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Transoral thyroidectomy-learning curve

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Professor Bertelli and cols. from Brazil have reported their early experience and complications during learning curve of transoral endoscopic thyroidectomy vestibular approach (TOETVA) (1). The authors have described their early experience of multiple institutions in Brasil having learned TOETVA either in Korea or in John Hopkins-Baltimore. They developed a study group, TOETVA-Bra. Two of the authors are from Johns Hopkins who have larger experience. The authors have reported a collective early experience of 93 patients undergoing TOETVA. Fifty-eight percent had total thyroidectomy, while 59% had benign pathology. The authors have reported 21.5% complications, 16% of which were minor.

It is commendable to organize a study group and report early experience with specific focus on safety and outcomes of adopting a new surgical procedure. The authors have not given the denominator from which these 93 patients were derived. However, at least 7 operating surgeons were directly involved in these procedures. They have used the same technique as popularized by Anuwong from Thailand (2). Even though the authors have titled the manuscript as "Learning Curve" it remains unclear as to the number of procedures required during the learning curve. The complication rate described by the authors is similar to many other publications on this subject. This technique was initially popularized in Thailand and subsequently commonly adopted in Korea and a few centers in the United States. However, there seems to be considerable interest in this surgical approach. The authors have used this for both benign and malignant problems. Approximately 2,000 transoral thyroidectomies have been performed worldwide, of which 400 were in the United States (3).

The entire idea of endoscopic thyroidectomy is to avoid a cervical scar. Whether avoiding a neck incision should be considered as a major indication for endoscopic thyroidectomy remains unclear. Initially the scarless thyroidectomy started in Korea, with transaxiliary approach and robotic instrumentation. There was a huge experience in Korea and selected centers in United States. The transaxiliary robotic surgery did not become very popular in United States partly due to higher rate of complications and a high learning curve and non approval of robotic instrumentation for thyroidectomy by FDA. It appears that there is considerable interest in transoral thyroidectomy; however, what is the exact learning curve remains unclear. And also, how many procedures should a surgeon initially perform under direct supervision of an expert mentor. The indications for this approach remain somewhat unclear and there is always a concern about lip issues and mental nerve injury. It is interesting to note that there are 324 publications on this subject in PubMed, most of which are in the last five years.

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It may be difficult to undertake these procedures without true hands-on training in a specialized center where these procedures are commonly performed. There are two interesting publications on learning curve for TOETVA. Liang and cols. reported 30 cases as learning curve while Cao and cols. reported 25 cases for learning curve (4,5). Lira and cols. reported a drop in surgical time from 167 minutes to 117 minutes after 15 cases (6). It is interesting to note that the transaxiliary endoscopic surgery had a learning curve of 60 cases (7).

The major question remains where should the surgeons experience this learning curve, in their own institution with start-up of this new procedure or a fellowship hands-on training in a specialized center? We always talk about learning curve in surgery, but sometimes we do not recognize higher incidence of complications during the early learning curve. This is primarily the reason why fellowship training is important for subspeciality expertise and perfecting surgical technique. There is no room for learning curve for a pilot in the airline industry, should we not adopt the same philosophy in human surgical procedures? We never ask the pilot if they are on the learning curve and also the airlines would not permit the learning curve. The entire idea of endoscopic thyroidectomies is truly not minimally invasive but probably maximally invasive and some of the complications may be difficult for the patient and the surgeon to handle. Endoscopic thyroidectomy is an evolving field and its true application outside of the major centers interested in this procedure remains unclear. The specialized instrumentation and robotics are not available in most parts of the world and direct cervical exploration is still the most chosen surgical procedure. However, the technology is having a direct impact in surgery and future will be evaluated as time goes by and surgical expertise developed in these new techniques around the world. The incidence of complications and cost are important considerations in application of the new technology. I would like to congratulate the authors of this manuscript for collaborative study and honest publication of their early experience.

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Thyroid surgery – Does the scar matter?

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Boog and cols. from São Paulo, Brazil, have reported the impact of scar on thyroidectomy patients (1). They have reviewed the aesthetic impact of scars on the lives of the patients. This is a retrospective analysis of 98 patients determining the scar impact through a qualitative questionnaire and categorizing three levels of dissatisfaction. Of 98 patients, 96 (98%) reported experiencing no functional or visual discomfort with their scars. There were 2 women who were unsatisfied to a moderate category. It would have been interesting if the authors had interviewed these two patients as to their reasons behind dissatisfaction and the examiner's impression about their scars. They have also discussed their impressions about conventional thyroidectomy against other extra cervical or scarless thyroidectomy. Their conclusions are consistent with the current literature regarding thyroidectomy scars. There may be some issues related to the methodology used by the authors, but the conclusions remain that most of the patients are quite happy with the thyroidectomy scars. I would like to take this opportunity to congratulate the authors for reconfirming our beliefs and would like to add some pertinent pointers about thyroidectomy scar.

There is always debate about the size of the incision; however, the wounds heal in a transverse fashion and there is hardly any cosmetic impact between smaller and larger incisions. What is critical is to get adequate exposure during surgery based on the size of the tumor, location of the tumor, the patient's configuration, and age of the patient. In a younger patient we prefer higher incision, as the scar gets pulled down over a period of time. The incision should be marked preoperatively based on the location of the cricoid cartilage and cervical creases. Surgery through small incision may be challenging, especially stretching the skin edges, and may require freshening the edges.

As the old aphorism goes, "The bold surgeons make small incisions, the scared surgeons make big incisions, but the good surgeons make adequate incisions". The incision is best made around or just below the cricoid cartilage and generally not recommended above the cricoid. If the patient requires a neck dissection, the incisions should be extended into the neck like a necklace at the level of cricoid. There is no need to make an apron or J-shaped incision. The surgical incision heals very well and is cosmetically readily accepted by the patients and their loved ones. The operating surgeon should spend more time in meticulous closure of the wound with appropriate subcuticular stitches. The platysma should be approximated well, which reinforces the skin and avoids wound separation. We generally use 5-0 monocryl subcuticular. Some surgeons use subcuticular prolean to be pulled out 3 days after surgery.

One needs to be extremely careful in location and closure of the incision in African American patients who have higher probability of developing a keloid or hyperplastic scar.

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The incision in the appropriate skin crease literally hides the incision in most of the patients. There are very few patients who are unhappy or who complain about the scars and most of the scars continue to get better in the postoperative period up to 9 to 12 months. Certain ancillary measures are quite helpful such as skin lotions-vitamin E, silicon, Scarguard, etc. Several patients demand wound closure by plastic surgeon even though it is not necessary, as essentially they perform the same surgical technique as thyroid surgeons.

Felix and cols. reported a high level of scar satisfaction (92%) after conventional thyroidectomy (2). Obviously some patients may be self-conscious of their incision. Kurumety and cols. published patient-reported data on thyroidectomy scar perception (3). They concluded older age and more than 2 years after surgery as predictive factors for scar perception. They also concluded "the impact of postoperative thyroidectomy neck appearance on quality of life to be mild and transient and returns to preoperative levels after 2 years".

There are newer surgical techniques of scarless thyroidectomy such as transaxiliary robotic thyroidectomy or the most recent addition of transoral thyroidectomy. These newer techniques are clearly surgical nuances with impact of technology; however, whether these are absolutely essential to avoid a scar will depend on variety of factors such as surgeon's experience, learning curve, patient's acceptance, and the cost. Prior to these newer techniques almost every thyroidectomy patient accepted the scar as is and we may need to revisit the whole issue of scar versus no scar. The newer techniques may have some application in highly selected patients. We need to revisit the extracervical scarless procedures in view of excellent patient satisfaction with standard open thyroidectomy.

Overall satisfaction rate in thyroidectomy scar is quite high and most of the patients learn to live with their scar and quite often enjoy it very much.

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Trans Oral Endoscopic Thyroidectomy Vestibular Approach (TOETVA) in Brazil: Safety and complications during learning curve

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ABSTRACT

Objective: The aim of this study was to address the first cases of TOETVA done in Brazil, by TOETVA-Bra study group, regarding safety and complications. **Materials and methods:** Series of the first 93 TOETVAs cases in Brazil. All authors except LPK, AJG JOR and RPT received TOETVA training including cadaveric hands-on in Thailand or United States (Johns Hopkins Medicine) during 2017. After they came back to Brazil and started doing their first TOETVA cases in the cities of Rio de Janeiro, Sao Paulo and Chapecó they agreed to collaborate and gather data using an online spreadsheet. All patients were submitted to the technique described by Anuwong. **Results:** A total of 93 patients underwent TOETVA. Most patients (58.1%) were submitted to total thyroidectomy and 59.1% had benign disease. Two patients (2.2%) needed conversion to open surgery. Five patients (9.3%) developed transient hypoparathyroidism and there were 3 (2.0%) temporary recurrent laryngeal nerve palsy. There was one (0.7%) permanent unilateral palsy. Twenty patients had some sort of complication, 16.1% were minor and 5.4% were major. A total of 73 patients (78.5%) had an uneventful recovery. **Conclusion:** The technique is reproducible with a low complication rate. While further studies are needed to confirm equivalency, early efforts suggest that TOETVA is not inferior to traditional open thyroidectomy in appropriately selected patients. Arch Endocrinol Metab. 2021;65(3):259-64

Keywords
Thyroid cancer; goiter; surgery; thyroidectomy; endoscopic

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INTRODUCTION

This decade has brought many modifications to the standard open thyroid surgery technique (1). From the beginning of remote access thyroidectomy, when the techniques focused on hiding the scar in more discrete locations (2-4), until now, scarless thyroid and central neck surgery has evolved and may be safely completed via the transoral vestibular approach (1,5-8).

