of T1D. There is evidence of changes in proinflammatory cytokines and oxidative stress in these patients, consistent with changes in glycemic stability and with changes in the miRNAs profile in according with long-standing hyperglycemia (30,31). This would be an interesting point for corroboration in future studies, with cell subpopulations to establish the true benefits and limitations of circulating miRNA as biomarkers of T1D.

Statement of human and animal rights: all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Statement of informed consent: informed consent was obtained from all patients for inclusion in the study.

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Increased insulin sensitivity in individuals with neurofibromatosis type 1

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ABSTRACT

Objects: To compare insulin resistance (IR) and metabolic aspects of patients with neurofibromatosis type 1 (NF1) and individuals without the disease. **Subjects and methods:** Forty patients with NF1 were matched by sex, age, and body mass index (BMI) to 40 controls from the community. Blood samples were collected for biochemical assessment. Homeostasis model assessment adiponectin (HOMA-AD), Homeostasis model assessment insulin resistance (HOMA-IR), and adiponectin/leptin ratio (ALR) were used to identify IR. **Results:** The median HOMA-IR values were similar between the groups. However, the HOMA-AD value was significantly lower and the ALR significantly higher in the NF1 group. Fasting blood glucose (FBG), leptin, and visfatin levels of patients with NF1 were significantly lower, although adiponectin levels were significantly higher than those in the controls. Fasting insulin and blood glucose levels 2 hours after administration of 75 g of dextrose, glycated hemoglobin, and resistin showed no significant differences between groups.The HOMA-AD correlated with BMI, FBG, blood glucose levels 2 hours after administration of 75 g of dextrose, fasting insulin, glycated hemoglobin, adiponectin, leptin, visfatin, ALR, and HOMA-IR. The ALR correlated with BMI leptin, visfatin, and adiponectin. **Conclusions:** Lower levels of FBG, leptin, visfatin, and HOMA-AD, and higher adiponectin levels and ALR may be related to increased insulin sensitivity and lower occurrence of type 2 diabetes mellitus in patients with NF1. Arch Endocrinol Metab. 2018;62(1):34-9

Keywords
Blood glucose; neurofibromatosis 1; diabetes mellitus; type 2; insulin resistance

INTRODUCTION

A rare disease is generally defined as a disease with a very low prevalence (1). Although considered individually rare, as a group, these diseases affect a significant percentage of the population and present a relevant health problem (2). Therefore, research is needed to better understand these diseases.

Neurofibromatosis type 1 (NF1) is a rare, autosomal-dominant disorder caused by inherited or new mutations on chromosome 17. These mutations result in dysfunction of the neurofibromin protein, which is involved in growth control and behavior of various tissues (3). It is one the most common human monogenic diseases, with an estimated prevalence of approximately 1: 3,500 births (4).

The clinical NF1 criteria include *café-au-lait* spots, neurofibromas, axillary and/or inguinal freckling, Lisch nodules in the iris, optical gliomas, specific bone dysplasia, and familial history (5). NF1 is a multisystem disease, affecting the musculoskeletal, cardiovascular, endocrine, and central and peripheral nervous systems as well as learning skills (6).

Data suggest a lower prevalence of type 2 diabetes mellitus (T2DM) in patients with NF1 (7,8). Madubata and cols. (9) showed that patients with NF1 had a lower chance of having DM compared to healthy individuals. Moreover, Martins and cols. (10) reported that patients with NF1 showed significantly lower fasting blood glucose (FBG) levels compared to controls without the disease.

T2DM (90% to 95% of all cases of diabetes) is associated with insulin resistance (IR) (11). The hyperinsulinemic euglycemic clamp technique is considered the gold standard to assess IR; however, it is a complex and expensive method that is often not available for studies and clinical practice (12). Another

IR validated index is the homeostasis model assessment for insulin resistance (HOMA-IR) (13). This index shows a strong correlation with hyperinsulinemic euglycemic clamp findings (14). However, there is some debate regarding its ability to detect IR in individuals with normal glucose or impaired glucose tolerance (15).

Matsuhisa and cols. (16) developed a modified HOMA index, the HOMA-adiponectin (HOMA-AD), by including adiponectin levels in the denominator. This modification makes the HOMA index a more sensitive marker of IR (16,17).

Mediators produced by adipose tissue, called adipocytokines (leptin, visfatin, resistin, and adiponectin) affect glucose homeostasis, appetite regulation, inflammation, and atherosclerosis and are associated with IR (18). Studies have shown increased levels of leptin, visfatin, and resistin in individuals with T2DM (18). In contrast, adiponectin levels are reportedly lower in patients with DM and metabolic syndrome (19) and studies have shown that their levels may be markers of the risk of prediabetes (20).

In addition, some studies have suggested that the adiponectin-leptin ratio (ALR) may be an effective parameter for the assessment of IR in individuals with or without diabetes (21).

The results of previous studies lead us to hypothesize that patients with NF1 may have (1) genetic differences that modify glucose utilization and cellular control and/or (2) phenotypic characteristics that result in increased insulin sensitivity, favoring the maintenance of lower levels of FBG and reducing the risk of developing T2DM. Thus, this study aimed to evaluate IR and metabolic aspects of patients with NF1 compared with control individuals without the disease.

MATERIALS AND METHODS

Population

The study included patients over 20 years of age with NF1 followed at the Reference Center on Neurofibromatoses at the Federal University of Minas Gerais (CRNF-UFMG) who met at least three criteria for the diagnosis of disease according to the National Institute of Health (22).

The control group consisted of volunteers from the community (e.g., university students, CRNF-UFMG officials, and companions of patients seen at the clinic) who were similar in terms of sex, age, and BMI to the included patients with NF1.

Individuals diagnosed with diabetes, liver disease or malignancy, and infection as well as those using steroids, antibiotics, statins, insulin, and oral hypoglycemic agents were excluded from both groups.

Sample

The sample size was calculated based on the results of the study by Martins and cols. (10), and we used a standard deviation of 13 units, a least significant difference of 10 units, and power of 90%, resulting in the requirement for a minimum of 37 participants in each group.

Ethical aspects

The study was approved by the Ethics and Human Research of the UFMG (number 258.325, year 2013) and all participants signed an informed consent form.

Procedures

Weight and height of the participants were measured, and BMI calculated based on protocols established by the World Health Organization (23).

We assessed the physical activity level by the International Physical Activity Questionnaire (24) and the diet by means of a 3-day non-consecutive dietary record. For each record, the total amounts of the following components were calculated using the food composition tables: calories, carbohydrates, lipids, proteins, fiber, cholesterol, saturated fatty acids, monounsaturated and polyunsaturated fatty acids, zinc, magnesium, selenium, and vitamin D.

Blood samples were collected from the participants for analysis of FBG, blood glucose 2 hours after administration of 75 g of dextrose, glycated hemoglobin (HbA1c), and fasting insulin, adiponectin, leptin, visfatin, and resistin according to the protocol used at the CRNF-UFMG.

Blood glucose and glycosylated hemoglobin (HbA1c) levels were measured using the Vitros equipment and reagents from Ortho-Clinical Diagnostics® (blood glucose coefficient of variation [CV] inter-assay, level 1: 2.24% and level 2: 2.23%; HbA1c CV, level 1: 5.11% and level 2: 6.04%). Insulin levels were measured using the Architect-Abbott® equipment, with Abbott® reagents (CV inter-assay, level 1: 4.4, level 2: 2.9, and level 3: 3.2).

The leptin assay was performed using the human leptin enzyme-linked immunoassay (ELISA) kit from Millipore (CV inter-assay: 3.7%; CV intra-assay: 4.4%, respectively). Resistin levels were measured using the human resistin ELISA from eBioscience® (CV inter-assay: 8.1%; CV intra-assay: 5.1%). Adiponectin levels were measured using the human adiponectin ELISA kit from eBioscience® (CV inter-assay: 3.1%; CV intra-assay: 4.2%, respectively). Visfatin was assayed using the human visfatin ELISA kit from MyBioSource® (CV < 10%). The assays were performed according to the kit protocols.

FBG, HbA1c, and glucose levels 2 hours after administration of 75 g of dextrose were categorized according to the cutoff values established by the American Diabetes Association (11).

The markers of IR included the HOMA-IR (HOMA-IR = fasting insulin (U/mL) × FBG (mmol/L)/22.5), HOMA-AD (HOMA-AD = fasting insulin (U/mL) × FBG (mmol/L)/Adiponectin (μ/mL) and ALR.

Statistical analysis

Continuous variables were described as means and standard deviations or as medians and 25th and 75th percentiles as appropriate. Categorical variables were described as absolute and relative frequencies.

The Kolmogorov-Smirnov test was used to test the normality of the variables. Paired t-tests were used to compare groups with normally distributed variables. The Wilcoxon test was used to compare groups with non-normal variables distribution. McNemar's test was used to compare proportions between two groups. When McNemar's test could not be used, we used chi-square or Fisher's exact tests.

The Spearman correlation test was used to verify the correlations between HOMA-AD and ALR and other variables.

To determine whether the differences and associations were statistically significant, we used a 5% level of significance. Thus, P-values ≤ 0.05 were considered to represent statistically significant differences.

Data were analyzed using the Statistical Package for Social Science (SPSS) version 13.0.

RESULTS

We evaluated 47 patients with NF1 and 47 controls. Seven individuals were excluded from the NF1 group (one because of the use of corticosteroids, one because of suspicion of malignancy, and five owing to unavailability of blood test results). The respective controls were also excluded, finally amounting to 40 participants in each group.

The general characteristics of patients with NF1 and controls are described in Table 1. The average weight and the median height were significantly lower in the NF1 group.

Table 1. General characteristics of patients with NF1 and controls

Variables	NF1 (n = 40)	Controls (n = 40)	P-value
Sex[a]			
Female	28 (70.0)	28 (70.0)	1.000[d]
Male	12 (30.0)	12 (30.0)	
Age[b]	40.7 (11.8)	40.4 (12.1)	0.390[e]
Weight (kg)[b]	60.6 (13.1)	68.5 (14.5)	< 0.001[e]
Height (m)[c]	1.57 (1.51–1.63)	1.62 (1.57–1.74)	< 0.001[f]
BMI (kg/m^2)[c]	24.2 (21.6–26.2)	24.6 (22.3–26.5)	0.096[f]
Physical activity level*			
Not active	9 (22.5)	14 (35)	0.343[d]
Not very active	3 (7.5)	5 (12.5)	
Moderately active	28 (70)	21 (52.5)	

[a] n (%); [b] Mean (standard deviation); [c] Median (percentiles 25-75); [d] McNemar's test; [e] Paired t-test; [f] Wilcoxon Test; BMI: body mass index. *According to the International Physical Activity Questionnaire (25).

There were no statistically significant differences between the NF1 and control groups regarding the intake of any of the evaluated nutrients.

Table 2 shows the IR markers and metabolic characteristics of patients with NF1 and controls. The HOMA-IR values were similar between groups. However, the HOMA-AD value was significantly lower and ALR was significantly higher in the NF1 group. The FBG, leptin, and visfatin levels in patients with NF1 were significantly lower than those in controls. The adiponectin levels was significantly higher in the NF1 group. No significant differences were observed between groups in the levels of resistin, post dextrose glucose, HbA1c, and fasting insulin.

The HOMA-AD values were correlated with BMI, FBG, post dextrose glucose, fasting insulin HbA1c, adiponectin, leptin, and visfatin levels, as well as ALR and HOMA-IR values (Table 3).

The ALR correlated with BMI (r = -0.239, p = 0.033), adiponectin (r = 0.652, p < 0.001), leptin (r = -0.869, p < 0.001) and visfatin levels (r = -0,329, p = 0,003).

Table 2. Insulin resistance markers and metabolic characteristics of patients with NF1 and controls

Variables	NF1 (n = 40)	Controls (n = 40)	P-value
HOMA-IR[a]	1.1 (0.7-1.6)	1.3 (0.9–2.3)	0.147[c]
HOMA-AD[a]	1.0 (0.5–1.7)	1.9 (1.0–4.1)	0.001[c]
ALR[a]	3.8 (2.1–12.6)	1.2 (0.7–2.1)	< 0.001[c]
FBG (mg/dL)[a]	83.5 (78.0–90.0)	86.0 (83.0–94.0)	0.008[c]
FBG classification[b]			
Normal (< 100 mg/dL)	40 (100.0)	35 (87.5)	0.027[d]
Impaired (≥ 100 mg/dL)	0	5 (12.5)	
Glucose level postdextrose administration (mg/dL)[a]	96.5 (85.3–112.5)	99.5 (88.0–118.3)	0.255[c]
Glucose level postdextrose administration classification[b]			
Normal (< 140 mg/dL)	38 (95.0)	34 (85.0)	0.219[e]
Impaired (≥ 140 mg/dL)	2 (5.0)	6 (15.0)	
HbA1c (%)[a]	5.2 (5.0–5.5)	5.4 (5.0–5.7)	0.100[c]
HbA1c classification[b]			
< 5.7%	30 (75.0)	26 (65.0)	
5.7–6,4%	10 (25.0)	14 (35.0)	0.503[e]
≥ 6.5%	0	0	
Fasting insulin (µU/mL)[a]	4.9 (3.6–11.6)	6.0 (4.4–15.7)	0.357[c]
Adiponectin (µg/mL)[a]	23.7 (17.0–39.4)	15.3 (11.3–20.9)	0.001[c]
Leptin (ng/mL)[a]	5.7 (3.1–11.7)	11,9 (6.8–20.7)	0.042[c]
Resistin (ng/mL)[a]	6.8 (3.4–9.0)	5,8 (2.4–9.8)	0.490[c]
Visfatin (ng/mL)[a]	118.2 (105.8–124.8)	138.2 (125.5–147.7)	< 0.001[c]

[a] Median (percentiles 25 and 75); [b] n (%); [c] Wilcoxon test; [d] Fisher's exact test; [e] McNemar's test. HOMA-IR: Homeostasis Model Assessment Insulin Resistance; HOMA-AD: Homeostasis Model Assessment Adiponectin; FBG: fasting blood glucose; HbA1c: glycated hemoglobin; ALR: adiponectin-leptin ratio.

Table 3. Variables significantly correlated with HOMA-AD values in all study participants (n = 80)

Variable	Correlation coefficient[a]	P-value
BMI	0.349	0.002
FBG	0.448	< 0.001
Fasting insulin	0.667	< 0.001
Post dextrose glucose	0.388	< 0.001
HbA1c	0.315	0.004
Adiponectin	-0.662	< 0.001
Leptin	0.300	0.007
Visfatin	0.269	0.016
ALR	-0.547	< 0.001
HOMA-IR	0.700	< 0.001

[a] Spearman correlation coefficient; FBG: fasting blood glucose; HbA1c: glycated hemoglobin; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; ALR: adiponectin-leptin ratio; HOMA-AD: Homeostasis Model Assessment-Adiponectin.

DISCUSSION

The results of the present study revealed that HOMA-IR was similar between the NF1 and control groups. However, the HOMA-AD values were significantly lower in patients with NF1 than those in the control group. FBG, leptin, and visfatin levels were significantly lower and adiponectin levels and ALR were significantly higher in patients with NF1 than those in the control group.

While the HOMA-IR is the most widely used index for the determination of IR, some authors have reported that this index fails to identify IR in individuals with normal glucose levels and impaired glucose tolerance (15). In this study, 100% of patients with NF1 showed FBG within normal levels and only 12.5% of controls had impaired FBG levels. Thus, the HOMA-IR may not be the most appropriate method for identifying IR in patients with NF1, in this sample.

HOMA-AD has been reported to be a suitable method for identifying IR in specific populations, and some authors consider it a more sensitive marker of IR (16,17). The results of this study revealed a significantly lower median HOMA-AD level in the NF1 group, suggesting that these patients may have greater insulin sensitivity than controls.

The ALR has also been reported to be a more efficient marker of IR than HOMA-IR, showing an inverse correlation with IR markers (21). In the present study, ALR was significantly higher in patients with NF1, also suggesting a greater sensitivity to insulin in this group of patients.

The mechanisms to explain increased insulin sensitivity in individuals with NF1 are unknown. It is known that excess adipose tissue plays an essential role in the development of IR. However, Pal and cols. (25) demonstrated that patients with mutations in the tumor suppressor phosphatase and tensin homologue, which causes a syndrome of predisposition to cancer, have increased insulin sensitivity and excess weight compared to controls. The authors have also shown that the excess weight found in the patients was not due to increased lean or bone mass, but rather to increased adiposity, and suggest that the excess weight may be due to the improved action of insulin in the adipose tissue.

Studies have shown that suppression of lipolysis is highly sensitive to insulin (26). This way, considering the conclusions of these studies, patients with NF1 who exhibit insulin sensitivity could be obese. However, our patients had BMI within the normal range, and other

studies have already demonstrated a lower BMI in NF1 patients, contradicting the findings of Pal and cols. Two hypotheses could explain these findings, insulin sensitivity in individuals with NF1 may be mild, and it may not be sufficient to induce obesity or insulin sensitivity that is specific to non-fat tissues.

We believe that the characteristics of body composition in NFI patients, demonstrated in other studies (27) and associated with increased adiponectin and lower visfatin and leptin levels, may be related to higher insulin sensitivity found in this group of individuals.

Regarding metabolic characteristics, the median values of FBG in patients with NF1 were significantly lower than those in control individuals; the number of individuals with impaired FBG was also significantly lower in the NF1 group than in the control group. These data agree with a previous study by our group (10), which evaluated 57 patients with NF1 and 171 controls and found significantly lower levels of FBG in patients with NF1.

HbA1c, blood glucose 2 hours after administration of dextrosol, and fasting insulin levels were also similar between the groups, suggesting that some mechanism involved in the control of fasting glucose may be responsible for the lower levels of FBG in the NF1 group. After a prolonged period of fasting, glucose levels in the blood decline, stimulating the pancreatic alpha cells to release glucagon. The result is hepatic glycogenolysis and gluconeogenesis to increase blood glucose levels (28). Thus, we hypothesize that the lower levels of FBG in NF1 could be a result of a change in the glycogenolysis or gluconeogenesis process.

Neurofibromin plays a role in the regulation of the hypothalamus and pituitary gland (29), which are involved in the regulation of energy balance (30). Neurofibromin could play a role in glucose metabolism, and its deficiency may cause reduced gluconeogenesis in patients with NF1. Moreover, the modified pathways resulting in insulin changes related to neurofibromin are not clear.

A second hypothesis to explain the lower levels of FBG in patients with NF1 is derivate of the production of insulin-like growth factor 2 (IGF2) by neurofibromas. Studies have shown that tumors produce IGF2, which increases the consumption of peripheral glucose and decreases the glucose production in the liver, resulting in hypoglycemia (31). However, further studies are needed to verify if the FBG levels in patients with NF1 can be accounted for by IGF2 produced by neurofibromas.

A third hypothesis that could explain the lower fasting glucose levels in patients with NF1 is related to adipocytokine levels. The results of this study showed that adiponectin levels in patients with NF1 were significantly higher and levels of visfatin and leptin were significantly lower than in controls. According to Yamauchi and cols. (32) one of the mechanisms through which adiponectin may decrease the risk of T2DM is the suppression of hepatic gluconeogenesis. Leptin can reduce hepatic glucose production by decreasing the synthesis of phosphoenolpyruvate carboxylase, which is the key enzyme in gluconeogenesis (33). However, Gutiérrez-Juárez and cols. (34) showed that administration of leptin in rats stimulated gluconeogenesis. Visfatin, in turn, appears to stimulate gluconeogenesis (35).

Thus, differences in the levels of these adipocytokines may contribute to lower hepatic gluconeogenesis and lower levels of FBG in patients with NF1. However, further studies are needed to validate this hypothesis. The reason for the changes in the levels of these adipocytokines in patients with NF1 remains unknown. No other studies have evaluated adipocytokine levels in NF1.

The correlation of HOMA-AD values with diabetes mellitus markers, FBG, post dextrose glucose, and HbA1c in addition to HOMA-IR values, levels of adiponectin, leptin, and visfatin, and ALR reinforce the hypothesis that the characteristics observed in patients with NF1 (lower levels of FBG, visfatin, and leptin and higher adiponectin and adiponectin/leptin levels) may explain the lower incidence of T2DM in these patients.

This study has several limitations, including the small number of patients involved, although NF1 is a rare disease for which surveys are usually performed with even fewer participants. In addition, IR was not evaluated using the gold-standard techniques.

In conclusion, the results of this matched case-control study suggested for the first time that NF1 individuals have increased insulin sensitivity, as well as lower levels of FBG, visfatin, and leptin and higher adiponectin levels, than those without the disease. These characteristics may be associated with the lower occurrence of T2DM in this group of patients.

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Higher fiber intake is associated with lower blood pressure levels in patients with type 1 diabetes

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ABSTRACT

Objective: The present investigation sought to evaluate the potential association between dietary fiber intake and blood pressure (BP) in adult patients with type 1 diabetes (T1D). **Subjects and methods:** A cross-sectional study was carried out in 111 outpatients with T1D from Porto Alegre, Brazil. Patients were predominantly male (56%) and white (88%), with a mean age of 40 ± 10 years, diabetes duration of 18 ± 9 years, BMI 24.8 ± 3.85 kg/m^2, and HbA1c 9.0 ± 2.0%. After clinical and laboratory evaluation, dietary intake was evaluated by 3-day weighed-diet records, whose reliability was confirmed by 24-h urinary nitrogen output. Patients were stratified into two groups according to adequacy of fiber intake in relation to American Diabetes Association (ADA) recommendations: below recommended daily intake (< 14g fiber/1000 kcal) or at/above recommended intake (≥ 14g/1000 kcal). **Results:** Patients in the higher fiber intake group exhibited significantly lower systolic (SBP) (115.9 ± 12.2 vs 125.1 ± 25.0 mmHg, p = 0.016) and diastolic blood pressure (DBP) (72.9 ± 9.2 vs 78.5 ± 9.3 mmHg, p = 0.009), higher energy intake (2164.0 ± 626.0 vs 1632.8 ± 502.0 kcal, p < 0.001), and lower BMI (24.4 ± 3.5 vs 26.2 ± 4.8, p = 0.044). Linear regression modelling, adjusted for age, energy intake, sodium intake, and BMI, indicated that higher fiber intake was associated with lower SBP and DBP levels. No significant between-group differences were observed with regard to duration of diabetes, glycemic control, insulin dosage, or presence of hypertension, nephropathy, or retinopathy. **Conclusion:** We conclude that fiber consumption meeting or exceeding current ADA recommendations is associated with lower SBP and DBP in patients with T1D. Arch Endocrinol Metab. 2018;62(1):40-7

Keywords
Fiber; type 1 diabetes; blood pressure; hypertension

The American Diabetes Association has specific dietary recommendations for individuals with type 1 diabetes (T1D) (1), which include: preferring whole grains and fiber; preferring fructose naturally current in fruits; restricted intake of saturated fats, trans fats, and cholesterol; and keeping daily sodium intake under 2,300 mg. In addition, regarding fiber in particular, the recommended intake is 14 g total fiber per 1000 kcal of energy intake, or approximately 25 g/day for adult women and 38 g/day for adult men. However, most studies evaluating fiber intake in persons with diabetes have been limited by short observation periods and small sample sizes, and by assessing combinations of fiber-rich diets and foods with low glycaemic index, which may have hindered analysis of the isolated effect of fiber as a determinant of improved glycaemic control in patients with diabetes (2,3).

Some observational studies have demonstrated an inverse relationship between total fiber intake and blood pressure (BP) levels (4,5). Intake of fiber from natural dietary sources, such as whole grains, fruits, and pulses, appears to have a beneficial effect on BP and on serum cholesterol levels in individuals with type 2 diabetes (T2D) (6,7). Furthermore, in patients with T1D and T2D, a high-fiber diet (40 g/day) was associated with significant reductions in fasting and post-prandial blood sugar levels (8). In T1D, dietary fiber plays important roles in lipid profile, glyceamic control, several parameters related to obesity, risk of cardiovascular disease (CVD), endothelial dysfunction, and markers of inflammation (9-12).

Current recommendations for the dietary treatment of hypertension in patients with diabetes, although evidence-based, are largely drawn from studies not conducted in diabetic populations. In fact, the effects of dietary fiber on BP in patients with T1D has been the subject of little research. One recent study of patients with T1D demonstrated a protective effect of

fiber, especially soluble fiber, against CVD and all-cause mortality (9).

The role of fiber in the presence of hypertension in patients with T1D has yet to be established definitively. Within this context, the aim of the present study was to evaluate a potential association between dietary fiber intake (in accordance with American Diabetes Association recommendations) and BP levels in adults with T1D.

SUBJECTS AND METHODS

Participants

This cross-sectional study was carried out on a cohort of patients with T1D recruited consecutively from the outpatient endocrinology clinic of Hospital de Clínicas de Porto Alegre, a large teaching hospital in Porto Alegre, Brazil. T1D was defined as diabetes with onset before 40 years of age, ketonuria or ketonaemia at the time of diagnosis, and dependence on insulin therapy to sustain life. Patients were selected on the basis of the following criteria: dietary counselling by a dietitian during the 6 months preceding study enrolment; age > 18 years; and duration of diabetes > 5 years. Patients with renal failure, symptomatic heart failure (NYHA class III or IV), acute cardiovascular events in the 6 months preceding study enrolment (stroke, myocardial infarction, or acute pulmonary oedema), or inability to complete weighed dietary records were excluded from the cohort. All ongoing medications were maintained, except for statins.

The recruitment process took place from January 2011 to December 2012, and all participants provided written informed consent for inclusion in the study. The study protocol was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee (number 08-470).

Clinical evaluation

All patients were evaluated by an endocrinologist and interviewed on past medical history, demographic data, medication use, and current lifestyle. Patients were classified by ethnicity as white or non-white. Smoking status was classified dichotomously (as smoker or non-smoker), as was current alcohol intake (present or absent). The frequency of physical exercise was graded into four levels using a standardised questionnaire, based on activities carried out during a typical day (13); level 1 corresponded to a sedentary lifestyle.

Sitting BP was measured in triplicate, in the left arm, after a 10-minute rest, using a digital sphygmomanometer (Omron® HEM-705 CP). Hypertension was defined as BP readings ≥ 140/90 mmHg on two separate occasions, or current antihypertensive therapy (14). All patients underwent a full physical examination.

The presence of diabetic nephropathy (DN) was classified according to the results of a random spot urine sample or 24-hour timed urinary collection (at least two samples obtained 6 months apart). Patients were considered normoalbuminuric when the urinary albumin excretion (UAE) rate was < 17 mg/L or < 20 µg/min; microalbuminuric when the UAE was 17-174 mg/L or 20-199 mg/min; and macroalbuminuric when the UAE was > 176 mg/L or > 199 mg/min on at least two occasions during a 2-month period (15).

Diabetic retinopathy (DR) was assessed by an ophthalmologist. DR was detected by dilated-pupil direct and indirect ophthalmoscopy, and graded by severity using the Global Diabetic Retinopathy Project Group scale (16) as 'absence of DR', 'mild non-proliferative DR' (NPDR), 'moderate NPDR', 'severe DR', or 'proliferative DR' (PDR). For purposes of analysis, patients were divided into two groups (absence or presence of DR, regardless of severity).

Nutritional evaluation

Nutritional evaluation was carried out as described previously (11). Briefly, dietary habits were assessed by means of 3-day weighed food records (two non-consecutive weekdays and one weekend day), as previously standardised (17,18).

The adequacy of dietary records was evaluated by comparing reported protein intake to protein intake estimated from 24-hour urinary urea nitrogen, collected on the third day of dietary records (17). The ratio of reported protein intake to estimated protein intake was considered acceptable when in the range of 0.79 to 1.26 (18).

The nutrient contents of dietary records were analised in NutriBase 2007 Clinical Nutrition Manager (Cybersoft, Phoenix, AZ, USA), version 7.14 (19). Nutrient intake was expressed as the percentage of total daily energy intake (%) and in grams amounts (g/day or mg/day). Nutrition facts for frequently consumed foods were updated and/or supplemented as necessary with data obtained from local manufacturers of specific industrialized foods.

The total, soluble, and insoluble fiber content was estimated according to data provided in the CRC Handbook of Dietary Fiber in Human Nutrition (20). Dietary sources of fiber were based on the foods described by patients in their 3-day weighed food records. The main sources of dietary fiber consumed by the cohort were pulses (beans, lentils, peas), whole-grain foods (granola, wholemeal bread, wholegrain biscuits/crackers, flaxseed, sesame seeds, sunflower seeds, quinoa, amaranth), rice, barley, potatoes, sweet potatoes, cassava (manioc), eddoes, taro, wheat, and fruits such as apple, banana, clementine, grape, mango, orange, papaya, peach, and pineapple. Vegetables were grouped in two categories on the basis of carbohydrate content (% of grams weight), with group A vegetables containing up to 5% and group B vegetables containing up to 10% carbohydrate (21).

Laboratory evaluation

HbA1c was measured by high-performance liquid chromatography (Merck-Hitachi 9100; Merck®, Darmstadt, Germany) (reference range 4.7-6.0%). Fasting plasma glucose was measured by the glucose-peroxidase enzymatic colorimetric method (Biodiagnostica®). Total serum cholesterol and triglycerides were measured by enzymatic colorimetric methods (ADVIA 1800® Autoanalyzer, Germany), and HDL cholesterol by the homogeneous direct method (ADVIA 1800® Autoanalyzer, Germany). High-sensitivity C-reactive protein (hs-CRP) was quantitated by the turbidimetric method (ADVIA 1800® Autoanalyzer, Germany), and fibrinogen was determined by the Clauss clotting method, which measures the rate of fibrinogen conversion to fibrin in a diluted sample under the influence of excess thrombin. Urinary urea was measured by an enzymatic ultraviolet assay (ADVIA 1800® Autoanalyzer, Germany).

Statistical analyses

Data are presented as mean ± standard deviation (SD), n (%), or median (interquartile range). Baseline characteristics were compared by fiber intake status by means of Student's *t*-test and Mann–Whitney *U* test. Patients were stratified into two groups on the basis of fiber intake in relation to American Diabetes Association recommendations: intake below recommendations (< 14 g/1000 kcal/day) or intake meeting/exceeding recommendations (≥ 14 g/1000 kcal/day). To determine the association between blood pressure and higher fiber intake (≥ 14 g/1000 kcal/day), a linear regression model was constructed with systolic (SBP) and diastolic (DBP) blood pressure values as the dependent variables. The multivariate model was adjusted for age, total calorie intake, BMI and sodium intake. All analyses were carried out in SPSS 18.0 (Chicago, IL, USA).

RESULTS

Of the 111 patients evaluated, 56% were male and 88% were white. The mean age was 40.0 ± 10.0 years, and mean duration of diabetes was 18.0 ± 9.0 years. Mean BMI was 24.8 ± 3.85 kg/m^2 and mean HbA1c was 9.0 ± 2.0%. Table 1 describes the clinical and laboratory characteristics of the sample stratified by fiber intake. There were no significant differences between the lower and higher fiber intake groups in terms of age, gender, ethnicity, duration of diabetes, physical activity level, or alcohol consumption. Patients in the high fiber intake group had a lower mean BMI than did those in the low fiber intake group (24.4 ± 3.5 vs 26.2 ± 4.8 kg/m^2, p = 0.044). There were no between-group differences in waist circumference or presence of hypertension. Regarding BP, patients in the higher fiber intake group (≥ 14 g/1000 kcal/day) had lower SBP (115.9 ± 12.2 vs 125.1 ± 25.0 mmHg, p = 0.016) and DBP (73.0 ± 9.2 vs 78.5 ± 9.3 mmHg, p = 0.009) than patients who consumed less fiber per day than recommended (< 14 g/1000 kcal/day). In addition, patients in the higher fiber intake group used lower doses of insulin. A similar, significant difference was found regarding antihypertensive therapy: fewer patients were on angiotensin-converting enzyme (ACE) inhibitors in the higher fiber intake group than in the lower fiber intake group (11.5% vs 20.8%, p < 0.001). There were no between-group differences in glycaemic control (HbA1c or serum glucose levels), presence of DN or DR, or lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides).

Daily macronutrient intake values, according to adequacy of fiber intake, are described in Table 2. Patients in the adequate fiber intake group had higher intakes of total energy, carbohydrates, protein, and fat than did patients who consumed less than the recommended adequate intake of fiber. Significant between-group differences were also found in terms

of fat fractions (saturated, monounsaturated, and polyunsaturated). Furthermore, patients in the higher fiber intake group had a significantly higher daily intake of sodium (2360.64 ± 919,81 vs 1676.59 ± 688.01 mg/day; p = 0.004). There were no between-group differences in intake of trans fats or cholesterol.

Table 3 describes the daily fiber intake, stratified by type and source, in our sample. As expected, patients in the high fiber intake group (≥ 14g/1000 kcal) consumed more total, soluble, and insoluble fiber than did their counterparts in the below-adequate fiber intake group. Analysis of the main sources of dietary

Table 1. Clinical and laboratory profile of patients with type 1 DM, stratified by adequacy of fiber intake (American Diabetes Association) (1)

	Below recommended fiber intake (< 14 g/1000 kcal/day)	At or above recommended fiber intake (≥ 14 g/1000 kcal/day)	P
N	24	87	-
Age (years)	37 ± 13	40 ± 11	0.161†
Sex (male)	29.2%	56.3%	0.180‡
Ethnicity (white)	91.7%	83.9%	0.451‡
Duration of diabetes (years)	19.4 ± 11.0	17.6 ± 8.6	0.394†
Physical activity: level 1* (%)	54.2	46.0	0.811‡
Alcohol intake (%)	54.5	56.2	0.893‡
BMI (kg/m²)	26.2 ± 4.8	24.4 ± 3.5	0.044†
Weight (kg)	70.40 ± 12.92	69.02 ± 10.73	0.632†
Height (cm)	162.95 ± 9.15	169.21 ± 9.21	0.004†
Waist circumference (cm)	83.0 ± 8.5	83.3 ± 9.8	0.856†
Males	78.40 ± 6.22	86.51 ± 9.14	0.059†
Females	83.30 ± 8.08	79.54 ± 9.44	0.210†
Hypertension (%)	40.9	38.2	0.815‡
SBP (mmHg)	125.1 ± 25.0	115.9 ± 12.2	0.016†
DBP (mmHg)	78.5 ± 9.3	72.9 ± 9.2	0.009†
Under treatment for hypertension	58.3%	32.2%	0.161
ACE inhibitor	20.8%	11.5%	< 0.001
ACE inhibitor + diuretic	12.5%	12.6%	0.813
Diuretic	4.2%	1.1%	0.250
Calcium channel blocker	4.2%	0.0%	< 0.001
ACE inhibitor + beta blocker + diuretic	8.3%	2.3%	0.043
Insulin dosage			
IU/kg body weight	53.88 ± 21.91	47.80 ± 19.34	0.240†
Diabetic nephropathy (%)	0.80 ± 0.26	0.67 ± 0.25	0.061†
Diabetic retinopathy (%)	20.8	12.6	0.312‡
HbA1c (%)	10.7	9.7	0.472‡
Plasma glucose (mg/dL)	90.7 ± 2.5	90.0 ± 1.8	0.079†
Cholesterol, total (mg/dL)	241.33 ± 160.0	193.0 ± 107.0	0.084†
Cholesterol, HDL (mg/dL)	194.2 ± 30	187.3 ± 37	0.408†
Cholesterol, LDL (mg/dL)	60.0 ± 17.0	59.5 ± 16.3	0.884†
Triglycerides (mg/dL)	116.6 ± 31.0	111.5 ± 35.0	0.511†
hs-CRP (mg/L)	98.5 (49-207)	88.1 (34.0-223.0)	0.315†
Fibrinogen (mg/dL)	2.5 (0.1-7.3)	2.1 (0.1-8.4)	0.566†
Urinary sodium excretion (mg/24h)	408.1 (225-672)	364.3 (208-667.0)	0.088†

Data expressed as mean ± SD, median (IQR), or n (%). † Student's *t* test. ‡ chi-square test. * Level 1: sedentary.

ACE: angiotensin-converting enzyme; BMI: body mass index; DBP: diastolic blood pressure; HbA1c: glycated haemoglobin; HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein; LDL: low-density lipoprotein; SBP: systolic blood pressure.

fiber revealed a significant between-group difference in the intake of fiber from pulses. Patients in the high fiber intake group consumed a greater amount of total fiber from pulses than did patients in the below-adequate fiber intake group (7.8 vs 1.6 g/day; $p < 0.001$). There were no significant between-group differences in terms of total fiber intake from vegetables (groups A+B), tubers, and whole grains.

Furthermore, we observed inverse correlations between total fiber, soluble fiber, and insoluble fiber intake with BP levels ($r = -0.219$, $p = 0.021$; $r = -0.221$, $p = 0.02$; and $r = -0.215$, $p = 0.02$ respectively).

Linear regression models (Table 4 and Table 5) were constructed to evaluate potential associations between BP levels and higher fiber intake (≥ 14g/1000 kcal). The models were adjusted for age, total energy intake, BMI and sodium intake. Analysis revealed that lower SBP and DBP levels were associated with higher fiber intake (beta -11.11, $p = 0.01$ and -6.21, $p = 0.005$ respectively), independent of the adjustments.

DISCUSSION

The present study demonstrated an association between increased dietary fiber intake and lower BP levels in patients with T1D. Patients whose daily fiber intake met or exceeded American Diabetes Association recommendations (≥ 14g/1000 kcal) exhibited lower SBP and DBP levels. These associations remained significant after adjustment for potential confounding factors (age, total energy intake, sodium intake, and BMI). Furthermore, we observed an association between fiber type and BP. Increased intake of soluble and insoluble fiber was negatively associated with BP levels. In this patient group, the mean total fiber intake was 19.8 ± 7.2 g/day (5.8 ± 2.4 g from soluble and

Table 2. Daily nutrient intake of patients with type 1 DM, stratified by adequacy of fiber intake (American Diabetes Association) (1)

	Below recommended fiber intake (< 14 g/1000 kcal/day)	At or above recommended fiber intake (≥ 14 g/1000 kcal/day)	P
n	24	87	-
Total energy intake (kcal/day)	1632.8 ± 502.0	2164.0 ± 626	0.001†
Total energy intake (KJ)	6821.76	9045.52	0.001†
Total energy intake (kcal/body weight/day)	24.09 ± 7.3	31.24 ± 8.0	0.001†
Carbohydrate			
g/day	201.7 ± 71.0	269.6 ± 86.5	0.001†
% of total energy intake	49.5 ± 7.6	50.2 ± 8.6	0.899†
Protein			
g/day	74.9 ± 27.1	98.1 ± 33.0	0.002†
% of total energy intake	18.4 ± 3.5	18.3 ± 3.6	0.742†
Fat, total			
g/day	57.6 ± 22.8	77.5 ± 34.5	0.009†
% of total energy intake	31.6 ± 8.3	31.7 ± 9.4	0.952†
Fat, saturated			
g/day	17.8 ± 9.0	24.2 ± 10.5	0.007†
% of total energy intake	9.5 ± 2.3	10.0 ± 3.1	0.648†
Fat, monounsaturated	21.0 ± 8.6	27.0 ± 12.6	0.031†
g/day	21.0 ± 9.8	27.6 ± 12.2	0.021†
% of total energy intake	9.5 ± 3.1	11.1 ± 3.5	0.627†
Fat, polyunsaturated			
g/day	12.0 (1.98-23.65)	19.0 (2.7-66.1)	0.026§
% of total energy intake	7.0 (1.73-12.78)	7.7 (1.8-27.3)	0.519§
Fat, trans (g/day)	0.2 (0.0-2.58)	0.3 (0.0-4.0)	0.770§
Cholesterol, dietary (mg/day)	224.7 ± 171.9	231.3 ± 109.0	0.818†
Sodium (mg/day)	1676.59 ± 688.01	2360.64 ± 919.81	0.004†

Data expressed as mean ± SD or median (IQR).† *t* test. § Mann-Whitney U.

14.0 ± 5.3 g from insoluble fiber). The main sources of dietary fiber associated with significant differences were pulses (beans, lentils, peas) and fruit (banana, apple, papaya, pineapple, clementine, orange, grape, mango, and peach).

The type of dietary fiber appears to play an important role in the risk of cardiovascular disease (CVD) in patients with T1D. Schoenaker and cols. (9) evaluated 2,108 patients with T1D aged 15 to 60 years and found that those who consumed at least 5 g of soluble fiber per day experienced a 16% reduction in CVD risk. In the present study, we evaluated parameters associated with CVD, such as lipid profile, and found no significant association between increased fiber intake (≥ 14g/1000 kcal/day) and serum levels of total cholesterol, HDL cholesterol, or LDL cholesterol.

In this sample of patients with T1D, the higher fiber intake group (≥ 14 g/1000 kcal/day) was also found to have higher energy and macronutrient (carbohydrate, protein, and fat) intake than patients in the lower fiber consumption group (< 14 g/1000 kcal/day). Interestingly, despite this increased energy and macronutrient intake, patients in the higher fiber intake group also had lower BMI, superior metabolic control of diabetes, and inferior use of medications to treat diabetes (insulin) and hypertension (ACE inhibitors). Patients in the higher fiber intake group also consumed more sodium than their counterparts in the lower fiber intake group, although without exceeding the recommended daily intake of sodium for diabetics (22).

Sacks and cols. (23) evaluated the effect of the Dietary Approaches to Stop Hypertension (DASH) diet in hypertensive and normotensive (but non-diabetic) individuals. Those who followed the DASH diet showed significant reductions in BP as compared with those who did not follow the diet. De Paula and cols. (24) analyzed the impact of fiber intake on the BP of 225 patients with T2D. Patients were stratified by BP tertiles, and patients in the subgroup with the highest BP levels (i.e., the top tertile) were found to consume less fruits and vegetables and fewer portions of dairy than recommended in the DASH diet.

A prospective study evaluated the association between quantity and variety of fruits and vegetables in the diet and incidence of diabetes (25), and found that increased intake of these foods was associated

Table 3. Daily fiber intake, types of fiber and their main dietary sources in patients with type 1 DM, stratified by adequacy of fiber intake (American Diabetes Association) (1)

	Below recommended fiber intake (< 14 g/1000 kcal/day)	At or above recommended fiber intake (≥ 14 g/1000 kcal/day)	P
n	24	87	-
Fiber (g/day)			
Total	10.4 ± 2.0	22.9 ± 7.4	< 0.001†
Soluble	3.2 ± 1.6	6.5 ± 2.2	< 0.001†
Insoluble	7.6 ± 2.0	15.8 ± 4.5	< 0.001†
Dietary sources (g/day)			
Fiber from fruit[a]	1.2 (0.0 – 3.5)	2.7 (0.0-10.2)	0.010§
Fiber from vegetables (A+B)[b]	1.2 (0.0 – 3.5)	2.8 (0.0-47.2)	0.208§
Fiber from tubers[c]	0.5 (0.0 – 2.2)	0.5 (0.0-3.5)	0.791§
Fiber from whole grains[d]	1.5 (0.0 – 6.5)	2.3 (0.0-12.4)	0.292§
Fiber from pulses[e]	1.6 (0.0 – 5.8)	7.8 (0.0-26.0)	< 0.001§

Data expressed as mean ± SD or median (IQR). † t test. § Mann–Whitney U.

[a] Fruit: apple, banana, clementine, grape, mango, orange, papaya, peach, pineapple.

[b] A vegetables: cabbage, chard, cress, cucumber, kale, lettuce, mustard greens, radish, rocket, spinach, turnip.

[b] B vegetables: aubergine (cooked), beets (cooked), broccoli, carrot (cooked), cauliflower (cooked), chayote (cooked), courgette (cooked), hearts of palm, onion, peppers, runner beans, squash/marrow (cooked).

[c] Tubers: potatoes, sweet potatoes, cassava (manioc), eddoes, taro.

[d] Whole grains: granola, wholemeal bread, wholegrain biscuits/crackers, wholemeal flour, oats, flaxseed, quinoa, amaranth, sunflower seeds.

[e] Pulses: beans, lentils, peas.

Table 4. Linear regression analysis: systolic blood pressure and adequate fiber intake (≥ 14g fiber/1000 kcal). Model adjusted for age, total energy intake, BMI, and sodium intake

Variables	β	P
≥ 14g fiber/1000 kcal	-11.11	0.010
Age (years)	0.03	0.810
Total energy intake (kcal/day)	- 0.004	0.200
BMI (kg/m²)	0.032	0.930
Sodium intake (mg/day)	0.03	0.031

BMI: body mass index.

Table 5. Linear regression analysis: diastolic blood pressure and adequate fiber intake (≥ 14g fiber/1000 kcal). Model adjusted for age, total energy intake, BMI, and sodium intake

Variables	β	P
	-6.21	0.005
≥ 14g fiber/1000 kcal	-0.18	0.040
Age (years)	0.005	0.009
Total energy intake (kcal/day)	0.14	0.550
BMI (kg/m²)	-0.001	0.310
Sodium intake (mg/day)	-6.21	0.005

BMI: body mass index.

with a lower risk of developing any diabetes (21%). Furthermore, increased consumption of fruits alone, vegetables alone, and both fruits and vegetables was associated with significant reductions in the risk of T2D (30%, 22%, and 39%, respectively). In the present study, we observed that fiber from beans/pulses and from fruit was a particularly important dietary component that may be associated with BP levels in patients with T1D.

The mechanisms by which dietary fibers lead to lower BP levels have not yet been elucidated (26). However, is well established in the literature that both soluble and insoluble fibers exert a protective and preventive effect in some pathologies such as metabolic syndrome, CVD and hyperlipidemia (27), in addition, it has an important role as evidenced in satiety and weight loss (28). Dietary fiber intake appears to improve serum lipids levels (29) and this reduction can be associated with improved endothelial vasodilation and consequently with reduced BP levels (27,29). Based on these findings and our observation that the group of patients with higher fiber intake also consumed more energy, including all macronutrients as well as sodium, we suggest that fiber intake may have a favorable effect on vasodilatation and on oxidative stress. Additional studies are needed to elucidate the mechanisms involved in the proposed beneficial effects of fiber intake on BP levels.

A potential limitation of the present study is that BP was only measured during the first study visit and when patients returned to submit their food records. In an ideal setting, 24-hour ambulatory BP monitoring would have been performed. Nevertheless, given the lack of studies demonstrating specific effects of fiber intake on BP level in patients with T1D, our study is highly relevant to clinical practice.

In conclusion, increased total fiber intake is associated with lower SBP and DBP in patients with T1D. Future studies, including randomized clinical trials should confirm the beneficial effects of dietary fiber (of different types and sources) on BP levels in patients with T1D.

Individual contributions: the authors' contributions were as follows: M. V. B. contributed to data collection and wrote the manuscript; F. R. B. and C. N. contributed to data collection and reviewed the manuscript; T. S. helped draft and review the manuscript; T. C. R. designed the study, contributed to data collection, and reviewed the manuscript.

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Influence of maternal weight gain on birth weight: a gestational diabetes cohort

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ABSTRACT

Objective: Our objective was to evaluate gestational weight gain (GWG) patterns and their relation to birth weight. **Subjects and methods:** We prospectively enrolled 474 women with gestational diabetes mellitus (GDM) at a university hospital (Porto Alegre, Brazil, November 2009-May 2015). GWG was categorized according to the 2009 Institute of Medicine guidelines; birth weight was classified as large (LGA) or small (SGA) for gestational age. Adjusted relative risks (aRRs) and 95% confidence intervals (95% CIs) were determined. **Results:** Adequate GWG occurred in 121 women [25.5%, 95% CI: 22, 30%]; excessive, in 180 [38.0%, 95% CI: 34, 43%]; and insufficient, in 173 [36.5%, 95% CI: 32, 41%]. In women with normal body mass index (BMI), the prevalence of SGA was higher in those with insufficient compared to adequate GWG (30% vs. 0%, $p < 0.001$). In women with BMI ≥ 25 kg/m², excessive GWG increased the prevalence of LGA [aRR 2.58, 95% CI: 1.06, 6.29] and protected from SGA [aRR 0.25, 95% CI: 0.10, 0.64]. Insufficient vs. adequate GWG did not influence the prevalence of SGA [aRR 0.61, 95% CI: 0.31, 1.22]; insufficient vs. excessive GWG protected from LGA [aRR 0.46, 95% CI: 0.23, 0.91]. **Conclusions:** One quarter of this cohort achieved adequate GWG, indicating that specific ranges have to be tailored for GDM. To prevent inadequate birth weight, excessive GWG in women with higher BMI and less than recommended GWG in normal BMI women should be avoided; less than recommended GWG may be suitable for overweight and obese women. Arch Endocrinol Metab. 2018;62(1):48-56

Keywords

Gestational diabetes mellitus; weight gain; birth weight

INTRODUCTION

Gestational diabetes mellitus (GDM) is typically diagnosed approximately 24-28 weeks using an oral glucose tolerance test (1). Adverse outcomes associated with GDM include increased risk of maternal hypertensive disorders and cesarean section as well as perinatal risks of macrosomia, shoulder dystocia and hypoglycemia (2).

Maternal obesity contributes to GDM and, in an independent fashion, to many other adverse maternal pregnancy outcomes, including pregnancy hypertensive disorders, cesarean section, weight retention, and postpartum diabetes. Adverse outcomes for offspring, including congenital anomalies, macrosomia and indicated preterm delivery, are also increased (3). Excessive weight gain *per se* contributes to an increased prevalence of large for gestational age (LGA) and macrosomia (4).

In 2009, the American Institute of Medicine (IOM) updated their recommendations on weight gain in pregnancy without a specific recommendation for GDM (4). Weight gain has been evaluated in several GDM cohort studies, with variable frequencies of adequate, insufficient or excessive weight gain being reported (5-8).

The objectives of this study were to evaluate how the 2009 IOM recommendations on gestational weight gain (GWG) applied to a contemporary cohort of GDM pregnancies and how the patterns of GWG in GDM impacted birth weight.

SUBJECTS AND METHODS

We studied a cohort of 508 women with GDM with singleton pregnancies, with at least one prenatal appointment, who delivered at Hospital de Clínicas de Porto Alegre (HCPA), a university hospital. HCPA is located in the Southern state of Brazil, Rio Grande do Sul (population ~11 million inhabitants) and provides medical care through the *Sistema Único de Saúde* (SUS), the national health system. In 2015, more than 600,000 general consultations were performed; approximately 4,000 babies were delivered; and the cesarean rate was 32.8% (9). From November 2009 to May 2015, all eligible women referred from primary care units were consecutively included; a multidisciplinary team provided prenatal care. Women gave their consent after being fully informed, and the authors signed the confidentiality document for data use. The hospital ethics committee approved the study protocol (number 2010-0364). We followed the STROBE statement for the study report (10).

Thirty-four women were excluded: one due to an abortion, one for having congenital achondroplasia and 32 due to missing data on maternal weight or infant birth weight. GDM was diagnosed with a 75-g oral glucose tolerance test (OGTT) using the criteria of fasting plasma glucose ≥ 110 mg/dL or 2-h plasma glucose ≥ 140 mg/dL in 232 women (49%) (11). After 2010, the International Association of Diabetes and Pregnancy Study Groups (IADPSG) recommendation was adopted; 242 (51%) of the women met these criteria (12).

Pregestational weight was self-reported, and pre-pregnancy BMI was classified according to the World Health Organization criteria (13). Weight and height were measured with light clothes and no shoes. All women were prescribed a normocaloric diet, emphasizing the intake of low glycemic index carbohydrates and fiber-rich food. Capillary glucose targets were ≤ 95 mg/dL for pre-meal and ≤ 120 mg/dL for 2-h postprandial measures (14). If goals were not met after 2 weeks of nutritional therapy, pharmacological treatment was initiated. Data on pregnancy evolution, delivery, and maternal and newborn outcomes were obtained from hospital registries.

Ethnicity was self-reported. Schooling was categorized as ≤ 11 years or > 11 years. Total weight gain was the weight measured at admission to delivery minus the pre-pregnancy weight; weight gains until diagnosis and from diagnosis to delivery were calculated. The 2009 IOM guidance on GWG by BMI was used, including the following ranges: underweight women, 12.5 to 18 kg; normal weight, 11.5 to 16 kg; overweight, 7 to 11 kg and obese, 5 to 9 kg (4). An A1C test was measured at booking (initial A1C test) and during the 3rd trimester (3rd trimester A1C test). Pregnancy-related hypertension disorders included any diagnosis of gestational hypertension or preeclampsia/eclampsia (15), and the composite of maternal risk factors included hypertensive disorders of pregnancy plus smoking.

Newborns were classified as small for gestational age (SGA) or as LGA according to the Alexander birth weight chart (16), which is routinely used at our hospital. Macrosomia was defined as a term birth weight ≥ 4,000 g, and preterm birth was defined as a delivery before the completion of 37 gestational weeks (17).

Plasma glucose was measured by the enzymatic method and an A1C test using HPLC (Variant II Turbo HbA1C, BioRad Laboratories, Hercules, CA, USA, aligned to DCCT recommendations).

Statistical analysis

We described the prevalence of adequate gestational weight gain as n (%) and evaluated the 95% confidence intervals [95% CIs]. Student's t test, χ^2 test, ANOVA, and Pearson correlation were applied as appropriate.

Relative risks (RR) and 95% CIs for SGA and LGA were determined according to maternal weight gain within normal or elevated BMI ranges (≥ 25 kg/m^2). Skin color, living with a partner, schooling, gravidity, weight gain grouped by IOM category, fasting glucose at the time of the diagnostic test, use of pharmacologic treatment, 3rd trimester A1C test, and the composite of maternal risk factors were explored in univariable and multivariable models for SGA. For LGA analysis, we added family history of diabetes to the models. Poisson regression with robust estimation was employed in multivariable analyses. Variables were included if a p value of at least < 0.10 was obtained in univariable analysis or if considered clinically important (gravidity and maternal risk factor).

We used SPSS software version 18.8 for statistical analyses. Statistical significance was set at 0.05, two-sided.

RESULTS

There were some slight differences in baseline clinical characteristics between women classified by the two

GDM diagnostic criteria (Table 1). We analyzed them as a single group because we assumed that dissimilarities were related to distinct profiles captured by each criterion. The main differences were observed for a family history of diabetes (54.3% vs. 42.6%, p = 0.011), pregestational BMI (29.4 ± 6.5 vs. 30.7 ± 7.0 kg/m^2, p = 0.046); the 2-hour glucose in the diagnostic OGTT (170.7 ± 29.1 vs. 148.5 ± 37.1 mg/dL, p < 0.001); the baseline A1C value (5.7% ± 0.8% vs. 5.4% ± 0.6%, p < 0.001), the latter measurement being within the range of laboratory references (6.0%); and weight at delivery (84.0 ± 17.3 vs. 89.1 ± 18.5 kg, p = 0.002). No differences were found regarding key maternal and perinatal outcomes.

Among the 474 women, only one had a BMI < 18.5 kg/m^2, and this case was analyzed in the normal BMI group; 119 had normal BMIs (n = 120, 25%, 95% CI: 21, 29%); and 354 (75%, 95% CI: 71, 79%) had BMIs ≥ 25 kg/m^2. Adequate weight gain occurred in 121 women [25.5%, 95% CI: 22, 30%], excessive in 180

Table 1. Comparison of women with gestational diabetes according to two diagnostic criteria

Characteristic/outcome	Brazilian criteria N = 232	IADPSG criteria N = 242	p
	Mean ± SD or %	Mean ± SD or %	
Maternal			
Age (years)	31.4 ± 6.2	31.2 ± 6.7	0.755
White ethnicity	75.4	78.9	0.365
Education (> 11 years)	49.6	47.5	0.656
With partner	63.4	45.5	< 0.001
Pregnancies (n)	2.7 ± 1.6	2.8 ± 1.7	0.789
Current smoker	0.9	3.3	0.064
Family history of diabetes	54.3	42.6	0.011
Previous GDM	13.4	13.2	0.964
Pre-pregnancy BMI (kg/m^2)	29.4 ± 6.5	30.7 ± 7.0	0.046
Systolic BP (mmHg)*	115.8 ± 11.9	117.0 ± 12.3	0.287
Diastolic BP (mmHg)*	72.5 ± 10.2	73.0 ± 9.8	0.591
Glycemia in the OGTT (mg/dL)**			
Fasting	100.0 ± 27	97.2 ± 16.4	0.177
1 hour (n = 180)	–	178.4 ± 35.8	–
2 hour	170.7 ± 29.1 n = 226	148.5 ± 37.1 n = 200	< 0.001
2nd trimester A1C test (%)***	5.7 ± 0.8	5.4 ± 0.6	< 0.001
3rd trimester weight gain (kg)	2.0 ± 4.0	2.7 ± 4.2	0.066
Weight at delivery (kg)	84.0 ± 17.3	89.1 ± 18.5	0.002
Total gestational weight gain (kg)	10.5 ± 7.5	10.1 ± 7.9	0.598
Hypertensive disorders of pregnancy	13.8	12.0	0.556
Cesarean section	58.6	52.1	0.151
Offspring			
Birth weight (g)	3,221.6 ± 578.7	3,246.6 ± 602.8	0.645
Birth weight category			0.681
SGA	9.9	12.0	
AGA	78.9	75.6	
LGA	11.2	12.4	
Macrosomia	7.3	8.3	0.704

IADPSG: International Association of Diabetes in Pregnancy Study Groups; GDM: gestational diabetes mellitus; BMI: body mass index; BP: blood pressure; OGTT: oral glucose tolerance test; SGA: small for gestational age; AGA: adequate for gestational age; LGA: large for gestational age.

* n = 230 for the Brazilian criteria and 241 for the IADPSG criteria; ** n = 226 for the Brazilian criteria and 200 for the IADPSG criteria; *** n = 206 for the Brazilian criteria and 207 for the IADPSG criteria.

[38%, 95% CI: 34, 43%] and insufficient in 173 [36.5%, 95% CI: 32, 41%]. Pre-pregnancy hypertension was present in 12.4% of the women, and preeclampsia/gestational hypertension was present in 9.5% of the women. The average gestational age at delivery was 38 ± 1.5 weeks (range: 30-41 weeks), and the rate of cesarean section was 55.3%. The baseline characteristics of the 474 women according to gestational weight gain categories are presented in Table 2.

When demographic and social characteristics across GWG categories (insufficient vs. adequate vs. excessive) were analyzed, maternal age was higher in women with insufficient GWG compared to those with excessive gain (Table 2). Fasting plasma glucose (mg/dL) in the OGTT was available for all women (98 ± 18 vs. 98 ± 26 vs. 100 ± 23, p = 0.455), 1 h glucose was available for 180 women (182 ± 35 vs. 185 ± 38 vs. 171 ± 34, p = 0.104) and 2 h glucose was available for 426 women (162 ± 31 vs. 162 ± 40 vs. 158 ± 35, p = 0.558). The initial A1C level was measured in 413 women at a mean gestational age of 31 ± 5.8 weeks and was similar across groups (5.6% ± 0.7% vs. 5.5% ± 0.8% vs. 5.7% ± 0.8%, p = 0.070).

Women who gained insufficient weight were more likely to be receiving pharmacological treatment (insulin or oral agents) compared to those with adequate gain (58% vs. 40%, p = 0.009). Metformin treatment was less frequent in those with adequate weight gain (31%) compared to those with insufficient weight gain (47%, p = 0.023), but in the excessive weight gain group (42%), the rates were not different compared to the two other groups. Insulin use was similar across all three groups (19.0% vs. 13.2% vs. 16.1%, p = 0.407).

The primary data on maternal weight gain by IOM category for the two BMI groups (normal and overweight/obese) are displayed in Table 3. Total weight gain increased significantly across the groups. Weight at delivery increased significantly across the three IOM categories in normal BMI women (63.3 ± 6.4 vs. 69.9 ± 6.7 vs. 80.7 ± 7.3 kg, p = 0.001). In women with BMI ≥ 25 kg/m^2, the average weight gain was almost 10 kg higher in the excessive weight gain group.

Table 3 depicts offspring outcomes according to GWG categories. The mean ± SD birth weight was 3.234 ± 591 g; 242 (51%) newborns were male; 52 (11%) were SGA and 56 (12%) LGA; and 37 (7.8%) were macrosomic. The preterm birth rate was 16.5% and was similar across groups (insufficient, 20%, adequate, 13% and excessive, 16%, p = 0.315).

The Pearson correlation (r) between GWG and birth weight in normal BMI women was weak, 0.47 (p < 0.001), with a coefficient of determination (r^2) of 0.22; in the group with overweight/obesity, r was lower, 0.24 (p < 0.001), and r^2 was 0.06. The overall r coefficient was 0.26 (p < 0.001), and r^2 was 0.07.

We could not run univariable analyses in the normal BMI group due to the lack of SGA babies and the presence of only one LGA baby in the adequate gain category (Table 3). Pharmacological treatment,

Table 2. Baseline characteristics of 474 women with gestational diabetes according to the 2009 Institute of Medicine weight gain categories

Characteristic	Weight gain category				p value
	Total n = 474	Insufficient n = 173 (36.5)	Adequate n = 121 (25.5)	Excessive n = 180 (38)	
Age (years)[&]	31 ± 6.4	33 ± 6.0	31 ± 6.6	30 ± 6.5	0.001
White ethnicity	366 (77)	138 (80)	99 (82)	129 (72)	0.073
Education, > 11 years	230 (49)	84 (49)	57 (47)	89 (49)	0.924
With partner	257 (54)	101 (58)	67 (55)	89 (49)	0.232
Pregnancies (n)	2.7 ± 1.7	2.8 ± 1.6	2.9 ± 1.6	2.6 ± 1.7	0.347
Current smoker	10 (2)	4 (2.3)	2 (1.7)	4 (2.2)	0.920
Family history of diabetes	229 (48)	74 (43)	63 (52)	92 (51)	0.185
Previous GDM	63 (13)	26 (15)	15 (12)	22 (12)	0.699
Pre-pregnancy BMI (kg/m^2)	30.1 ± 6.8	30.7 ± 7.8	29.2 ± 6.5	29.9 ± 5.8	0.194
Systolic BP (mmHg), n = 471	116 ± 12	115 ± 13	116 ± 12	118 ± 12	0.120
Diastolic BP (mmHg), n = 471	73 ± 10	73 ± 10	71 ± 9	74 ± 11	0.163

GDM: gestational diabetes mellitus; BMI: body mass index; BP: blood pressure.

Data represent the mean ± standard deviation (SD) or n (%). ANOVA and Tukey's test for multiple comparisons χ^2 test and Z test for proportion with Bonferroni adjustment.

[&] Insufficient and excessive groups are significantly different.

excessive total GWG and excessive gain in the 3rd trimester were all statistically significant in the BMI ≥ 25 kg/m² group and were included in the multivariable model, as were gravidity and 3rd trimester A1C. A maternal risk factor composite was added to SGA model, and the fasting plasma glucose at the OGTT was added to the LGA model.

Analyses were run including the total GWG and 3rd trimester gain separately, with adequate weight gain as the reference category. Comparisons of the effect of insufficient GWG on LGA risk were also performed, with excessive GWG as reference. Overall, we analyzed 328 pregnancies with 34 SGA and 42 LGA infants in multivariable models.

As observed in Table 4, the SGA risk decreased 75% with excessive total GWG and 23% with each kilogram gained during the third trimester but was not enhanced by insufficient weight gain. The LGA risk increased independently with total GWG; each kilogram gained in the 3rd trimester increased the risk by 10%. In

Table 3. Pregnancy outcomes according to pre-pregnancy body mass index and 2009 Institute of Medicine weight gain categories in 474 women with gestational diabetes

Outcome according to BMI	Weight gain category			p value
	Insufficient	Adequate	Excessive	
BMI < 25 kg/m² (n = 120)	**n = 54**	**n = 34**	**n = 32**	
Maternal				
3rd trimester weight gain#	-0.7 ± 4.6	2.4 ± 2.3	4.1 ± 5.3	< 0.001
Weight at delivery (kg)¶	63.3 ± 6.4	69.9 ± 6.7	80.7 ± 7.3	< 0.001
Total weight gain (kg)¶	6.8 ± 3.0	13.3 ± 1.3	22.6 ± 5.0	< 0.001
Hypertensive disorders of pregnancy	7 (13)	2 (6)	2 (6)	0.427
Cesarean section	24 (44)	18 (53)	16 (50)	0.722
Maternal risk factors	12 (22)	2 (6)	4 (13)	0.101
Offspring				
Birth weight (g)#	2868 ± 478	3284 ± 368	3413 ± 454	< 0.001
Birth weight category				< 0.001
SGA#	16 (30)	0 (0)	1 (3)	
AGA£	37 (69)	33 (97)	25 (78)	
LGA&	1 (2)	1 (3)	6 (19)	
BMI ≥ 25 kg/m² (n = 354)	**n = 119**	**n = 87**	**n = 148**	
Maternal				
3rd trimester weight gain¶	0.7 ± 2.8	2.2 ± 3.4	4.1 ± 4.3	< 0.001
Weight at delivery (kg)†	88.0 ± 18.0	88 ± 16.0	98 ± 14	< 0.001
Total weight gain (kg) ¶	1.8 ± 3.1	8.2 ± 1.8	16.4 ± 5.8	< 0.001
Hypertensive disorders of pregnancy	11 (9)	12 (14)	27 (18)	0.110
Cesarean section	66 (56)	49 (56)	89 (60)	0.715
Maternal risk factors*	35 (29)	23 (26)	43 (29)	0.882
Offspring				
Birth weight (g)†	3157 ± 642	3157 ± 569	3425 ± 586	< 0.001
Birth weight category				< 0.001
SGA§	12 (10)	17 (20)	6 (4)	
AGA	94 (79)	64 (74)	113 (76)	
LGA§	13 (11)	6 (7)	29 (20)	

* Maternal risk factors include: hypertensive disorders of pregnancy plus smoking.

BMI: body mass index; n: number; SGA: small for gestational age; AGA: adequate for gestational age; LGA: large for gestational age. Data represent the mean ± standard deviation (SD) or n (%).

ANOVA and Tukey's test for multiple comparisons χ^2 test and Z test for proportion with Bonferroni adjustment.

insufficient group is significantly different from the adequate or excessive groups; £ insufficient and adequate groups were different; & insufficient and excessive groups were different; † insufficient and adequate groups were different from the excessive group; ¶ all groups were different; § adequate and excessive groups were different.

addition to weight gain, pharmacological treatment increased the LGA risk in the model with total GWG (RR 2.60, 95% CI: 1.11, 5.81). In the model with 3rd trimester weight gain, the risks were also independently increased with pharmacological treatment (RR 2.38; 95% CI: 1.10, 5.28) and 3rd trimester A1C (RR 1.72; 95% CI: 1.28, 2.31). Both adequate and insufficient weight gain had a protective effect upon LGA risk when compared to excessive weight gain.

Table 4. Risk for small and large for gestational age babies in women with gestational diabetes with BMI ≥ 25 kg/m^2 according to gestational weight gain

Risk factor	Multivariable analysis*	
	aRR [95% CI]	p value
	Small for gestational age	
Total GWG (adequate as reference)		
Excessive	0.25 [0.10,0.64]	0.004[a]
Insufficient	0.61 [0.31,1.22]	0.161
3rd trimester weight gain (kg)	0.87 [0.81,0.95]	0.001[a]
	Large for gestational age	
Total GWG (adequate as reference)		
Excessive	2.58 [1.06,6.29]	0.037[b]
Insufficient	1.17 [0.42,3.29]	0.768
3rd trimester weight gain (kg)	1.11 [1.05,1.17]	< 0.001[b]
Total GWG (excessive as reference)		
Adequate	0.39 [0.16,0.95]	0.037[b]
Insufficient	0.46 [0.23,0.91]	0.026

aRR [95% CI]: adjusted relative risk (95% confidence interval); GWG: gestational weight gain.
* Poisson regression with robust estimation.
[a] Adjusted for gravidity, pharmacological treatment, maternal risk factors, and 3rd trimester A1C.
[b] Adjusted for gravidity, fasting plasma glucose in the oral glucose tolerance test, pharmacological treatment, and 3rd trimester A1C.

COMMENTS

In this GDM cohort, adequate weight gain was attained by only one quarter of women. Those with normal BMI had increased SGA rates with insufficient weight gain. In women with overweight or obesity, excessive GWG increased the LGA risk and protected from SGA, while there was a trend towards a decreased risk of LGA when GWG was insufficient.

Overweight and obesity were frequent in our cohort (75%), with a rate quite similar to that described for type 2 diabetes pregnancies, 80% (18), reflecting the pattern described in 49.1% of non-pregnant Brazilian women in a recent survey (19). Variable rates of obesity, 17 to 71%, have been reported in GDM cohorts; different study populations and diagnostic criteria may explain this wide range (5-7,20,21). Maternal and offspring outcomes can be worsened if women enter pregnancy in the overweight or obese categories; deleterious effects are further magnified by excessive weight gain (22). The high frequency of excessive GWG found (38%) was expected, since up to 65% women were reported gaining more weight than recommended in several cohort studies in GDM (5,7,8,22). This is not exclusive to GDM pregnancies; a similar rate (32.9%) was observed in women in a large Brazilian cohort (n = 2,244) (23). Less than recommended GWG was 33.4% in the latter cohort, close to our rate (36.5%) and to those described in GDM (up to 40% of women) (7,8). The influence of GWG on GDM outcomes may therefore be uncertain, leading to the conclusion by a National Institutes of Health committee that evidence was insufficient "because of inconsistency across studies and imprecise effect estimates" (24).

We observed higher weight gain until GDM diagnosis followed by lower gain thereafter, as described in other studies (25,26); this reinforces the idea that more than intervention *per se*, being labeled GDM may increase treatment compliance (27). Moreover, we suppose that excessive weight might be perceived as deleterious by GDM mothers, since overweight and obese women gained approximately 5 kg less than their normal BMI counterparts along pregnancy. Women with insufficient weight gain in our cohort frequently needed pharmacological treatment; this association was also found in the Atlantic-DIP cohort, where insulin use was common in women with lower weight gain (5), in contrast to what was previously described (25). A possible explanation could be the presence of a more severe degree of metabolic disorder. We could not further explore this possibility because we did not measure insulin or C-peptide. We can speculate that this may in part also explain the interesting finding that women gaining insufficient weight were older than those with excessive weight gain. Another explanation for this latter finding could be ascribed to the presence of some degree of placental insufficiency in the oldest group or to compliance being greater due to their previous life experiences.

Although excessive and insufficient weight gain were frequent, maternal outcomes seemed unaffected in our study, opposing findings described by other authors: a higher weight gain in GDM pregnancies increased risks of cesarean section (7,22) and pregnancy-related

hypertension by almost two-fold in Irish women with excessive weight gain (5).

Regarding offspring outcomes, we found that only 7% of birth weight could be explained by maternal GWG. Despite this, normal BMI women who gained less than the recommended GWG delivered more SGA babies, while in overweight/obese women we did not observe this association. Furthermore, the SGA rate was close to that of the LGA rate for the whole group, an unexpected finding. We could speculate that close surveillance of diet and weight gain could eventually be an explanation for both an increased rate of SGA in normal BMI women and a decreased rate of LGA in women with adequate or insufficient weight gain, while in women with excessive GWG, high rates of LGA remained. In non-diabetic pregnancies, delivery of SGA or low birth weight babies (< 2,500 g) is associated with multiple factors, such as hypertension, smoking and insufficient weight gain (27). No difference in hypertension or smoking rates across the weight gain groups was found. High rates of SGA were not expected, as it is well established that GDM treatment *per se* does not increase this risk (28). However, 22% of birth weight was ascribed to GWG in normal BMI women in our study, which could partially explain our findings. Weight gain below recommendations was not related to increased rates of SGA in other GDM cohorts (8,25) nor was it in a type 2 diabetes cohort (18); of note, the results were not adjusted by pre-pregnancy BMI categories. Weight loss in GDM women with BMI ≥ 25 kg/m^2 resulted in increased SGA in a large American cohort, despite protecting against LGA and macrosomia, although in the study, the last weight measurement was taken around a mean gestational age of 34.8 weeks (29). The linkage between SGA and poor maternal weight gain, which is stronger in underweight women, is not well established yet for other BMI categories in non-GDM pregnancies and not even in GDM, though it is described in large cohort studies and remains positive when adjusted for confounders such as smoking and hypertension (4). It is tempting to speculate that other factors such as vitamin D deficiency might play a role. Maternal vitamin D deficiency and an increased rate of SGA births have been previously described in our cohort (30).