The Transoral Endoscopic Thyroidectomy Vestibular Approach (TOETVA) is the first remote access technique of thyroid surgery to become widely popular in the Western Hemisphere (9-13). Before this procedure, a few surgeons were performing other minimally invasive

techniques such as minimally invasive video-assisted thyroidectomy (MIVAT) or axillary, breast or post-auricular approaches, but without reproducible results (2,3,14). These techniques were not widely adopted for a number of reasons (15). The aim to avoid a neck scar was the fuel to develop, improve and spread remote access thyroid surgery. Anuwong and cols., in 2016, published a series of the first 60 human cases with good results (16). The technique was adopted by some US head and neck surgery centers (9).

This article was planned by a team of Brazilian surgeons trained in TOETVA in the United States and Thailand. Each one did the training program individually and founded the TOETVA-Bra study group aiming to collect data from the first Brazilian cases. All Brazilian authors work in high volume head and neck surgery centers that perform more than 50 thyroidectomies per year.

The aim of this study was to address the first cases of TOETVA performed in Brazil, by TOETVA-Bra study group, with a specific focus on safety and outcomes of adoption in high volume centers.

MATERIALS AND METHODS

All authors except LPK, AJG, JOR and RPT received TOETVA training including cadaveric hands-on in Thailand or United States (Johns Hopkins Medicine) during 2017. After they came back to Brazil and started doing their first TOETVA cases in the cities of Rio de Janeiro, Sao Paulo and Chapecó. They agreed to collaborate and gather data using an online spreadsheet shared with Numbers® (Apple inc). RT and JOR are Head and Neck Surgeons experienced with TOETVA who participated in the authors training. This study has IRB approval from all the institutions collecting data: Santa Casa de São Paulo Faculty of Medical Sciences – main institution (3.897.377) (CAAE: 27131119.2.0000.5479), A.C. Camargo Cancer Center (1913/14), Federal Hospital of Bonsucesso (2.825.617), Brazilian National Cancer Institute (89042418.7.0000.5274), State University of Rio de Janeiro (07678819.0.0000.5259), and Unimed Chapecó Hospital (089/2018).

The surgical technique employed was that described by Anuwong in 2016 (1) using 3 endoscopic ports through the oral vestibule, dissecting a subplatysmal pouch after hydrodissection and dilation, insuflating this pocket with high flow and low pressure CO2, opening the raphe to identify and divide the isthmus, and resecting the thyroid gland. It also involves identifying both parathyroid glands and the recurrent laryngeal nerve on each side using conventional laparoscopic instruments. Nerve monitoring (Neurosoft, Ivanovo, Russia or Medtronic, Dublin, Ireland) was used in all surgeries to help visual identification and preservation of the recurrent laryngeal nerve. In some cases, the nerve monitor was also used for superior laryngeal nerve preservation and vagal stimulation. Ultrasonic or advanced bipolar laparoscopic devices (Johnson & Johnson, New Jersey, USA or Medtronic, Dublin, Ireland, respectively) were used to seal thyroid vessels in all cases. The specimen was always taken out with an endoscopic bag through the midline incision. All procedures were done under general anesthesia.

The data included was demographic information, size of dominant nodule, Fine Needle Aspiration Cytology (FNAC) result, extension of surgery, need for conversion to open surgery, final pathology results and occurrence of complications during the first 30 postoperative days. Complications were divided in minor and major. Minor complications were temporary events without sequelae and major complications were considered permanent events with some sequelae or events that threaten life or may need hospitalization.

RESULTS

A total of 93 patients underwent TOETVA from June 2017 to January 2019. Of these, 79 (85.0%) were women and 14 were men. It was estimated the participation of 40 surgeons in all procedures. The total number of leading surgeons was 7 (Table 1). As the procedures occurred in a high number of different hospitals in Brazil, including teaching institutions with residents, it wasn't possible to determinate the exact number of doctors involved in these surgeries. Most patients

Table 1. Number of procedures for each leading surgeon

Surgeon	n
LGR (RJ)	34
RBL (SP)	20
MAST (SC)	11
AATB (SP)	8
GDS (RJ)	8
MAG (RJ)	8
ICS (RJ)	4
Total	93

(n = 54, 58.1%) were submitted to total thyroidectomy and 39 (41.9%) to lobectomy (Table 2). Median age of patients was 41 years (ranging from 15 to 69 years). Median nodule size was 1.8 cm (ranging from 0.6 cm to 6.0 cm). A total of 55 (59.1%) patients had benign disease and 38 (40.8%) had malignant disease, mostly T1 (32 cases) and six T2 papillary carcinomas.

Indications for surgery based on Bethesda classification and nodule size are shown in Table 3. Two patients (2.2%) needed conversion to open surgery because of bleeding with no other complications reported.

Complications are shown in Table 4. Considering 54 total thyroidectomies, 5 patients (9.3%) developed transient hypoparathyroidism. There was no permanent hypoparathyroidism. Considering 147 nerves at risk, there were 3 (2.0%) temporary recurrent laryngeal nerve palsy (all have function returned after 3 months). There was one (0.7%) permanent unilateral palsy. No laryngeal nerves were known to be severed during this study. Three patients (3.2%) had small skin burns (Figure 1). Two patients (2.1%) had suture dehiscence of central incision that healed spontaneously after two weeks. One patient (1%) had surgical site infection treated with needle aspiration and antibiotics with no need for reoperation. One patient (1%) had a small tracheal tear (2 mm). This lesion was identified intraoperatively and endoscopically sutured after removing the thyroid.

Table 2. Extension of thyroidectomy performed

	Benign	%	Malignant	%	Total	%
TT	24	44	30	79	54	58
Lobectomy	31	56	8	21	39	42
Total	55	100	38	100	93	100

TT: total thyroidectomy.

Table 3. Indications for surgery based on Bethesda Fine Needle Aspiration Biopsy (FNAB), corresponding pathology report and median nodule size

FNAB (Bethesda)	n	Pathology report	Median nodule size (cm)
I	1	Benign	
II	14	All benign	4,0
III	21	4 malignant (19%)	2,4
IV	21	5 malignant (24%)	1,8
V	16	13 malignant (81%)/3 NIFTP*	1,5
VI	15	All malignant (100%)	1,0
Ignored	5	1 malignant, 4 benign	
Total	93		

*NIFTP: Noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

Table 4. Complication rates in TOETVA

Complications	n	%	Note
No	73	78.5	
Yes	20	21.5	
Transient hypopara	5	9.3	54 TT
Transient RLN palsy	3	2.0	147 nerves at risk
Permanent RLN palsy	1	0,7	147 nerves at risk
Skin burn	3	3.2	
Mental nerve palsy	1	1.1	
Infection	1	1.1	
Bleeding/conversion	2	2.2	
Suture dehiscence	2	2.2	
Tracheal lesion	1	1.1	
Mandibular palsy	1	1.1	

TT: total thyroidectomy.

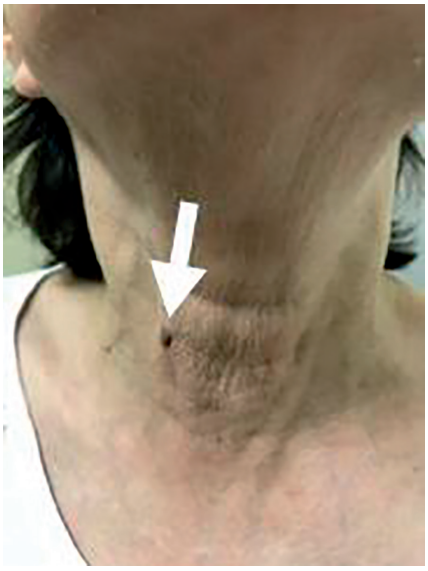

Figure 1. Example of skin burn (white arrow) due to space creation using hook monopolar device.

The patient healed well after one week with a suction drain. One patient had a temporary unilateral marginal mandibular nerve palsy for 3 months. It was identified a minor lip weakness in the first day post-operatively and the patient evolved with full spontaneous recovery. One patient (1%) had transient mental region anesthesia that was resolved after 5 months. There was no hematoma in this series.

Considering lobectomies and total thyroidectomies (93 cases), 20 had some sort of complication (21.5%), 16.1% were minor complications (transient hypoparathyroidism, transient recurrent laryngeal nerve palsy, skin burn, suture dehiscence, mental nerve and marginal mandibular nerve transient palsy) and

5.4% were major complications (permanent vocal fold palsy, permanent hypoparathyroidism, surgical site infection, tracheal perforation and conversion). A total of 73 patients (78.5%) had an uneventful recovery.

DISCUSSION

This study represents the initial experience of Brazilian surgeons with a new thyroid surgery technique – TOETVA. All surgeons involved in the 93 cases are pioneers in this new procedure in Brazil. This technique has evolved in eastern countries such as Thailand and South Korea due to a common goal to avoid the visible scar and also optimize access to the central neck (1,17,18). It has brought better cosmetic results and quality of life according to some authors in the earliest series of cases (1,19).

Although other minimally-invasive techniques for thyroid surgery were performed before in Brazil, TOETVA is the first one to reach a high number of surgeons. It is probably associated with better acceptance by Brazilian surgeons due to its wide operative indications (20), reproducibility and its lower cost. The instrumentation is also widely available, as it requires only standard laparoscopic instruments (1).

The beginning of the learning curve is tricky mostly because is hard to choose the first cases. Although TOETVA shows itself as a technique with wide operative indications, surgeons take some time to get used to it. Perform some and learn with more experienced colleagues drives the surgeon to improve skills and start to indicate more. Commonly the learning curve starts with single small benign nodules, treated with lobectomies. Since the daily main routine of Head and Neck surgeons in Brazil are thyroid nodules, its guaranteed that we still perform a much higher number of conventional thyroidectomies.

Of note, our series showed that most patients (N = 54, 58.1%) underwent total thyroidectomy and 40% were performed for malignant disease. This suggests that Brazilian surgeons felt comfortable progressing quickly from the indications for benign to malignant disease. The low median age (41 years old) is consistent with our anecdotal experience that young patients usually show more interest in TOETVA. The low median nodule size (1.8 cm) demonstrates that patient selection was followed by the surgeons, although some 6 cm (benign) nodules were also addressed. In those patients who had benign FNAC findings, the median nodule size was higher (4.0 cm), similar to traditional indications for thyroidectomy in benign nodules.

The sample is in accordance with the indications determined by the literature, being a good indication for TOETVA: thyroids up to 10 cm wide, benign nodules up to 6 cm and malignant nodules up to 2 cm (1). As the working space has its limitations and the specimen removal should be performed trough the central inferior lip incision the nodule size and the thyroid width are the most limiting aspects of the technique. Fragmentation of the thyroid is reported and other incisions are been used in some centers to remove large specimens, however, these doesn't solve the limited space issue and fragmentation of nodules are not recommended.

Despite the aesthetic appeal, it is a new technique in Brazil and in the first cases it was necessary to present the procedure to patients, however, it has become increasingly common to seek the technique as it has been consolidated. Surprisingly 15% of patients are male, confronting the belief that good cosmetic results in this population are not relevant. As these patients were not the first cases, the vast majority sought the technique on their own.

Only 2 patients needed conversion to open surgery due to bleeding and it occurred in the first cases. In one, arterial bleeding started when the surgeon was ligating the superior pole and in the other the bleeding emerged from the thyroid and the leaking spot was not clearly identified. We believe that the magnification of the image and the video equipment can induce the surgeon to overestimate the bleeding. We also think that the lack of expertise with instruments and the laparoscopic technique, specially the 2D view, were major factors for the conversion in these cases.

The complication rate of TOETVA was very similar to the complication rates of the traditional open technique described by other authors (1,16,18,21,22). This series presented a 9.2% rate of transient hypoparathyroidism with no cases of permanent hypoparathyroidism. The rates of transient and permanent inferior laryngeal nerve palsy were similar to other studies of TOETVA (1,16,17,18,21,22). It is likely that there is a selection bias due to cases of benign disease, predominantly small nodules, and carefully selected early stage cancer, which could all be associated with improved outcomes when compared to series of traditional open thyroidectomy with broader operative indications (23). It should be noted that the video magnification and smoothness of

dissection due to small and delicate instrumentals add safety to recurrent laryngeal nerve dissection, especially after learning curve has ended, although it is not clear if this will result in an improvement over the excellent safety profile in traditional thyroid surgery. Future studies will help to elucidate this point.

Every new procedure brings new complications. This is especially true with remote access surgery, where development of a working space can introduce novel complications relevant to the local anatomy. With TOETVA, this anatomy stretches from the oral vestibule to the central neck and complications may include (17) skin burns, hypoesthesia due to mental nerve trauma, marginal mandibular nerve palsies and neck infection. The skin burns occurred because an energy device was used close to the skin, usually a monopolar hook. These complications generally occurred early in the surgeon's operative experience, and they generally healed well (24). Hypoesthesia due to mental nerve trauma was temporary and occurred only once, during the first cases, when we were still placing the incisions closer to the mandible. Modifications were made, and all surgeons started placing the central incision closer to the lip and the lateral incisions closer to the oral commissure. The group decided to do this modifications after conversations with more experienced surgeons in the US and Thailand, who observed that the lower incisions were closer to mental nerve foramen. Since that time, there have been no more mental nerve injuries (1,17,24). The marginal mandibular nerve palsy is interesting, as it is not common in other series. It may have occurred due to lip musculature being traumatized during placement of the lateral trochar rather than a true marginal mandibular nerve weakness. Although it could be explained by incorrect placement of lateral trochars, connected to some anatomic variation, this seems less likely due to the position of the ports and the likelihood that any injury would be to no more than a terminal branch of the marginal mandibular nerve. Possibly, the use of permanent metallic trochars contributed, as they may be more likely to permit energy conduction. These trochars are frequently used in Brazil due to cost savings. Wound infection isn't common in traditional open surgery, and neither is it common in TOETVA as shown by other authors (16-18). The patient who had an infection recovered promptly with antibiotic treatment with amoxicillin with clavulanate and needle aspiration. Bacterial cultures were negative. One tracheal lesion occurred which is also a possible complication in open surgery. In this situation, however, the corresponding surgeon believes that the decreased tactile sensation during endoscopic surgery contributed to this injury. Finally, there were two episodes of suture dehiscence in the mouth that were not treated with any intervention. No long term effects were noted in those patients.

More recently novel technique without CO2 insufflation was developed based on TOETVA (25). It is important to differentiate both procedures, since TOETVA is getting popular worldwide and day by day more surgeons are starting to do it. The CO2 free technique is still in progress. A few number of surgeons are performing it and additional specific instrumentation are needed (25). Although good results are observed without CO2 insufflation, this results are still note reproducible in a worldwide scale and more data is needed.

TOETVA is a feasible and safe procedure in high volume thyroidectomy centers of Brazil (26,27). It is reproducible and the learning curve is around 10-15 cases (1,17,24,26,28). It seems to be spreading quickly and reaching many Brazilian surgeons because of excellent cosmetic results. Cultural factors and patient demand will likely continue to drive requests for the procedure. More experience in this field should bring more applicability and innovation to the technique and further studies should focus on advanced procedures such as central neck dissection. TOETVA is a novel method of performing central neck surgery without a cutaneous incision that is becoming more popular and has demonstrated good outcomes thus far in Brazil.

In conclusion, the first 93 cases of TOETVA reported in Brazil demonstrates a complication rate similar to that of traditional open thyroidectomy. Its adoption is feasible and safe in high volume centers. More experience with this technique should bring more applicability of the endoscopic central neck vestibular approach technique.

Disclosure: AATB is speaker for Johnson & Johnson and consultant for Delta Medical; RPT is consultant for Medtronic and RGS Healthcare; JOR is consultant for Baxter Scientific; LGR is consultant for RGS Healthcare, Medical Health and speaker for RF medical; others have nothing to disclose.

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Conventional thyroidectomy: what is the impact of the scar on the lives of operated patients?

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ABSTRACT

Objective: This study was aimed at investigating the aesthetic impact of scars on the lives of patients who undergo conventional thyroidectomy. **Materials and methods:** This cross-sectional study was based on a retrospective analysis of 98 electronic medical records of patients who underwent conventional thyroidectomy performed by the same surgeon. The impact was determined through a qualitative question and categorized into three levels of dissatisfaction. **Results:** Among the 98 patients, 96 (97.95%) reported experiencing no functional or visual discomfort with their scars. The two unsatisfied individuals were women, and both classified their discomfort as moderate. Although the diseases that indicated surgery varied, papillary thyroid carcinoma predominated. **Conclusion:** The sample's satisfaction level indicates that, in line with the current literature, the decision to opt for cosmetically appealing methods is not justified by aesthetic complaints about scars. The benefits of lower cost and fewer complications make conventional thyroidectomy an old but reliable option for afflictions of the thyroid gland that require surgery. Arch Endocrinol Metab. 2021;65(3):265-8

Keywords
Thyroidectomy; surgery; scar; aesthetics; social impact

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INTRODUCTION

The anatomical description of the thyroid gland began in 1500 with Leonardo da Vinci. Nonetheless, until the 19th century, thyroidectomy was associated with high levels of complications such as dyspnea and difficulty breathing, hoarseness, hemorrhage, and infection. At the beginning of the 1850s, thanks to the parents of thyroidectomy, Theodor Billroth and Theodor Kocher, there was a remarkable methodological improvement in terms of antisepsis, vascular clamping, dissection techniques, and anesthesia (1). The method these pioneers created effectively reduced mortality from 40% to 2.4%. It consisted of a transverse incision starting at the anterior border of the sternocleidomastoid muscle and continuing up to its contralateral side (2). To minimize scar visibility, the incision is performed on the natural necklines. However, some problems persisted, such as the tetany caused by the loss of the parathyroids. These complications were systematically reduced by Anton Wolfer and Jan Radecki (3). Throughout the 20th century, new and better tools were created to guide and assist surgical results, such as histopathology and drugs intended to replace thyroid hormones (4).