Excessive birth weight is conditioned by maternal obesity and hyperglycemia as well as by excessive weight gain (21,31,32). The association of birth weight with maternal weight gain has been described in women with type 2 diabetes (18), in obese-only women (31), and in obese GDM women (33), reflecting the independent role of those factors. Gestational diabetes leads to excessive birth weight irrespective of diagnostic criteria (2), while proper treatment has been associated with decreased risk (28). In our cohort, as in others, higher rates of LGA were associated with excessive weight gain mainly in overweight and obese women (7,8,25). In the Atlantic-DIP cohort, adjusted risks of similar magnitude to ours were reported for LGA (5), while in another cohort, LGA was increased in obese, but not in overweight, women (6). It is worthy of consideration that those studies calculated GWG from the time of booking. The independent effects of hyperglycemia and weight gain on birth weight were recently quantified: an A1C test < 5.0% avoided 47% of LGA, while adequate GWG avoided 52% of LGA (32). Finally, total GWG was not the only important factor in our study; weight gain in the third trimester also independently influenced LGA risk. A trend towards LGA risk was previously reported in women with excessive GWG after GDM diagnosis (34).

Insufficient GWG and its influence on LGA have been less commonly evaluated. A tendency toward a decreased LGA risk was previously reported in obese GDM women, as well as in overweight women (aRR 1.05, 95% CI: 0.68, 4.19), with a risk magnitude similar to ours (aRR 1.17 (95% CI: 0.42, 3.29) (6). The protective effect of lower GWG on LGA risk compared to excessive gaining was expected, as we compared two extremes, but it is worth saying that women with insufficient GWG had a trend toward delivering more LGA babies than those gaining adequate weight. We believe that the independent effects of hyperglycemia and increased BMI could prevail over that of GWG because their additive effects, which are mediated through maternal hyperlipidemia and relative insulin insufficiency, further stimulate insulin secretion by the fetal pancreas, promoting intrauterine overgrowth (21). Appropriate treatment strategies, including reduced weight gain, potentially counteract these metabolic effects (35).

The main strength of our study is the possibility to evaluate the 2009 IOM recommendation on GWG in a mixed ethnic cohort with a typical GDM profile of excessive BMI at the beginning of pregnancy. In addition to the well-known effects of excessive GWG, we demonstrated that less than recommended GWG might not be deleterious in GDM pregnancies.

A study limitation, the influence of treatment on pregnancy outcomes, is inherent to the study design and is mitigated by the similar antenatal care offered throughout the time period.

In conclusion, only one quarter of this cohort achieved weight gain within the 2009 IOM guidance, perhaps indicating that specific ranges should be tailored for GDM pregnancies. Less than currently recommended GWG should be avoided in normal BMI women, although it may be suitable for overweight and obese women because it prevents excessive birth weight. Excessive GWG should be prevented in overweight and obese women to reduce the risk of large for gestational age babies.

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Assessing endocrine and immune parameters in human immunodeficiency virus-infected patients before and after the immune reconstitution inflammatory syndrome

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ABSTRACT

Objective: The present study compares immune and endocrine parameters between HIV-infected patients who underwent the Immune Reconstitution Inflammatory Syndrome (IRIS-P) during antiretroviral therapy (ART) and HIV-patients who did not undergo the syndrome (non-IRIS-P). **Materials and methods:** Blood samples were obtained from 31 HIV-infected patients (15 IRIS-P and 16 non-IRIS-P) before ART (BT) and 48 ± 2 weeks after treatment initiation (AT). Plasma Interleukin-6 (IL-6) and Interleukin-18 (IL-18) were determined by ELISA. Cortisol, dehydroepiandrosterone sulfate (DHEA-S) and thyroxin concentrations were measured using chemiluminescence immune methods. **Results:** Concentrations of IL-6 (7.9 ± 1.9 pg/mL) and IL-18 (951.5 ± 233.0 pg/mL) were significantly higher ($p < 0.05$) in IRIS-P than in non-IRIS-P (3.9 ± 1.0 pg/mL and 461.0 ± 84.4 pg/mL, respectively) BT. Mean T4 plasma level significantly decreased in both groups of patients after treatment ($p < 0.05$). In both groups cortisol levels were similar before and after ART ($p > 0.05$). Levels of DHEA-S in IRIS-P decreased AT (1080.5 ± 124.2 vs. 782.5 ± 123.8 ng/mL, $p < 0.05$) and they were significantly lower than in non-IRIS-P (782.5 ± 123.8 vs. 1203.7 ± 144.0 ng/mL, $p < 0.05$). IRIS-P showed higher values of IL-6 and IL-18 BT and lower levels of DHEA-S AT than in non-IRIS-P. **Conclusion:** These parameters could contribute to differentiate IRIS-P from non-IRIS-P. The significant decrease in DHEA-S levels in IRIS-P after ART might suggest a different adrenal response in these patients, which may reflect the severity of the disease. Arch Endocrinol Metab. 2018;62(1):57-64

Keywords

HIV; interleukins; immune reconstitution syndrome; cortisol; dehydroepiandrosterone sulfate

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INTRODUCTION

The immune reaction to different diseases elicits an endocrine response which influences the course of the process (1). In inflammatory processes, pro-inflammatory cytokines (besides their immunological effects) are known to affect the function of crucial neuroendocrine mechanisms, which, in turn, can modulate the immune response (2,3). Such mechanisms include the actions of cytokines on the hypothalamus-pituitary-adrenal (HPA), -gonadal and -thyroid axis (2,4).

Viral infections, in general, are physiologically stressful, as shown by the concomitant activation of the HPA axis, and it has become clear that cytokine-HPA axis interactions are fundamental for immune regulation during these infections (3,5). Among the pro-inflammatory cytokines, Interleukin-18 (IL-18) was shown to have an important role in the immune response to intracellular pathogens in acute infections and it may participate in the regulation of the HPA axis (6). Another pro-inflammatory cytokine, interleukin-6 (IL-6), can activate the HPA axis, leading to the final production of steroid hormones by the adrenal gland (7).

Glucocorticoids have a critical role in maintaining the balance between the beneficial and detrimental effects of pro-inflammatory cytokines as part of the bidirectional communication between the immune system and the HPA axis (3,8). Regarding the adrenal steroids, glucocorticoids can promote Th2 cytokine acquisition profile, facilitating Th2 activities, whereas dehydroepiandrosterone (DHEA) is able to favor Th1 related cytokine production and interferes with Th2 associated cytokine synthesis (9,10).

Some human immunodeficiency virus (HIV)-infected patients undergo a clinical deterioration during the antiretroviral therapy (ART), which occurs regardless of the increase of $CD4^+$ T lymphocyte counts and the decrease of plasma HIV-1 viral loads. This clinical condition, known as immune reconstitution inflammatory syndrome (IRIS), reflects an exacerbated inflammatory response to opportunistic pathogens and/or tumor antigens in HIV-infected patients (11,12). This disorder occurs after the initiation of ART and is temporally related to an increase in the host $CD4^+$ lymphocyte count (11,13). The mechanisms involved in IRIS are not fully understood but they appear to be associated with the restoration of the immune response against pre-existent pathogens related to sub-clinical infections (14). The HIV-infected patients that will undergo IRIS during their treatment could present a more marked unbalance in their immune endocrine regulation (1,15).

Based on the mentioned data, the aim of the study was to assess parameters of adrenal and thyroid responses and immune pro-inflammatory reaction in HIV-infected patients receiving highly active ART. The results obtained from patients who suffered from IRIS (IRIS-P) and the ones who did not undergo the syndrome (non-IRIS-P) during treatment were compared in order to evaluate potential differences of the studied parameters between both groups of patients.

MATERIALS AND METHODS

Patients and ethics

All patients signed a written consent to participate in the study, and the protocol was approved by the Ethical Committee of CAICI Institute (Center for Assistance and Comprehensive Clinical Research, Rosario, Argentina). Patients with endocrine pathologies and hormonal treatments were excluded from the study.

This was a case-control study including 31 HIV-infected patients: 16 patients with normal response to ART (non-IP; 48 ± 11 years old), and 15 patients who underwent IRIS during the treatment (IP; 52 ± 12 years old). Both groups did not differ in age and sex composition ($p > 0.05$).

The diagnosis of IRIS was based on the criteria proposed by French and cols. (16), in patients who were infected with HIV and underwent a rapid clinical deterioration shortly after starting ART, despite having effective viral suppression. This was associated with co-infections caused by a diverse array of pathogens, and by tumor development. The diagnosis of IRIS was made by exclusion, ruling out other possible causes of disease after starting ART.

Blood sample collection

Ethylenediaminetetra-acetic acid (EDTA)-treated blood samples were obtained from patients at 8:00 a.m, before treatment initiation and 48 ± 2 weeks after ART initiation. Following plasma separation and addition of aprotinin (100 U/mL, Sigma-Aldrich Inc, USA), samples were preserved at -20°C until used in the assays.

T lymphocyte subsets count

T lymphocyte subsets (CD4, CD8) in patients' blood samples were quantified by standard flow cytometry techniques. Fluorochrome-labelled antibodies (anti-CD8-fluorescein isothiocyanate isomer, anti-CD3-phycoerythrin, and anti-CD4-PE-Cy5, Becton Dickinson, Heidelberg, Germany) that specifically bind to lymphocyte surface antigens were added to aliquots of blood samples. After incubation, a fixative solution (Becton Dickinson) was added and sample analysis was performed on a Becton Dickinson FACSCALIBUR flow cytometer (Four-Colors; Becton Dickinson, Heidelberg, Germany). The analysis provided absolute counts of $CD4^+$, $CD8^+$, $CD3^+$ lymphocytes and the $CD4^+/CD8^+$ ratio.

The absolute lymphocyte count was generated by a SYSMEX 2000i hematology analyzer (dual platform method, Roche, Basel, Switzerland).

Viral load quantification

Total RNA was extracted from the patients' samples and analyzed by the Amplicor HIV-1 Monitor test (Roche, Branchburg, NJ, USA) following the manufacturer's instructions.

Assays of IL-6 and IL-18

Both IL-6 and IL-18 plasma concentrations were assayed by Quantitative Elisa Kits from R & D Systems (Minneapolis, MN, USA), following the manufacturer's instructions. The sensitivities of the assays were 0.7 pg/mL and 12.5 pg/mL, respectively. The intra-assay variation coefficients for IL-6 and IL-18 assays were 5.0 % and 8.0 %, respectively.

Hormone measurements

Plasma concentrations of cortisol, DHEA-sulfate (DHEA-S), and thyroxin (T4), were determined using an Immulite 1000 Immunoassay System (Siemens, USA). The intra-assay variation coefficients were always lower than 5.0%.

Statistical analysis

Results from patients with and without IRIS or before and after ART were compared using the Student t-test or the alternative nonparametric Mann-Whitney test when required. Pearson's correlation coefficient (r) was used to analyze relationships among paired data. The Receiver Operating Characteristics (ROC) curve analysis was used to compare IL-18 values between IRIS-P and non-IRIS-P before the treatment. Results were expressed as media ± standard error (SE). A $p < 0.05$ was considered statistically significant.

RESULTS

The following disorders were associated with the IRIS that suffered the IRIS-P: *Herpes zoster* infection, tuberculosis, hepatitis B, toxoplasmosis, polyarthritis, and Kaposi's sarcoma. These disorders appeared 5.0 ± 0.6 months after ART initiation and usually in a sequential rather than a concurrent way. No patient had an active disease or opportunistic infection at the time of testing for this study, i.e., before the treatment and after 48 ± 2 weeks of ART initiation.

Immune parameters

The results indicated that all patients achieved a significant increase in their $CD4^+$ T cell counts after treatment. The values of $CD4^+$ T lymphocytes increased significantly after ART both in IRIS-P ($p < 0.01$) and in those who did not suffer from the syndrome ($p < 0.01$), compared to pre-treatment values (Table 1). The $CD4^+/CD8^+$ ratio was also significantly higher 48 ± 2 weeks after treatment initiation both in IRIS-P ($p < 0.05$) and in non-IRIS-P ($p < 0.01$). There were no significant differences in $CD4^+$ - $CD8^+$ cell counts or $CD4^+/CD8^+$ ratio between IRIS-P and non-IRIS-P, neither before nor after the treatment (Table 1).

Before treatment, the mean values of viral load in IRIS-P (261333 ± 117474 copy number/mL) and in non-IRIS-P (159954 ± 35324 copy number/mL) were not significantly different (Table 1).

Statistical analysis showed a significant difference in IL-6 mean plasma concentrations between IRIS-P and non-IRIS-P, before ART ($p < 0.05$, Table 2 and Figure 1A). In addition, in IRIS-P, the IL-6 values were significantly reduced after 48 ± 2 weeks after ART initiation ($p < 0.01$, Figure 1A) with respect to the values before the treatment. In the other patients, the decrease in IL-6 after ART did not reach statistical significance.

Before ART, mean plasma IL-18 levels in IRIS-P were higher than those in patients who did not suffer from the syndrome ($p < 0.05$, Table 2, Figure 1B). Based on a ROC curve analysis, a IL-18 value of 695 pg/mL before ART would allow to differentiate IRIS-P from patients who did not undergo IRIS in the present study, with 80% of specificity and 57% of sensitivity ($p = 0.08$).

After 48 ± 2 weeks of ART initiation, the IL-18 values tended to be higher in IRIS-P than in the other patients ($p = 0.09$). Plasma concentrations of IL-18 before ART were significantly higher than those after treatment both in IRIS-P and non-IRIS-P ($p < 0.05$ and $p < 0.01$, respectively, Figure 1B).

Table 1. Measured parameters in HIV-infected patients, before ART or after 48 ± 2 weeks of treatment initiation

	BT		AT	
	IRIS-P SRI	Non-IRIS-P +/-SE	IRIS-P	Non-IRIS-P +/-SE
$CD4^+$ (cel/mL)	221.4 ± 40.2[a]	262.3 ± 43.7[b]	447.5 ± 67.8[a]	429.8 ± 41.7[b]
$CD8^+$ (cel/mL)	760.4 ± 152	919.0 ± 100.7	715.7 ± 107.1	965 ± 134.1
$CD4^+/CD8^+$	0.46 ± 0.22[c]	0.40 ± 0.15[d]	0.59 ± 0.11[c]	0.51 ± 0.06[d]
VL (copy number/mL)	261332 ± 117474	159954 ± 35324	167 ± 37	50 ± 0.0

The table shows the mean results of the measured parameters in IRIS-P and in non-IRIS-P, before treatment initiation (BT) or after 48 ± 2 weeks (AT) of ART initiation. Results were expressed as media ± SE. $CD4^+$: $CD4^+$ T lymphocytes counts. $CD8^+$: $CD8^+$ T lymphocytes counts.

VL: viral load.

The same letters indicate mean values which are significantly different: [a] $p < 0.01$; [b] $p < 0.01$; [c] $p < 0.05$; [d] $p < 0.01$.

Table 2. Measured parameters in HIV-infected patients, before treatment initiation (BT) or after 48 ± 2 weeks (AT) of ART initiation

	BT		AT	
	IRIS-P SRI	Non-IRIS-P +/-SE	IRIS-P	Non-IRIS-P +/-SE
IL-6 (pg/mL)	7.9 ± 1.9[a,b]	3.9 ± 1.0[a]	3.2 ± 0.6[b]	2,8 ± 0.6
IL-18 (pg/mL)	951.5 ± 233.0[a,b]	461.0 ± 84.4[a,c]	270.4 ± 72.7[b]	30.7 ± 36.5[c]
Cortisol (µg/dL)	20.1 ± 1.5	20.3 ± 2.3	21.4 ± 2.5	21.0 ± 2.3
DHEA-S (µg/mL)	1080.5 ± 124.2[a]	1222.4 ± 1.7	782.5 ± 123.8[a,b]	1203.7 ± 144.0[b]
T4 (µg/dL)	8.9 ± 0.4[a]	7.8 ± 0.5[b]	7.2 ± 0.3[a]	6.9 ± 0.2[b]
DHEA/Cortisol	53.7 ± 6.5	66.2 ± 9.7	41.3 ± 7.9[a]	72.2 ± 11.9[a]

The table shows the mean results of the measured parameters in IRIS-P and in non-IRIS-P, before treatment initiation (BT) or after 48 ± 2 weeks (AT) of ART initiation. Results were expressed as media ± SE.

The same letters indicate mean values which were significantly different: IL-6: interleukin 6 ([a] $p < 0.05$; [b] $p < 0.01$); IL-18: interleukin 18 ([a] $p < 0.05$, [b] $p < 0.05$, [c] $p < 0.01$); DHEA-S: dehydroepiandrosterone sulfate ([a] $p < 0.05$, [b] $p < 0.05$); DHEA-S/Cortisol ratio ([a] $p < 0.05$); T4: thyroxin ([a] $p < 0.05$, [b] $p < 0.05$).

Endocrine measurements

Mean plasma concentrations of T4 in patients from each group are shown in Table 2, before and after the initiation of ART. Despite a significant decrease in the mean T4 plasma level after 48 ± 2 weeks of treatment in both groups of patients ($p < 0.05$, Figure 1C), the T4 concentrations always remained within the normal range. In addition, the mean values of T4 between the two groups, before or after ART, were not significantly different.

Table 2 shows the mean plasma concentration values of cortisol found in IRIS-P and in the other patients. In both groups, cortisol levels were similar both before and after ART initiation (Figure 2A).

Before ART, a significant correlation ($r = 0.59$, $p < 0.05$) between the values of $CD4^+$ lymphocytes count and cortisol plasma levels was observed in the patients who did not undergo IRIS. However, no association was found between $CD4^+$ cell count and cortisol concentrations in IRIS-P before the treatment, nor between these parameters after ART initiation in both groups of patients.

The mean plasma DHEA-S concentrations significantly decreased after treatment in IRIS-P ($p < 0.05$; Table 2 and Figure 2B). Statistical analysis indicated a significant difference in mean plasma levels of DHEA-S between the IRIS-P and non-IRIS-P, after receiving ART ($p < 0.05$, Table 2 and Figure 2B). However, before ART, plasma levels of DHEA-S in IRIS-P were not different from the values in non-IRIS-P.

No significant correlation between the mean values of $CD4^+$ cell count and DHEA-S plasma levels was found neither in IRIS-P nor in the other patients, neither before nor after ART initiation.

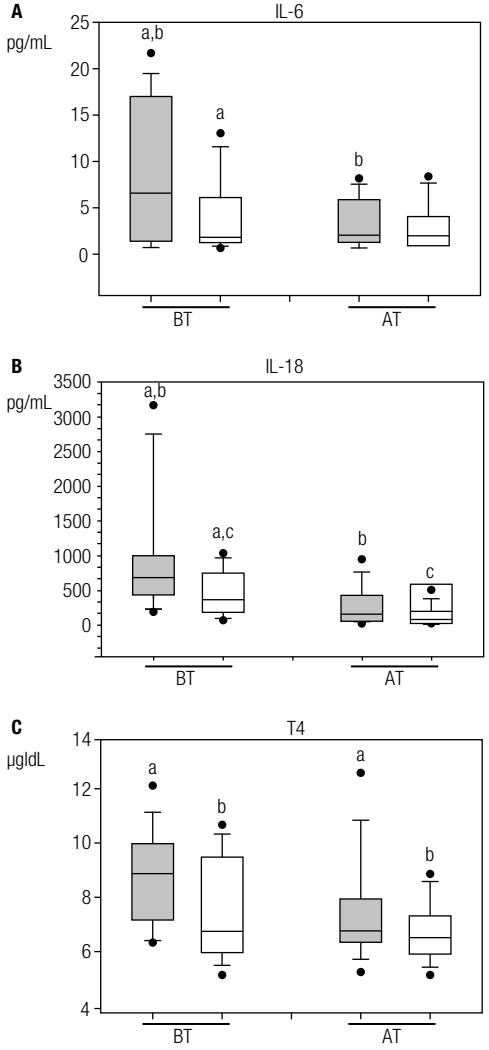

Figure 1. Box plots show plasma levels before (BT) or 48 ± 2 weeks after (AT) of ART initiation in IRIS-P (grey boxes) and non-IRIS-P (white boxes): A) interleukin 6 (IL-6, a: $p < 0.05$, b: $p < 0.01$); B) interleukin 18 (IL-18, a: $p < 0.05$, b: $p < 0.05$, c: $p < 0.01$); C) thyroxin (T4, a: $p < 0.05$, b: $p < 0.05$). The same letters indicate the groups that were compared statistically in the corresponding graph. Line inside box: median; limits of box: 75th and 25th percentiles.

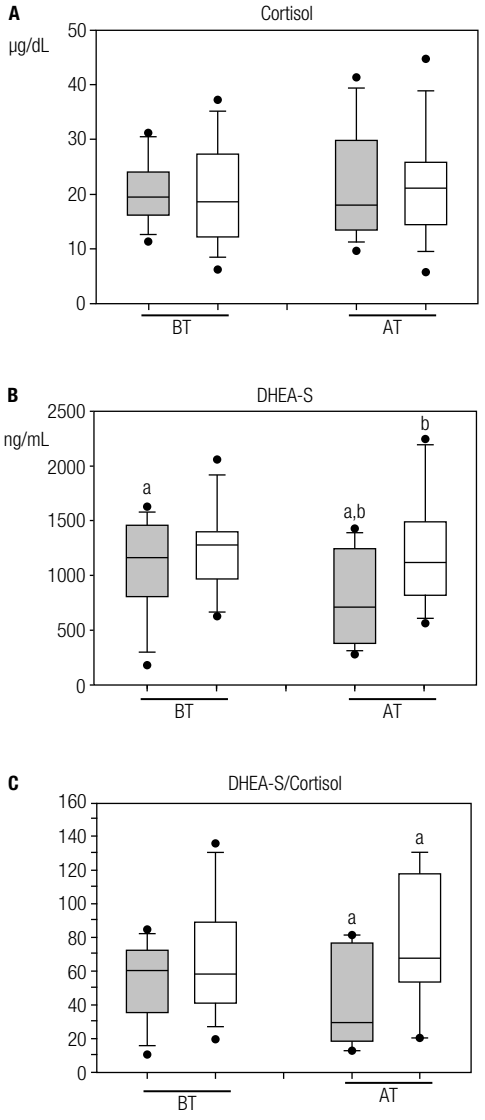

Figure 2. Box plots show plasma concentrations before (BT) or 48 ± 2 weeks after (AT) of ART initiation in IRIS-P (grey boxes) and non-IRIS-P (white boxes): A) cortisol; B) dehydroepiandrosterone sulfate (DHEA-S; a: $p < 0.05$, b: $p < 0.05$). C) Box plot indicates values of DHEA-S/Cortisol ratio before (BT) or after 48 ± 2 weeks (AT) of ART initiation in IRIS-P (grey boxes) and non-IRIS-P (white boxes, a: $p < 0.05$). The same letters indicate the groups that were compared statistically in the corresponding graph. Line inside box: median; limits of box: 75th and 25th percentiles.

The statistical analysis did not reveal a significant difference in the values of DHEA-S/Cortisol ratio between the two groups of patients before ART (Table 2 and Figure 2C). However, after ART initiation, the values of DHEA-S/Cortisol ratio were significantly lower in IRIS-P ($p < 0.05$; Table 2 and Figure 2C) than the values observed in the other patients studied.

DISCUSSION

It is known that treatment-induced immune improvement may increase the risk of an exacerbated immune response in some patients, worsening infections already present in the host, and leading to IRIS (12,17).

It has been reported that HIV-infected patients with a lower $CD4^+$ cell count before ART initiation are at a higher risk of undergoing IRIS (18,19). However, in the present study there were no significant differences in $CD4^+$ - $CD8^+$ cell counts, $CD4^+/CD8^+$ ratio or in viral loads between IRIS-P and non-IRIS-P before treatment. This lack of difference in $CD4^+/CD8^+$ rate between the two groups may be due to the small sample size. The results indicated that all patients responded to ART, increasing $CD4^+$ cell counts, which is in agreement with previous studies (20,21). It has been reported that the increase in $CD4^+$ cell count with ART was not a risk factor for IRIS because it can occur without an appreciable $CD4^+$ cell increase (22).

A previous study reported that a low $CD4^+/CD8^+$ ratio was an independent predictor for IRIS (23). They concluded that patients with a $CD4^+/CD8^+$ ratio less than 0.15 were more likely to have an IRIS event than were patients with a ratio greater than 0.30. However, in the present study $CD4^+/CD8^+$ ratios did not differ between IRIS-P and non-IRIS-P. In addition, 6 IRIS-P and 6 non-IRIS-P patients presented $CD4^+/CD8^+$ ratios less than 0.15 before ART.

The results showed that after one year of ART initiation, IL-6 plasma levels were significantly reduced in IRIS-P respect to pre-treatment values. Previous studies have also shown that ART decreases most markers of inflammation (24,25). The present study indicated that ART also caused a significant decrease in plasma levels of IL-18 in all the patients studied with respect to pretreatment values.

Before treatment, both IL-6 and IL-18 plasma concentrations in IRIS-P were significantly higher than in non-IRIS-P. Other authors also reported higher levels of inflammatory cytokines in IRIS-P than in non-IRIS-P previous to ART (24). The increased levels of these cytokines might be thought to be a characteristic of patients at risk of suffering from IRIS during ART. The higher values of IL-6 might reflect a resistance to glucocorticoids, which are normally involved in the decrease of the cytokine level and can promote a Th2 cytokine acquisition profile (15).

Interleukin-18 produced by macrophages is known to drive the differentiation of Th cells toward the Th1 type (6). It has been suggested that the higher levels of IL-18 in HIV-infected patients co-infected with TB may contribute to the sudden recovery of Th1 responses in those conditions (26). Thus, the higher IL-18 concentrations observed in IRIS-P before ART could also reflect this possibility. In the present study, an attempt to identify patients at potential risk of developing IRIS, a cut-off value of IL-18 ≥ 695 pg/mL before ART was chosen with 80% of specificity and 57% of sensitivity. Despite the fact that the study showed significant differences of IL-18 and IL-6 values between IRIS-P and non-IRIS-P, it would be necessary to carry out studies with larger number of patients to define cut-off values of both cytokines that could differentiate, with higher specificity and sensitivity, both types of patients from the general population of HIV patients prior to receiving ART, standardizing the pre-analytical and analytical variables.

Before ART, a positive correlation between $CD4^+$ cell count and levels of cortisol was found in patients who did not suffer from IRIS. This correlation was suggested to indicate a more controlled clinical response of the HIV-infected patients (27). The results showed that plasma cortisol concentrations were similar before and after ART in all the patients studied. It was suggested that patients experiencing IRIS could present an inadequate HPA axis response (1). Previous reports have suggested an intra-adrenal shift from DHEAS towards the cortisol production during critical illness (28-30), as could be the case of HIV infected patients who suffered IRIS. It has been proposed that an exacerbated proinflammatory response could result from the suppression of the HPA axis and of adrenal failure or reflect glucocorticoid tissue resistance as well (31,32). This clinical disorder is known as critical illness-related corticosteroid insufficiency resulting from an inadequate corticosteroid production or action for such severe disease (32,33). In agreement with the previous idea, patients with adrenal insufficiency could present an altered regulation of the immune system, which has been linked to IRIS (34). The fact that plasma levels of DHEA-S, which are mainly of adrenal origin, did not significantly change in non-IRIS-P after ART, but rather decreased more than 20% in IRIS-P with respect to values before treatment, could reflect a more impaired adrenal function in IRIS-P than in non-IRIS-P. These results are consistent with other studies in chronic diseases, such as tuberculosis, suggesting that the decrease in DHEA-S levels were associated with worse prognosis of the disease (35). An inadequate adrenal steroid production could be thought to aggravate the inflammatory process, and to contribute to the development of IRIS in HIV-infected patients. Supporting this idea, the DHEA-S/Cortisol ratios were significantly lower in IRIS-P after ART than in the other patients. This decrease has been associated with the altered metabolic pathways of adrenocorticoids synthesis (33,34,36).

It has been reported that acquired immune deficiency syndrome patients with decreased levels of DHEA-S show excessive cytokine production by Th2 cells (IL-4, IL-5, IL-6, and IL-10) and suppression of other cytokines (IL-2, IFN-γ, IL-12) (37). This would negatively affect these patients' evolution.

Abnormal thyroid function tests are more frequent in HIV-infected patients than in the general population (38). In the present study, the results showed that the plasma concentrations of T4 decreased significantly after ART, but the levels always remained within the normal range in all the patients.

Despite an intense search for hormonal or immune markers, which could predict which HIV-infected patients may be at risk of suffering IRIS after ART initiation, no reliable markers have been reported so far. The results of this study indicated that mean levels of IL-6 and IL-18 in IRIS-P almost duplicate the respective values in non-IRIS-P before ART. In addition, the decreased DHEA-S plasma levels and DHEA-S/cortisol ratio in IRIS-P with respect to values in non-IRIS-P, after ART initiation, could suggest a mild but critical adrenal deficiency in HIV-infected patients who undergo IRIS during ART. Based on these results, it could be useful to test the adrenal function of patients before they receive ART, in order to correlate the results with the potential development of IRIS.

In recent years, the enormous progress of ART has changed the survival expectations of HIV-infected patients. However, around 10% of the patients treated will suffer from IRIS during ART (18,39). Since the syndrome represents an important clinical problem, more studies on risk factors involving a larger number of patients, as well as the development of strategies for detection of patients at higher risk for IRIS are needed.

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Cardiovascular risk in rural workers and its relation with body mass index

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ABSTRACT

Objective: Evaluate the propensity of cardiovascular risk in rural workers and, through the Framingham Risk Score (FRS), relate this risk with the classification of Body Mass Index (BMI). **Subjects and methods:** This study is characterized as descriptive and exploratory, with the participation of 138 subjects, ranging between 25-73 years old. Clinical and laboratory analysis of the risk factors contained in the FRS were performed, in addition to the determination of BMI, blood pressure, smoking and physical inactivity. **Results:** The procedures indicated a low risk of a coronary event in 10 years with 70.3% of the population. In contrast, 88.4% of the subjects were overweight. It was evidenced a risk improvement as the BMI increased, since 96.4% of high-risk cases were overweight or obese. **Conclusion:** Results suggest larger prevalence of intermediary or high FRS for women with higher BMI, which was not observed in men. Arch Endocrinol Metab. 2018;62(1):65-71

Keywords

Agribusiness; mortality; cardiovascular diseases

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INTRODUCTION

Cardiovascular diseases are responsible for the high number of deaths, and recorded worldwide data in 2011 of 17 million, since 2000 leading the ranking of the ten leading causes of mortality. Mortality and morbidity levels in adults from 2008 to 2011, reported by WHO, show a significant increase of deaths due to cardiovascular disease, with production losses, and expenses with medical care, treatment and rehabilitation. Predictions are that by 2025 the risk of coronary heart disease and stroke will have increased by 120% for women and 137% for men living in developed countries (1). These figures reinforce the importance of effective primary measures for the prevention of these diseases (2).

Hypertension, diabetes mellitus, dyslipidemia, obesity and some habits related to lifestyle (poor diet, smoking, alcohol consumption, physical inactivity) are factors that predispose to cardiovascular risk (3). The Brazilian Society of Cardiology data indicate that 80% of individuals of the Brazilian adult population is sedentary and 52% are overweight, 11% obese, which reinforces the propensity of increased morbidity and mortality, since obesity is an independent risk factor associated with cardiovascular diseases (4).

These aspects combined with regional characteristics of the population and the changes processed in the labor world, with numerous effects on health and epidemiological profile of the working population (5), broaden the spectrum of health problems. Because, in addition to unhealthy lifestyles, inadequate working conditions, such as long working hours and high occupational stress, may predispose workers to high risk of cardiovascular diseases (6).

Rural workers have presented health problems. There is evidence that, by underreporting, the prevalence of diseases is higher in rural than in urban areas. Many factors could be related to health worsening, including restricted access to health system and work economic dependence, which subjects workers to long working days, frequent physical efforts, low income, assiduity even at physical adversities, and long term labor (increasing age group for this population) (7). For the International Labour Organization (ILO) rural work is of great significance, and more dangerous than other activities. It is estimated that millions of farmers suffer from serious health problems (8). Therefore, prevention measures are an important issue in promoting the health of these workers.

Given the complexity of these interactions, it is performed, in 1960, the first cohort study focusing

on cardiovascular disease by identifying risk factors and pathophysiology, the Framingham Heart Study (FHS), a time when mortality and the incidence of cardiovascular disease showed progressive increases (9). As a result of advances in the FHS, which occurred from 1998, it is possible to stratify the risk of coronary events in 10 years, through a specific score for each risk factor, using clinical variables of daily practice. This indicative stratification of cardiovascular disease risk propensity is classified as low, intermediate and high risk (10). According to the Ministry of Health, the Framingham Score identifies the potential for developing cardiovascular disease even before the onset of symptoms (11).

In this context, the objective of this study is to evaluate the cardiovascular risk in farmers and their relation to the classification of Body Mass Index.

SUBJECTS AND METHODS

This is a descriptive exploratory study, developed from the database project "Screening of risk factors related to overweight in agribusiness workers using new analytical and health information technologies" developed at the University of Santa Cruz do Sul, approved by the Ethics Committee in Research with Human Beings, under the Protocol 2509/10. We evaluated 138 subjects with a mean age of 51.32 years (SD 10.32), who met the following inclusion criteria: be 18 years old and not be carriers coronary artery disease, stroke, heart failure and acute myocardial infarction.

Labor activities of the sample individuals are those typical of family farming workers, such as planting, farming maintenance, harvesting, animal husbandry, manufacturing of homemade products and trading of the production. Sample was obtained among residents in the municipalities of Santa Cruz do Sul, Passo do Sobrado, Vale Verde, Rio Pardo, Candelária, Encruzilhada do Sul e General Câmara, in the State of Rio Grande do Sul, Brazil. According to Bertê and cols. (12), the region has its rural sector dedicated mainly to monoculture, but receives incentives aiming to diversification of production and development of family agroindustry.

The screening of the subjects was performed by external seminar with the institutions involved in the study. Opportunity in which voluntary participants were informed about the purpose of the study and the conditions of involvement, when the lifestyle questionnaire was applied. Sampling was non-probabilistic and by convenience.

A survey form proposed by the Brazilian Association of Survey Companies (ABEP – 2014) (13) was used for economic classification, which consists on collecting data of movable and immovable property, domiciliary goods, as well as the graduation level of the householder. There are eight classification levels for Brazilian Criterion: A1, A2, B1, B2, C1, C2, D and E. In this study, subjects were divided in two groups, one of them formed by the members of A1, A2, B1 and B2, and the other one formed by the remaining categories.

Anthropometric, biochemical and physiological assessments were subsequently applied at the University. The body mass index (BMI) was calculated from the body mass obtained on the scale (Welmy) and height measured in a stadiometer, and calculated from dividing weight (kg) by height (m) squared. Systolic blood pressure (SBP) was measured after five minutes rest, with the subject seated, and the data categorized as normal SBP (< 129 mmHg), borderline (between 130 and 139 mmHg) or hypertension (above 140 mmHg) according to the VI Brazilian Guidelines of Hypertension (2010) (14).