The current trend consists of attempts to improve aesthetic aspects, decrease the length of hospital stays, and reduce possible complications. In this sense, minimally invasive thyroidectomy techniques were created, usually following the orientation of incisions smaller than 3 cm (5).

Among the described methods, minimally invasive video-assisted thyroidectomy (MIVAT) stood out. This technique, which consists of a 1.5-cm transverse incision 2 cm above the sternal manubrium, has limitations and is not indicated for patients with thyroid volume greater than 30 mL, nodules larger than 30 mm in diameter, or tumors larger than 20 mm (6). Another technique presented is endoscopy, which is performed by two periareolar incisions, followed by dissections and tissue detachments to reach the anterior neck, providing a view similar to that of open surgery. However, this method is associated with increased post-operative complications and discomfort due to the large area of displaced tissue (7).

The rise of robotic surgery introduced another technique involving a 5- to 6-cm incision in the posterior part of the pectoral muscle in the axillary

region. Prohibited in the United States, this operation also has considerable limitations, such as the high costs, complications, and time of surgery (8). In the 2010s, a new method created by Anuwong constituted a relevant innovation. It is based on an incision made in the oral cavity, followed by dissection and detachment of the subplatysmal region and subsequent insufflation of the region with CO_2, creating a space to operate from the upper border of the sternal manubrium to the oral vestibule (9).

The aesthetic appeal of these operative techniques is remarkable, especially concerning the absence of visible scars on the anterior neck region, but their limitations should not be ignored. For this reason, in this study, we investigated how much traditional thyroidectomy scars can socially and aesthetically impact the lives of patients who undergo this procedure.

MATERIALS AND METHODS

In this cross-sectional observational study, we conducted a systematic and retrospective analysis of 98 electronic medical records of patients who underwent conventional thyroidectomy performed by the same surgeon at least six months before the interview.

To understand the impact of the scars resulting from these procedures on the patients' quality of life, each person's degree of satisfaction with their scar was explored in the medical records through the question "Does the scar bother you? If so, how much?" To categorize the complaints, the patients were asked to choose between "minimally" (no issues with the scar), "moderately" (self-conscious about the scar), or "considerably" (desire to change the scar).

The sample was composed of men and women, and the operative act was indicated due to the most diverse pathologies. There were no restrictions regarding age or any other selection criteria. Data regarding the patient's characteristics were collected and included the following variables: sex, age, disease that motivated the surgery, subtype of thyroidectomy procedure (e.g., lobectomy, total thyroidectomy), surgery date, and last recorded consultation date.

The study was approved by the Research Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP), São Paulo, Brazil (CAAE 40285320.1.0000.0068).

RESULTS

Our sample included 98 Brazilian patients who underwent conventional thyroidectomy between 2003 and 2019. The mean age among the participants was 47.7 years (standard deviation = 13.7). The median age was 46 years (range 19-80). The group was composed of 33 (33.67%) men and 65 (66.32%) women, and 96 of the patients (97.95%) underwent medical monitoring in 2019, the year when the satisfaction data were collected. The conventional thyroidectomy models performed were as follows: 75 total thyroidectomies (76.5%), 8 partial thyroidectomies (8.2%), 2 total thyroidectomies with lymphadenectomy (2%), and 13 total thyroidectomies with neck dissection (13.3%). These sample characteristics results are shown in Figure 1. Although the diseases that indicated the surgery varied, papillary thyroid carcinoma predominated.

Among 98 patients, 96 (97.95%) reported experiencing no functional or visual discomfort with the scar. The patients reported to their surgeon. Both unsatisfied individuals were women submitted to total thyroidectomy, and both classified their discomfort as moderate.

All scars were inspected on physical examination. Twenty-one patients allowed the use of non-identifiable photographs to illustrate the results of their thyroidectomy scars. None of these individuals reported any discomfort. Four pictures were selected and included in Figure 2.

DISCUSSION

The assessment of our sample's general satisfaction and the current evidence (10) indicate that the decision to opt for minimally invasive methods, with their higher costs and longer operative times, is not justified by aesthetic complaints. In this regard, other factors gain importance, such as the experience of the surgeon, the length of the incision, and the post-operative care.

An interesting finding of our study was that the proportion of patients dissatisfied with the scars resulting from more invasive operations, such as associated neck dissection or lymphadenectomy, was not higher than that of the other members of the study.

The literature indicates that the social impact of the scar is minimal, as seen in approximately 90% of female patients, who did not feel the desire to hide the scar with accessories or request surgical revisions of the cicatrix (11). Indeed, the satisfaction rate in our investigation (97.85%) was similar to those reported in previous observational studies, such as 91.3% and 91.7% (11,12).

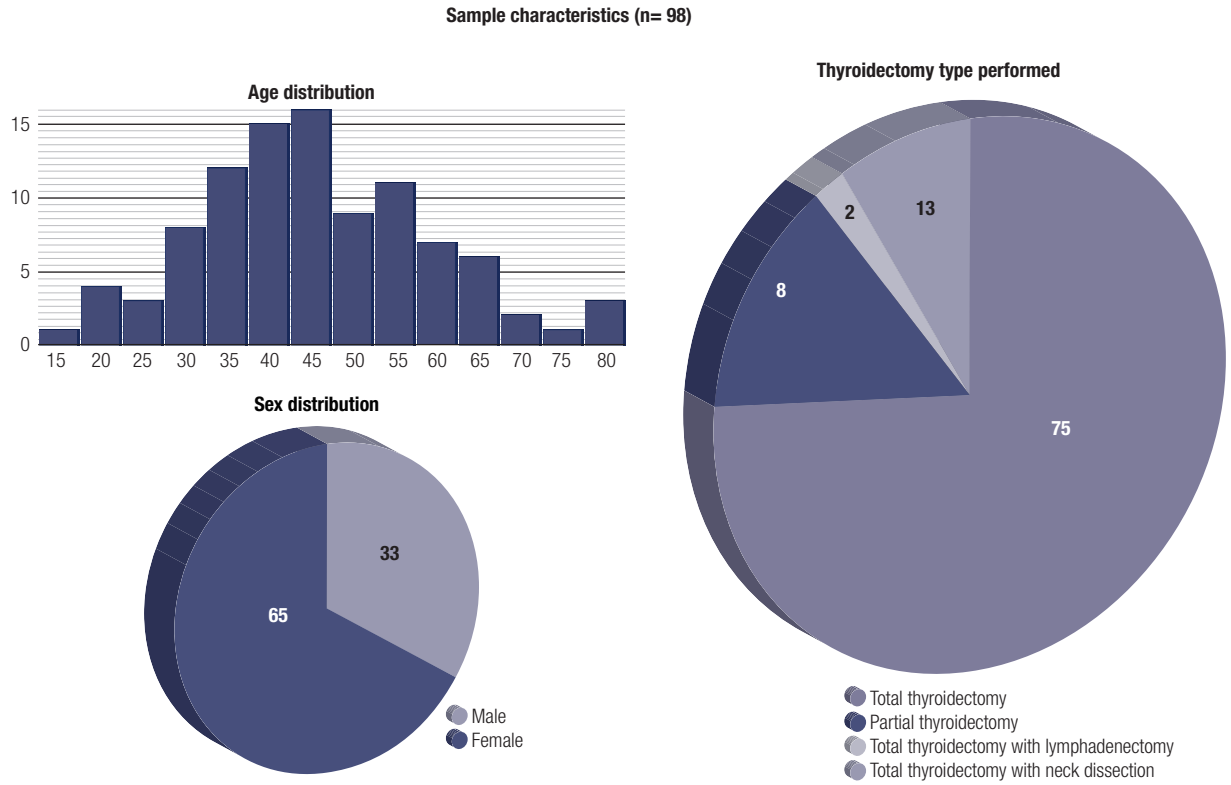

Figure 1. Sample characteristic graphs (age/sex distribution and thyroidectomy type performed).

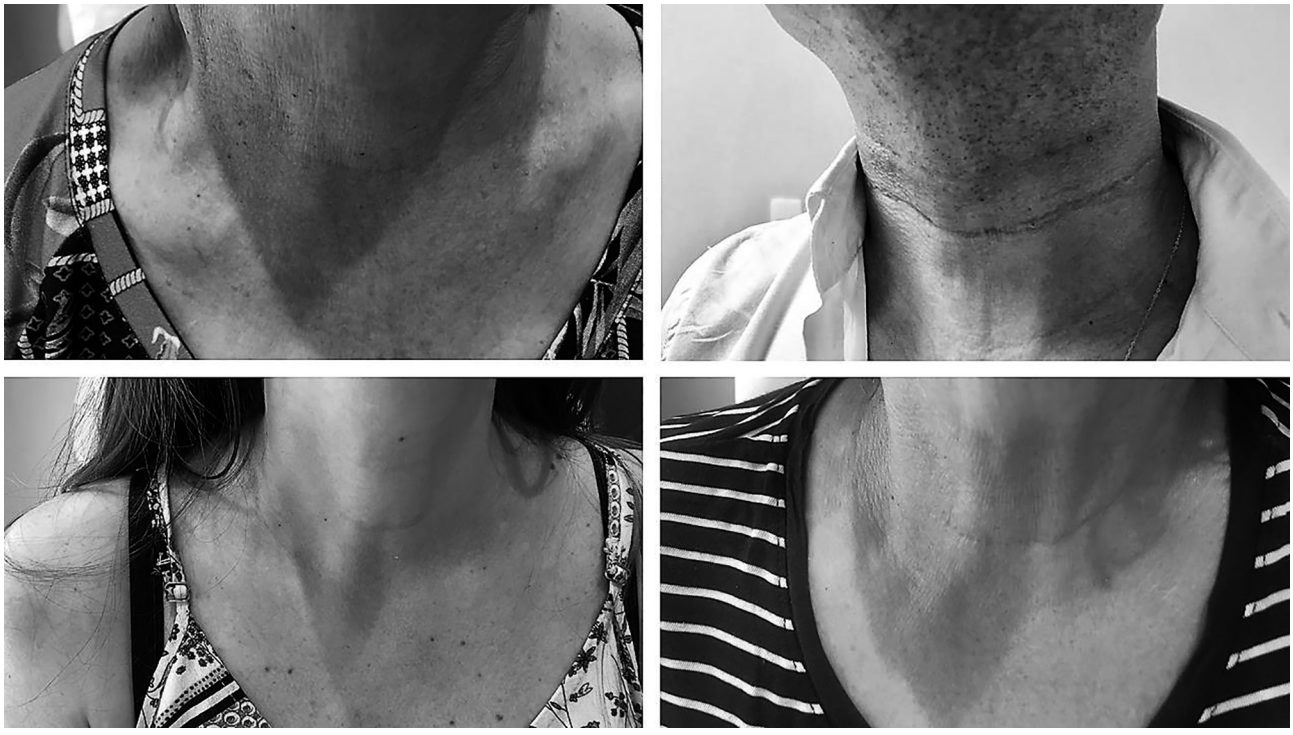

Figure 2. Four examples of patients satisfied with their conventional thyroidectomy scars.

To optimize cosmetic outcomes, some general scar care guidance was provided to the patients. Silicon gel application on the incision region and specific massage techniques, such as circular manipulation, medial to lateral movements, and punctual compressions, are recommended for four months. In addition, the scar should be protected with sunscreen for at least one year.

There are some limitations to be considered. The sample was predominantly composed of women ages 40-60 affected by papillary carcinoma, and the low number of benign cases might influence the subjective perception of discomfort. In addition, we did not use any quantitative tool to assess cosmetic satisfaction, which might affect the study's reproducibility.

Our study's results indicate that conventional thyroidectomy remains the first choice of surgery for the most prevalent pathologies, carcinomas, and goiters of various categories, as well as any other thyroid diseases that cannot be resolved with pharmacological therapy. Even though the remote access techniques have an aesthetic appeal, the social and cosmetic implications of the conventional scar cannot justify these methods, because the cicatrix's impact on the patient's life is very low. The benefits of lower cost and fewer complications make conventional thyroidectomy an old but reliable option for afflictions of the thyroid gland that require surgical treatment.

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Inverse association of plasma hydrogen sulfide levels with visceral fat area among Chinese young men: a cross-sectional study

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ABSTRACT

Objective: To investigate the association between plasma Hydrogen Sulfide (H_2S) levels and visceral fat area (VFA) among Chinese young men. **Subjects and methods:** This cross-sectional study involved 156 Chinese male subjects, aged 18–45 years, who visited the First Hospital of Qinhuangdao (Hebei, China) in 2014 for annual health check-up. Participants were categorized into: low (VFA < 75.57 cm^2), medium (75.57 $cm^2 \leq$ VFA<100.37 cm^2), and high (VFA ≥ 100.37 cm^2) (n = 52/group). We estimated VFA and plasma H_2S levels by using bioelectrical impedance analysis and a fluorescence probe-based approach, respectively. The associations of H_2S with VFA and obesity anthropometric measures were assessed. **Results:** In the high VFA group, the body mass index (BMI, 30.4 ± 2.45 kg/m^2), total body fat (TBF, 27.9 ± 3.23 kg), plasma H_2S (3.5 µmol/L), free fatty acid (FFA, 0.6 ± 0.24 mmol/L), triglyceride (TG, 2.0 mmol/L), and total cholesterol (TC, 5.5 ± 1.02 mmol/L) levels were significantly higher than that of those of the low and medium VFA groups, respectively ($P < 0.05$). Plasma H_2S levels were found to be inversely correlated with VFA, TBF, waist circumference, BMI, FFA, LnFINS, LnHOMA-IR, LnTG, TC, and LDL-C ($P < 0.05$). Multiple backward stepwise regression analysis revealed an inverse correlation of plasma H_2S levels with FFA ($\beta = -0.214$, $P = 0.005$) and VFA ($\beta = -0.429$, $P < 0.001$), independent of adiposity measures and other confounding factors. **Conclusion:** VFA was independently and inversely associated with plasma H_2S levels among Chinese young men. Therefore, determining plasma H_2S levels could aid in the assessment of abnormal VAT distribution.
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Keywords
Hydrogen sulfide; visceral fat; correlation study; bioelectrical impedance; free fatty acids

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INTRODUCTION

It is well-known that the fat tissue in our body is distributed into two main compartments – subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), each having distinct metabolic features (1). Owing to its ubiquitous involvement in different medical pathologies, most of the research has focused on visceral adiposity. Visceral obesity or central obesity, characterized by an excessive accumulation of VAT, has been linked to different pathologies including, metabolic syndromes, chronic inflammation, coronary artery disease (CAD), deranged glucose and lipid metabolism, insulin resistance (2,3), increased predisposition to cancers of the colon (4), breast (5), and prostate (6). Moreover, it has been shown to be associated with prolonged hospital stays, infections and non-infectious complications, and in-hospital mortality (7). Abnormal VAT accumulation predisposes an individual to ischemic heart disease, arterial hypertension, and/or comprehensive cardiovascular risk (3,8-10). The bulk

of evidence suggests that visceral fat is the key mediator between the multiple facets of the metabolic syndrome: glucose intolerance, hypertension, dyslipidemia, and insulin resistance (11). However, due to the presence of metabolic heterogeneity among obese patients with similar levels of VAT, individual genetic susceptibility may be responsible in modulating the risk associated with the excessive accumulation of VAT (12). Owing to its potential association with worse prognosis, metabolic abnormalities, and degree of disease activity in various chronic diseases, it is necessary to quantify VAT from total adipose tissue.

To date, several techniques have been developed for measuring visceral adiposity ranging from simple, indirect methods of evaluation, such as body mass index (BMI), waist-to-hip circumference ratios (WHR), and waist circumference (WC) to imaging techniques, such as computed tomography (CT) that not only estimates the amount of abdominal visceral fat but also measures multi-compartment body fat (1,13,14). Anthropometric measures and bioelectrical impedance analysis (BIA) were developed to provide measures of body composition. BIA is an easily accessible, safe, and a cost-effective method for estimating body composition (15,16). Apart from measuring whole-body fat content, BIA determines fat-free mass. Significant correlations were observed when BIA was employed to assess the amount of abdominal subcutaneous and visceral fat, in comparison with precise imaging techniques such as CT (17,18).