Blood tests were performed at the Laboratory of Clinical Biochemistry at the University of Santa Cruz do Sul, using reactive of Labtest brand in semiautomated system (LabMax Progress – LabTest), observing, to the glycemic index the recommendations of the American Diabetes Association (15). Total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c) reference values of the Brazilian Cardiology Society (16). Blood collection was performed in the brachial vein, preceded by fasting for 12 hours using two *vacutainer*, one of them containing fluoride\oxalate (to obtain plasma) and another without additive (to obtain serum). Blood samples deposited in *vacutainer* without anticoagulants were incubated at 37°C for 15 minutes and centrifuged at 2500 rpm for five minutes to collect serum. The plasma samples were subjected to the determination of glucose (GLU), and the serum samples submitted to the determination of the TC and HDL-c. Diagnosis of diabetes was determined after the results of the glucose test.

According to the criteria proposed by the Framingham Heart Study (FHS) the following risk factors were considered: age, sex, diabetes, TC, HDL-c, smoking and SBP. Through the Framingham score

(FRS), the risk of occurrence of coronary events in 10 years was calculated. For each risk factor was applied a specific score as suggested by the FHS, obtaining the final score by the sum of individual points. The results were classified as low risk (LR) score < 10%, intermediate risk (IR) score between 10 and 20% and high risk (HR) > 20%. It is important to highlight that all those identified as diabetics were considered automatically as HR (6).

Data were analyzed using the Statistical Software for Social Sciences (SPSS, version 20.0), using descriptive statistics with central tendency and dispersion measures, for numeric variables, and frequency and percentage, for categorical variables. Pearson's chi-square test was used to the analytical statistics of the frequencies distribution between FRS and BMI as well as between FRS and sociodemographic characteristics, considering $p < 0.05$. In order to evaluate the prevalence ratio of BMI over the FRS, a Poisson Regression Test was used.

RESULTS

Most of the subjects is aged 40-55 (44.2%), married (75.4%), from C1-C2-D economic classes (58%), up to 7 years educated (73.2%) and women (62.3%). Regarding BMI, it is noteworthy that 88.4% of employees have some level of overweight (overweight or obese). Obesity prevails among women (51.2%) and overweight among men (65.4%). Regarding the FRS, 81.4% of women and 51.9% of men have low risk (Table 1).

Regarding the biochemical characteristics and BP, it can be seen that 58.0% has increased level of TC, HDL values within the desired standard in 83.3% of cases. Yet the glucose levels of 43.5% of workers are in the range of pre-diabetic and 15.9% in the diabetes range (Table 1).

By analyzing the average values of the indicators that compose the estimate of FRS and the average of risk score, we observed that the factors that had higher scores on the FRS were the mean age 51.32 ± 10.32 years and the total cholesterol 211.81 ± 48.98 mg/dL. It is important to state that 93.5% of workers had no smoking habit. As regards the systolic pressure, the mean value obtained was 131.67 ± 17.00 mmHg. The average score of 11.09 ± 6.19 points represents low risk for myocardial infarction or coronary heart disease within 10 years (Table 2) which is confirmed by the percentage of individuals rated as LR (Table 1).

Table 1. Characterization of the sample

Variables	Gender		Total* 138 (100)
	Female* 86 (62.3)	Male* 52 (37.7)	
Age range			
< 40 years	14 (16.3)	11 (21.2)	25 (18.1)
40-55 years	41 (47.7)	20 (38.5)	61 (44.2)
> 55 years	31 (36.0)	21 (40.4)	52 (37.7)
Marital status			
Single	6 (7.0)	8 (15.4)	14 (10.1)
Married	65 (75.6)	39 (75.0)	104 (75.4)
Others	15 (17.4)	5 (9.6)	20 (14.4)
Economic level			
B1-B2	32 (37.2)	26 (50.0)	58 (42.0)
C1-C2-D	54 (62.8)	26 (50.0)	80 (58.0)
Schooling			
Up to 7 years	64 (74.4)	37 (71.2)	101 (73.2)
8 to 10 years	9 (10.5)	6 (11.5)	15 (10.9)
11 years or more	13 (15.1)	9 (17.3)	22 (15.9)
BMI			
Recommended range	8 (09.3)	8 (15.4)	16 (11.6)
Overweight	34 (39.5)	34 (65.4)	68 (49.3)
Obesity	44 (51.2)	10 (19.2)	54 (39.1)
Total cholesterol			
Desirable	36 (41.9)	22 (42.3)	58 (42.0)
Increased	50 (58.1)	30 (57.7)	80 (58.0)
HDL			
Desirable	75 (87.2)	40 (76.9)	115 (83.3)
Decreased	11 (12.8)	12 (23.1)	23 (16.7)
SBP			
Recommended range	34 (39.6)	26 (50.0)	60 (43.5)
Bordering	22 (25.6)	10 (19.2)	32 (23.2)
Hypertension	30 (35.0)	16 (30.8)	46 (33.3)
Glucose			
Desirable	36 (41.9)	20 (38.5)	56 (40.6)
Pre-diabetes	38 (44.2)	22 (42.3)	60 (43.5)
Diabetes	12 (14.0)	10 (19.2)	22 (15.9)
FRS			
Low risk	70 (81.4)	27 (51.9)	97 (70.3)
Intermediate risk	1 (1.2)	12 (23.1)	13 (9.4)
High risk	15 (17.4)	13 (25.0)	28 (20.3)

*: frequency (percentage); BMI: body mass index; HDL: high density lipoprotein; SBP: systolic blood pressure; FRS: Framingham Risk Score.

When stratifying the risk of coronary artery disease, according to the FRS, it was observed that 70.3% of workers has low risk, 8.6% has intermediate risk and

22.3% has high risk When analyzing the risk related to BMI classification, statistical significance was found (p = 0.035) indicating relationship between the variables, while obesity is a preponderant factor in the definition of risk, there are other intervening variables that were not evidenced in this study. Although most of the subjects were rated low risk, it is noteworthy that among subjects with intermediate risk 69.2% are overweight, since those with high risk in 96.4% of the patients present weight excess: overweight (39.3%) or obese (57.1%). Another fact to highlight is the occurrence of high risk in the subject with recommended weight range, which was due to the presence of diabetes mellitus (Table 3).

When analyzing the prevalence ratio between BMI and FRS, it can be observed that overweight women have 14.7% greater risk prevalence than those rated as normal BMI. Obese women showed risk prevalence 25% greater than the eutrophic. This fact was neither observed for the male population, nor in whole sample (Table 4). From Table 5 it comes that, among sociodemographic characteristics, gender and age group are related to FRS, an expected fact, since this factors are component variables of FRS.

Table 2. Average values in the indicators that composse the Framingham Risk Score

FRS variables	Mean value (SD)	Mean score (SD)
Age	51.32 (10.32)	4.75 (5.51)
Cholesterol level	211.81 (48.98)	3.70 (2.55)
Smoke* [yes/no]	9 (6.5)/129 (93.5)	0.35 (1.51)
HDL	49.96 (10.97)	0.48 (0.94)
SBP	131.67 (17.00)	1.83 (1.88)
FRS	11.09 (6.19)	-

*: frequency (percentage); FRS: Framingham Risk Score; HDL: high density lipoprotein; SBP: systolic blood pressure; SD: standard deviation.

Table 3. Relation between BMI and the Framingham Risk Score

BMI	FRS			p*
	Low risk** 97 (70.3)	Intermediate risk** 13 (9.4)	High risk** 28 (20.3)	
Recommended range	12 (12.4)	3 (23.1)	1 (3.6)	
Overweight	48 (49.5)	9 (69.2)	11 (39.3)	0.035
Obesity	37 (38.1)	1 (7.7)	16 (57.1)	

*: Pearson's chi-square; **: frequency (percentage); FRS: Framingham Risk Score; BMI: body mass index.

Table 5. Relation between sociodemographic characteristics and the Framingham Risk Score

	FRS			p*
	Low risk** 97 (70.3)	Intermediate risk** 13 (9.4)	High risk** 28 (20.3)	
Gender				
Male	27 (27.7)	12 (92.3)	13 (46.4)	< 0.001
Female	70 (72.2)	1 (7.7)	15 (53.6)	
Age range				
< 40 years	20 (20.6)	-	5 (17.9)	
40-55 years	53 (54.6)	3 (23.1)	5 (17.9)	< 0.001
> 55 years	24 (24.7)	10 (76.9)	18 (64.3)	
Marital status				
Single	10 (10.3)	-	4 (14.3)	
Married	74 (76.3)	12 (92.3)	18 (64.3)	0.382
Others	13 (13.4)	1 (7.7)	6 (21.4)	
Economic level				
B1-B2	43 (44.3)	4 (30.8)	11 (39.3)	0.615
C1-C2-D	54 (55.7)	9 (69.2)	17 (60.7)	
Schooling				
Up to 7 years	69 (71.1)	11 (84.6)	21 (75.0)	
8 to 10 years	12 (12.4)	1 (7.7)	2 (7.1)	0.806
11 years or more	16 (16.5)	1 (7.7)	5 (17.9)	

*: Pearson's chi-square; **: frequency (percentage); FRS: Framingham Risk Score.

Table 4. Prevalence ratio of FRS high risk in relation to BMI

BMI	General	Gender				
		Male		Female		
	PR (CI 95%)	p	PR (CI 95%)	p	PR (CI 95%)	p
Recommended range	1	-	1	-	1	-
Overweight	1.035 (0.86-1.25)	0.719	0.961 (0.74-1.24)	0.762	1.147 (1.03-1.27)	0.010
Obesity	1.052 (0.87-1.28)	0.610	1.067 (0.79-1.44)	0.672	1.25 (1.13-1.38)	< 0.001

FRS: Framingham Risk Score; BMI: body mass index; PR: prevalence ratio; CI: confidence interval.

DISCUSSION

The results of this cross-sectional study show the prevalence of overweight and obesity among workers classified with intermediate and high cardiovascular risk. Regardless of overweight or obesity present in most of sample, the subjects were often classified with low cardiovascular risk. From the factors considered in the FHS, the ones who obtained the highest scores in the formation of the risk score were age and TC. Variables that may be related because the increase in TC may be associated with increased age, as in this study the subjects had higher mean age (51.32 years) compared with those obtained by workers in other economic sectors, such as studies industrialists (36.27 ± 10.21) (17) and commercial workers (27.65 ± 9.38) (18).

This mean age is characteristic of the demographic profile of Brazilian rural workers marked by continuous aging (19). This phenomenon stems from the migration of youth to urban centers, as has also been identified in France (20). This happens due to the migratory flow, which is common in contemporary societies, according to Dasre and cols. (21).

However, in addition to issues related to the logic of the organization and dynamics of production centered on family labor, factors that contribute to male dominance is the aging of the rural population. However, external elements to the family play an important role in the movement of young people to the cities, such as the increasing difficulty of access to basic health and education services (22).

With respect to the average value of TC, it was observed that this was above the recommended as desirable in half the subjects in this study. This result shows resemblance to a survey of urban bus drivers in Teresina (Piauí), in which approximately half of subjects had TC at levels above 200 mg/dL (10), as well as 39.4% of 678 drivers, working in alternating shifts in a mining company in Minas Gerais, had increased TC (23). A bi-racial study with women and African-American and white men, found above the recommended cholesterol (> 200 mg/dL) among white women (24).

Regarding HDL-c most of the subjects were classified at levels considered adequate (50.03 ± 10.96). Similar average results were found in a study applied with 2037 subjects (24). It is important to highlight that the small number of smokers (6.5%) led to the disregard of this variable in this study.

Although the highest percentage of subjects has presented systolic blood pressure in the proper range, a third of them (33.1%) was classified with hypertension. This finding may be associated with the age of the workers, since study in two rural communities identified 42.9% of hypertensive in 18-94-year-old subjects (25). These findings confirm the scientific evidence that high blood pressure increases progressively with age, being a common condition in people with older age, especially in people over 60 years old (26). The Framingham Heart Study states that the prevalence of hypertension increases from 27.3%, in patients younger than 60 years, to 74.0% in those aged over 80 years old (27).

In addition to the primary prevention, it is necessary more control and treatment of hypertension, especially in critically ill patients, as shown in a study of medical records of patients from the Cardiology Clinic of Anapolis/GO. The same authors stress the urgency of greater control over risk factors, such as physical inactivity and obesity, in order to avoid the emergence of CVD associated with SAH (28).

This recommendation is based on the influence of overweight in the prevalence of hypertension due to the risk that these changes represent a condition found in 87.8% of workers. This finding overcomes the data from a study that observed overweight and obesity in 65.5% of subjects in the hospital area (29) and 40.7% of farmers in Minnesota, United States (30). It is possible to observe that one of the factors prevalent among hypertensive individuals is the obesity (31).

Regarding BMI, studies show that the prevalence of most chronic diseases are associated with the increase of this anthropometrical measure. In addition, the cardiometabolic risk factors have been affecting both men and women with overweight and obesity (32), with this measure related to increased risk of premature death in subjects with severe obesity or above 35 kg/m^2 (33).

It is worth noting the risk posed by visceral fat and body fat for the development of coronary atherosclerosis, before the presence of comorbidities resulting from cardiovascular diseases (34). Thus, the Framingham score, for its ability to anticipate the identification of individuals at risk of developing cardiovascular events, is an important method for the primary prevention. Preventive interventions should be proposed and are indispensable in order to prevent the occurrence of any event.

Regarding the groups, no significant differences were observed between the clinical variables. According to

the Framingham risk score, the average value found was 10.93 points, featuring intermediate risk of coronary events in 10 years. The results found in this study show that 22.3% of the subjects are at high risk of developing coronary event. Unlike the study of 309,955 workers in different economic sectors in Spain, with an average age of 36.5 years, in which 5.9% of the subjects were classified with high risk and 0.9% with moderate risk, those classified with high cardiovascular risk 7.6% were men and 1.7% were women, with a higher occurrence in agricultural (11.3%) and construction workers (8.2%), when compared to the industrial and services sector (35).

Finally, it is worth noting the presence of abnormal glucose (59.0%) with diabetic (15.8%) and pre-diabetic patients (43.2%) in the group assessed, highlighting the importance of primary actions that may reverse health complications through the assessment of cardiovascular risk in rural workers, using the Framingham risk Score, and stratify its distribution according to the classification of body mass index.

We considered the high number of diabetics in the sample was due to several factors, such as the advanced age of the individuals, the great number of overweight subjects and the fact that they referred not having regular attendance for this marker. Another point to be highlighted is the fact that men does not present risk increase as BMI increase. This fact points to weakness of BMI for part of this group, which could have been influenced by factors as lean mass increasing, what is not discriminated by BMI. Thus, we are sendend towards further investigations related to body composition and cardiovascular risk addressing the specifities of this population, especially regarding to the high average age.

The subjects of the study have a labor fairly active, mainly the men, which are more involved in heavy farm activities. Thus, men BMI might be biased, which could be explained by the overweight caused by lean body mass instead of fat body mass, since BMI does not tell which kind of body mass. This hypothesis could be investigated in future researches, using different anthropometric variables.

This study showed the presence of low risk for coronary events in the study population, estimated by the Framingham Risk Score. The influence of BMI on increasing cardiovascular risk was observed in the women workers. However, this fact was not identified in men. Neverthless, these results show the importance of this variable when implementing actions that can stimulate healthier lifestyles, especially when addressed to a population with limited access to health care networks, as in the case of rural workers.

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A possible association between the -2518 A>G *MCP-1* polymorphism and insulin resistance in school children

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ABSTRACT

Objective: Monocyte chemoattractant protein 1 (MCP-1) has been suggested to be involved in the pathophysiology of insulin resistance (IR); therefore, variants in the *MCP-1* gene may contribute to the development of this disease. The aim of this study was to analyze the relationship of the -2518 A>G *MCP-1* (rs1024611) gene polymorphism with insulin resistance in Mexican children. **Subjects and methods**: A cross-sectional study was performed in 174 children, including 117 children without insulin resistance and 57 children with IR, with an age range of 6-11 years. Levels for serum insulin and high-sensitivity C-reactive protein were determined. The -2518 A>G *MCP-1* polymorphism was identified by the polymerase chain reaction-restriction fragment length polymorphism method. Insulin resistance was defined as a HOMA-IR in the upper 75th percentile, which was ≥ 2.4 for all children. **Results:** Genotype frequencies of the rs1024611 polymorphism for the insulin-sensitive group were 17% AA, 48% AG and 35% GG, and the frequency of G allele was 59%, whereas frequencies for the insulin-resistant group were 12% AA, 37% AG and 51% GG, and the frequency of G allele was 69%. The genotype and allele frequencies between groups did not show significant differences. However, the GG genotype was the most frequent in children with IR. The GG genotype was associated with insulin resistance (OR = 2.2, P = 0.03) in a genetic model. **Conclusion:** The -2518 A>G *MCP-1* gene polymorphism may be related to the development of insulin resistance in Mexican children. Arch Endocrinol Metab. 2018;62(1):72-9

Keywords
Insulin resistance; MCP-1; polymorphism; obesity; children

INTRODUCTION

The marked increase in pediatric obesity in the past decade has resulted in unprecedented increases in the incidence of type 2 diabetes mellitus among children and adolescents (1,2). There is substantial evidence that obesity is the main determinant of insulin resistance in children and that it increases the risk not only for the metabolic syndrome in adulthood but also for cardiovascular disease and type 2 diabetes later in life (3,4).

Obesity is associated with a chronic low-grade inflammatory state, characterized by enhanced production of multiple cytokines and chemokines. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine produced by adipose tissue and other tissues. The production of MCP-1 in obesity is triggered when adipocytes are exposed to inflammatory cytokines and fatty acids. Other cells that produce MCP-1 in obesity include hepatocytes, skeletal muscle cells, monocytes, vascular smooth muscle and endothelial cells. MCP-1 production results in initiation and propagation of the inflammatory response in obesity (5,6). Some studies have found that plasma levels of MCP-1 are increased in obese adults (7) and in obese children (8) compared to lean controls. Furthermore, MCP-1 signaling has a direct role in the development of obesity. Younce and cols. reported that MCP-1-induced adipogenesis in 3T3-L1 cells is independent of PPAR gamma activation (9).

Mice with CCR2 deficiency had attenuated deposition of visceral fat and insulin resistance when challenged with a high fat diet (10). Moreover, *MCP-1* had an angiogenic effect on endothelial cells (11); therefore, it can contribute to the expansion and remodeling of adipose tissue (12).

In diabetic subjects, the high glucose-induced inflammatory process is characterized by the cooperation of a complex network of inflammatory molecules, such as cytokines, adhesion molecules, growth factors, and chemokines. The high glucose concentration induces an increase in the synthesis and release of MCP-1 by endothelial cells (EC) and smooth muscle cells (SMC) (13). MCP-1 is a potent chemotactic factor that regulates monocyte and macrophage migration and infiltration at sites of inflammation (14). The interaction of MCP-1 with its receptor CCR2 is considered pivotal in obesity-induced insulin resistance. Several groups have reported that mice with targeted deletions in the genes for *MCP-1/CCL2* and its receptor *CCR2* have reduced adipose tissue macrophage (ATM) content, decreased inflammation in fat and protection from high-fat (HF) diet-induced insulin resistance (15). Conversely, mice overexpressing MCP-1 in adipose tissue have an increased number of ATMs along with insulin resistance (16). Therefore, the MCP-1-CCR2 axis is of central importance for promoting ATM recruitment and insulin resistance in mice. Zineh and cols. reported that serum levels of MCP-1 increased in children with type 1 diabetes compared with the control group (17). In another study in adult subjects, serum concentrations of MCP-1 were higher in patients with type 2 diabetes than in normal subjects (18). The glucose and insulin appear to exert effects on MCP-1 secretion, and this interaction might be important for the development of insulin resistance in children (19).

A single nucleotide polymorphism characterized by a chance of A>G at position -2518 in the *MCP-1* distal promoter regulatory region affects MCP-1's transcription activity in response to IL-1β (20). This polymorphism has been associated with the development of chronic diseases such as obesity, hypertension, atherosclerosis, type 2 diabetes and insulin resistance (21-23). Briefly, in a German population, the *MCP-1* -2518G allele was associated with decreased prevalence of insulin resistance and type 2 diabetes (24). Another study carried out in a Japanese population found that in obese diabetics, -2518AA carriers had increased insulin resistance compared to -2518GG carriers (23). Alternatively, in an adult population of western Mexico, it was found that subjects without insulin resistance presented the -2518A allele more frequently than their counterparts with IR (25). In light of these contradictory results, it is important to perform replication studies in different populations to determine the association of this polymorphism with insulin resistance. In the Mexican population, the distributions of genotypic frequencies of this polymorphism in adults and its relations with bladder cancer and tuberculosis have been reported (26,27). Genotypic frequencies and their possible relationship to insulin resistance have not been identified in children. The aim of this study was to investigate whether the -2518 A>G *MCP-1* polymorphism is associated with insulin resistance in Mexican children.

SUBJECTS AND METHODS

Subjects

We analyzed a total of 174 unrelated children (86 girls and 88 boys), with an age range of 6-11 years, who were divided into two groups: 117 children without IR and 57 children with IR. The participants were all born in the State of Guerrero, Mexico, with a family history of ancestors, at least back to the third generation, born in this state. All children with evidence of infectious disease or with any treatment that could influence biochemical or hematological parameters were excluded from the study. Informed written consent was obtained from all parents or guardians before enrollment of children in the study according to the ethical guidelines of the 2008 Declaration of Helsinki. Approval for the study was obtained from the Research Ethics Committee of the University of Guerrero.

Clinical and anthropometric measurements

Body weight was determined in light clothes and without shoes using a body composition monitor (Tanita BC-553, Arlington, USA). Height and body circumferences were measured with a stadiometer and anthropometric tape (Seca, Hamburg, Germany), respectively. The classification of obesity was made using the 2000 Centers for Disease Control and Prevention growth charts, with normal weight defined as the 5th-85th percentiles and obesity as ≥ 95th percentile (28). The four skinfold thicknesses (triceps, biceps, subscapular and suprailiac) were measured with a plicometer

(Dynatronics Co, Salt Lake City, USA) and blood pressure (BP) with an aneroid sphygmomanometer (Riester CE 0124, Jungingen, Germany). Hypertension was defined as systolic or diastolic blood pressure in the 95th percentile or higher for age and sex, using an average of 2 measurements (29).

Laboratory assessment

A fasting blood sample was obtained from each child by antecubital venipuncture. Serum glucose levels were analyzed with semi-automated equipment (COBAS MIRA), and insulin levels were measured using an enzyme-linked immunosorbent assay (GenWay INS-EASIA kit). Intra-assay and inter-assay variation coefficients were 4.8% and 8.1%, respectively, and the detection limit was 0.17 µU/mL. The homeostasis model assessment of insulin resistance was used to determine insulin resistance in children. This score was calculated with the following formula: fasting serum insulin (µU/mL) x fasting plasma glucose (mmol/L)/22.5 (30). Insulin resistance was defined as a HOMA-IR at or above the 75th percentile, which was ≥ 2.4 for all children. Leukocyte and platelet counts were determined using an ADVIA-60 (Bayer Diagnostics). High-sensitivity C-reactive protein (hsCRP) levels were measured by a turbidimetry assay (BioSystems SA) with a sensitivity of 0.06 mg/L and intra-assay and inter-assay variation coefficients of 1.8% and 3.6%, respectively.

Genotyping analysis

Genomic DNA (gDNA) was extracted from peripheral leukocytes obtained from whole blood samples, according to the Miller method. The analysis of the -2518 A>G *MCP-1* (rs1024611) polymorphism was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, using the following primers: 5'CACAGAGAGAGTCTGGCCACGT3' (forward) and 5'CCAACAGAGGACTCTTGGTCT3' (reverse). The reaction was carried out in a final volume of 20 µl, adding buffer 1X to 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer, 2.0 U/µl Taq polymerase (Invitrogen Life Technologies) and 0.1 µg/mL of gDNA. PCR was performed with an initial denaturation at 94 °C for 5 min, followed by 25 cycles of amplification consisting of 94 °C for 30 s of denaturation, 63 °C for 30 s of annealing and 72 °C for 30 s of extension, and a final extension step at 72 °C for 5 min. The amplified product of 234 bp was digested with PvuII restriction enzyme (New England Biolabs) for 2 hours at 37 °C and analyzed by electrophoresis in 6% polyacrylamide gel (Invitrogen™ life technologies) stained with silver nitrate. The AA genotype lacking the PvII site migrated as a 234 bp fragment, whereas the GG genotype was cleaved and appeared as 159 bp and 75 bp fragments.

Statistical analysis

The analysis was performed with STATA software (V.9.2). Differences in variables between groups were evaluated using a chi-square test for categorical variables, Student's *t* test for continuous variables with symmetrical distributions (data are presented as the mean and standard deviation), and a Mann-Whitney U test for variables without symmetrical distributions (data are presented as median and 5th to 95th percentiles). The genotype and allele frequencies were determined by direct counting, and a chi-square test was used to calculate the Hardy-Weinberg Equilibrium in case and control groups. To evaluate the association between insulin resistance and the polymorphism under investigation, we used a logistic regression model that was adjusted by age, gender, obesity and hypertension. P values < 0.05 were considered significant.

RESULTS

The study was conducted in a total of 174 children, divided into two groups: 117 children without IR were recruited as a control group, and 57 children with IR were the case group. The descriptive characteristics of the participants are summarized in Table 1. The median age of the children was 9 years, without differences between groups in age or gender. The insulin-resistant children had increases in all measures of central and peripheral adiposity and in both systolic and diastolic blood pressure compared to the group with insulin sensitivity. We found a high prevalence of obesity (73.7%) in the insulin-resistant group compared with the insulin-sensitive group (36.8%). The group with IR also showed an increase in serum hsCRP levels but not in leukocyte or platelet counts.

Demographic, clinical and metabolic variables were compared between children grouped according to their genotypes of the -2518 A>G *MCP-1* polymorphism. Although three genetic models were performed,

Table 1. Clinical and laboratory variables of the studied groups

Variables	All children (n = 174)	Group without IR (n = 117)	Group with IR (n = 57)	*P* value
Age (years)‡	9 (6-11)	9 (6-11)	9 (6-11)	0.08
Gender % (n)*				0.36
Male	50.6 (88)	53 (62)	45.6 (26)	
Female	49.4 (86)	47 (55)	54.4 (31)	
Weight (kg)‡	33.2 (20.1-59.1)	30.7 (19.7-48.8)	44.4 (23-68.2)	< 0.001
Height (cm)†	132.6 ± 11.3	131.1 ± 10.6	137.2 ± 12.1	0.001
BMI (kg/m^2)‡	18.4 (14.3-27)	17.3 (14.1-24.9)	23.5 (14.9-29.9)	< 0.001
Obesity % (n)*				< 0.001
No	51.1 (89)	63.2 (74)	26.3 (15)	
Yes	48.9 (85)	36.8 (43)	73.7 (42)	
Waist circumference (cm)‡	67 (54-88)	66 (53-84)	80.3 (58-96)	< 0.001
Hip circumference (cm)‡	75 (62-96)	73 (62-89)	85 (66-104)	< 0.001
Waist-to-hip ratio‡	0.9 (0.8-0.97)	0.9 (0.8-0.97)	0.9 (0.8-1.0)	0.001
Arm circumference (cm)‡	21 (16-28)	20 (16-26)	24.5 (17-33)	< 0.001
Biceps skinfold (mm)†	15.5 ± 4.8	14.8 ± 4.8	17.6 ± 4.3	< 0.001
Triceps skinfold (mm)‡	15 (8.5-21.5)	13.5 (8-21.5)	18 (10-22)	< 0.001
Subscapular skinfold (mm)‡	13 (6-22)	12 (5.5-21)	18.5 (8-25)	< 0.001
Suprailiac skinfold (mm)†	17.9 ± 5.5	17.0 ± 5.5	20.7 ± 4.7	< 0.001
SBP (mmHg)‡	98 (81-115)	96 (81-110)	101 (80-124)	< 0.001
DBP (mmHg)‡	58 (48-71)	57 (48-68)	60 (44.5-78)	< 0.001
Glucose (mg/dL)‡	96 (74-112)	95 (72-112)	98 (78-114)	0.06
Insulin (μU/mL)‡	6.9 (0.8-22.4)	4.7 (0.6-9.2)	13.7 (9.9-31.9)	< 0.001
HOMA-IR‡	1.2 (0.-4.9)	0.6 (0-2.1)	3.1 (2.5-7.3)	< 0.001
hsCRP (mg/L)‡	0.8 (0.1-6.5)	0.6 (0.1-6.0)	1.6 (0.1-8.3)	0.004
Leukocytes (10^3/mm^3)‡	7.7 (5.0-12.3)	7.6 (5.0-12.5)	7.9 (5-11.5)	0.08
Platelets (10^3/mm^3)‡	308 (219-413)	306 (219-440)	316 (218-410)	0.22

* Values are expressed as percentages and *n*. Chi-square test. † Values are expressed as the means ± SD. Student's *t* test. ‡ Values are expressed as median (p5-p95). Mann-Whitney test.
IR: insulin resistance; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostasis model of assessment-insulin resistance; hsCRP: high sensitivity C-reactive protein.

the table only includes the comparison that shows significant differences. All children were divided into two groups, carriers and non-carriers of the A allele (AA+AG vs. GG). Interestingly, the G allele was the most frequent in children with insulin resistance (69%). However, we found that some A allele carriers also had insulin resistance (31%) (Table 2).

The -2518 A>G *MCP-1* polymorphism was found in Hardy Weinberg Equilibrium in the total population (X^2 = 0.57, P = 0.45), in cases (X^2 = 1.03, P = 0.31) and in controls (X^2 = 0.014, *P* = 0.91). The comparative analysis of genotype and allele frequencies between groups did not show significant differences; however, we found an association of the GG genotype with insulin resistance (OR = 2.2, 95% CI, 1.1-4.5; P = 0.03), determined by an adjusted genetic model (Table 3).

DISCUSSION

In this study, an association between the -2518 A>G *MCP-1* polymorphism and insulin resistance is shown in a sample of Mexican children. Our results indicate that children with the GG genotype have a 2.2-fold higher risk of developing IR in comparison to those with the AA or AG genotype.

In contrast, a study in a German population found a low prevalence of insulin resistance and type 2 diabetes in carriers of the G allele compared with subjects homozygous for the A allele (24). In another study in a Chinese population, the G allele proved to be protective (adjusted OR=0.49, 95% CI, 0.32-0.77; P < 0.0001) against type 2 diabetes compared with subjects homozygous for allele A (31). These results

Table 2. Clinical and laboratory variables according to *MCP-1* genotypes

Variables	All children (n = 174)	AA+AG (n = 104)	GG (n = 70)	*P* value
Age (years)‡	9 (6-11)	9 (6-11)	9 (6-11)	0.39
Gender % (n)*				0.42
Male	50.6 (88)	48.1 (50)	54.3 (38)	
Female	49.4 (86)	51.9 (54)	45.7 (32)	
Weight (kg)‡	34.1 (20.1-59.2)	32.1 (20.3-59.1)	36.2 (20.1-64.9)	0.13
Height (cm)†	133.4 ± 11.5	132.6 ± 11.1	134.6 ± 12.2	0.26
BMI (kg/m^2)‡	18.9 (14.3-28.2)	18.4 (14.3-25.9)	19.7 (14.5-29.1)	0.08
Obesity % (n)*				0.57
No	51.1 (89)	53 (55)	49 (34)	
Yes	48.9 (85)	47 (49)	51 (36)	
Waist circumference (cm)‡	67.5 (54-90)	67 (54-89)	70 (56-94)	0.10
Hip circumference (cm)‡	76 (62-96.5)	74 (62-96)	79 (62-96.5)	0.15
Waist-to-hip ratio‡	0.9 (0.8-0.97)	0.9 (0.8-0.97)	0.9 (0.8-0.97)	0.24
Arm circumference (cm)‡	21 (16-29)	21 (16.5-28)	22 (16-30)	0.20
Biceps skinfold (mm)†	15.6 ± 4.6	15.7 ± 4.9	15.5 ± 4.1	0.76
Triceps skinfold (mm)‡	15 (8.5-21.5)	15 (8.5-21)	15 (7.5-22)	0.97
Subscapular skinfold (mm)‡	13 (6-22.5)	12.8 (5.5-21.5)	13.5 (6-23)	0.65
Suprailiac skinfold (mm)†	18.2 ± 5.6	18.1 ± 5.5	18.2 ± 5.9	0.95
SBP (mmHg)‡	98 (80-115)	98 (80-117)	98 (81-110)	0.82
DBP (mmHg)‡	58 (49-71)	58 (49-72)	58.5 (50-70)	0.83
Hypertension % (n)*				0.47
No	92.5 (161)	91.3 (95)	94.3 (66)	
Yes	7.5 (13)	8.7 (9)	5.7 (4)	
Glucose (mg/dL)‡	96 (73-109)	96.5 (72-109)	95.5 (78-112)	0.71
Insulin (μU/mL)‡	6.9 (0.8-22.4)	6.6 (1.0-21.5)	8.2 (0.8-22.4)	0.17
HOMA-IR‡	1.7 (0.2-5.5)	1.5 (0.2-4.8)	1.9 (0.2-5.8)	0.15
Insulin resistance % (n)*				0.04
No	67.2 (117)	73.1 (76)	58.6 (41)	
Yes	32.8 (57)	26.9 (28)	41.4 (29)	
hsCRP (mg/L)‡	0.8 (0.1-6.5)	0.8 (0.1-8.0)	0.9 (0.1-6.3)	0.99
Leukocytes (10^3/mm^3)‡	7.6 (4.8-11.5)	7.7 (4.8-12.3)	7.6 (5.1-11.2)	0.64
Platelets (10^3/mm^3)‡	308 (219-412)	310.5 (224-413)	306 (205-410)	0.66

* Values are expressed as percentages and *n*. Chi-square test. † Values are expressed as the means ± SD. Student's t test. ‡ Values are expressed as median (p5-p95). Mann-Whitney test.
IR: insulin resistance; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostasis model of assessment-insulin resistance; hsCRP: high sensitivity C-reactive protein.

suggest a protective role of the G allele for the development of insulin resistance and type 2 diabetes in the Chinese population. These differences may be attributed to the ancestry of the population; it is known that the Mexican population originated from mixed racial ancestry consisting of individuals from Europe (mainly Spain) or Africa who migrated to America where Native Americans of this region were living, giving origin to the Mexican mestizo population, who present a greater genetic diversity. This diversity can cause marked differences in allelic frequencies and patterns of linkage disequilibrium across their genome (32).

There are few studies reporting an association of -2518 A>G polymorphism with insulin resistance and type 2 diabetes, but other investigators have found associations of the GG genotype with high serum MCP-1 levels, hypertension, lupus nephritis and tuberculosis (27,33,34).

Table 3. Association of insulin resistance with genotype and allele frequencies of *MCP-1* polymorphism

Genotype/allele	Group without IR (n = 117) % (n)	Group with IR (n = 57) % (n)	*P*†	OR crude (95% CI); *P*	OR adjusted (95% CI); *P*‡
Frequencies			0.13		
AA*	17 (20)	12 (7)		1	
AG	48 (56)	37 (21)		1.1 (0.4-2.9); 0.89	
GG	35 (41)	51 (29)		2.0 (0.8-5.4); 0.16	
Frequencies			0.08		
A*	41 (96)	31 (35)		1	
G	59 (138)	69 (79)		1.6 (1.0-2.6); 0.06	
Dominant model			0.41		
AA*	17 (20)	12 (7)		1	1
AG+GG	83 (97)	88 (50)		1.5 (0.6-3.7); 0.41	1.6 (0.6-4.3); 0.37
Recessive model			0.04		
AA+AG*	65 (76)	49 (28)		1	
GG	35 (41)	51 (29)		1.9 (1.0-3.6); 0.04	2.2 (1.1-4.5); 0.03
Additive model	-	-	-	1.5 (0.96-2.5); 0.07	1.7 (0.98-2.7); 0.05

* Reference category; † Chi square test. ‡ Genetic model adjusted by age, gender, obesity and hypertension.
IR: insulin resistance; OR: odds ratio.