Growing evidence sheds light on the multifaceted roles of hydrogen sulfide (H_2S) in adipose tissue. Cystathionine γ lyase (CSE)-derived H_2S expressed by adipocytes regulates several biological activities in adipose tissue, including inflammation, apoptosis, insulin resistance, adipokine secretion, and adipocyte differentiation (19). H_2S, a novel endogenous gaseous signal transducer (gasotransmitter) is naturally synthesized by CSE, cystathionine β-synthetase (CBS), 3-mercaptopyruvate sulfur transferase (3-MST) and cysteine aminotransferase (CAT). Additionally, enzymatic production of H_2S occurs *in vivo* from L-cysteine (LC) (20). Alterations in H_2S levels or H_2S synthetase expression have been implicated in the pathogenesis of many pathophysiological processes, such as neurological systems, vascular function, energy metabolism and biogenesis, obesity, and ageing (20). Consequently, in recent years, there has been a surge in research on adipose tissue-derived endogenous H_2S and its pathophysiological roles in adipose tissue, with a focus related to its effects on adipose tissue inflammation, apoptosis, adipokine secretion, glucose and lipid metabolism, and vascular tension (21). Despite this, the complex role of H_2S in the regulation of adipose tissue metabolism has not been fully understood. Several published data have highlighted the importance of H_2S in the physiology and pathophysiology of the nervous, cardiovascular, and gastrointestinal systems via its antioxidant, anti-inflammatory (22), antinociceptive, antihypertensive, neuromodulative, and cytoprotective effects (23). In addition, beneficial roles of H_2S in anti-apoptosis of cardiomyocytes and other cardiovascular processes have also been reported (24,25). Further, reduced plasma levels of H_2S have been observed in patients with ischemia (26), diabetes (27), high-fat diet-induced cardiomyopathy (28), and hypertension (29).

A recent study has utilized computed tomography (CT) to evaluate visceral obesity by measuring visceral fat area (VFA), and reported significant associations between VFA and metabolic disturbances (9). However, studies investigating the relationship between plasma levels of H_2S and VFA and obesity anthropometric measures are rare. Therefore, in view of the above, this study aimed to analyze the associations of plasma levels of H_2S with VFA (measured by BIA) and obesity anthropometric measures among Chinese young men, and further speculated whether determining plasma H_2S levels could aid in the assessment of abnormal VAT distribution.

SUBJECTS AND METHODS

Study design and subjects

This cross-sectional study involved 156 Chinese male subjects (N = 156), aged 18–45 years, who visited the First Hospital of Qinhuangdao (Hebei, China) in 2014 for annual health check-up and who had maintained a stable body weight (< 2.5 kg) for over 3 months prior to enrollment. Subjects with a previous medical history of diabetes, dyslipidemia, or coronary artery diseases, secondary obesity (hypophyseal tumor, hypothyroidism, or drug-induced obesity), uncontrolled hypertension (>160/90 mmHg), cardiovascular and/or peripheral vascular diseases, malignant tumors, severe hepatic or renal dysfunction (> 1.5-fold elevation of alanine aminotransferase and aspartate aminotransferase, or serum creatinine > 115 μmol/L), acute/chronic inflammation and/or fever were excluded from this

study. Those who were current or former smokers (participants who have a smoking history equivalent to at least one cigarette per day for more than 6 months were defined as smokers), and/or were heavy drinkers (participants who consumed more than 80 g of alcohol at least once per day for two weeks or had been drinking more than 40 g of alcohol for over 5 years) were not included in this study. Further, we excluded those who underwent specific treatment for metabolic abnormalities (consumption of weight-loss products, in the context of medicine, health, or physical fitness), or those who took medications known to affect glucose and lipid metabolism, such as statins, glucocorticoids, thyroid hormones, and thiazide diuretics. Of note, in this study, participants were enrolled according to strict inclusion and exclusion criteria. This study was conducted according to the STROBE guidelines (30).

Participants were categorized into low (VFA < 75.57 cm^2), medium (75.57 cm^2 ≤ VFA<100.37 cm^2), and high (VFA ≥ 100.37 cm^2) VFA groups (n = 52/group). The cut-off values for VFA groups were determined based on tertile distribution. This study was approved by the ethics committee of the First Hospital of Qinhuangdao. Written informed consent was obtained from all subjects prior to enrollment.

Blood sampling and plasma collection

After 10-hours overnight fasting, blood samples were collected from the antecubital vein into K2-EDTA tubes. Plasma specimens were obtained immediately after collection by centrifuging the samples for 10 min at 3,500 rpm at 4°C and were stored at -80°C until further use.

Estimation of plasma H_2S levels

Plasma H_2S levels were measured by using a modified fluorescence probe-based approach, previously described by Wu and cols. (31). Briefly, 100 pmol of H_2S sensitive probes in 20 μL ethanol were added in 96-well microplates by using an interlaced model in the plate. An equal volume of ethanol was also added into the uncoated wells. Subsequently, the plates were allowed to air dry in dark for 1 h and were then stored in a sealed condition at -20°C. Equal volumes (150 μL) of plasma sample and saturated ammonium sulfate buffer (pH 7.8) were mixed and then centrifuged at 25,000×g for 15 min at 4°C. The supernatant was transferred into a new tube and re-centrifuged. Subsequently, 100 μL of supernatant was added into the probe-coated and uncoated wells, respectively. Following incubation in a dark environment at 37°C for 2 h, the fluorescence intensity in each well was acquired with excitation at λEX (340 nM) and emission at λEM (445 nM) by using a FLUOstar® OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). The discrepant fluorescence intensity values between the coated and uncoated wells were measured, and the corresponding plasma H_2S concentrations were determined on the basis of standard calibration curves constructed with several known H_2S concentrations and sodium hydrogen sulfide (NaHS).

Measurement data

Physical measurements of height and weight of each subject were obtained by using an electronic digital scale (HGM-800, Henan Shengyuan Industrial Co., Ltd.). During all measurements, subjects wore light clothing and were barefoot. The WC was measured from the front at the mid-point between the rib cage and the lateral iliac crest after full expiration, while the subject was breathing gently. Electronic sphygmomanometer (HBP-9020; Omron, Osaka, Japan) was used to record blood pressure levels [systolic blood pressure (SBP); diastolic blood pressure (DBP)] of seated participants. Two consecutive readings were taken 10 min apart and the average was used for analysis. BMI was calculated as weight (kg) divided by square of height (m^2).

A bioelectrical impedance analysis (BIA) device, InBody S10 (Inbody Co., Ltd., Seoul, Korea) was used to measure total body fat (TBF) and VFA. All measurements were performed on subjects in seated position. Typically, this device includes eight electrodes and uses thumb and middle finger of both the hands and both feet. The whole process took about 5 min.

Levels of fasting blood glucose (FBG), plasma free fatty acids (FFA), and plasma lipids (total cholesterol (TC), total triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured by using the glucose oxidase-phenol 4-aminoantipyrine peroxidase method, and enzymatic colorimetric assays with a biochemical auto-analyzer (Hitachi 7600 automated analyzer, Tokyo, Japan). Fasting insulin concentration (FINS) was measured by using an enzyme linked immunosorbent assay (ELISA) kit (intra-assay coefficient of variation (CV%) < 3 and inter-assay CV%

< 4; USCN Life Science Inc., USA) on a microplate reader (Model 680; Bio-Rad, USA). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index was computed as follows: [fasting insulin level (mIU/L) × fasting glucose level (mmol/L)]/22.5.

Statistical analyses

All analyses were performed by using the SPSS statistical software (SPSS for Windows, Version 14.0 SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Non-normally distributed data were presented as median and interquartile range (IQR) and were analyzed after logarithmic transformation. Differences between groups were evaluated by using analysis of variance (ANOVA) and Pearson's Chi-Square test. Multiple comparisons with statistically significant variables were performed by using least significant difference (LSD) test. The association of plasma H_2S levels with various parameters was determined by using multiple backward stepwise linear regression models, with plasma H_2S levels as the dependent variable, and age, VFA, TBF, WC, BMI, SBP, DBP, FFA, FINS, HOMA-IR, TG, TC, and LDL-C as independent variables. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the study population

A total of 156 male subjects (N =156) were included in our analyses. There were 52 subjects (n = 52) in each of the three groups (low, medium, and high VFA groups). The mean ages of subjects in low, medium, and high VFA groups were 35.6 ± 6.02, 36.2 ± 6.19, and 34.5 ± 6.41 years, respectively, while the mean weights were 72.4 ± 6.62, 83.4 ± 6.71, and 96.1 ± 10.81 kgs, respectively. There were no significant differences in age ($P = 0.345$) and DBP ($P = 0.105$). The anthropometric measures, BMI (30.4 ± 2.45 kg/m^2), and WC (102.2 ± 6.12 cm) of the high VFA group were significantly higher than those of the low and medium VFA groups, respectively ($P < 0.05$). Similarly, in the high VFA group, TBF (27.9 ± 3.23 kg), plasma H_2S (3.5 µmol/L), FFA (0.6 ± 0.24 mmol/L), FBG (5.9 ± 1.09 mmol/L), FINS (12.9 uIU/mL), TG (2.02 mmol/L), TC (5.5 ± 1.02 mmol/L), HDL-C (1.1 ± 0.24 mmol/L), and LDL-C (3.8 ± 1.10 mmol/L) levels and HOMA-IR (3.4) were significantly higher than those of the low and medium VFA groups, respectively ($P < 0.05$). Table 1 summarizes clinical and laboratory characteristics of Chinese male subjects in the three VFA groups.