We found that the GG genotype was associated with an increased risk for developing IR when compared with children carrying other genotypes, which suggest that IR may also be related to increased serum *MCP-1* levels. In other studies, it was observed that insulin induces substantial expression and secretion of MCP-1, both *in vitro* in insulin-resistant 3T3-L1 adipocytes and *in vivo* in insulin-resistant obese mice; thus, MCP-1 was identified as an insulin-response gene (6). This result should be considered with caution, as it is necessary to perform a replication study in a larger sample to confirm these results.

Obesity and IR are characterized by chronic systemic low-grade inflammation. Markers of inflammation, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), CRP and MCP-1, are increased in peripheral blood levels in obesity and are associated with IR and may predict the development of type 2 diabetes (35). In this study, the children with IR showed increased hsCRP levels but not leukocyte or platelet counts. The GG genotype carriers did not present an increase in these markers, which could be an indicator of systemic inflammation not related to the development of insulin resistance in the studied children.

We observed that children with IR showed increases in all measures of central and peripheral adiposity compared to children without IR. Insulin resistance is a hallmark of obesity, emerging early in the metabolic syndrome, and is highly associated with increased visceral adipose tissue mass (36). Adipose tissue in obese subjects is characterized by macrophage infiltration, which is an early event contributing to the development of systemic insulin resistance. Indeed, transgenic mice that overexpress MCP-1 specifically in adipocytes develop adipose tissue inflammation and insulin resistance without obesity (37). This finding indicates that the subcutaneous adiposity may be an important predictor of IR in children.

Two main limitations should be considered in our investigation. First, the small sample size limited the statistical power. Second, MCP-1 levels were not measured, therefore the association of -2518 A>G polymorphism with MCP-1 levels remains uncertain in our population. Thus, future studies in Mexican children are necessary to determine MCP-1 levels.

In conclusion, the -2518 A>G *MCP-1* gene polymorphism may be related to the development of insulin resistance in Mexican children.

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Association of different biomarkers of renal function with D-dimer levels in patients with type 1 diabetes mellitus (renal biomarkers and D-dimer in diabetes)

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ABSTRACT

Objective: This study aimed to evaluate the association between different renal biomarkers with D-Dimer levels in diabetes mellitus (DM1) patients group classified as: low D-Dimer levels (< 318 ng/mL), which included first and second D-Dimer tertiles, and high D-Dimer levels (≥ 318 ng/mL), which included third D-Dimer tertile. **Materials and methods:** D-Dimer and cystatin C were measured by ELISA. Creatinine and urea were determined by enzymatic method. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI equation. Albuminuria was assessed by immunoturbidimetry. Presence of renal disease was evaluated using each renal biomarker: creatinine, urea, cystatin C, eGFR and albuminuria. Bivariate logistic regression analysis was performed to assess which renal biomarkers are associated with high D-Dimer levels and odds ratio was calculated. After, multivariate logistic regression analysis was performed to assess which renal biomarkers are associated with high D-Dimer levels (after adjusting for sex and age) and odds ratio was calculated. **Results:** Cystatin C presented a better association [OR of 9.8 (3.8–25.5)] with high D-Dimer levels than albuminuria, creatinine, eGFR and urea [OR of 5.3 (2.2–12.9), 8.4 (2.5–25.4), 9.1 (2.6–31.4) and 3.5 (1.4–8.4), respectively] after adjusting for sex and age. All biomarkers showed a good association with D-Dimer levels, and consequently, with hypercoagulability status, and cystatin C showed the best association among them. **Conclusion:** Therefore, cystatin C might be useful to detect patients with incipient diabetic kidney disease that present an increased risk of cardiovascular disease, contributing to an early adoption of reno and cardioprotective therapies. Arch Endocrinol Metab. 2018;62(1):80-6

Keywords
D-Dimer, type 1 diabetes mellitus, cystatin C, creatinine, albuminuria

INTRODUCTION

Diabetic kidney disease is defined as a progressive increase in urinary albumin excretion (UAE), leading to glomerular filtration declining and, eventually, renal failure (1). It is the most important cause of end-stage renal disease, is an independent risk factor for cardiovascular disease and is responsible for increased mortality. It is estimated that nearly 30% of patients with diabetes develop renal disease (2,3).

Several biomarkers can be used to evaluate renal function in patients with diabetes, such as creatinine, urea, glomerular filtration rate (GFR), UAE and cystatin C. Creatinine is derived from metabolism of creatine and phosphocreatine of muscle cells, while urea is the major nitrogenous metabolite derived from the degradation of proteins (4,5). However, various factors can influence their levels aside from renal disease; therefore, the estimative of GFR is more often used in clinical

practice (4,5). UAE is an important biomarker of renal injury, which is used for the diagnosis and prognosis of diabetic kidney disease, since it enables early detection of renal parenchyma injury (4,6). Cystatin C is a low molecular weight protein synthesized by all nucleated cells, whose function is to regulate cysteine proteases. It has been shown to be very promising in detecting early stages of renal disease in diabetic patients (6,7).

D-Dimer is a specific degradation product of cross-linked fibrin clots. It is a classic hypercoagulability biomarker, useful in the diagnosis of thromboembolic events (8). There is an association between D-Dimer levels with the development of atherothrombosis and cardiovascular complications in patients with diabetes, indicating that D-Dimer can be useful in evaluating the risk of cardiovascular disease in these patients (8-10). D-Dimer levels also increase with the progression of renal disease in patients with diabetes, indicating that hypercoagulability could be a link between diabetic kidney disease and the increased risk of cardiovascular outcomes (11-14).

A biomarker that is capable of detecting early stages of renal function decline and, therefore, could be simultaneously associated with a higher hypercoagulability status could be of great value. This is because it could be useful for detecting patients with incipient diabetic kidney disease that present an increased risk of cardiovascular disease, contributing to an early adoption of reno and cardioprotective therapies and, consequently, to a reduction of mortality.

Therefore, this study aimed to evaluate the association between D-Dimer levels and different biomarkers to assess the relationship between hypercoagulability and renal disease in patients with type 1 diabetes mellitus (DM1).

MATERIALS AND METHODS

The study was performed in accordance with the 2000 Declaration of Helsinki. It was approved by the Research Ethics Committee of *Universidade Federal de Minas Gerais* (CAAE – 0392.0.203.000-11), and informed consent was obtained from all participants.

Clinical records of 240 consecutive DM1 patients being assisted at Endocrinology Ambulatories of the Hospital das Clínicas and Santa Casa de Misericórdia of Belo Horizonte, Brazil, from November 2011 to September 2012, were analyzed. After the application of exclusion criteria, 125 patients with clinical and laboratorial diagnosis of DM1 (15), 18 to 60 years of age, were selected for this study. Criteria of exclusion consisted of hepatic disease, alcoholism, coagulation or hemostatic abnormalities, malignant diseases, acute infectious, history of kidney transplantation, pregnancy and hemodialysis. Data regarding age, sex, weight, height, time of diagnosis of DM1, use of antihypertensive, statin and acetylsalicylic acid were obtained from medical records.

Serum creatinine and urea were determined using an enzymatic method, serum albumin was assessed using a colorimetric method and HbA1c was determined through an immunoturbidimetric method, using dry chemistry technology. Cystatin C and D-Dimer were measured by ELISA. UAE was determined in urine samples collected after at least 4 hours of urinary retention, and urinary albumin was normalized by urinary creatinine. Urinary albumin was evaluated using an immunoturbidimetric method and urinary creatinine was assessed using an enzymatic method, using dry chemistry technology. UAE ≥ 30 mg/g of creatinine was confirmed in two out of three occasions during a period between three and six months, and the median was calculated (6). The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation (16).

Statistical analysis was performed using SPSS software v. 20.0. Patients were divided into tertiles based on D-Dimer levels and were classified into two groups: low D-Dimer levels (< 318 ng/mL), which included first and second D-Dimer tertiles, and high D-Dimer levels (≥ 318 ng/mL), which included third D-Dimer tertile (17). The Shapiro-Wilk test was used to test the normality of the variables. Data normally distributed were expressed as mean ± SD and were compared using ANOVA and a t-est. Data not normally distributed were expressed as median (percentiles 25%-75%) and were compared using the Kruskal-Wallis H test and Mann-Whitney U test. Categorical variables were expressed as frequencies and compared using a chi-square test (χ^2). The presence of renal disease was evaluated using each renal biomarker; these were dichotomized using cutoff of ≥ 1.3 mg/dL, ≥ 40 mg/dL, ≥ 0.92 mg/L and ≥ 30 mg/g, for creatinine, urea, cystatin C and UAE, respectively (18-20). Bivariate logistic regression analysis was performed to assess which dichotomized renal biomarkers are associated with high D-Dimer levels and an odds ratio was calculated. Multivariate logistic regression analysis was performed to assess

which dichotomized renal biomarkers are associated with high D-Dimer tertiles after adjusting for sex and age, and an odds ratio was calculated. The correlation between non-categorized renal biomarkers and D-Dimer levels were evaluated using the Spearman Correlation. Differences were considered significant when $p \leq 0.05$.

RESULTS

The characteristics and clinical variables of the DM1 patients included in this cross-sectional study are presented in Table 1.

Patients with high D-Dimer levels were older ($p = 0.003$) and presented an increased frequency of antihypertensive use than those with low D-Dimer levels ($p = 0.001$). The frequency of males was decreased in the high D-Dimer group compared to the low D-Dimer group ($p = 0.003$). There were no significant differences among groups regarding BMI, time of diagnosis, HbA1c levels, use of statin and use of AAS. Reduced serum albumin was observed in patients of the high D-Dimer group when compared to the low D-Dimer group ($p = 0.006$). Patients with high D-Dimer levels presented more increased levels of creatinine, eGFR, urea, cystatin C and UAE than patients with low D-Dimer levels ($p = 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.004$, respectively).

Bivariate logistic regression analysis has demonstrated that patients with cystatin C ≥ 0.92 mg/L showed a better association with high D-Dimer levels [OR of 9.0 (3.8–21.1)], than patients with UAE ≥ 30 mg/g, creatinine ≥ 1.3 mg/dL, eGFR < 60 mL/min/1.73 m² and urea ≥ 40 mg/dL [OR of 5.0 (2.2–11.4), 5.3 (2.1–13.3), 6.0 (2.3–15.7) and 3.3 (1.5–7.3), respectively] (Table 2). A multivariate logistic regression analysis has shown that the association between all renal biomarkers and high D-Dimer levels remained, even after adjusting for sex and age (Table 2). After the adjustment for sex and age, patients with cystatin C ≥ 0.92 mg/L remained, presenting a better association with high D-Dimer levels [OR of 9.8 (3.8–25.5)] than patients with UAE ≥ 30 mg/g, creatinine ≥ 1.3 mg/dL, eGFR < 60 mL/min/1.73 m² and urea ≥ 40 mg/dL [OR of 5.3 (2.2–12.9), 8.4 (2.5–25.4), 9.1 (2.6–31.4) and 3.5 (1.4–8.4), respectively].

Cystatin C levels correlated better with D-Dimer levels ($r = 0.476$, $p < 0.001$) than other renal biomarkers ($r = 0.174$, $p = 0.070$ for creatinine; $r = 0.238$, $p = 0.012$ for urea; $r = -0416$, $p < 0.001$ for eGFR; $r = 0.314$, $p = 0.005$ for albuminuria) (Figure 1).

Table 1. Characteristics of patients with diabetes classified according to D-Dimer levels

	Low D-Dimer Group	High D-Dimer Group	p
Number of individuals (n)	82	43	
Age (years)	32 (24 – 37)	35 (30 – 45)*	0.003
Sex/male (n, %)	37 (45.1)	8 (18.6)*	0.003
BMI (kg/m²)	24 ± 3	23 ± 3	NS
Time of diagnosis (years)	18 ± 8	20 ± 6	NS
Use of antihypertensive (n, %)	44 (53.7)	36 (83.7)*	0.001
Use of statin (n, %)	22 (26.8)	18 (41.9)	NS
Use of AAS (n, %)	10 (12.2)	11 (25.6)	NS
HbA1c (%)	8.5 (7.5 – 9.8)	8.4 (7.6 – 8.4)	NS
Creatinine (mg/dL)	0.81 (0.66 – 0.92)	1.02 (0.71 – 1.45)*	0.001
eGFR (mL/min/1.73 m²)	112 (91 – 123)	76 (43 – 104)*	< 0.001
Urea (mg/dL)	31 ± 7	42 ± 17*	< 0.001
Albumin (g/dL)	4.1 ± 0.4	3.8 ± 0.4*	0.006
Cystatin C (mg/L)	0.74 (0.64 – 0.85)	1.11 (0.86 – 1.97)*	< 0.001
UAE (mg/g of creatinine)	8 (4 – 18)	44 (6 – 157)*	0.004
D-Dimer (ng/mL)	191 (134 – 233)	484 (381 – 639)*	< 0.001

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies n (%) and compared using the chi-square test ($\chi 2$).
* $p < 0.05$ for high D-Dimer group compared to low D-Dimer group.
NS: not significant. BMI: body mass index. UAE: urinary albumin excretion. AAS: acetylsalicylic acid.

Table 2. Association between renal biomarkers and high D-Dimer levels

Variable	Odds ratio (95% confidence interval) unadjusted	p*	Odds ratio (95% confidence interval) adjusted for sex and age	p*
Creatinine ≥ 1.3 mg/dL	5.303 (2.106 – 13.357)	< 0.001	8.374 (2.464 – 28.459)	< 0.001
eGFR < 60 mL/min/1.73 m^2	6.048 (2.335 – 15.666)	< 0.001	9.110 (2.643 – 31.408)	< 0.001
Urea ≥ 40 mg/dL	3.340 (1.535 – 7.271)	< 0.001	3.480 (1.433 – 8.453)	0.008
Cystatin C ≥ 0.92 mg/L	9.018 (3.853 – 21.109)	< 0.001	9.844 (3.796 – 25.527)	< 0.001
UAE ≥ 30 mg/g	5.042 (2.222 – 11.440)	< 0.001	5.285 (2.160 – 12.926)	< 0.001

Data was evaluated by bivariate and multivariate logistic regression analysis and are presented as odds ratio (95% confidence interval). NS = not significant.
* p < 0.05 for high D-Dimer group compared to low D-Dimer group.

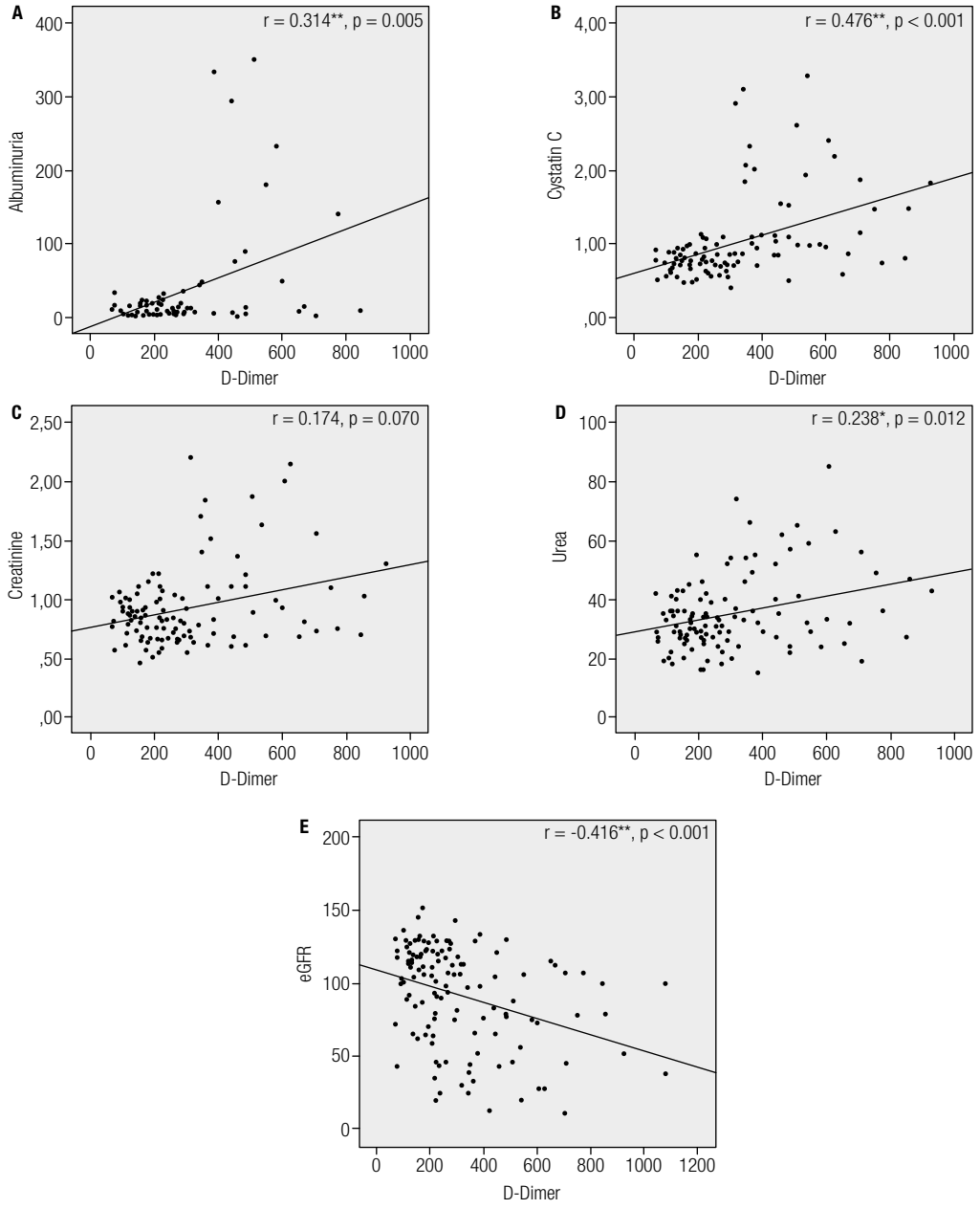

Figure 1. Spearman correlation of albuminuria (**A**), cystatin C (**B**), creatinine (**C**), urea (**D**) and (**E**) eGFR with D-Dimer levels.
* Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level.

DISCUSSION

Diabetic kidney disease is associated with an increased mortality, mainly due to cardiovascular outcomes (6). It has been demonstrated that the risk of cardiovascular death gradually increases with progressing stages of kidney disease (21,22). Therefore, simultaneous evaluation of early stages of renal function and hypercoagulability status using a unique biomarker would be of great value. Here, we have evaluated the association between different biomarkers of renal function with hypercoagulability status as assessed by D-Dimer levels in DM1 patients.

Some studies have found an association between increased D-Dimer levels and the presence of increased UAE levels and reduced eGFR in patients with diabetes (12-14,23). In this study, increased levels of different renal biomarkers, such as creatinine, urea, cystatin C, eGFR and UAE, were observed in patients with high D-Dimer levels. The association between renal dysfunction and increased levels of D-Dimer in DM1 patients may be explained by the increased synthesis of D-Dimer, but not by the reduced loss of D-Dimer in urine, since it has been shown that patients with diabetic kidney disease show higher urinary levels of D-Dimer than healthy individuals due to proteinuria (24). Proteinuria should also be responsible for the loss of important natural anticoagulant proteins, such as antithrombin, protein C and protein S, intensifying the hypercoagulability status and the production of D-Dimer (25).

It was verified that the decline of GFR also results in endothelial dysfunction and the release of the von Willebrand factor, which promotes platelet adhesion and aggregation and, consequently, microthrombi formation and increased D-Dimer levels (26). Endothelial dysfunction also impairs the activation of protein C, which depends on the endothelial protein C receptor and thrombomodulin, whose expression is reduced in damaged microvasculature, enhancing hypercoagulability status (27).

Haase and cols. (28) has demonstrated that D-Dimer plasma levels are higher in older adults and in females, which was also found in this study. These findings could be explained by the development of age-related changes in microcirculation and blood coagulation, which contribute to generate a hypercoagulability status and a gradual increase of D-Dimer levels with aging, as well as by the use of hormonal contraceptives in most of women. Such contraceptives can promote clotting mechanisms and increase D-Dimer levels (29,30). Increased frequency of antihypertensive use was observed in patients with high D-Dimer levels, which was expected, since these patients also showed an increased frequency of renal disease. Antihypertensive is commonly prescribed to patients with diabetes who have kidney disease to protect renal function (31). These patients also showed reduced serum albumin, which is in accordance to the increased UAE.

The relationship between increased UAE levels with a higher risk of cardiovascular disease in DM1 patients has been demonstrated by several authors (21,32,33). After the onset of proteinuria, median survival is only about seven years, and this increased mortality is mainly due to cardiovascular death rather than renal failure (34). High levels of serum creatinine and reduced eGFR have also been demonstrated to be indicative of progressive cardiovascular disease among diabetic patients (35,36), and increased levels of cystatin C have been associated with the development of cardiovascular events (37-40). Some authors have even shown that cystatin C is a stronger predictor of cardiovascular outcomes in patients with diabetes and elderly adults than creatinine and eGFR (39,40).

In agreement in this study, cystatin C presented a better association – when assessed by odds ratio analysis – with higher D-Dimer levels than urea, creatinine, eGFR and UAE, after adjusting for sex and age, which are variables that can influence D-Dimer levels (28). Cystatin C levels also presented a better correlation with D-Dimer levels than other renal biomarkers. These results suggest that cystatin C presents a better association with hypercoagulability status than other renal biomarkers and that it might be able to detect hemostatic changes that are not completely captured by measurements of urea, creatinine, eGFR and UAE. Cystatin C has been demonstrated to be a better biomarker to detect early stages of chronic kidney disease than creatinine and eGFR (41,42). This could partially explain the better association of this renal biomarker with higher D-Dimer levels, since cystatin C could detect a decline of renal function, and consequently a hypercoagulability state, that is not detected by other biomarkers. However, further longitudinal studies that directly assess the development of cardiovascular disease are still necessary to confirm the superiority of cystatin C to predict this risk in comparison to other renal biomarkers.

Some studies have reported that arteries with atherosclerosis contain more cysteine proteases

than normal arteries, which may contribute to the degradation of atherosclerotic plaque (43,44). Cystatin C is a protein responsible for inhibit cysteine proteases and is a biomarker able to detect early stages of chronic kidney disease (41,42). Therefore, patients with renal disease present high levels of cystatin C, which may inhibit proteases that promote the degradation of atherosclerotic plaque. This contributes to the development of atherosclerosis and cardiovascular disease and explains why cystatin C is the renal biomarker that presents a better association with hypercoagulability status. However, this hypothesis should be further evaluated.

In conclusion, all renal biomarkers showed a good association with D-Dimer levels and, consequently, with hypercoagulability status. However, cystatin C showed the best association among them. These findings suggest that cystatin C might present an important utility to simultaneously evaluate renal function decline and the hypercoagulability status in DM1 patients.

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A review of Cushing's disease treatment by the Department of Neuroendocrinology of the Brazilian Society of Endocrinology and Metabolism

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ABSTRACT

The treatment objectives for a patient with Cushing's disease (CD) are remission of hypercortisolism, adequate management of co-morbidities, restoration of the hypothalamic-pituitary-adrenal axis, preservation of fertility and pituitary function, and improvement of visual defects in cases of macroadenomas with suprasellar extension. Transsphenoidal pituitary surgery is the main treatment option for the majority of cases, even in macroadenomas with low probability of remission. In cases of surgical failure, another subsequent pituitary surgery might be indicated in cases with persistent tumor imaging at post surgical magnetic resonance imaging (MRI) and/or pathology analysis of adrenocorticotropic hormone-positive (ACTH+) positive pituitary adenoma in the first procedure. Medical treatment, radiotherapy and adrenalectomy are the other options when transsphenoidal pituitary surgery fails. There are several options of medical treatment, although cabergoline and ketoconazole are the most commonly used alone or in combination. Novel treatments are also addressed in this review. Different therapeutic approaches are frequently needed on an individual basis, both before and, particularly, after surgery, and they should be individualized. The objective of the present review is to provide the necessary information to achieve a more effective treatment for CD. It is recommended that patients with CD be followed at tertiary care centers with experience in treating this condition. Arch Endocrinol Metab. 2018;62(1):87-105

Keywords
Cushing's disease; Cushing's syndrome; treatment

INTRODUCTION

Cushing's syndrome is associated with a high mortality risk (1-10). A meta-analysis has found a standardized mortality ratio (SMR) of 2.22 (range, 1.45 – 3.41; confidence interval, CI, 95%) in patients with Cushing's syndrome compared to the general population (7). The major causes of mortality in these patients are cardiovascular diseases (ischemic heart disease and cerebrovascular diseases), diabetes mellitus (secondary to hypercortisolism), and infections (due to immunosuppression).

Clearly, the mortality rate is influenced by the disease activity. The SMR is higher in patients with persistent disease when compared to patients in clinical remission of the hypercortisolism: 5.50 (range, 2.69 – 11.26) vs. 1.20 (range, 0.45 – 3.18), respectively (7).

Nevertheless, even after the resolution of hypercortisolism, there may not be a complete reversal of cardiovascular risk factors or, alternatively, a complete reversal may take more than 5 to 6 years to occur (8,11,12). In addition, patients with active Cushing's disease (CD) present with poorer quality of

life and lower scores might persist even after surgical remission (13).

One factor contributing to the onset and progress of associated co-morbidities is the time spent since the recognition of the disease, its diagnostic confirmation (involving complex and expensive laboratoty and imaging studies), and correct definition of the etiology (1,14-17). Accordingly, a recent study showed increased mortality in patients with longer exposure to hypercortisolism (8).

Thus, to improve the prognosis of patients with Cushing's syndrome and to help reverse morbidities, it is important to identify the disease and to achieve eucortisolism as soon as possible.

The objective of the present review is to provide the necessary information to achieve a more effective treatment of CD. The specific therapeutic approach of the several co-morbidities associated with CD is beyond the scope of this review. The present manuscript highlights the importance of centers of excellence with a highly experienced multidisciplinary team for long-term follow-up of these patients

TREATMENT

The goals of the treatment of CD are as follows: (i) remission of hypercortisolism, (ii) adequate management of co-morbidities and cardiovascular risk factors, (iii) restoration of the hypothalamic-pituitary-adrenal axis, (iv) preservation of fertility and maintenance of pituitary function and (v) improvement of visual defects in cases of macroadenomas with suprasellar extension. However, it is common that one or more objectives need to be sacrificed to achieve remission in a patient (18).

Although surgical treatment generally results in high remission rates at short term in series from specialized centers (~70-80%), recurrence is observed in a substantial proportion of patients who then need other therapies to control hypercortisolism.

Surgical treatment

Despite advances in drug treatment and progress in other therapeutic modalities, such as stereotactic radiotherapy, surgical treatment is still the principal definitive treatment for CD (19).

Some recommendations regarding the preoperative management of patients with CD should be taken into account. Due to a higher risk of cardiovascular complications, such as coronary heart disease, it is important to conduct a careful cardiac assessment of these patients. However, the risk stratification does not differ from that in individuals without Cushing's syndrome. There is also increased risk of thromboembolic events, both pre- and postoperatively (PO) (20-25). Thus, the analysis of coagulation factors is very important, although there are no formal guidelines to define which factors should be analyzed in addition to those traditionally considered (i.e., thrombin time, prothrombin time, thromboplastin activated time, platelets) or which coagulation protocol should be recommended before and after surgery (26). In patients treated with antiplatelet drugs (to prevent ischemic events), antiplatelet therapy should be withdrawn at least seven days before surgery.

Usually, drug treatment for hypercortisolism is unnecessary during the preoperative period. Nevertheless, in some cases, it is necessary to initiate a specific medical treatment during the preoperative period, for instance, in patients with more severe disease and increased preoperative risk and when surgical treatment can not be immediately performed. The most commonly used approach is therapy with adrenal steroidogenesis inhibitors, particularly ketoconazole, which has a shorter half-life than the dopaminergic agonist cabergoline, which acts on corticotroph tumors and causes less interference with cortisol measurements during the PO period (27).

Many factors influence transsphenoidal surgery remission rates, including characteristics of the pituitary adenoma (e.g., tumor size, location, extension, aggressiveness, histological type and radiological identification), surgical procedure (e.g., the quality of the equipment and surgical technique), variability of the remission criteria used, and, particularly, the surgeon (e.g., experience, surgical identification of the tumor) (28).

All factors mentioned above contribute to the wide range of PO remission rates reported on by numerous case studies. In general, the mean remission rate ranges from 70% to 90% in several reviews (19,27,29,30). However, within the same case series, the remission rate may vary, depending on the subset of the patient analyzed, e.g., whether patients had a micro or a macroadenoma, whether tumor was identified on preoperative pituitary magnetic resonance imaging (MRI) or during surgery; and whether patients had been previously submitted to bilateral and simultaneous

petrosal sinus sampling (BIPSS). Importantly, recurrence may occur several years later.

In cases of macroadenoma (≥ 10 mm), the remission rate ranges from 50% to 70% (27) and is generally lower than the microadenoma remission rate (19). These percentages can vary depending on the size and, particularly, the degree of invasion of the adenoma into the adjacent tissues. A study performed on a small sample (n = 40) identified tumor size as the main factor responsible for post surgical remission rate. The authors observed remission rates of 84% for microadenomas (21/25), 92% for macroadenomas (11/12; mean of 15 mm; only 2 with invasion) and no remission for patients without visible tumor during surgery (n = 3) (31).

The remission rate in patients submitted to BIPSS is also lower than that observed in visible microadenomas on MRI and ranges from 50% to 70% (27,32). Interestingly, a study that specifically assessed remission in these cases did not show any difference between positive and negative MRI findings (33). In this subset, the main factor influencing remission rate was the intraoperative identification of the tumor.

Other predictive factors of a better surgical outcome include absence of invasion of the duramater or cavernous sinuses, histological confirmation of adrenocorticotropic hormone-positive (ACTH+) adenoma, low serum cortisol levels during the PO period, and prolonged secondary adrenal insufficiency (> 12 months) (27).

In pediatric patients, the number of reported cases is lower, but remission rates are similar or slightly increased in some cases, ranging from 83% to 98% (34,35). A recent study identified histopathology with an ACTH+ pituitary adenoma and no invasion as early predictors of remission in children. Young age, smaller tumors and no invasion of duramater or cavernous sinuses were predictive of long-term remission (35).

Almost all patients undergo pituitary surgery via transsphenoidal endonasal approach, including those with macroadenoma (29,30). Craniotomies are the exception and are indicated in rare selected cases. The most studied is the microscopic technique, but the endoscopic approach has been increasingly used in the last decade. The remission rate is similar for both techniques, particularly in the case of microadenomas (30,36-40). For macroadenomas and invasive tumors, the endoscopic technique has a potential advantage in offering a greater angular field of view, and therefore visualizing and removing tumors impinging the cavernous sinus or extending beyond the sellar boundaries. Other techniques that can improve intraoperative tumoral localization, such as ultrasonography, neuronavigation, rapid pathological and/or hormonal analysis and intraoperative MRI, are not available in most centers and therefore their usefulness cannot be properly evaluated so far.

Adenomectomy is the most performed surgery, although exploration of the entire gland is justified in most cases. In patients with no identified tumor, an ipsilateral hemi-hypophysectomy (partial hypophysectomy) is usually performed in the side suggested by BIPSS with lower remission rates. Total hypophysectomy is rarely justified due to a limited remission rate in such cases and expected hypopituitarism (27).

Pituitary surgery has a low mortality rate (0% to ≤ 1.5%), (29,41) comparable to the outcomes observed in simpler surgical procedures. The most common complications are endocrine: transient diabetes insipidus in 3% to 9% of patients (polyuria and/or hypernatremia), hyponatremia that can result from secondary adrenal insufficiency, particularly in patients not taking glucocorticoids, or syndrome of inappropriate antidiuretic hormone secretion (SIADH), and other pituitary deficiencies (growth hormone deficiency, hypogonadotropic hypogonadism, central hypothyroidism). Other complications include cerebrospinal fluid fistula (< 8%), bleeding or hematomas (range, 1-6%), epistaxis, infections (particularly sinusitis), and thromboembolic events (29). Due to the risk of thromboembolic events, it is recommended to perform active prophylaxis, including pneumatic compression of the lower limbs, low-molecular-weight heparin treatment as soon as possible (24h after surgery), and early mobilization during the hospital stay. However, there is no current specific anticoagulation protocol for Cushing's disease. The rates of complications derived from microsurgery and endoscopic techniques are similar.

Criteria for remission, glucocorticoid replacement therapy, and prediction of recurrence risk

Several clinical and laboratory criteria are used to define PO remission, but there is no consensus or ideal method that guarantees a recurrence-free follow-up period (29). However, a patient who develops adrenal insufficiency with very low serum cortisol levels (< 2 mg/dL) and requires glucocorticoid

replacement therapy clearly exhibits PO remission. However, these "rigid" laboratory criteria are not found in up to 20% of patients who show long-term remission and exhibit "normal" PO cortisol levels (42). Other factors used to define post surgical remissions are reversal of hypercortisolism, need of glucocorticoid replacement therapy and normalization of cortisol parameters, particularly urinary free cortisol (UFC) lasting at least 6 months after surgery.

Adrenal insufficiency is not clear in all cases, particularly when early glucocorticoid replacement therapy is routinely performed or in previously treated patients who undergo surgery and who exhibit eucortisolism. The symptoms that indicate adrenal insufficiency are asthenia, appetite loss, nausea, skin peeling, joint and muscle pain, weight loss, low blood pressure and/or postural hypotension. Mild hyperthermia, a transient increase in TSH levels, and hyponatremia might also occur. Although ACTH also stimulates aldosterone secretion, it should be highlighted that severe hypotension and hyperkalemia are not common due to the integrity of the renin-angiotensin-aldosterone system.

The most studied and utilized laboratory parameter is serum cortisol. In a recent guideline, a cortisol level < 5 µg/dL in the first PO week was stated as indicative of remission (19). Other authors have attempted to identify a more accurate "ideal" cortisol level (43). However, it is known that even undetectable levels of serum cortisol are not a guarantee of long-term remission (44), and an important study observed recurrence in 20% of patients at 5 years, even among those with cortisol levels < 2 µg/dL (45). Thus, more important than determining an "ideal value" is to understand that there are different levels of recurrence associated with serum cortisol values, as follows: < 2, low risk; 2 – 5, intermediate risk; > 5 µg/dL, high risk (46). Another important finding is that 5.6% of patients present a gradual decline in cortisol levels after the first week ("late remission") (47). For this reason, other cortisol samples must be collected, particularly during the first PO month. The two most cited explanations for this fact are the persistence of cortisol secretion due to chronically stimulated adrenal glands and the subsequent post surgical necrosis of corticotropic tumor cells. Interestingly, one study has shown increased long-term recurrence in patients from this late remission subgroup (47).