Table 1. Clinical and laboratory characteristics of Chinese male subjects in different groups

Characteristic variable	VFA			P value
	Low <75.57 cm^2 (n = 52)	Medium 75.57-100.37 cm^2 (n = 52)	High ≥100.37 cm^2 (n = 52)	
Age (years)	35.6 ± 6.02	36.2 ± 6.19	34.5 ± 6.41	0.345
Weight (kg)	72.4 ± 6.62	83.4 ± 6.71	96.1 ± 10.81	<0.05*
TBF (kg)	15.3 ± 2.93	21.9 ± 2.43#	27.9 ± 3.23#§	<0.05*
WC (cm)	84.3 ± 7.98	95.8 ± 4.73#	102.2 ± 6.12#§	<0.05*
BMI (kg/m^2)	23.8 ± 2.42	27.8 ± 1.96#	30.4 ± 2.45#§	<0.05*
SBP (mmHg)	123.1 ± 13.71	129.9 ± 13.13#	130 ± 13.12#	0.012*
DBP (mmHg)	81.3 ± 9.59	83.7 ± 10.94	85.6 ± 9.78	0.105
H_2S (µmol/L)	4.9 (4.08, 6.15)	3.9 (3.30, 4.18)#	3.5 (2.91, 3.96)#§	<0.05*
FFA (mmol/L)	0.4 ± 0.16	0.5 ± 0.19#	0.6 ± 0.24#§	0.012*
FBG (mmol/L)	5.5 ± 0.51	5.5 ± 0.56	5.9 ± 1.09#§	0.005*
FINS (uIU/mL)	8.8 (7.54, 11.51)	10.6 (9.04, 13.71)	12.9 (9.11, 17.84)#§	0.001*
HOMA-IR	2.2 (1.74, 3.00)	2.7 (2.21, 3.36)	3.4 (2.36, 4.81)#§	<0.05*
TG (mmol/L)	1.2 (0.96, 2.09)	1.6 (1.12, 2.71)#	2.0 (1.42, 2.75)#	<0.05*
TC (mmol/L)	4.9 ± 0.72	5.2 ± 1.12	5.5 ± 1.02#	0.004*
HDL-c (mmol/L)	1.2 ± 0.30	1.1 ± 0.23#	1.1 ± 0.24#	0.001*
LDL-c (mmol/L)	3.0 ± 0.77	3.5 ± 0.90#	3.8 ± 1.10#	<0.05*

VFA: Visceral fat area; TBF: total body fat; WC: waist circumference; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; H_2S: hydrogen sulfide; FFA: free fatty acid; FBG: fasting blood glucose; FINS: fasting insulin; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TG: triglyceride; TC: total cholesterol; HDL-c: high density lipoprotein-cholesterol; LDL-c: Low density lipoprotein-cholesterol.

Normally distributed variables were expressed as means ± SD. Non-normally distributed variables were expressed as medians (inter-quartile range, IQR). Comparisons were done between the three groups using analysis of variance (ANOVA). Multiple comparisons with statistically significant variables were performed by using least significant difference (LSD) test. *$P < 0.05$ was considered significant. #Compared with low VFA group. §Compared with medium VFA group.

Univariate regression analysis

Univariate analysis was performed to analyze the association of plasma H_2S levels with anthropometric indices of obesity and other laboratory parameters (Table 2). Plasma H_2S levels were found to be negatively correlated with VFA (r = -0.502, $P < 0.05$), TBF (r = -0.403, $P < 0.05$), WC (r = -0.430, $P < 0.05$), BMI (r = -0.460, $P < 0.05$), FFA (r = -0.298, $P < 0.05$), LnFINS (r = -0.283, $P = 0.003$), LnHOMA-IR (r = -0.240, $P = 0.003$), LnTG (r = -0.207, $P = 0.009$), TC (r = -0.221, $P = 0.006$) and LDL-C (r = -0.289, $P < 0.05$).

Multiple linear regression analysis

Multiple backward stepwise regression analysis revealed that plasma H_2S levels were independently and inversely associated with FFA (β = -0.214, $P = 0.005$) and VFA (β = -0.429, $P < 0.001$) after adjusting for age, BMI, WC, TBF, FINS, HOMA-IR, TG, TC, and LDL-C (Table 3).

Table 2. Univariate analysis of correlations between plasma H_2S levels and other variables

Variable	r	*P* value
Age (years)	-0.045	0.576
VFA (cm^2)	-0.502	<0.05*
TBF (kg)	-0.403	<0.05*
WC (cm)	-0.430	<0.05*
BMI (kg/m^2)	-0.460	<0.05*
SBP (mmHg)	-0.154	0.056
DBP (mmHg)	-0.157	0.051
FFA (mmol/L)	-0.298	<0.05*
FBG (mmol/L)	-0.071	0.377
LnFINS	-0.283	0.003*
LnHOMA-IR	-0.240	0.003*
LnTG	-0.207	0.009*
TC (mmol/L)	-0.221	0.006*
HDL-C (mmol/L)	0.074	0.391
LDL-C (mmol/L)	-0.289	<0.05*

H_2S: hydrogen sulfide; VFA: visceral fat area; TBF: total body fat; WC: waist circumference; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FFA: free fatty acid; FBG: fasting blood glucose; LnFINS: the natural logarithm (Ln) of fasting insulin; LnHOMA-IR: the natural logarithm of Homeostatic Model Assessment of Insulin Resistance; LnTG: the natural logarithm of triglyceride; TC: total cholesterol; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol.
Non-normally distributed variables were analyzed after logarithmic transformation. *$P < 0.05$ was considered significant.

DISCUSSION

Our study reveals independent, inverse associations of plasma H_2S levels with VFA and FFA among Chinese young men, and further demonstrated that plasma H_2S levels were negatively correlated with VFA, TBF, WC, BMI, FFA, LnFINS, LnHOMA-IR, LnTG, TC, and LDL-C.

Adipose tissue is one of the largest, complex endocrine organs that secretes a variety of factors (32), which play a significant role in the development of systemic oxidative stress (33). Recent studies have demonstrated that H_2S is synthesized by cystathionine γ-lyase CSE in perivascular adipose tissue (PVAT) (34). VAT is a hormonally active component of TBF and its abnormal high deposition leads to visceral obesity (1). VAT has been associated with the metabolic consequences of obesity (35). Further, in obese patients, VAT has been shown to abnormally release adipokines and FFAs (36), thus promoting systemic oxidative stress. In contrast to subcutaneous adipocytes, visceral adipocytes are characterized by a hyperlipolytic profile, and individuals with more VAT tend to have a high concentration of circulating FFAs (37), which is consistent with the finding of this study. It has been hypothesized that visceral fat is largely a VAT marker for excess FFA release. Therefore, metabolic abnormalities resulting as a consequence of increased visceral adiposity may be due to the exposure of lean tissues to high FFA concentrations (38). Of note, high FFA levels have been shown to stimulate the production of reactive oxygen species (ROS), including hydroxyl radicals, superoxide anions, and hydrogen peroxide (H_2O_2) in the endothelial and vascular smooth muscle cells (39), thus generating high levels of H_2O_2 in the mitochondria (40). On the contrary, endogenous H_2S, which has

Table 3. Multiple linear regression analysis

Variables	Unadjusted β-coefficient	S.E.	β-coefficient[a]	t	*P* value	r^2
VFA	-0.006	0.001	-0.429	-5.685	<0.001*	0.256
FFA	-0.382	0.135	-0.214	-2.834	0.005*	0.040

VFA: visceral fat area; FFA: free fatty acid; S.E: standard error. Unstandardized β-coefficients were derived from multivariate linear regression.

[a]The estimates were adjusted for the confounding effects of age, BMI (body mass index), WC (waist circumference), TBF (total body fat), FINS (fasting insulin), HOMA-IR (Homeostatic Model Assessment of Insulin Resistance), TG (triglyceride), TC (total cholesterol), and LDL-C (low density lipoprotein-cholesterol). *$P < 0.05$ was considered significant.

the ability to scavenge ROS, protects the cells against any oxidative damage (41). Therefore, during stress, there is a high consumption of endogenous H_2S for the elimination of excessive ROS, which may lead to decreased plasma H_2S levels in individuals with high FFA. This hypothesis is further supported by the independent inverse correlation of plasma H_2S levels with FFA demonstrated in this study. Taken together, these findings indicate that plasma H_2S levels may reflect VAT distribution and FFA metabolism. Delving further, low plasma H_2S levels have been found in patients with type 2 diabetes or obesity, among other metabolic derangements (19).

It has been reported that adiposity rather than diabetes status is a major determinant of plasma H_2S levels (42), which highlights the role of H_2S metabolism in obesity. Consistent with a previous study, adiposity measures (WC, TBF, and BMI) showed a negative correlation with plasma H_2S levels among Chinese young men, which implies the existence of an inverse relationship between the degree of obesity and H_2S production (42). Further lending strength to this finding, a previous study has demonstrated that plasma H_2S levels were lowest in subjects with obesity and type 2 diabetes, and suggested that adiposity may be the key driving force for predicting low plasma H_2S levels (43). Another study showed that adipose tissue-derived low levels of endogenous H_2S may contribute to adipose tissue inflammation associated with obesity/metabolic syndrome, or conversely excess H_2S levels might result in insulin resistance in metabolic syndrome (21).