A very important factor for the analysis of serum cortisol levels in the PO period is glucocorticoid replacement therapy. Generally, two replacement strategies have been used and they do not include glucocorticoids during anesthetic induction. In one strategy, routine replacement therapy is not performed during the immediate PO period and in the initial days. Despite the short half-life of cortisol (range, 50–70 minutes) and intense reduction of its serum concentration after a successful adenoma removal, the patient does not usually show adrenal insufficiency too early – 24-48 hours – after surgery (48-50). Thus, the measurement of morning cortisol or a 6/6-hour curve starting in the immediate PO period is performed during the first days, and glucocorticoid replacement is initiated only after suggestive symptoms of adrenal insufficiency (with measurement being performed immediately before) and/or when low levels of cortisol are detected (< 5 µg/dL). Endocrinologists should closely assess the patient, if possible, until replacement is initiated. In this strategy, one advantage is that serum cortisol measurements are not influenced by exogenous corticosteroids. The second strategy consists of initiating routine glucocorticoid replacement therapy during the immediate PO period, preferably with short half-life corticoids, such as hydrocortisone (immediate PO, 25–50 mg intravenously three times per day), followed by oral hydrocortisone (from the 1st PO day forward: 20 mg early in the morning, 10 mg at 2 PM). In this way, serum cortisol measurements are performed only in the morning from 8 to 9 AM on any given day, under fasting condition, 18 to 24 hours after the last dose. The main advantages of this strategy are (i) easy applicability; (ii) patient safety; (iii) a lower incidence of adrenal insufficiency symptoms, although relative adrenal insufficiency can still occur with replacement therapy; (iv) reduced suppression of the hypothalamic-pituitary-adrenal axis (good for the pediatric population) compared with longer half-life drugs, such as prednisone or dexamethasone; and (v) easy measurement of serum cortisol, which allows the physician to observe the progressive increase of serum cortisol levels along with the recovery of the axis, which occurs 6 to 18 months PO.

However, although it is the most used glucocorticoid in this clinical setting, hydrocortisone is commercialized in Brazil in just one tertiary center (hydrocortisone, 20 and 5 mg tablets). The advantage of using dexamethasone (dose: 0.25 – 1 mg once daily; tablets with 0.5, 0.75 and 4 mg) is that it does not usually interfere with the serum and urine cortisol measurement, and it has been

used in some centers (29). However, due to its longer half-life, even when used at low doses, it is not possible to exclude the suppression of the axis, potentially leading to underestimated cortisol levels. The only advantage of prednisone (dose: 2.5 – 5 mg once daily; tablets, 5 and 20 mg) is the diffuse availability of the product; however, this drug can cause interference with the cortisol measurement and may suppress the axis with chronic use, although the risk of suppressing the axis is lower when compared to dexamethasone. Prednisone should be withdrawn at least 48 h before serum and/urine cortisol measurements, leading to an increased need of observation for the risk of adrenal insufficiency. Recently, another form of oral dual-release hydrocortisone has been studied in patients with adrenal insufficiency (51). However, there is no data on advantages of these formulations in this subgroup of PO patients with CD in order to normalize the hypothalamic pituitary adrenal axis.

In addition to serum cortisol, other laboratory criteria have been used, albeit infrequently, to define short-term remission (40). Among these criteria are lower than normal UFC (47), lower than normal plasma ACTH (52) and cortisol suppression after a low dose of dexamethasone (15).

Among these parameters, ACTH measurement is currently the most studied and is primarily used to predict the risk of long-term recurrence (49,53). A study that analyzed patients in initial PO remission (serum cortisol < 3 µg/dL) showed lower PO ACTH in patients with long-term remission compared to those with recurrent disease (11.9 vs. 34.3 pg/mL, $p < 0.0001$, respectively) (49).

One study investigated late-night salivary cortisol and found this parameter to be more accurate in predicting PO remission in comparison to serum cortisol and UFC (54). Late-night salivary cortisol was measured starting from 6 months PO. Further studies are necessary to show the utility of this method in assessing initial remission. However, there are studies showing a good utility of this method for earlier diagnosis of recurrence, even before the UFC (55,56).

Other methods are used to predict the risk of long-term recurrence. Primary among these methods are measurements of ACTH or cortisol following administration of corticotropin-releasing hormone (CRH) (46), metyrapone, thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), loperamide and desmopressin. The rational of these tests derives from the incapacity of normal corticotropes, suppressed by hypercortisolism, to secrete ACTH. In this way, early PO responses suggest the presence of residual tumor cells and, consequently, an increased risk of recurrence. The response to desmopressin is the most used test, with several studies showing similar results (57-64). One of the major problems with this test is the definition of ACTH and cortisol responses after desmopressin administration in the PO period. Several authors have used criteria similar to those used in the preoperative period (i.e., serum cortisol increased by > 20% and ACTH increased by > 30% to 50%) (57-61,63,64). These definitions, however, may overestimate any observed increase; for example, a change in serum cortisol from 1 to 2 µg/dL could correspond to an increase of 100%. A previous study assessed the risk of recurrence if the variation of serum cortisol is > 7 µg/dL (variation (Δ): peak minus time 0) after IV desmopressin 10 µg administration approximately 2 weeks PO. This method had a specificity of 100% and a sensitivity of 33%. It should be noted that in this previous study, only patients with low serum cortisol (< 6 µg/dL approximately 6 days PO) were subjected to the test, excluding patients with a risk of recurrence due to increased PO cortisol levels (62). Another factor to be kept in mind as associated to lower risk of recurrence is the higher length of postoperative glucocorticoid replacement, such as more than 12 months (41).

Surgery following initial surgical failure

After an initial surgical failure, the clinical case must be entirely reviewed. A diagnosis of CD should be confirmed by histopathological examination of the pituitary adenoma, which should be ACTH-positive on immunohistochemistry. If no adenoma has been found and the pituitary gland is reported as normal, then the diagnosis might be confirmed through PO remission or through the central to peripheral ACTH gradient at BIPSS. BIPSS might be performed with the sole objective of confirming a central origin not yet proven, considering that lateralization cannot be trusted to predict tumor localization. Another important issue is the description of the surgery as reported or registered by the surgeon. For example, a report of partial tumor resection due to invasion of the cavernous sinus in a macroadenoma patient limits the indication for subsequent surgery (29). The confirmation of

pituitary adenoma is also important in deciding upon a new pituitary surgical procedure. This should be evaluated with a new pituitary MRI performed at least 3 months after transsphenoidal surgery showing a residual tumor (65). These aspects need to be carefully analyzed before referring patients for another pituitary surgical procedure, especially if the first operation was not performed by an experienced surgeon (29). The remission rate is lower than observed for the first surgery, ranging from 40% to 70% (27,66,67), a rate that is similar to that obtained with current medical treatment. In addition, it is important to highlight that the rate of complications, including cerebrospinal fluid fistula and hypopituitarism, is higher when compared to the first surgery.

Some authors recommend early re-operation at 3 to 15 days after initial surgical failure, defined by serum cortisol > 2 µg/dL (53,68-70). However, as late remission can occur in 5.6% of patients 30 to 50 days after the initial surgery (47), this strategy is not commonly used.

Medical treatment

Medical treatment can be classified as primary or secondary. Primary therapy is used to lower cortisolemia and improve preoperative clinical conditions, or in cases of surgical contraindication or refusal, or before other definitive approaches. Secondary treatment after surgical failure is much more common, and is indicated for patients with relapse and no indication for a new surgery, as well as in patients who undergo pituitary radiotherapy. The drugs are classically divided into three classes: a) acting on the ACTH-secreting tumor, b) adrenal steroidogenesis inhibitors, and c) cortisol receptor antagonists. The first two classes comprise several drugs, some of which are currently in use and several others are not available or are under development. The limited number and availability of these drugs reflect the difficulty of controlling cortisol levels in CD patients. No ideal treatment is available, as reviewed in several recent articles (18,22,23,71-80).

Drugs acting on the corticotropic tumor

Cabergoline

Due to the very frequent expression of dopaminergic subtype 2 receptors (DRD2) on the surface of tumor cells in several types of pituitary adenomas, dopamine agonists, particularly cabergoline, have been used to treat prolactinomas (first option), acromegaly (adjuvant), clinically non-functioning adenomas and CD (adjuvant).

A study on corticotropic tumors elegantly demonstrated the expression of DRD2 in more than 80% of tumor samples, which exhibit binding affinity and *in vitro* inhibition of ACTH secretion in response to dopamine agonists (50).

For the treatment of CD, bromocriptine was initially used with limited efficacy and common side effects due to the need of high doses (range, 3.75 – 30 mg). Thus, the use of bromocriptine is no longer justified. Cabergoline, a better-tolerated and more potent and specific DRD2 receptor agonist, has been assessed for efficacy in some studies (50,81-85). UFC normalization, the primary endpoint for most studies, was observed in 25 – 40% of the 72 patients analyzed (4 studies with at least 10 cases), with mean dose of 3 mg/week (range, 1 – 7) and an average treatment period of 18 months (range, 3 – 60) (50,81-83). No long-term response predictor has been identified and there have been few cases studying the correlation between the *in vivo* response and DRD2 receptor tumor expression (50). The efficacy of cabergoline declines with increasing treatment duration, an effect that is primarily due to tachyphylaxis, which occurs due to unknown mechanisms and is observed in 18 – 30% of cases (81,82), even with prolonged use (1 – 5 years) (81,82). Cabergoline therapy is usually initiated at 1 mg/week (0.5 mg twice per week, at night; tablets, 0.5 mg), with a monthly increase of 1 mg if UFC normalization does not occur. One study defined unresponsiveness as a reduction in UFC lower than 25% after 3 months of treatment with increasing doses (81). A reduction in tumor size/volume was poorly assessed in previous studies. A reduction of at least 25% of the tumor size occurred in 50% of cases in one study (81), although case reports have demonstrated significant reductions (86,87). There is a recommendation for monitoring cardiac valves by echocardiogram during cronic use of cabergoline due to potential risk of thickening described with larger doses of cabergoline in Parkinson's patients (88). However, there are no data on echocardiographic changes after prolonged use in Cushing´s patients. Finally, although studies have shown the effectiveness of cabergoline in a subset of patients with CD, its use is off-label, and it is not approved for CD treatment in Brazil.

Pasireotide

The first-generation somatostatin analogues octreotide and lanreotide, traditionally used to treat patients with acromegaly, are not indicated for the treatment of CD due to the low expression of the subtype 2 somatostatin receptor (SSTR2) as a consequence of down regulation by hipercortisolism in corticotrope tumors (89,90). The development of new somatostatin analogues with a different affinity profile to SSTR subtypes has opened new therapeutic possibilities. Pasireotide is the first specific drug approved for the treatment of CD in Europe, USA and recently in Brazil (ampules for subcutaneous use: 300, 600 and 900 mg). In comparison to first-generation somatostatin analogues, pasireotide has increased affinity to SSTR1 (> 30 times), SSTR3 (> 3 times) and, particularly, SSTR5 (> 40 times) (27), which is the most expressed receptor in tumor corticotropes (91). This new analogue has been tested both *in vitro* (92,93) and *in vivo* studies (94). The core study employed in the pasireotide approval was published in 2012 (95). This was a prospective, multicenter, double-blind study on the use of pasireotide in patients with persistent or recurrent CD or naïve patients who could not undergo surgical treatment. Two doses were tested (600 or 900 µg SC twice per day) for 12 months, and a total of 162 patients were assessed. A significant UFC reduction (at least > 50%, primary endpoint) was observed in 49% of patients at 6 months, with UFC normalization in 28.8% of patients who were treated with 900 µg of pasireotide, a response that was maintained up to 12 months with no relapses during this period. A subsequent extension study showed a similar and sustained response after 24 months (96) and even in longer studies up to 72 months (73,91,97).

The therapeutic response to pasireotide depends on the intensity of hypercortisolism. Complete response was noted in 50% of patients with baseline UFC up to twice the upper limit versus 8% in those with baseline UFC > 5 times the upper limit (95). In addition, an early response at 2 to 3 months predicted a sustained response at 12 months. Parameters such as weight, blood pressure, lipid profile and quality of life improved in patients with reduced UFC, even when normalization was not achieved. An analysis of tumor volume was performed in a subset of patients and showed a reduction of 43.8% (compared to initial volume) at 12 months in patients who are treated with 900 mg. Side effects were very common and similar to those of other somatostatin analogues (i.e., diarrhea, nausea, cholelithiasis, mild increase of liver enzymes, bradycardia, and others). At 12 months, 73% of patients experienced adverse events related to hyperglycemia, but most of the events were considered mild to moderate (95). These events are due to a concomitant inhibition of insulin, glucagon-like peptide 1 (GLP1) and glucose-dependent insulinotropic polypeptide (GIP), as well as to only a slight inhibition of glucagon (43,98). Thus, in addition to metformin (usually used in CD patients), dipeptidyl peptidase 4 (DPP4) inhibitors or GLP1 analogues should be the first options to treat hyperglycemia in this situation. Another undesired effect observed in a short-term study (80 days) was a reduction in insulin-like growth factor 1 (IGF1) levels to lower than normal in > 50% of patients (99).

Adrenal steroidogenesis inhibitors

Ketoconazole

Ketoconazole is one of the most prescribed drugs for the treatment of CD, despite being used off label. This compound is an imidazole antifungal drug that reversibly inhibits adrenal steroidogenesis through the action of several enzymes (i.e., cholesterol desmolase, 17αOH and 11β-hydroxylase). Ketoconazole also inhibits androgen production resulting in hypogonadism in men (gynecomastia, decreased libido and erectile dysfunction); in women, in contrast, it might improve hyperandrogenism.

Two studies published in 2008 and 2014 provided the best assessment of ketoconazole use in CD (100,101). In the oldest study, 38 cases were analyzed and 6 series were reviewed, totalizing 99 patients. The series included patients who had undergone previous radiotherapy (102), small numbers of cases (6 – 8 cases) (103-107), or short-term follow-ups (range, 15 – 30 days) (104-107). Of the 38 patients, 51.5% achieved a normal UFC with a mean treatment period of 22 months (6 – 72) and a mean dose of 529 mg/day (range, 200 – 1000 mg). Five patients (13%) interrupted treatment due to side effects (nausea, diarrhea, and increased levels of transaminases by five-fold in one patient) during the first week. A few cases without visible tumors were primarily treated with ketoconazole. One-third of these patients (5/15) showed visible lesions during follow-up (after 12 – 30 months) and were then submitted to surgery (101). No tumor progression

occurred in patients who already had a lesion visible by MRI, although the study did not report the proportion of micro- and macroadenomas. Similar results were found in the more recent multicenter retrospective study with a larger group of 200 patients. Normal UFC was observed in 49.3% of patients with mean dose of 600 mg/day during mean time of observation of 24 months (100).

Another study showed similar control of disease activity in 9/17 patients (53%) during the preoperative period of patients with Cushing's syndrome (pituitary or adrenal in 85% of cases) treated with 200 – 1000 mg/day over a mean period of 4 months (108). Usually, treatment begins with 400 mg/day (200 mg twice daily; tablets: 200 mg), with the medication not taken near meals, as an acidic pH is needed for absorption. Accordingly, the use of proton pump inhibitors decreases drug availability. The dose is increased monthly, up to 1200 mg/day, to achieve UFC normalization, although it is uncommon to reach the maximum dose. Similar to cabergoline, tachyphylaxis may occur in up to 33% of patients following prolonged use of ketoconazole (101,108). Mild side effects are relatively common and include headache, nausea, and rash. Another important side effect is increased levels of hepatic transaminases by as much as 3 times the upper limit. Such increase is usually asymptomatic and reversible with drug interruption or dose reduction. Thus, it is important to monitor hepatic transaminases during the first month of treatment and thereafter. Idiosyncratic severe hepatic insufficiency has been described on rare occasions (19,109).

Metyrapone

Metyrapone is used to assess the sufficiency of the hypothalamic-pituitary-adrenal axis and to treat CD. A reduction of hypercortisolism is achieved by blocking adrenal steroidogenesis via the inhibition of 11β-hydroxylase. This enzyme converts 11-desoxicortisol (compound S) to cortisol, and treatment with metyrapone can result in a rebound of ACTH levels (73). Treatment usually begins at 250 – 500 mg, 3 – 4 times per day, with a maximum dose of 4 – 6 g/day (capsules, 250 mg). In addition, the drug acts rapidly (from hours to days). Due to ACTH rebound and a shift in the production of other steroids, metyrapone increases androgen production and commonly causes hirsutism and acne. This drug may also cause mineralocorticoid effects, such as hypertension and hypokalemia. In general, short-term studies show control of the cortisol excess in a significant number of patients (73). A study showed control in 75% of patients treated with a mean dose of 2250 mg/day (110). One study assessed the use of metyrapone during the preoperative period in patients with Cushing's syndrome (85% pituitary) and showed UFC control in 26% of patients (6/23) treated with 750 – 4500 mg/day for an average of 4 months (108). In the larger multicenter retrospective study, normal UFC was found in 43% of Cushing's syndrome patients from all etiologies (CD, Ectopic ACTH syndrome (EAS), adrenal diseases) in a mean of 8 months (3 days – 12 years) (111). Long-term studies with a large number of CD cases are necessary to better assess the effects of this drug. In addition, this drug is not available in Brazil. Metyrapone is currently available in USA and Europe.

Etomidate

Etomidate is an intravenous anesthetic (imidazole carboxylate derivative) that decreases cortisol levels by inhibiting 11β-hydroxylase (112,113). The main advantage of etomidate is its rapid time of action, allowing reduction or normalization of serum cortisolin less than 24 hours. Thus, etomidate is useful for severe cases of Cushing's syndrome, generally patients with ectopic ACTH syndrome (EAS). Treatment is performed in hospitalized patients, especially in intensive care units, due to clinical severity and the need for close monitoring, although the dose used is usually safe and does not cause severe sedative effects. Treatment assessment is primarily performed by the measurement of serum cortisol (113), and care should be taken to avoid adrenal insufficiency. 'Block and replace' therapy with hydrocortisone IV can be used. Treatment is performed by continuous intravenous infusion and may consist of an initial bolus of 0.03 mg/kg followed by 0.1 – 0.3 mg/kg/hour (ampules, 2 mg/mL). Intermittent use for several hours with periodic intervals has been described. A review published in 2012 including 18 studies (mostly case reports) with a total of 12 patients with CD found cortisol normalization in virtually all cases when used from 5 hours to 56 days (113).

Mitotane

Also known as o,p'-DDD (dichloro-diphenyl-dichloro-ethane), mitotane is an oral chemotherapy used to treat

patients with adrenal carcinoma. Mitotane is considered an adrenolytic compound due to mitochondrial toxicity that causes cellular necrosis. In addition, mitotane inhibits the adrenal production of cortisol by acting on enzymes involved in steroidogenesis (i.e., 11β-hydroxylase and cholesterol desmolase) (114). Mitotane is a lipophilic drug, has a slower mechanism of action than other inhibitors, and has a very long half-life due to storage in adipose tissue (range, 18 – 159 days). The established dose for the treatment of adrenal cancer is high (approximately 8 – 12 g/day), and the effective dose is verified by mitotane levels > 14 – 20 μg/mL. However, for the treatment of CD, lower, non-adrenolytic doses are prescribed, i.e., approximately 2 – 4 g/day (115), depending on the patient's profile and UFC. Usually, treatment is initiated at 500 mg at bedtime, with doses increasing every 1 – 4 weeks (according to tolerance to treatment) up to 2 – 3 g/day in fractionated doses at meals (tablets, 500 mg). In contrast to the effective dose for adrenal cancer treatment, there is no target mitotane concentration for CD, and UFC monitoring is the most important parameter. In one study, mitotane levels > 8.5 μg/mL were associated with normal UFC levels during follow-up (114). Use of mitotane is limited due to relatively common and severe side effects, such as nausea, vomiting, anorexia, rash, diarrhea, ataxia, gynecomastia, arthralgia, leukopenia, hepatotoxicity and hypercholesterolemia. In addition, mitotane may cause adrenal insufficiency, which can occasionally be underestimated due to an increase in cortisol binding globulin (CBG) levels. Due to increased corticoid metabolism, higher doses might be needed for adrenal insufficiency replacement. One study that assessed the use of mitotane in 76 patients with CD showed UFC normalization in 72% of patients with a mean treatment duration of 6.7 months (range, 5.2 – 8.2; mean dose, 2.6 ± 1.1 g/day) (114). Similar to the ketoconazole study (101), 25% of cases without a visible pituitary tumor developed a visible lesion during follow-up, which allowed patients to undergo surgical treatment (114). Due to limited availability, difficult management and high cost, this option is rarely used in Brazil for the treatment of CD.

Cortisol receptor antagonist mifepristone

Mifepristone is an antiprogestogen that, at high doses, rapidly and competitively antagonizes the cortisol receptor, resulting in a rebound increase of ACTH and cortisol plasma levels (116). Thus, monitoring of mifepristone therapy in Cushing's syndrome should be performed using clinical and biochemical parameters such as serum glucose, and not ACTH or cortisol levels. Mifepristone was approved in 2012 in the USA to control hyperglycemia (diabetes mellitus or glucose intolerance) in patients with endogenous Cushing's syndrome (tablets, 300 mg). The approval was based mainly on the results of the SEISMIC trial (71), a prospective, multicenter, 24-week study of patients with endogenous Cushing's syndrome, and diabetes mellitus/glucose intolerance or isolated high blood pressures, who were unresponsive to other therapies (71). The trial involved 50 patients: 43 with CD, 4 with EAS, and 4 with adrenal carcinoma. All of the patients received initially 300 mg/day, and the dose was increased to 600, 900, and 1200 mg/day every 4 weeks if clinical improvement was not observed. The primary endpoints were (i) a decrease in the area under the curve (AUC) of glucose of at least 25% on the 75 g oral tolerance test and (ii) a reduction by > 5 mmHg of diastolic blood pressure. With a mean dose of 600 mg/day, improvement in glycemia (AUC) was observed in 60% of patients (mean reduction, 36%), and the HbA1c levels decreased from 7.43 ± 1.52 to 6.29 ± 0.99%. Diastolic blood pressure improved in 38.1% of patients. In addition, improvements were observed in weight (-5.7%), waist circumference, and insulin sensitivity. The main side effects, primarily mild or moderate, were nausea, fatigue, headache, hypokalemia (effect of cortisol on mineralocorticoid receptor), arthralgias and endometrial thickening/menorrhagia. Others concerns about the use of mifepristone is the adrenal insufficiency not biochemically detected but amenable to be treated with high dose of dexamethasone while withholding mifepristone, and the possible risk of tumor enlargement recently published (117).

Combination therapy and perspectives

Given that the control rate is limited with the currently used drugs, especially in patients with severe Cushing's syndrome, combinations of different medications have been used as an alternative approach to control hypercortisolism (118). Combination therapy can be performed with medications from the same therapeutic class (e.g., combined use of steroidogenesis inhibitors) or from different classes (e.g., cabergoline + ketoconazole).

Combined use of adrenal steroidogenesis inhibitors

At least two recent studies assessed the effect of the combined use of adrenal steroidogenesis inhibitors. Using ketoconazole and metyrapone (doses: 200 – 1000 mg and 750 – 4500 mg, respectively) for an average of 4 months (range, 1 – 30), one study showed control (defined as UFC, clinical parameters and morbidity normalization) in 23% of patients with Cushing's syndrome (5/22) in the preoperative period (108). The second study assessed the effect of a triple combination of mitotane, metyrapone and ketoconazole as an alternative to adrenalectomy in 11 patients with severe ACTH-dependent Cushing's syndrome (UFC: 853 – 22605 µg/24 h, reference 10 – 65), 4 patients with CD and 7 with EAS. Significant and rapid (24 – 48 hours) improvements in clinical and laboratory parameters were observed in all cases, with a reduction of UFC from 2737 to 50 µg/24 h (range, 18 – 298) and normalization achieved in 64% of patients. The treatment was initiated with all three drugs simultaneously: 2250 mg/day metyrapone, 800 mg/day ketoconazole, and 3000 mg/day mitotane. The doses were adjusted according to clinical severity, UFC, and tolerability (119).

Combined use of drugs targeting corticotrope tumors and adrenal steroidogenesis inhibitors

There is few published data concerning combined use of cabergoline with ketoconazole. One of the first studies on this subject was published in 2010 (83), involving 12 patients with CD (microadenomas) and active disease after surgical failure. The protocol consisted of initiating treatment with 1 mg/week of cabergoline (0.5 mg twice/week), with monthly adjustments of 1 mg/week according to UFC or up to 3 mg/week (1.5 mg twice/week) for 6 months. UFC normalization occurred in 3 patients (25%). In the remaining 9 uncontrolled patients, low dose of ketoconazole (100 mg/day) was added, with monthly adjustments of 100 mg or up to 400 mg/day (200 mg twice/day). Control was achieved in 6/9 patients (66%) using both drugs. The remaining 3 patients, those with higher pre-treatment UFC levels (range, 882 – 991 µg/24 h, reference 10–90), presented a lower response to cabergoline only. Thus, after 12 months, UFC was controlled in 75% of patients with cabergoline only or cabergoline + ketoconazole, without escapes during treatment (83). A recent prospective study also assessed this combination therapy in 14 patients with CD by comparing two different regimens: cabergoline followed by ketoconazole (n = 6) vs. ketoconazole followed by cabergoline (n = 8) (84). Cabergoline was initiated at doses of 0.5 – 1 mg/week up to a maximum of 3 mg with the objective of normalizing UFC and late-night salivary cortisol (NSC). Alternatively, ketoconazole was initiated at a dose of 200 mg/day up to a maximum dose of 600 mg/day. Regimens were maintained for 6 months. After this treatment period, patients who achieved UFC and NSC normalization were maintained on monotherapy; patients with values outside of normal ranges received combination therapy (either with cabergoline or ketoconazole) for an additional 6 months. In the first 6 months, none of the patients achieved control (UFC and NSC normalization) with cabergoline, although 33% of patients (2/6) achieved UFC normalization. Ketoconazole monotherapy induced control in 62.5% of patients (5/8) at 6 months. UFC normalization occurred in 79% of patients who were treated with combination therapy, and no differences were observed between the two different combination regimens (84).

Another study evaluated a triple combination regimen with pasireotide, cabergoline and ketoconazole in the preoperative period of 17 patients with CD (99), with the objective of normalizing UFC in 80 days. The treatment was initiated with 100 µg SC pasireotide, 3 times per day, up to a maximum of 250 µg SC, 3 times per day. The increases were performed according to UFC, and the treatment continued for 30 days. After this period, for patients who did not achieve control, cabergoline was added to the treatment at a dose of 1.5 mg/week up to a dose of 4.5 mg/week for 30 days. Following this period, patients who did not exhibit a normalized UFC in 60 days with pasireotide+cabergoline received ketoconazole at a dose of 600 mg/day for 20 days. Overall, control was achieved in 29% of patients with pasireotide monotherapy, 47% of patients with pasireotide+cabergoline, and in 88% of patients with the triple combination. However, the achieved control of 88% does not necessarily mean that all of these patients needed the triple combination. The side effects were worsening of HbA1c levels from 5.8±0.2 to 6.7 ± 0.3% ($p < 0.01$) and reduction of IGF1 to levels lower than reference in 53% (9/17) (99). Although a high rate of control was obtained over a short-term period, this was a small study with an unusual and expensive regimen requiring rapid adjustments, with a potential for

significative adverse events. Moreover, the outcome of monotherapy with cabergoline and with ketoconazole was not tested. For this reason, more studies enrolling a larger number of patients and, perhaps, exploring different therapeutic sequences are necessary.

Other medications and perspectives

Temozolomide is an oral, imidazotetrazine alkylating chemotherapy agent used primarily for the adjuvant treatment of cerebral gliomas. This compound has been increasingly used to treat aggressive/atypical pituitary adenomas and pituitary carcinomas. The antitumor activity of temozolomide occurs through its active form, monomethyl-triazen-imidazole-carboxamide (MTIC), which promotes DNA methylation. This action can be neutralized by the presence of the DNA repair enzyme O-6-methylguanine-DNA methyltransferase (MGMT), an effect that can be assessed by immunohistochemistry or real-time reverse transcription PCR (RT-PCR) on tumor tissue. Although most patients who are responsive to temozolomide show low expression of MGMT, recent studies have shown some cases of dissociation between enzyme expression and drug action (120,121). Temozolomide is usually prescribed in monthly 5-day cycles at a dose of 200 mg/m^2/day (tablets, 5/20/100/140/180/250 mg). A review study showed clinical improvement (hormonal and tumor reduction) in 50% (4/8) of patients with corticotrope adenomas and in 83% (5/6) of ACTH-producing pituitary carcinomas (122). Temozolomide is generally well tolerated, and the most significant side effects are leukopenia and thrombocytopenia. This medication is reserved for refractory and aggressive cases, not only ACTH-producing tumors, and tumor escape from its salutary effects may occur (121). It is not approved for CD in either Brazil or the USA, and it is rather costly.

Retinoic acid is known to have *in vitro* effects on corticotrope tumors and in an animal model of CD (canine) (123-125). The action of retinoic acid is likely mediated by a decrease in ACTH secretion and pro-opiomelanocortin (POMC) synthesis, as demonstrated in a murine corticotrope cell line by the inhibition of POMC transcription (123). In addition, retinoic acid has an antiproliferative action. In a recent prospective, proof of concept study, 7 patients with CD were treated with retinoic acid (tretinoin) at a dose of 80 mg/day for 6 – 12 months. A significant UFC reduction (> 50%) in 71% of patients (5/7) with normalization in 43% of cases (3/7) was observed (126) and the drug was well tolerated. Another study with 16 CD patients showed UFC normalization in 25% of cases with isotretinoin use (20-80 mg/day) for 6-12 months (127).

Other drugs have been studied *in vitro* and in animal models, showing action on corticotrope tumors but still lacking results in patients with CD. These drugs include bexarotene (retinoic acid receptor (RXR) agonist) (72), (α1-adrenergic receptor antagonist) (128) and gefitinib (EGF receptor antagonist) (129).

Osilodrostat (LCI699) is a new developed steroidogenesis inhibitor. This drug was initially described as an aldosterone synthase inhibitor with potential for the treatment of hypertension (130). Osilodrostat is a potent inhibitor of 11β-hydroxylase and 18-hydroxylase (98). In a small proof-of-concept study, 10/11 patients with mild to severe CD achieved UFC normalization after 70 days of treatment with 5 – 10 mg twice per day. The main side effects were fatigue, nausea, headache, and an ACTH level increase of more than 2-fold in 5 cases (43). More recent and prolonged study lasting 22 weeks observed normal UFC in 89.5% (17/19) of CD patients (131).

Levoketoconazole is the 2S, 4R enantiomer of ketoconazole, purified from racemic ketoconazole. In *in vitro* studies, levoketoconazole was shown to be a more potent inhibitor than the 2R,4S enantiomer (132). An open-label, phase III, dose-titration study evaluating levoketoconazole in patients with Cushin's syndrome is ongoing.

Table 1 provides a summary of the drugs used for the treatment of CD.

Radiotherapy

While used less frequently in patients with GH and prolactin-producing pituitary tumors, radiotherapy is still an important option for adjuvant or rescue treatment in patients with ACTH-producing tumors (133,134).

Pituitary radiotherapy for CD is classically indicated as a secondary treatment after surgical failure. Radiotherapy is also used in uncontrolled patients receiving drug treatment, particularly those with residual or non-resectable tumors (e.g., cavernous sinus invasion). Radiotherapy is rarely used as a primary treatment in cases where surgical treatment is contraindicated. A study performed nearly 40 years

Table 1. Drug treatment in Cushing's disease

Drug	Initial dose	Maximum Dose	Control*	Duration	Observations
Act on the corticotrope tumor					
Cabergoline	0.5 mg OR 2x/week	3 mg/week (1-7)	25-40%	18 months (3-60)	Escape from treatment in 18-30%; *off label*
Pasireotide	600 μg SC 2x/day	1800 μg/day	29%	12 months	Frequent hyperglycemia
Steroidogenesis inhibitors					
Ketoconazole	200 mg OR 2-3x/day	1200 mg/day	52%	22 months (6-72)	Escape from treatment in 33%; mild common increase in ALT/AST; improves hirsutism; hypogonadism in men
Metyrapone	250 mg OR 3-4x/day	4 -6 g/day	26-75%	4 months	Rebound increase of ACTH: hirsutism/ acne, HAS/hypokalemia; N/A
Etomidate	IV bolus 0.03 mg/kg; 0.1 mg/kg/h	0.3 mg/kg/h	100%	7 days (5 h-56 days)	Used in severe cases; hospital use (monitoring)
Mitotane	500 mg OR/day	2-3 g/day	72%	7 months	Frequent side effects; difficult handling; high cost
Cortisol receptor antagonist					
Mifepristone	300 mg OR/day	1200 mg/day	60% (glycemia AUC)	24 weeks	Approved for the control of DM in Cushing's syndrome; very high cost; N/A
Combination therapies					
Ketoconazole + metyrapone	200/750 mg/day	1000/4500 mg/day	23%	4 months	Metyrapone N/A
Mitotane + metyrapone + ketoconazole	3000/2250/800 mg/day	3000/2250/800 mg/day	100%	< 6 months	Critically ill patients; effect in 24-48 h
Cabergoline + ketoconazole	1 mg/week/100 mg/day	3 mg/week/400 mg/day	75-79%	12 months	Short-term studies
Pasireotide + cabergoline + ketoconazole	100 μg SC 3x/day/1.5 mg/week/600 mg/day	250 μg SC 3x/day/4.5 mg/week/600 mg/day	88%	80 days	> 50% IGF1 reduction; frequent hyperglycemia

* Control commonly defined as the normalization of 24-hour urinary cortisol; OR: oral route; SC: subcutaneous; ALT/AST: alanine aminotransferase/aspartate aminotransferase; ACTH: adrenocorticotropic hormone; AUC: area under curve; DM: diabetes mellitus; IGF1: insulin-like growth factor 1; N/A: not available.

ago showed better efficacy in the pediatric population than in the adult population and for this reason, radiotherapy has been more utilized in children (135), although this approach is not currently accepted. In addition to hormonal control, another objective of radiotherapy is tumor mass control, either via reduction or stabilization/prevention of growth (i.e., the "oncologic" indication). Usually, the efficacy of tumor mass control is higher than hormonal control, ranging from 83% to 100% (29,134,136).

As hormonal control is initiated at least 6 months after radiotherapy (mean, 18 – 24 months), there is a need for medical treatment in this interval (19,29,134). In other words, patients should not be followed without medication waiting for the effects of radiotherapy. In patients with effective concomitant clinical treatment, the effect of radiotherapy may be assessed by biannual withdrawn of drug treatment to measure cortisol (e.g., UFC, NSC and/or low dose dexamethasone suppression test (LDDST)) (19,133).

Most data regarding the control rates and prevalence of complications of radiotherapy are derived from older studies that used conventional methods. More modern stereotactic radiotherapy techniques have been developed (i.e., fractionated or single dose), which are potentially more effective and induce less morbidity.

Radiotherapy is usually performed on a well-defined therapeutic target, particularly in the context of single-dose stereotactic radiotherapy (i.e., radiosurgery). However, it is occasionally performed without a clear target lesion in CD and radiation may encompass the entire pituitary tissue, provided that a diagnosis of central origin has been confirmed (e.g., ACTH+ pituitary adenoma, previous PO remission, or central to peripheral ACTH gradient at BIPSS). In cases in which the target is well-defined, single dose

stereotactic radiotherapy may be performed in lesions up to 4 cm in diameter, although it is recommended to allow a minimum distance in relation to optic structures (nerves and chiasm) of at least 3 – 5 mm. In lesions that are close to these structures, fractionated radiotherapy is safer.

Generally, hormonal control is achieved within 2 – 5 years (mean follow-up period, 5 – 10 years) in approximately 50 – 60% of cases (27,29,136), regardless of the technique. More recent studies performed with radiosurgery showed a remission rate of approximately 50% (45,116,134,137) over a similar period (4 – 8 follow-up years). There is no good evidence of a more rapid effect of radiosurgery in comparison to conventional techniques (136). Some studies recommend drug treatment interruption during radiotherapy sections due to the risk of decreased efficacy (138), but this is a controversial issue.

The main concern regarding radiotherapy is related to potential side effects. The most common is hypopituitarism, which occurs in over 50% of patients in the long-term. Other reported effects are optical lesions (neuritis, 1 – 2%), radionecrosis of the brain parenchyma (< 1%), radioinduced secondary tumors (1.5%) (139), neurocognitive disorders (< 1%), and cerebrovascular diseases (< 5%). However, stereotactic techniques, especially radiosurgery that is focused on the lesion and results in less radiation exposure to adjacent tissues, has shown lower complication rates (range, 0 – 1.3%), with the exception of hypopituitarism (72% in 17 years) (140). It is actually not known whether stereotactic radiotherapy is also associated with an increased risk of secondary tumors since this is a relatively novel treatment modality and long-term dara are lacking.

Adrenalectomy

Bilateral adrenalectomy is considered a 100% effective hypercortisolism treatment (27,141). The main advantage of this method is the immediate normalization of cortisol levels. Currently, the surgical procedure is performed through a laparoscopic approach with reduced rate of PO complications and reduced time of hospitalization (141). Some cases may present recurrence of hypercortisolism due to a vicarious increase of ectopic adrenal tissue or adrenal residues after incomplete surgical resections (142).

Adrenalectomy is generally indicated as the last therapeutic option in refractory cases following unsuccessfully surgical, drug and/or radiation treatment. This treatment may also be primarily indicated in severe cases of CD, for which rapid clinical resolution is required, as well as in patients with EAS (143). Finally, adrenalectomy can be performed in women of reproductive age who wish to become pregnant without hormonal stimuli and for whom repeated pituitary surgery and, particularly, radiotherapy could induce hypogonadotropic hypogonadism. One criticism brought up by some authors is related to the long time for adrenalectomy indication, exposing the patients for prolonged hypercortisolism and its catastrophic effects. This situation can be mitigated with treatment optimization based on rapid changes in the therapeutic approach, particularly with medical treatment.

The disadvantages of bilateral adrenalectomy are permanent adrenal insufficiency with the consequent need of lifelong gluco- and mineralocorticoid replacement, the risk of acute crisis in stress situations, and the development of corticotrope tumor progression – Nelson's Syndrome (NS) (144).

Corticotrope tumor progression may occur following adrenalectomy in 21% of cases over 3 – 5 years (145). Predictive risk factors for occurrence of corticotrope tumor progression are younger age, no previous radiotherapy, the presence of pituitary tumor residues or invasive tumors, higher UFC levels and mainly ACTH increase in the first year after adrenalectomy (145). However, there has been controversy regarding the ACTH cutoff level indicating increased risk. Some authors suggested that an increase of plasma ACTH levels ≥ 600 or ≥ 1000 pg/mL on the first year after adrenalectomy might indicate tumor progression (144,146). Due to inconsistent data, radiotherapy has not been used as prophylaxis, especially with available pituitary MRI imaging and ACTH measurements that are systematically assessed during the follow-up in order to identify corticotrope tumor progression. Interestingly, although the suggested risk of corticotroph tumor progression has been around 50% (140), many of these patients do not develop clinical features of Nelson's syndrome with mass effect or skin hyperpigmentation. Thus, is seems that clinical Nelson's syndrome is less common than corticotroph tumor progression after bilateral adrenalectomy.

Finally, an old study compared remission rate of pituitary surgery with a unilateral adrenalectomy protocol associated with conventional primary pituitary radiotherapy. This report found a similar result of 64% remission of both strategies (142). However, due to the absence of similar data and a lack of improved surgical remission rates, the clinical applicability of this approach is limited.

Pregnancy

Pregnancy during active CD is very rare and difficult to handle. The reason for the low prevalence is that hypercortisolism interferes with fertility, particularly due to changes in LH/FSH pulsatility, causing menstrual changes/amenorrhea and anovulation. In addition, an increase in androgen production usually occurs due to ACTH stimulation, which inhibits the normal dynamics of gonadotropins. Perhaps, for this reason, most pregnant patients with CD are carriers of adrenal adenomas (40 – 50%) (147-150).

Although rare, the identification and correct treatment of this condition is important due to the increased risk of materno-fetal complications (> 70%) (147,148).

The most common treatment approaches are pituitary surgery, medical therapy, and bilateral adrenalectomy. Expectant approach is also possible when the Cushing's syndrome diagnosis is made at the end of pregnancy, with careful management of associated co-morbidities, such as hypertension and diabetes (148). When treatment is indicated, it is usually performed during or after the second trimester due to the time needed to establish the diagnosis. During the gestation period, most pituitary surgeries have a good outcome, and for this reason, pituitary surgery should be the first treatment of choice. Alternatively, during or after the second trimester, drug treatment may be chosen. In this case, metyrapone has been the most reported drug but it is not available in Brazil. The use of has been reported in few cases without the occurrence of congenital malformations (96). However, ketoconazole has been shown to be teratogenic in animal studies and should only be used when metyrapone is not available or induces side effects. There is a single report of cabergoline use in a pregnant patient with CD (151). In more severe and unresponsive cases, as well as in adrenal-related disease, adrenalectomies have been performed in several patients (147,148) and are generally effective.

Table 2 summarizes indications for therapeutic options in CD, and Figure 1 shows a proposed treatment algorithm.

Role of the authors: the authors of this review comprise an expert committee that was gathered in order to provide an updated review of the treatment of Cushing's disesase through the contribution of each author.

Review methods: The lead author performed a PubMed search of CD treatment and discussed with the senior author. The review was then drafted and circulated to the members of the committee who provided with numerous contributions to the final text.

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Table 2. Therapeutic options in Cushing's disease

Type	Control*	Advantage	Disadvantage	Observations
Pituitary surgery	MIC: 70 – 90% MAC: 50 – 70%	Rapid; direct	Lower remission in invasive macros, and when tumor not visible in image	General treatment of choice
Subsequent pituitary surgery	40 – 70%	Possibility of definitive resolution	Lower remission compared to first surgery; increases the risk of cerebrospinal fluid fistula and hypopituitarism	Best suited for patients with persistence of tumoral image
Drug treatment	40 – 100%	Noninvasive; allows patients to undergo surgery if needed	Chronic use; side effects	Mainly indicated in surgical failure, and after RTX
Stereotactic radiotherapy	50 – 60%	Direct treatment	Slow start; side effects (hypopituitarism)	Associated with drug treatment
Bilateral adrenalectomy	100%	Immediate control	Risk of corticotrope tumor progression; permanent gluco- and mineralocorticoid insufficiency	Indicated in severe cases, refractory cases, and when pregnancy is desired

* Control of urine free cortisol; MIC: microadenoma; MAC: macroadenoma; RTX: radiotherapy.

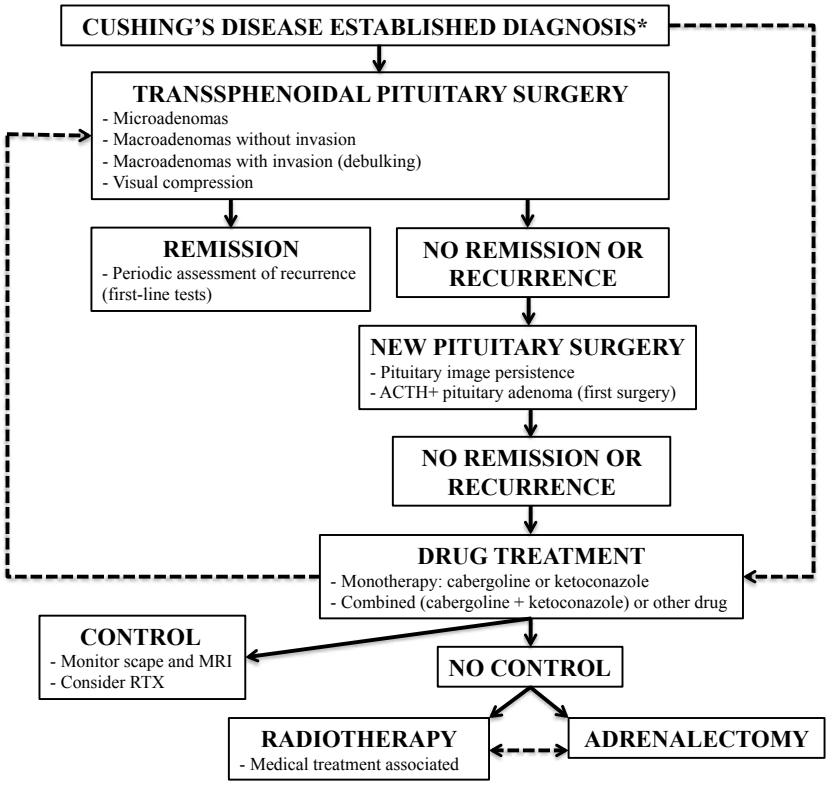

Figure 1. Treatment algorithm for the treatment of Cushing's disease.

* Primary clinical treatment may be considered in patients with a contraindication to surgery, those who need to improve preoperative clinical conditions, those who refuse surgical treatment, and in case an experienced surgeon is unavailable. Pituitary surgery should be performed in tertiary centers by experienced surgeons, and patient referral should be considered when these conditions cannot be achieved. Very severe cases may undergo initial bilateral adrenalectomy.

RTX: pituitary radiotherapy.

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Diagnosis and treatment of hypoparathyroidism: a position statement from the Brazilian Society of Endocrinology and Metabolism

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ABSTRACT

Objective: To present an update on the diagnosis and treatment of hypoparathyroidism based on the most recent scientific evidence. **Materials and methods:** The Department of Bone and Mineral Metabolism of the *Sociedade Brasileira de Endocrinologia e Metabologia* (SBEM; Brazilian Society of Endocrinology and Metabolism) was invited to prepare a document following the rules set by the Guidelines Program of the *Associação Médica Brasileira* (AMB; Brazilian Medical Association). Relevant papers were retrieved from the databases MEDLINE/PubMed, LILACS, and SciELO, and the evidence derived from each article was classified into recommendation levels according to scientific strength and study type. **Conclusion:** An update on the recent scientific literature addressing hypoparathyroidism is presented to serve as a basis for the diagnosis and treatment of this condition in Brazil. Arch Endocrinol Metab. 2018;62(1):106-24

Keywords
Hypoparathyroidism; hypocalcemia; calcitriol; PTH; guideline; diagnosis; treatment

INTRODUCTION

Serum calcium concentration is maintained within a narrow physiological range by complex controlling mechanisms involving the parathyroid hormone (PTH), active vitamin D (1,25(OH)$_2$D), and calcium sensor receptors (CaSRs) acting in renal, intestinal, parathyroid, and bone tissues to maintain mineral homeostasis. When these homeostatic mechanisms fail or are not fully compensated, hypocalcemia occurs (1).

Inappropriately low (insufficient) circulating PTH levels, which in adults occurs mainly after thyroid surgery, is the most common cause of hypocalcemia. Current standard treatment of low PTH levels comprising vitamin D analogs and calcium supplementation is challenging as it does not involve replacing the missing hormone (2).

Over the past ten years, we have gained a greater understanding of hypoparathyroidism regarding its epidemiology, genetics, associated skeletal disease, and therapies. A major therapeutic challenge in hypocalcemia is effectively balancing calcium levels while avoiding hypercalciuria and other complications (3).

This document is a result of efforts by the Department of Bone Metabolism of the *Sociedade Brasileira de Endocrinologia e Metabologia* (SBEM; Brazilian Society of Endocrinology and Metabolism) for the development of recommendations based on the current evidence available in the scientific literature regarding the diagnosis and treatment of hypoparathyroidism. The objective of this document is to answer routine questions and serve as a guideline for endocrinologists and clinicians in Brazil.

MATERIALS AND METHODS

We elaborated this guideline motivated by SBEM's Practical Guidelines Program. The model applied to this document followed the Guidelines Program of the *Associação Médica Brasileira* (AMB; Brazilian Medical Association) and *Conselho Federal de Medicina* (CFM; Federal Medical Council). After selecting collaborators with a significant role and relevant publications in the area of hypoparathyroidism, we elaborated clinical questions for discussion. We searched the databases MEDLINE/PubMed and SciELO/LILACS for relevant publications, and categorized each publication according to the level of evidence, as recommended by the Oxford Centre for Evidence-Based Medicine. These recommendations evaluate the study design and consider the best available evidence for each question to attribute a recommendation level or evidence strength to each article (4,5). In this document, we report the levels of recommendation and evidence as:

A: experimental or observational studies with consistent results.
B: experimental or observational studies with less consistent results.
C: case reports (uncontrolled studies).
D: opinion is lacking critical evaluation or is based on guidelines, physiological studies, or animal models.

ETIOLOGY

1. What are the causes and differential diagnoses of hypocalcemia?

Decreases in serum ionized calcium are recognized by CaSRs in the parathyroid glands, eliciting PTH release from preexisting pools and stimulating PTH production and secretion. Serum calcium levels are then restored by PTH-mediated decreases in urinary calcium excretion, and increases in bone resorption and intestinal calcium absorption, the later in association with increased $1,25(OH)_2D$ (calcitriol) synthesis in the renal tubules. Thus, the causes of hypocalcemia may be divided into those associated with PTH deficiency or resistance (addressed separately), and those not directly associated with hypoparathyroidism, as listed in Table 1 (6-9).

Although hypoalbuminemia is the most common cause of low serum total calcium levels, it has no effect on the ionized calcium fraction and, therefore, no clinical significance. Thus, measurement of serum albumin is always recommended during the investigation of hypocalcemia, along with correction of the total calcium values, which is achieved by adding 0.8 mg/dL to the total calcium level for each 1.0 g/dL decrease in albumin below 4.0 g/dL or by the formula: Calcium corrected = Calcium measured + [(4.0 - albumin) x 0.8] (6-9).

Severe hypomagnesemia decreases PTH secretion and increases resistance to PTH effects in bone and kidney. The occurrence of hypomagnesemia should be considered in all patients with hypocalcemia and low or inappropriately normal PTH levels (6-9).

Severe calcium and/or vitamin D deficiency could be associated with hypocalcemia and lead to secondary hyperparathyroidism. Measurement of plasma 25-hydroxyvitamin D [25(OH)D], the main vitamin D metabolite stored in the body, is recommended. The active metabolite of this vitamin [$1,25(OH)_2D$] has a short plasma half-life and does not reflect the vitamin D status (6-14).

In acute pancreatitis, the action of pancreatic lipase generates free fatty acids that avidly chelate the insoluble calcium salts present in the pancreas, resulting in calcium deposition in the retroperitoneum (7).

In acute hyperphosphatemia, phosphate binds avidly to calcium leading to calcium deposition, mostly in bone but also in extraskeletal tissues. Hypocalcemia is commonly found in patients with chronic kidney disease in association with low $1,25(OH)_2D$ levels and secondary hyperparathyroidism (15).

The hungry bone syndrome may occur after surgical cure of severe hyperparathyroidism, leading to hypocalcemia and hypophosphatemia due to a rapid increase in skeletal mineralization. Hypocalcemia is directly associated with the severity of bone disease and concomitant vitamin D deficiency (16).

Intravenous bisphosphonates (17) and subcutaneous denosumab (18), potent antiresorptive agents used to treat osteoporosis, may lead to clinical hypocalcemia, mainly in vitamin D deficient patients. This side effect of antiresorptive agents is most commonly seen in high bone turnover states such as Paget's disease of bone.

Several other drugs may aggravate hypocalcemia by acting through diverse mechanisms, and their concomitant use should be evaluated in patients with low calcium levels. Some anticonvulsants accelerate the breakdown of vitamin D, limiting bone mineralization. Proton pump inhibitors and H_2 blockers reduce the production of gastric acid, interfering with calcium absorption. Loop diuretics and glucocorticoids induce hypercalciuria; glucocorticoids also have detrimental effects in the intestinal action of vitamin D. Antiviral drugs have also been associated with a negative impact on calcium, vitamin D, and bone metabolism. A review of the subject by Liamis and cols. is recommended (19).

Highlights SBEM: In normal conditions, about 50% of the total calcium circulates bound to albumin. Low albumin concentrations may inaccurately indicate hypocalcemia, but the ionized calcium fraction is normal. Thus, total calcium concentration should always be corrected by albumin level during the investigation of hypocalcemia **(A)**.

2. What are the causes of hypoparathyroidism?

From a functional point of view, hypoparathyroidism arises from an inability of the parathyroid glands in secreting PTH and/or impaired PTH action, directly impacting the homeostasis of calcium and phosphorus (Table 2). Hypoparathyroidism resulting from peripheral resistance to PTH action is known as pseudohypoparathyroidism. Thus, the term "hypoparathyroidism" is commonly reserved to

Table 1. Causes of hypocalcemia other than hypoparathyroidism and pseudohypoparathyroidism

	Total calcium	Ionic calcium	PTH	Symptoms of hypocalcemia	Bone manifestations	Associated diseases
Hypoalbuminemia	Low	Normal	Normal	Absent	None	Liver cirrhosis, nephrotic syndrome, burn, malnutrition, sepsis
Hypomagnesemia	Low	Low	Variable	Variable	None	Diuretics, chronic diarrhea, small bowel bypass or resection
Calcium deficiency	Low	Low	High	Variable	Rickets/osteomalacia	Severe malnutrition, Roux-en-Y gastric bypass (RYGB)
Vitamin D deficiency	Low	Low	High	Variable	Rickets/osteomalacia increased resorption (decreased density)	Decreased sun exposure, obesity, dark skin, RYGB, aging, severe malnutrition, celiac disease, pancreatic diseases, steatorrhea, antiepileptic drugs
Hyperphosphatemia (acute)	Low	Low	Variable	Variable	Dependent on the etiology	Phosphate-containing enemas, renal failure, intense tissue breakdown (rhabdomyolysis or tumor lysis)
Chronic kidney disease (CKD) and GFR < 30 mL/min/1.73 m^2	Low	Variable	High	Variable	Hyperparathyroidism (eventual low turnover)	Severe hypertension, diabetes mellitus, and others
Hungry bone syndrome	Low	Low	Variable	Moderate/ severe	Hyperparathyroidism	Surgical cure of hyperparathyroidism (primary or secondary to CKD)
Drugs: Antiresorptives (bisphosphonates, denosumab), calcimimetics (cinacalcet), calcium chelators (EDTA, citrate, foscarnet), antiepileptics (phenytoin, phenobarbital, carbamazepine), proton pump inhibitors, loop diuretics, chemotherapeutic drugs	Low	Low	Variable	Variable	Dependent on the etiology	Osteoporosis, primary or secondary hyperparathyroidism, blood transfusion, epilepsy, peptic ulcer, neoplasias, etc.
Acute pancreatitis	Low	Low	High	Variable	None	Alcohol abuse, gallbladder stones

describe a situation in which the parathyroid glands are unable of properly producing PTH **(A)** (1-3,20).

2.1. Causes of hypoparathyroidism

The main cause of hypoparathyroidism is the surgical destruction of the parathyroids **(A)**. Autoimmunity is considered the second most frequent etiology of hypoparathyroidism (1,2,21-23). Rare causes of hypoparathyroidism, such as parathyroid destruction by neoplastic infiltration or heavy metals, irradiation, radioiodine therapy, Riedel's thyroiditis and genetic diseases affecting the development of the parathyroids and/or the production of PTH are listed in Table 2. Hypoparathyroidism may also result from deregulation of PTH secretion secondary to disorders of magnesium homeostasis or abnormal activation of CaSRs due to a genetic or autoimmune cause (23).

2.1.1. Postsurgical hypoparathyroidism

Surgical manipulation of the anterior cervical region is the most frequent cause of hypoparathyroidism, corresponding to 75% of the cases of the acquired form of the disease **(A)** (2,22-24). The destruction of the parathyroids may occur due to an aggressive surgical treatment of cervical cancer or may be accidental as a result of truncal ligation of the inferior thyroid arteries or inadvertent removal of the parathyroids (25-27). Relevant aspects of surgical-related hypoparathyroidism will be discussed separately in Section 4.

2.1.2. Autoimmune hypoparathyroidism

Autoimmune aggression to the parathyroids is considered the second most common cause of hypoparathyroidism in adults **(A)** and may occur as an isolated endocrinopathy or as part of the autoimmune polyglandular syndrome type 1 (APECED) (2,22). Isolated autoimmune hypoparathyroidism has been related to antiparathyroid and anti-CaSR antibodies, but the pathogenic role of these antibodies is still poorly characterized. The prevalence of antiparathyroid and anti-CaSR antibody positivity in individuals with suspected autoimmune hypoparathyroidism is variable (between 25 to 40%), and the measurement of these antibodies is generally limited to research studies (2,28). In clinical practice, the presence of other autoimmune manifestations helps the identification of autoimmune hypoparathyroidism in individuals who develop nonsurgical hypoparathyroidism **(D)**. The autoimmune polyglandular syndrome type 1 is a rare autosomal recessive disease caused by mutations in the *AIRE* gene, characterized mainly by mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency; several other autoimmune manifestations may also occur **(C)** (29).

2.2. Hypoparathyroidism due to deregulation of PTH secretion

Changes in magnesemia may lead to functional hypoparathyroidism (2,21). Magnesium participates in

Table 2. Causes of hypoparathyroidism

Hypoparathyroidism due to PTH deficiency	Parathyroid destruction	Following cervical surgery
		Cervical radiotherapy or radioiodine therapy
		Autoimmune: isolated or associated with polyglandular disease [*AIRE*]
		Parathyroid infiltration by neoplastic, granulomatous, or storage diseases (Wilson's disease and hemochromatosis)
		Riedel's thyroiditis
	Genetic diseases affecting parathyroid development and/or PTH production	Isolated hypoparathyroidism [*PTH*, *GCM2*, *SOX3*]
		Genetic syndromes associated with hypoparathyroidism (e.g. DiGeorge, CHARGE, Kenny-Caffey, Sanjad-Sakati, HDR, etc.) [*TBX1*, *NEBL*, *GATA3*, *TBCE*, *FAM111A*, *CHD7*, *SEMA3E*]
		Mitochondrial diseases
	Regulation change in PTH secretion	Autosomal dominant hypocalcemia type 1 [*CASR*] and type 2 [*GNAS11*]
		Activating anti-CASR antibodies
		Chronic hypomagnesemia and hypermagnesemia
Functional hypoparathyroidism due to peripheral resistance to PTH action	Pseudohypoparathyroidism	Genetic defects in PTH post-receptor signaling [*GNAS*, *STX16*, *NESP55*, *PRKAR1A*, *PDE4D*]

Genes associated with hypoparathyroidism are shown in square brackets. Compiled from (23,31,32).

both processes of PTH secretion and action through the adenylcyclase system. Thus, conditions of chronic magnesium depletion (chronic diarrhea, alcoholism, poorly controlled diabetes mellitus, and chronic use of proton pump inhibitors and diuretics) cause hypocalcemia with inappropriately normal or overtly low PTH levels. On the other hand, acute hypermagnesemia (due to the excessive parenteral administration or renal insufficiency) also leads to hypocalcemia since magnesium may activate CaSRs and suppress PTH secretion (22). Rare genetic defects of factors involved in the homeostasis of magnesium (*TRMP6*, *CLND16*, *CLDN19*) may also cause hypoparathyroidism (22). Therefore, serum magnesium levels must be determined upon evaluation of hypocalcemia, to exclude functional hypoparathyroidism **(A)**. PTH secretion may also be deregulated by anti-CaSR antibodies activating the CaSR receptor by mimicking calcium and inhibiting the secretion of this hormone; this is a rare cause of hypoparathyroidism (28).

2.2.2 Causes of pseudohypoparathyroidism

Peripheral resistance to PTH action resulting in functional hypoparathyroidism is known as pseudohypoparathyroidism. This condition is caused by genetic defects in postreceptor PTH signaling and is characterized in laboratory tests by hypocalcemia and hyperphosphatemia in the presence of elevated PTH levels in patients with normal renal function **(A)**. In pseudohypoparathyroidism, the production of PTH by the parathyroids is normal, and the biochemical disorder resulting from hormonal resistance (hypocalcemia and hyperphosphatemia) stimulates increased PTH production. The main genetic defects causing pseudohypoparathyroidism are inactivating mutations of the alpha subunit of the stimulatory G protein, coded by the *GNAS* gene, which in physiological conditions acts by coupling to the PTH receptor and propagating the stimulation arising from the binding of the hormone to the receptor **(A)** (30). Thus, in pseudohypoparathyroidism, defects in the *GNAS* gene interfere with PTH signaling in peripheral target tissues, particularly in the kidneys. Other molecular defects (for example, modification in *GNAS* methylation) or defects in other mediators of PTH signaling in target tissues (for example, PRKAR1A) may also cause pseudohypoparathyroidism **(A)** (23). The diagnosis and management of pseudohypoparathyroidism are beyond the scope of this article.

Highlights SBEM: In patients without a history of conditions leading to parathyroid destruction, other causes of hypocalcemia must be considered. Clinical and laboratory evaluation of these patients is fundamental since, in most cases, it is not difficult to identify the cause of the hypocalcemia **(A)**, although differentiating autoimmune from idiopathic hypoparathyroidism may be clinically challenging.

EPIDEMIOLOGY

3. What is the prevalence of hypoparathyroidism?

Hypoparathyroidism is a rare disorder with an estimated prevalence of 0.25 per 1,000 individuals **(B)** (33,34). Most patients with hypoparathyroidism had their parathyroids incidentally removed or injured during thyroid surgery (35). Transient postsurgical hypoparathyroidism is common, due to functional parathyroid impairment after acute manipulation, with subsequent spontaneous recovery. However, it may, more rarely, be definitive **(C)** (22). Despite literature reports of recoveries occurring more than six months after surgery, hypoparathyroidism is considered definitive when lasting more than six months from the surgical event **(D)** (2,3,20,36).

The actual prevalence of postsurgical hypoparathyroidism is probably underestimated, due to lack of clear definitions, inappropriate follow-up, conflicts of interest in reports of individual series, and different strategies in calcium and vitamin D supplementation, among other causes. Thus, the prevalence of transient hypoparathyroidism varies widely from 3% to 52%, while that of definitive hypoparathyroidism ranges from 0.4% to 13% (24,37-44). Since postsurgical hypoparathyroidism is due to direct parathyroid injury, treatment with total thyroidectomy is associated with increased rates of hypoparathyroidism when compared with partial thyroid surgeries (subtotal thyroidectomy and hemithyroidectomy) (Table 3). On the other hand, these rates are even higher with central compartment (level VI) lymph node dissections and in thyroid and parathyroid reoperations, especially in parathyroid hyperplasia **(B)** (45,46). Although the Brazilian literature in this regard is scarce, the results of Brazilian studies are aligned with those described above (25,47-52) (Table 3).

Table 3. Epidemiology of post-surgical hypoparathyroidism

Reference	Author, year	Country	Prevalence of hypoparathyroidism	Comments
27	Underbjerg and cols., 2013	Denmark	22/100,000 (general population)	Data from 1988 to 2012
33	Powers and cols., 2013	USA	5.0%	120,000 surgeries in one year
35	Rosato and cols., 2004	Italy	1.7%	15,000 thyroidectomies with a 5-year follow-up
37	Youngwirth and cols., 2010	USA	0.74%	12% of patients had transient hypoparathyroidism, only 2 required calcitriol in the long term
38	Snyder and cols., 2013	USA	1.0% (for Graves' disease) 1.8% (for other types of hyperthyroidism) 0% (for other benign diseases)	780 total thyroidectomies for different reasons
39	Pelizzo and cols., 2014	Italy	3.3%	233 patients after a second thyroid surgery
40	Puzziello and cols., 2014	Italy	0.9%	2,631 thyroidectomies 28.8% had transient hypocalcemia
41	Ritter and cols., 2015	USA	1.9%	1,054 thyroidectomies
42	Wei and cols., 2014	China	0.9% (3/321) – autotransplantation 3.8% (6/156) – parathyroid glands preserved in place	477 thyroidectomies with (n = 321) and without (n = 156) parathyroid autotransplantation
43	Ito and cols., 2014	Japan	8%	154 completion thyroidectomies after a previous thyroidectomy
44	Lorente-Poch and cols., 2015	Spain	4.6%	657 thyroidectomies, 1-year follow-up
45	Nawrot and cols., 2014	Poland	8.5%	401 thyroidectomies
46	Järhult and cols., 2012	Sweden	6% of the total thyroidectomies	265 thyroidectomies in patients with Graves' disease
47	Dedivitis and cols., 2010	Brazil	6.6%	91 thyroidectomies, 6 patients had biochemical hypoparathyroidism after 1 month
48	Accetta and cols., 2011	Brazil	0/66 – no case	66 thyroidectomies
49	Vanderlei and cols., 2012	Brazil	2.5%	40 thyroidectomies
50	Molinari and cols., 2015	Brazil	0.3%	3,411 thyroidectomies
51	Ywata de Carvalho and cols., 2015	Brazil	11.8% of patients with central neck dissection 2.3% of patients without central neck dissection	Two different groups: total thyroidectomy with (n = 106) and without central neck dissection (n = 478)
52	Montenegro and cols., 2012	Brazil	28%	83 patients with hyperparathyroidism due to MEN-1/all with parathyroid autotransplantation

Highlights SBEM: There are no consistent data on the prevalence of permanent hypoparathyroidism after surgery. The risk of postsurgical hypoparathyroidism should be considered upon recommendation of thyroid/parathyroid surgeries, along with the extent of the procedures **(C)**.

SURGICAL ASPECTS

4. Is it possible to prevent postsurgical hypoparathyroidism?

Injury to the parathyroid glands during thyroid surgery may be due to direct tissue trauma (mechanical or thermal), injury to the vascular pedicle, inadvertent removal of the parathyroids during surgery, or even intentional removal of the glands for oncologic reasons (for example, presence of metastases in the central compartment, which upon resection could also remove the parathyroids from their location) (53,54).

Maintaining intact parathyroids in place is the most important factor in preventing hypoparathyroidism (44,55). This requires from the surgeons a broad knowledge of the anatomy and embryology of the parathyroids, meticulous surgical technique, and command of parathyroid autotransplantation techniques in case the glands are removed (43,56,57).

There is no standard and widely used technique in terms of handling of the parathyroids during thyroidectomy, but some aspects may be highlighted: ligation of the inferior thyroid artery branches should

be performed as close as possible to the thyroid capsule to avoid vascular lesions; direct cauterization of the parathyroids or within millimeters from them must be avoided; careful dissection of the thyroid capsule should be performed, gently moving away the parathyroids without causing tissue or vascular trauma; when central compartment lymph node dissection is required (where the inferior parathyroids are at greater risk), start by identifying and maintaining the superior parathyroids intact and in place; inspect the thyroid after removal in search of parathyroids that may have been inadvertently resected. In this case, autotransplantation of small fragments of the removed parathyroid (approximately 2 mm^3) is recommended, followed by their implantation usually in the homolateral sternocleidomastoid muscle (43,56,57), although the actual effectiveness of this procedure has been questioned by several authors **(B)** (44,58).

There have been recent attempts at the development of auxiliary methods to identify the parathyroid glands during thyroidectomy, such as the injection of nanoparticles of carbon into the thyroid (59,60) and detection of the natural autofluorescence of the parathyroid with near-infrared imaging (61-63) or in association with methylene blue (64) or indocyanine green (65). In spite of these techniques improving the identification of the parathyroids, they have been unable to reduce the rates of hypoparathyroidism after total thyroidectomy **(B)** (66).

Highlights SBEM: The maintenance of intact parathyroids in place is the most important factor in preventing hypoparathyroidism. Extensive knowledge of anatomy and embryology, meticulous surgical technique, and command of parathyroid autotransplantation techniques may minimize the risk of hypoparathyroidism. To date, no technology for intraoperative identification of the parathyroids has proven effective in reducing the rates of permanent hypoparathyroidism **(B)**.

5. Is it possible to predict the risk of hypocalcemia after thyroidectomy?

Efforts have been directed toward the identification of patients at higher risk of developing hypocalcemia after thyroidectomy. These efforts were initially focused on protocols recommending serial blood sample collection for measurement of calcium levels (67-69) and, more recently, also include measurement of PTH levels (49,70-85). The literature describes several protocols for measurement of serum PTH levels (49,73,80,82), with variations in the time of blood collection, criteria for the diagnosis of hypocalcemia, number of collected blood samples, and different PTH cutoff levels. The decay of PTH levels in samples collected before and 1 hour after surgery has been associated with the occurrence of hypocalcemia **(B)** (49,83). A single postoperative measurement has also shown to correlate with the occurrence of hypocalcemia. Samples can be collected from the end of the surgery (49,70,71,78,79,81) till the first postoperative day **(B)** (72,76,84). None of these studies has guaranteed 100% accuracy with such measurements. Severe hypocalcemia is unlikely with a normal PTH value measured after surgery (75). Although the time period, PTH value, and ideal protocol have not been defined yet, perioperative PTH measurements appear to be useful in the postoperative management of patients undergoing thyroidectomy who later develop hypoparathyroidism.

Highlights SBEM: The use of perioperative PTH values may be useful in predicting the risk of hypocalcemia in patients undergoing thyroidectomy. However, the protocol must be established according to the experience of each institution **(B)**.

CLINICAL MANIFESTATIONS

6. What are the acute and chronic clinical manifestations of hypocalcemia?

Hypocalcemia may be associated with several signs and symptoms, considering that calcium has distinct roles in various organs and tissues, including the central nervous system, heart, skeletal muscle, and kidneys (2,3,22). The severity of the symptoms depends on the duration and intensity of the hypocalcemia and on how fast the manifestations emerge **(D)**. Classically, low serum calcium manifests acutely with paresthesias in the face or distal extremities, weakness, and muscle pain accompanied or not by increased levels of the enzyme creatine phosphokinase (CPK) **(C)** (86,87). Hypocalcemia is associated with increased neuromuscular excitability; therefore, symptoms such as carpopedal spasms, cramps, and muscle contraction are characteristic of this metabolic disorder.

On physical examination, the characteristic signs of hypocalcemia reflecting a status of neuromuscular excitability are Trousseau's and Chvostek's signs. The first is an involuntary contraction of the forearm muscles with flexion of the wrist and metacarpophalangeal joint, extension of the interphalangeal joints, and adduction of the thumb ("obstetrician's hand" or carpopedal spasm) when the cuff of the sphygmomanometer is inflated 10 to 20 mmHg above the systolic pressure for about 3 minutes causing an occlusive pressure (88). Although this sign is very specific of hypocalcemia, it may also be present in up to 4% of the normal individuals **(B)**. The Chvostek's sign is characterized by an ipsilateral contraction of the muscles around the lips or other facial muscles triggered by the tapping of the facial nerve over its trajectory anterior to the ear (89). This signal may be also positive in up to 10% of the normal individuals **(D)**.

In severe cases, excessive muscle contraction may lead to spontaneous tetany or trigger generalized seizures, which may be the initial presentation of hypoparathyroidism **(C)** (90). In addition, acute and severe hypocalcemia can potentially lead to sudden dyspnea followed by laryngeal stridor, characterizing laryngospasm (91), or result in papilledema associated or not with increased intracranial pressure. All these situations are reversible with normalization of serum calcium levels **(C)** (92,93).

Since calcium plays a fundamental role in myocardial excitation and contraction, severe hypocalcemia triggers electrocardiographic changes such as prolongation of the QT interval, which may progress to ventricular fibrillation and cardiac arrest **(C)** (94). There have been reports of dilated cardiomyopathy with systolic dysfunction reversing after normalization of calcium levels **(C)** (95,96).

Some dermatological manifestations secondary to acute hypocalcemia have been reported, including piodermitis and acute generalized pustular psoriasis (psoriasis of von Zumbusch) **(C)** (97-99). The reason for the association between hypocalcemia and psoriasis, specifically, is the role of the intracellular calcium in regulating the proliferation and differentiation of keratinocytes (97-99).

In contrast to the manifestations in acute hypocalcemia, neuromuscular symptoms may be milder in chronic hypocalcemia **(D)** (2,3,22). Some chronic manifestations such as cataract and cerebral calcification will be discussed in the session dedicated to chronic complications (100-102).

Hypocalcemia of long duration has been associated with psychiatric manifestations, such as mood changes, anxiety, depression, and, more rarely, hallucinations and psychotic episodes **(C)** (103).

Other symptoms including tinnitus and dizziness have been reported in patients with hypocalcemia and cerebral calcification **(C)** (104). Nonspecific signs may be present, such as dry and rough skin, weak and brittle nails, and dry hair (105). Dental abnormalities, especially during childhood, such as delayed teeth eruption, dental hypoplasia, and enamel and tooth root defects, have also been reported **(C)** (106).

Highlights SBEM: The most frequent symptoms of acute hypocalcemia are paresthesias, cramps, pain, and muscle weakness. In cases of severe hypocalcemia, seizures, tetany, papilledema, and laryngospasm may be included in the clinical manifestations. On physical examination, the presence of Trousseau's and Chvostek's signs suggest hypocalcemia. Chronic hypocalcemia may be asymptomatic **(C)**.

DIAGNOSIS

7. How is hypoparathyroidism diagnosed?

Although hypoparathyroidism is suspected on clinical grounds, its diagnosis is based on laboratory tests demonstrating inappropriately low PTH levels in the presence of hypocalcemia **(A)** (1-3,9,21). Of note, the definite calcium and PTH values for diagnosis of hypoparathyroidism have not been defined yet.

The diagnosis of hypoparathyroidism should take into account the presence of suggestive clinical manifestations, history of surgery or cervical irradiation, and factors that might suggest the etiology of the disease, such as concomitant autoimmune conditions or syndromic manifestations (2,20). The physical examination must include a careful inspection of the cervical region in search of signs of prior surgery and assessment of Chvostek's and Trousseau's signs (1-3).

The laboratory evaluation must include serum measurement of total calcium corrected for albumin, PTH, phosphorus, magnesium, creatinine, and 25(OH)D, in addition to 24-hour urinary calcium **(A)** (1-3,9,21).

Some laboratory features of hypoparathyroidism are noteworthy. Although ionized calcium is the

physiologically active calcium fraction, its measurement requires standardized blood collection and pre-analytical care, both of which are not always followed. In clinical practice, the most reliable alternative to ionized calcium measurement is the determination of total calcium corrected for albumin, which is calculated by adding 0.8 mg/dL for each 1.0 g/dL decrease in albumin level below 4.0 g/dL (6-9). The diagnosis of hypocalcemia is established in the presence of calcium levels below the normal range, but in hypoparathyroidism, the levels are usually below 7.5 mg/dL **(C)**. In cases of rapid development, the symptoms may precede the hypocalcemia **(C)** (107).

The values of intact PTH are low or undetectable. In the presence of hypocalcemia, values below 20 ng/mL are diagnostic of hypoparathyroidism (20). PTH measurement requires certain care with blood collection and storage (108).

Levels of phosphorus in chronic hypoparathyroidism are usually increased, but in the presence of concomitant hungry bone syndrome, they may be within the normal range or decreased. Measurement of magnesium levels is important to rule out functional hypoparathyroidism. The determination of serum creatinine and 25(OH)D levels are useful during follow-up.

In the presence of a normal renal function, the 24-hour urinary calcium reflects the nutritional intake of calcium. During follow-up, it is important to monitor the occurrence of hypercalciuria (urinary calcium greater than 4 mg/kg/day), due to the absence of PTH during treatment with calcium and vitamin D **(A)** (109,110).

The etiologic diagnosis of nonsurgical hypoparathyroidism is challenging. So far, no autoantibody has been standardized for this purpose in clinical practice, although the determination of anti-IFNω and anti-NALP5 has proven to be useful in this regard (111). The diagnosis of autosomal dominant hypocalcemia through molecular CaSR analysis is important during management of this condition to prevent nephrolithiasis/nephrocalcinosis **(B)** (111).

Highlights SBEM: Hypoparathyroidism is suspected on clinical grounds, but its diagnosis is based on laboratory tests indicating inappropriately low PTH levels in the presence of hypocalcemia **(A)**. The patient's laboratory evaluation must include total serum calcium corrected for albumin, PTH, phosphorus, magnesium, creatinine and 25-hydroxyvitamin D, in addition to 24-hour calciuria **(A)**.

TREATMENT

8. How should patients with hypoparathyroidism be treated? How should therapeutic failure with conventional treatment be defined?

Treatment of hypoparathyroidism is aimed at correcting hypocalcemia and hyperphosphatemia, reducing symptoms, and preventing chronic complications resulting from the disease or its treatment. To avoid long-term side effects, the goal of the treatment is to maintain the total calcium close to the lower normal range. Persistent hypomagnesemia after normalization of calcium levels must be corrected (112). Two different strategies may be used, depending on the rate of development of hypocalcemia and presence of symptoms **(A)**.

8.1 Treatment of acute hypocalcemia

Acute manifestations threatening the patients' lives such as tetanic seizures, laryngospasm or bronchospasm, seizures, bradycardia, prolongation of the QT interval, or congestive heart failure, require urgent treatment with intravenous calcium **(A)** (1-3,7,20,21,107,112,113). The most used salt among us is 10% calcium gluconate, which contains approximately 90 mg of elemental calcium per 10 mL of solution. One to two ampoules should be diluted in 50 to 100 mL of 0.9% saline solution (SS) or 5% glycosylated solution (GS) and administered slowly via intravenous infusion over 10 to 20 minutes **(B)**. Rapid infusion may trigger cardiac arrhythmias and cause a severe inflammatory reaction in the venous path (phlebitis). Overflow may lead to calcification of local soft tissues, especially when serum phosphorus concentrations are increased. To preserve normocalcemia, a continuous infusion of elemental calcium 0.5 to 2.0 mg/kg of body weight/hour diluted in 5% GS may be required until the effect of the long-term oral medications on calcium levels becomes established **(B)**. Serum calcium concentrations must be periodically monitored for titration of the infusion dose; the heart rhythm should also be monitored, especially in patients on digitalis **(A)** (1-3,7,20,21,107,112,113).

8.2 Treatment of chronic hypocalcemia

Calcium concentrations must also be corrected in patients with slowly developing hypocalcemia (chronic), but intravenous calcium infusion is not required if the patient is mildly symptomatic or asymptomatic.

Maintenance of calcium concentrations in the long term has been achieved with the use of vitamin D or its active form, calcitriol, associated with oral calcium salts **(A)** (1-3,7,20,21,107,112,113).

The conversion of 25(OH)D into its active metabolite ($1,25(OH)_2D$ or calcitriol) is catalyzed by the enzyme 1α-hydroxylase in renal tubular cells, usually stimulated by PTH and inhibited by hyperphosphatemia (11). Therefore, the production of $1,25(OH)_2D$ is reduced in hypoparathyroidism. For this reason and due to a shorter half-life, treatment with oral calcitriol is preferable **(B)**, although vitamin D (cholecalciferol or ergocalciferol) may also be used **(B)** (114). The effects of these hypercalcemic drugs arise mainly from their action on the absorption of calcium in the intestine, but when administered in excessive doses, they stimulate bone resorption and promote the release of calcium from the bone **(A)** (115). In severe PTH deficiency, calcium levels can only be normalized with calcitriol or high doses of vitamin D **(C)**. With a long biological half-life (4 to 6 hours), calcitriol may be administered every 12 hours, and its initial doses range from 0.5 to 1.0 μg/day divided into at least two daily doses. Serum calcitriol peak is achieved 4 to 6 hours after administration, and elevations in calcium levels may be observed 1 to 3 days after treatment initiation. The dose of calcitriol should be titrated according to calcium levels and varies among individuals, in some cases exceeding 2.0 μg/day **(B)** (1-3,7,20,21,107,112,113). High doses of vitamin D (ergocalciferol or cholecalciferol) may also be used to treat hypoparathyroidism and have often been used in the past when access to calcitriol was restricted. The effects of these agents in increasing calcium levels take longer (approximately ten days), and their biological half-life is 2 to 3 weeks. Since these doses are very high, situations of severe intoxication manifesting with hypercalcemia may occur in the long term and are usually very prolonged **(A)** (114). Due to that, there is a preference for the use of calcitriol, whose shorter half-life enables faster correction of calcium levels, both in cases of hypocalcemia as well as hypercalcemia secondary to intoxication **(B)**. There is no evidence that the use of vitamin D supplementation doses associated with calcitriol is effective in controlling calcium when compared to calcitriol alone.

The use of oral calcium salts is essential in the treatment of hypocalcemia and has two objectives: to offer calcium for absorption by the intestinal cells under the effect of vitamin D, and to sequester radicals containing phosphorus present in the food, indirectly reducing phosphatemia (1-3).

The most commonly used calcium salts are carbonate and citrate. Calcium carbonate has a larger amount of elemental calcium per gram of salt (40%) and a lower cost. However, the calcium requires gastric acidity to dissociate from the salt and be absorbed. In cases of achlorhydria, low acidity (use of proton pump inhibitors), or gastrectomy, calcium citrate is preferred despite a lower concentration of elemental calcium per gram of salt (21%) and a higher cost (113). Other calcium formulations may be used, such as lactogluconate and citrate malate, but their use has limited scientific evidence. The daily amount of required elemental calcium varies greatly among patients, from as little as 1 g to as much as 9 g (21), but most of patients can be well controlled with daily doses ranging from 1 to 3 g, divided in three times a day over meals **(A)** (1-3,7,20,21,107,112,113). Due to the absence of the phosphaturic PTH action, it is recommended to limit the amount of phosphates and calcium phosphate salts in the diet. For this reason, increased intake of dairy products, which are rich in calcium but have a high phosphorus content, should not be encouraged **(D)**.

The use of intestinal phosphate chelating agents may be rarely necessary, in addition to the use of calcium carbonate, which also performs this function **(B)** (1-3). Unfortunately, vitamin D derivatives do not fully replace the effects of PTH, so this treatment is not substitutive. An important PTH effect is an increase in tubular calcium reabsorption. When calcium levels are increased during vitamin D administration, the supply of calcium to the glomerular filtrate increases. Without the effect of PTH to induce tubular calcium reabsorption, hypercalciuria may occur and lead in the long term to complications such as nephrolithiasis, nephrocalcinosis, and renal insufficiency **(A)** (24,116,117). To correct hypercalciuria during hypoparathyroidism treatment, thiazide diuretics including chlorthalidone and hydrochlorothiazide may be used, although this approach has variable efficacy. The dose of hydrochlorothiazide varies between 25 to 50 mg administered in one or two doses a day and the association with amiloride may help spare potassium, avoiding hypokalemia secondary to prolonged use of such diuretics **(B)** (1-3,7,20,21,107,112,113).

Magnesium has metabolic pathways very similar to those of calcium, and its level may decrease, especially in the occurrence of hypocalcemia. Usually, magnesium levels correct in parallel to those of calcium, but in some cases, supplementation with magnesium salts is required, particularly in hypoparathyroidism resulting from activating CaSR mutation (1-3,113). Commercially available magnesium pidolate has 130 mg of elemental magnesium in each vial and the dose varies between 1 to 2 vials a day, but manipulated formulations may also be used.

Treatment of hypoparathyroidism with calcium salts and vitamin D has some challenges. The success of the treatment depends on the patient ingesting several pills many times a day **(B)** (1-3,113).

Some clinical situations may destabilize a well-adjusted treatment or even hinder a proper control of calcium levels, including gastrointestinal infections, urgent hospitalizations, medications affecting the absorption of nutrients or other medications (orlistat, cholestyramine, and glucocorticoids, among others), as well as conditions associated with spontaneous malabsorption (inflammatory bowel disease, celiac disease, or malabsorption due to other causes) or iatrogenic (such as post-bariatric surgeries) and difficulties in the ingestion of medications by mouth, as seen in wide surgical resections due to laryngeal cancer (2,118-123). The metabolic control during pregnancy and lactation in patients with hypoparathyroidism will be discussed in Section 10.

The use of PTH and its derivatives has been tested with some success in the treatment of hypoparathyroidism, and the use of PTH (1-84) has been approved by American and European agencies (FDA and EMA) and will be discussed in Section 9 (124). Furthermore, perspectives point out to the use of allografts of macroencapsulated parathyroid cells, but studies in this regard are still in experimental phases (125).

Considering that a hypocalcemia emergency may be potentially lethal, patients with chronic hypoparathyroidism must carry an identification card indicating their diagnosis and treatment, in the case of emergency **(D)**.

Highlights SBEM: Treatment of hypoparathyroidism aims at correcting hypocalcemia and hyperphosphatemia, reducing symptoms, and preventing chronic complications **(B)**. The treatment goal is to maintain the patient asymptomatic, with total calcium levels close to the lower normal range, phosphorus levels close to the high normal range and 24-hour urinary calcium should be maintained within the normal range (below 300 mg/day) or below 4 mg/kg/day. Treatment of acute hypocalcemia is aimed at controlling situations leading to imminent life threat and requires an intravenous infusion of calcium. The treatment of chronic hypocalcemia involves the use of active vitamin D and calcium salts **(B)**.

9. What evidence supports the use of PTH analogs in the treatment of hypoparathyroidism?

Hypoparathyroidism persists as the last classic hormone deficiency in which the conventional treatment is not done with replacement of the missing hormone **(D)** (1-3). There has been some accumulated experience with the use of PTH analogs since 1996, when Winer and cols. followed up adults treated with PTH (1-34,126-128) and studies with the duration of 3 years compared this type of treatment with the conventional one in children and adults (5-70 years). Serum calcium levels were more consistent, and no differences in urinary calcium were observed **(B)** (129,130). Studies with PTH (1-34) administered with a continuous infusion pump, compared with subcutaneous PTH (1-34) twice daily in adults and children, showed a more physiological control of serum calcium and reduction in urinary calcium **(B)** (131,132). It is important to highlight that PTH (1-34) is only approved for the treatment of osteoporosis in adults and for 18 to 24 months, and has not been approved by regulatory agencies for the use in hypoparathyroidism.

In January 2015, the FDA approved the use of PTH (1-84) for the treatment of hypoparathyroidism. From a pharmacokinetic standpoint, this molecule has a longer half-life, which allows for a single daily application. Studies have shown that the dose of PTH (1-84) must be titrated between 25, 50, 75, and 100 µg, thus enabling a reduction in the doses of calcium and vitamin D. Serum calcium must be monitored since episodes of hypercalcemia have been described, mostly asymptomatic, especially with the dose of 100 µg. In some cases, calcium and vitamin D supplements could be discontinued **(B)** (133-135).

The REPLACE study (a double-blind, randomized, phase 3, placebo-controlled trial) has shown that 53% of the adult patients (18-85 years) treated with up to 100 µg of PTH (1-84) were able to decrease by 50%

their doses of calcium and vitamin D after 24 weeks compared with 2% in the placebo group **(A)** (136). A study with longer duration was published by Rubin and cols. with 33 patients followed up for six years with dose titration. There was a reduction in urinary calcium and maintenance of serum calcium, but a decrease in radial bone mineral density (BMD) in 33% **(B)** (124).

The use of PTH analogs to treat hypoparathyroidism is promising, but several limiting aspects must be considered: these agents must be administered by injection, are very expensive, and have not been approved in Brazil yet (until November 2017). Additionally, their efficacy in preventing the emergence of chronic complications has not been proven so far, there are no studies in children, and the supplementation with calcium and vitamin D has not been entirely suspended in most patients. However, some situations have been considered as potential indicators of benefit from their use in the USA, where the drug has been approved (113,137).

Highlights SBEM: PTH analogues may be used to treat hypoparathyroidism in specific cases. As far, there is no evidence showing their effectiveness in preventing chronic complications in the long term **(D)**.

10. How should hypoparathyroidism be managed during pregnancy and lactation?

The unique physiological environment during pregnancy, determined by cardinal transient factors such as the presence of the placenta (which is equipped with an endocrine machinery) and the demand for building blocks for the development of fetal organs and tissues (including bone), challenge adaptations in mineral metabolism. First, we must call attention to prominent differences in the consequences of vitamin D and PTH deficiencies in pregnant woman and the fetus (138,139).

Physiological adaptations during pregnancy and lactation affect the management of maternal hypoparathyroidism. Calcium absorption is facilitated during pregnancy by increased calcitriol synthesis and sensitivity, among other factors. At this phase, maternal PTH secretion is inhibited, but serum calcium corrected for albumin remains normal. In contrast, the action of the PTH-related protein (PTHrP) produced by the mammary gland tends to mobilize calcium from the skeleton during lactation (138,139).

Another important consideration in the management of hypoparathyroidism during pregnancy is the effect of hemodilution on serum albumin levels and circulating total calcium. Due to that, ionized calcium is the preferred control parameter and should be measured every 2-3 weeks. However, the technique to measure ionized calcium is very sensitive and requires rigid but often neglected pre-analytical protocols, leading to inaccurate results (140). In this context, the determination of total calcium corrected for albumin is an advisable alternative.

Hypoparathyroidism is a risky condition affecting the maternal/neonatal survival (spontaneous abortion, stillbirth, and premature labor), and impairing fetal bone development. Hypocalcemia in pregnant women with poorly controlled hypoparathyroidism leads to fetal secondary hyperparathyroidism with devastating effects on fetal bone, expressed as bone demineralization and fractures. In this case, concomitant maternal hyperphosphatemia is an additional worsening factor **(C)** (138,139).

The literature about the management of hypoparathyroidism during pregnancy and lactation is scarce and mostly based on case reports without randomized controlled studies comparing optional therapies (2). Frequent evaluations are necessary to assess symptoms and serum levels of albumin-corrected total calcium. Circulating calcium levels must be maintained in the lower normal range. During lactation, serum calcium levels must be closely verified due to the production of PTHrP by the breast and increased bone resorption, while medication tapering usually becomes necessary **(C)** (141).

Activated vitamin D analogs (calcitriol or 1 α-calcidiol) are preferable, but when unavailable, pharmacological doses of D_2 or D_3 may be used. The occurrence of an alternate source (placental) of $1,25(OH)_2D$ and PTHrP during pregnancy indicate a potential need for lowering the medication dose. However, there is usually no need to reduce the doses of calcium and calcitriol in clinical practice. Indeed, the increased demand of calcium for skeletal mineralization in the last trimester increases the requirement of calcitriol to avoid hypocalcemia. In case reports, oral elemental calcium varies from 800 to 1,500 mg at the beginning of pregnancy to 2,000 to 3,200 mg in the third gestational trimester **(C)** (142,143).

During pregnancy, the daily recommended dose of calcitriol is between 0.25-3 µg. At the beginning of lactation, the dose has to be individually reduced to avoid hypercalcemia. After weaning, the woman usually resumes the medication dose used before pregnancy.

Pediatricians must be advised about the management of maternal hypoparathyroidism during pregnancy. When maternal overtreatment results in hypercalcemia, which in turn suppresses fetal PTH secretion, neonatal hypocalcemia must be assessed. On the other hand, maternal hypocalcemia may lead to fetal secondary hyperparathyroidism and skeletal abnormalities.

The authors encourage the readers to review the insightful articles by Kovacs and cols. about mineral metabolism in this singular period of a woman's life (138,139).

Highlights SBEM: Treatment of hypoparathyroidism during pregnancy and lactation requires special care and more frequent control for dose adjustment, which must be individualized **(C)**.

COMPLICATIONS

11. What are the chronic complications of hypoparathyroidism? How should they be monitored?

The chronic complications of hypoparathyroidism are associated with the progression of the disease and the implemented therapy and include clinical comorbidities of varying severity **(D)** (22). Since for many years hypoparathyroidism remained a neglected disease, data on the long-term complications of this condition are rare.

11.1 Renal manifestations

Renal complications arise from chronic hypocalcemia and hyperphosphatemia due to lost regulation of calcium and phosphorus metabolism **(D)** (144). Treatment with large amounts of calcium and active vitamin D (calcitriol) leads to hypercalciuria, in addition to increasing the intestinal absorption of phosphorus, intensifying the hyperphosphatemia and increased calcium-phosphorus product and predisposing to nephrolithiasis and nephrocalcinosis. In a study by Mitchell and cols. in patients with hypoparathyroidism, higher serum calcium levels were associated with higher urinary calcium values, prevalence of 38%, while lithiasis and nephrocalcinosis were observed in 31% of the patients. Renal function was decreased in 52% of the patients and was associated with age, disease duration, and proportion of time with relative hypercalcemia **(A)** (116). In a study by Lopes and cols., the prevalence of renal complications in patients with hypoparathyroidism was 25%; although the levels of urinary calcium were within the normal range in most of the cohort, the levels in the group with renal complications were higher (around 3.3 mg/kg/day) (24). Renal manifestations occur independently from the etiology of the hypoparathyroidism (34,27) and are already present at birth in children affected with this disorder (117).

Although it is unclear in the literature whether prophylactic measures can prevent renal impairment, measurement of urinary calcium and serum creatinine are recommended every six months. The 24-hour urinary calcium should be maintained within the normal range for gender (below 300 mg/day) (21,109) or below 4 mg/kg/d (20,110). Periodic imaging evaluations of the kidney and urinary tract are not supported in the literature. However, based on the high prevalence of renal manifestations, both European and American consensus recommendations include periodic imaging evaluations **(D)** (2,20).

11.2 Cardiovascular manifestations

Two studies with historical and controlled cohorts in patients with nonsurgical hypoparathyroidism and postsurgical hypoparathyroidism have addressed the occurrence of cardiovascular complications. Although the general mortality in these cohorts was not increased, ischemic heart disease, stroke, and arrhythmia were more frequent in patients with nonsurgical hypoparathyroidism compared with the general population **(A)** (34). These complications should be individually monitored, and no established routine has been recommended.

11.3 Ocular manifestations

Epidemiological studies have shown that patients with nonsurgical hypoparathyroidism have a four-fold increased risk and earlier onset of cataract when compared with the general population **(A)** (145). Annual clinical monitoring and metabolic control are recommended, despite the lack of studies confirming

an advantage of routine assessment in patients with hypoparathyroidism **(D)** (2).

11.4 Neuropsychiatric manifestations

Chronic complications of hypoparathyroidism affect the central nervous system and include cerebral calcifications, decreased seizure threshold, seizures, depression, and decreased quality of life (146). Cerebral calcifications vary in prevalence from 12% to 74% and are associated with the duration of the hypocalcemia **(A)** (103). Regardless of the etiology of the hypoparathyroidism, the occurrence of seizures is frequent and associated with a greater risk of hospitalization **(A)** (34,145). Therefore, determination of serum calcium is recommended during the etiological investigation of all patients with epilepsy **(D)**.

Decreased quality of life is a common and important chronic complication of hypoparathyroidism and is associated with a multifactorial etiology. Although undervalued, studies report that decreased quality of life affects 32% to 65% of the patients with hypoparathyroidism **(A)** (34,103). Physical complaints (muscle spasms, decreased muscle strength, fatigue, myalgia, and paresthesia), cognitive symptoms ("brain fog" and difficult concentration), and depression and/or anxiety are associated with decreased quality of life **(D)** (22). Depression and affective disorders are twice as frequent in postsurgical hypoparathyroidism and are associated with a feeling of poor health **(A)** (147). In nonsurgical hypoparathyroidism, there is a higher risk of hospitalization due to psychiatric diseases and a tendency to depression **(A)** (145). Traditional treatment of hypoparathyroidism is unable to prevent these manifestations **(B)** (18), and results obtained with PTH replacement show discrepancies between studies **(B)** (148,149).

11.5 Musculoskeletal manifestations

The absence of PTH leads to decreased remodeling in both trabecular and cortical bone and a consequent increase in BMD. Peripheral quantitative computed tomography shows increased cortical and trabecular volume and decreased cortical porosity, without evidence of changes in bone strength **(A)** (150). Histomorphometry shows increased trabecular volume and width, with the maintenance of the trabecular number and spacing, decreased bone formation, and a lower bone resorption rate, which indicates a deep reduction in bone turnover rate in hypoparathyroidism **(A)** (151,152). Retrospective epidemiological studies have shown conflicting results concerning fractures of the superior limbs, showing a lower risk of fracture of the proximal humerus in postsurgical hypoparathyroidism and increased risk of fracture of the forearm in nonsurgical hypoparathyroidism **(A)** (34,145). The risk of fractures, in general, has been described as comparable to that in control populations (34,145). A pioneer prospective study with radiological assessment of vertebral fractures has shown increased morphometric fractures in women with postsurgical hypoparathyroidism **(B)** (153), a finding confirmed by other authors (154). Monitoring of bone mass using dual-energy X-ray absorptiometry (DXA) has little value during long-term follow-up of patients with hypoparathyroidism, noting that the BMD tends to increase with the duration of the disease (155). With the recent findings of a higher prevalence of morphometric vertebral fractures in patients with hypoparathyroidism, periodic radiologic monitoring of the spine is recommended in patients at risk.

Highlights SBEM: Chronic complications of hypoparathyroidism are associated with the progression of the disease and its treatment, and include renal manifestations (hypercalciuria, nephrocalcinosis, nephrolithiasis, and renal insufficiency), cataract, cerebral calcifications, cognitive and affective manifestations, changes in quality of life, vertebral fractures, and increased cardiovascular risk **(A)**. Periodic monitoring of such complications is recommended and should be individualized **(D)**.

CONCLUSIONS

Hypoparathyroidism is a neglected disease, and the scarcity of data regarding this condition restricts potential recommendations for its ideal management. The most common etiology of hypoparathyroidism is the surgical resection of the parathyroids and, therefore, the risk of postsurgical hypoparathyroidism should always be considered when thyroid and parathyroid surgeries are recommended, and the extent of these procedures is planned. From a surgical point of view, the maintenance of intact parathyroids in place is the most important factor in preventing hypoparathyroidism.

Acute hypocalcemia is associated with frequent symptoms including paresthesias, cramps, pain, and muscle weakness, seizures, tetany, and laryngospasm, while chronic hypocalcemia may present with fewer manifestations. Hypoparathyroidism is suspected on clinical grounds but is diagnosed based on laboratory tests indicating inappropriately low PTH levels in the presence of hypocalcemia.

The treatment of hypoparathyroidism is focused on correcting hypocalcemia and hyperphosphatemia, reducing symptoms, and preventing chronic complications. The treatment goal is to maintain the patient asymptomatic with a total calcium level close to the lower limit of the normal range, with the administration of active vitamin D and calcium salts. Special situations such as pregnancy and lactation require frequent and individualized monitoring.

The chronic complications of hypoparathyroidism are related to the disease progression and its treatment and include renal, ocular, cardiovascular, bone, and neuropsychiatric manifestations. Monitoring these complications is recommended. More studies are needed to evaluate their progression and response to treatment in order to improve the management of hypoparathyroidism.

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Humoral hypercalcemia of pregnancy treated with bisphosphonates

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SUMMARY

Hypercalcemia can be hazardous during pregnancy, most cases being due to primary hyperparathyroidism. We report a case of hypercalcemia with suppressed PTH levels necessitating treatment with bisphosphonates during pregnancy. A 38-year-old woman at the 26th week gestation was admitted because of symptomatic hypercalcemia. She did not take any medication that could influence her calcium levels. Physical examination was unremarkable. Laboratory tests on admission were: calcium 12.7 mg/dL (8.5-10.5 mg/dL), phosphorus 1.8 mg/dL (2.5-4.5 mg/dL) and PTH on 3 consecutive tests 1.2, 1.3 and 1.2 pg/mL (15-65 pg/mL). Her 24h urine calcium was 900 mg, 25-OH-D 40 ng/mL (30-58 ng/mL) and 1,25-OH-D 99 pg/mL (80-146 for women in the third trimester). Abdominal ultrasound revealed multiple hypervascular liver lesions consistent with hemangiomas by MRI. Breast and neck ultrasound were normal, and chest CT revealed few non-significant 0.3-0.7 cm pulmonary nodules with no change after an interval of 3 months. She was treated with isotonic saline, loop diuretics and calcitonin. Despite this treatment, calcium levels remained high (14.1 mg/dL), and pamidronate was initiated. On 35th week gestation, she underwent a cesarean section complicated by hypocalcemia of the newborn. Eight weeks after delivery, her calcium levels are 9.4 mg/dL and PTH 18 mg/dL. According to the extensive workup and the post-partum normalization of PTH and calcium levels, we conclude that excessive secretion of placental PTHrP was the cause of hypercalcemia in this patient. No significant adverse effect of bisphosphonate on the mother or baby were seen at the short term follow up. Arch Endocrinol Metab. 2018;62(1):125-8

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INTRODUCTION

Hypercalcemia can be hazardous during pregnancy, leading to maternal and fetal complications. Hypercalcemia is challenging to diagnose during this period because physiological changes such as hemodilusion, hypoalbuminemia, an elevated glomerular filtration rate, hypercalciuria and placental transfer of calcium to the fetus may all lead to lower blood calcium levels. Most cases of hypercalcemia diagnosed during pregnancy are due to primary hyperparathyroidism (PHPT) caused by a solitary adenoma. Less commonly, hypercalcemia is caused by multiple parathyroid adenomas, diffuse hyperplasia and parathyroid carcinoma (1). When maternal PTH levels are suppressed, the diagnostic challenge is further increased. Several cases have been reported of women with hypercalcemia secondary to milk alkali syndrome (2,3), and cases of parathyroid-related-protein (PTHrP) mediated hypercalcemia have been described during pregnancy in women with uterine leiomyoma (4), neuroendocrine tumor of the pancreas (5) and ovarian clear cell carcinoma (6). Rare cases of humoral hypercalcemia of pregnancy, in which the PTHrP source was suspected to be the placenta, have also been described previously in the literature (7,8).

It is probable that most cases of hypercalcemia due to PHPT go unrecognized and are not associated with maternal or fetal complications (9). When symptomatic, women can present with nausea, vomiting, confusion, agitation, nephrolithiasis, pancreatitis, hyperemesis gravidarum and preeclampsia. Hypercalcemic crisis has been reported with calcium levels above 14 mg/dL leading to uremia, coma and maternal death. The most serious fetal complications involve suppressed parathyroid gland with severe hypocalcemia, tetany and fetal or neonatal demise (10-13).

We report a case of hypercalcemia with suppressed parathyroid hormone (PTH) levels necessitating treatment with bisphosphonates during pregnancy.

CASE REPORT

A 38-year-old woman at the 26th week gestation was admitted to hospital because of elevated calcium levels. Her first pregnancy 16 years prior to presentation was uneventful. She was a carrier of the hemophilia gene and was generally healthy. Her symptoms consisted of pruritus, polidypsia and polyuria. She did not take any medication or food supplement that could influence her calcium levels. Physical examination was unremarkable. Laboratory tests were as follows: calcium 12.7 mg/dL (reference range, 8.5-10.5 mg/dL), albumin 3.6 mg/dL (3.5-5.2 g/dL), ionized calcium 7.9 mg/dL (4-4.9), magnesium 1.21 (1.8-2.6 mg/dL), phosphorus 1.8 mg/dL (2.5-4.5 mg/dL) and PTH levels on 3 consecutive tests 1.2, 1.3 and 1.2 pg/mL (15-65 pg/mL). Her 24h urine calcium was 900 mg, 25-hydroxyvitamin (25OH) D- 40 ng/mL (30-58 ng/mL), 1,25OH vitamin D- 99 pg/mL (16-80 pg/mL for the general population, 80-146 for women in the third trimester (14). Thyroid function tests and angiotensin-converting enzyme levels were normal. Further evaluation included abdominal ultrasound, which revealed multiple hypervascular liver lesions that were later diagnosed as liver hemangiomas by MRI; breast and neck ultrasounds, which were normal; and chest CT, which revealed small pulmonary nodules (0.3-0.7 cm) that had not changed at a follow up study 3 months afterward. She was treated with isotonic saline (up to 250 mL/hour) and loop diuretics (40 mg/day) and received calcitonin (300 units twice a day, than 200 units twice daily for 2 days). Urinary output reached 13.5 liter/day. Since calcium levels rose up to 14 mg/dL (corrected to albumin) after this vigourus therapy, treatment with pamidronate was initiated. A total of 90 mg was given in three divided doses with a good response. At the 35th week gestation, after calcium levels began to rise again, induction of delivery was ensued. Due to no progression, she underwent a cesarean section complicated by hypocalcemia of the newborn. Eight weeks after delivery, her calcium levels were 9.4 mg/dL, phosphorus 3.4, 1,25 OH vitamin D 49 mg/dL and PTH 18 pg/mL. Four months after delivery, maternal and infant's calcium levels were within the normal range, and the infant's height and weight growth were adequate.

DISCUSSION

Regulation of calcium and phosphorus during fetal life is of utmost importance for proper bone development and mineralization. This regulation is dependent on maternal PTH and PTHrP (15). During pregnancy, maternal intestinal absorption of calcium more than doubles as a response to the increasing demand. Factors responsible for this are calcitriol and placental lactogen among other things. Several changes become apparent more obviously during the 3rd trimester and during lactation: free calcitriol levels rise (16), maternal bone resorption becomes evident (due to an increase in urine cross-linked N-telopeptides of type I collagen, especially during the winter) (17), and PTHrP levels, which rise steadily during pregnancy, undergo further elevation during lactation (18).

PTHrP can be secreted physiologically from the lactating breast, placenta, pregnant uterus and various benign and malignant tumors. It has an effect on chondrocyte differentiation and an anabolic function on bone. PTHrP signaling is required for the formation of the mammary glands and is related to calcium transfer across the placenta (19). PTHrP can reach the circulation and cause hypercalcemia (7,20). Its role in maternal bone resorption and osteoporosis of pregnancy and lactation has not been elucidated yet (16,21).

After the extensive workup, and in view of the normalization of calcium levels after delivery, it is our opinion that the cause of the presented patient's hypercalcemia was overproduction of PTHrP in the placenta. It is possible that PTHrP was secreted from mammary glands, or from another unrevealed source. Another possibility is that this is a case of aberrant calcium homeostasis not related to pregnancy. Unfortunately, PTHrP measures are unavailable to us.

Calcitriol levels rise during normal pregnancy. PTH is normally the dominant regulator of Cyp27b1 in adults. During pregnancy the marked increase in calcitriol occurs while PTH is often suppressed to low levels, which suggests that PTH is not responsible for the upregulation of Cyp27b1 (22). Reference values for pregnancy have been suggested and are significantly higher than for the general population and even higher during the third trimester (14).

It is suggested that estradiol, prolactin and placental lactogen, which are elevated during pregnancy, may in part stimulate Cyp27b1, as suggested by animal data. Although the placenta was previously considered to be the source of calcitriol during pregnancy, it is nowadays believed that it is mainly secreted from the maternal kidney (23). In the present case calcitriol levels were not elevated when compared to pregnancy reference values, and hence, it is our view that PTHrP might be a reasonable explanation for the hypercalcemia presented in this case. Other pregnancy-related hormones working excessively or aberrantly might have been the culprit, but the limited laboratory possibilities prevented us from reaching the final conclusion.

Bisphosphonates are synthetic analogues of pyrophosphate that inhibit bone resorption. They are absorbed into the mineral surface of the bone where they interfere with the action of osteoclasts (24). The slow release of bisphosphonates from the bone causes detectable levels in urine for many weeks and months after discontinuing the drug (25).

Bisphosphonates are not considered an accepted treatment during pregnancy, as animal studies found adverse effects on the fetus's skeleton. In rats, bisphosphonates were found to cross the placenta, accumulate in the skeleton of the fetus and decrease fetal weight and bone growth (26). It was also shown to cause symptomatic hypocalcemia of the dams and even fetal demise (27). It is noted that doses administered in animal studies were much higher than those used in clinical practice.

Data regarding the effects of bisphosphonates on human reproduction are scarce. Case reports of inadvertent exposure during pregnancy or pre-pregnancy administration had no apparent adverse effect on the embryo or fetus, although the newborn can develop hypocalcemia in the first few days of life (28). In a review of 65 maternal-fetal pairs with a wide variety of agents and doses, bisphosphonates were related to a small decrease in gestational age and birthweight and hyper or hypocalcemia of the newborn. No long-term maternal or neonatal adverse effects were reported (29). In this case, decision making was established by a multidisciplinary team constituted by endocrinologists, gynecologists, a nephrologist and a clinical pharmacologist. As calcium levels were elevated and tended to further increase despite the reception of accepted treatment, it seemed prudent to make another, more significant intervention even though the data was not sufficient.

In conclusion, hypercalcemia during pregnancy constitutes a diagnostic and management challenge. We add our experience in evaluating hypercalcemia with decreased PTH levels and the use of bisphosphonates during pregnancy with good results.

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