Our study is the first-of-its-kind to demonstrate an inverse correlation between VFA and plasma H_2S levels, independent of adiposity measures and other confounding factors. BMI and WC are the most commonly used central obesity anthropometric measures for assessing adiposity-related risk and body fat distribution (44). Despite the fact that WC reflects visceral and subcutaneous fat of VAT, VFA showed a strong, inverse association with plasma H_2S levels, even after accounting for BMI and WC. Our finding is in line with a previous study which showed that VAT area, but not WC, was strongly associated with an unfavorable metabolic risk profile (3). As high VFA has been identified as a critical risk factor for metabolic syndromes and obesity-related complications, our observations further indicate that low plasma H_2S levels might reflect the presence of metabolic abnormalities, and hence may be used as a potential early biomarker for metabolic syndromes and obesity-related diseases. In addition, reduced plasma H_2S concentration was found to be associated with increased FINS, HOMA-IR, TG, TC, and LDL-C levels. However, these findings were not significant after adjusting for measures of adiposity (VFA, BMI, WC, and TBF). This could be possibly due to the inclusion of relatively younger subjects without any severe metabolic disorders. However, lack of independent correlations between plasma H_2S levels and these indexes suggest that determining plasma H_2S levels may not aid in the complete understanding of mechanisms through which H_2S acts on adipose tissue metabolism, such as glycolipid metabolism. Interestingly, in this study, the association of plasma H_2S levels with body fat distribution (TBF) shifted towards visceral fat accumulation, thus indicating that the visceral fat proportion of total body fat is more important for assessing the impact on metabolic disease (45). This finding is supported by a recent study, which showed that H_2S reduced lipolysis of adipocytes in HFD mice without increasing total fat mass and body weight (19). However, the mechanism underlying the regulation of plasma H_2S levels in presence of excess VFA remains elusive.

This study has a few limitations that should be considered when interpreting the results. As this was a cross-sectional study, the associations between VFA and plasma H_2S levels cannot be construed as causal. Large population studies employing longitudinal methods would enable researchers to determine causal pathways and validate the directionality of this association. In addition, the heterogeneity of this association with respect to race, ethnicity, gender needs to evaluated. As this study design involved convenience sampling, and only male subjects aged between 18 and 45 years were recruited, generalization of our findings to other populations may be impeded. Further the lack of diversity among research participants may pose some limitation in the present study.

In conclusion, our study demonstrated that plasma H_2S levels progressively declined ($P < 0.05$) in correlation with the degree of VFA among the three groups of Chinese young men. VFA was independently and inversely associated with plasma H_2S levels among Chinese young men. Therefore, determining plasma H_2S levels could aid in the assessment of abnormal VAT distribution.

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Are clinicopathological features of the isthmic thyroid nodule different from nodules in thyroid lobes? A single center experience

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ABSTRACT

Objective: Thyroid nodules located in isthmus were found less prevalent, although papillary thyroid cancer in this location was reported to be more aggressive behaviour in some studies. Our aim was to evaluate hormonal,ultrasonographic, and cytopathologic features of nodules located in isthmus (isthmic nodules). **Subjects and methods:** Patients who underwent thyroidectomy between 2006-2014 reviewed retrospectively. Hormonal, ultrasonographic, and cytopathologic features compared between patients with isthmic (Group-1) and with lober (non-isthmic, Group-2) nodules. **Results:** Group-1 and Group-2 consisted of 251 and 2076 patients, respectively. 260 isthmic (5.5%) and 4433 non-isthmic (94.5%) nodules were compared.However,most ultrasonographical features such as presence of microcalcification and halo, diameters, echogenicity, texture, margin, and vascularity were similar between groups, macrocalcification rate was lower in isthmic nodules (18.8%, 25.9%; p = 0.012). Cytologic results were also similar.Although malignancy rate was lower in isthmic nodules (6.2%, 12.5%; p = 0.002), type of thyroid cancer was similar in isthmic and non-isthmic nodules.When malignant isthmic (n = 16,2.8%) and malignant non-isthmic nodules (n = 553, 97.2%) were compared, diameter and type of tumor, lymphovascular and capsular invasions, extrathyroidal extension and multifocality rates were not statistically significant.Malignant isthmic nodules (n = 16, 6.2%) had smaller size [10.1 (7.5-34.5) mm, 19.95 (8.4-74.1) mm; p = 0.002], and higher hypoechogenicity rate (31.3%, 5.7%, p = 0.003) compared to benign isthmic nodules (n = 244, 93.8%). Negative predictive value was higher and positive predictive value was lower in isthmic nodules compared to non-isthmic nodules (p = 0.033, p = 0.047, respectively). **Conclusion:** Isthmic nodules appear to be indolent because of having lower malignancy rate. FNAB might be required in isthmic nodules even if it has relatively small size. The surgery with limited extent or follow-up might seem to be reliable in the management of patients having isthmic nodules especially with indeterminate cytology. Arch Endocrinol Metab. 2021;65(3):277-88

Keywords
Isthmus; ultrasonography; cytology; histopathology; thyroid cancer

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INTRODUCTION

The prevalence of having a thyroid nodule is 3-7% by palpation in population (1), and the lifetime risk for developing a palpable thyroid nodule is estimated as 10% (2). Nodule diagnose has been increased in clinical practice, especially with advancement of diagnostic

techniques. Its prevalence is reached to 19-68% by high-resolution ultrasound (US) (2,3).

Thyroid fine needle aspiration biopsy (FNAB) is the most accurate and reliable diagnostic technique for diagnosis of malignancy in thyroid nodules. It helps to select candidates for surgery, however this is an invasive procedure. US is a non-invasive, sensitive, cheap, and easy method for evaluating the nodules. Some US features were found to be associated with higher malignancy risk (4). These are hypoechogenity, solid texture, micro- and/or macrocalcification, lack of peripheral halo, irregular margin, taller than wider shape, and increased vascularity (5). Also, Thyroid Imaging Reporting and Data System (TIRADS) is the assessment tool that categorizes thyroid nodules and stratifies their malignancy risk usually using a score according to their US features (6). Recently, whether nodule localization is a malignancy predictor like these suspicious US features has become the focus of interest (7-10).

The isthmus is the smallest part of thyroid gland which connects right and left lobes. Prevalence of thyroid nodules located in isthmus was found as 4.2-6.4% (7,9,11). The incidence of isthmic papillary thyroid cancer (PTC) ranges from 1% to 12.3% in different studies (12-21). Although PTC had an indolent course, isthmic PTCs were reported to have more aggressive behaviour, including multifocality, capsular invasion, and frequently having metastasis to lymph nodes in some studies (12,16). The features of other histopathologic types of thyroid carcinomas arising from isthmus are unknown. There is no specific suggestion for diagnosis and follow-up of isthmic thyroid nodules in clinical guidelines. There have been a few studies regarding cytologic and ultrasonographic features of isthmic thyroid nodules in the literature. Therefore, in the present study, our aim is to evaluate whether thyroid nodules located in the isthmus are different from located in thyroid lobes according to their clinicopathological features.

SUBJECTS AND METHODS

Patient selection

The records of 2441 patients who underwent thyroidectomy between 2006-2014 were screened for this study. Exclusion criteria were previous history of thyroidectomy and radiotherapy to head and neck region, absence data of cytopathological and histopathological features, presence of nodules without coupled cytopathologic and histopathological results, and incidentally found malignant parenchymal lesions. Finally, 2327 patients were enrolled. Patients divided into two groups: having isthmic nodules (Group-1) and non-isthmic nodules (located in right or left thyroid lobes) (Group-2). Group-1 had 251 (10.8%) and Group-2 had 2076 (89.2%) patients. Demographic features, laboratory, cytology and histopathology results were compared between groups. Furthermore, the tumour characteristics of malignant nodules such as the type, size, multifocality, vascular invasion, lymphatic invasion, capsular invasion, extrathyroidal extension, lymph node metastasis and TNM stages (22), radioactive iodine (RAI) administration and RAI doses, and stimulated thyroglobuline (Tg) levels on 6th month of malignant patients were recorded.

Isthmic and non-isthmic nodules were compared in terms of US, cytopathologic and histopathologic features. Also, malignant nodules separated into isthmic and nonisthmic. Furthermore, isthmic nodules were analysed in two groups as benign and malignant nodules.

Local Ethical Committee of the School of Medicine, Yildirim Beyazit University approved this study.

Laboratory

Serum thyroid stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4), anti-thyroid peroxidase (anti-TPO), anti-Tg antibody, and Tg measurements were made by chemiluminescence methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA, and the UniCel DxI 800, Beckman Coulter, CA, USA). Reference ranges for TSH, fT3, fT4, Tg were 0.4 - 4.0 uIU/mL, 1.57-4.71 pg/mL, 0.61-1.12 ng/dL, and 0-78 ng/mL, respectively. Anti-TPO higher than 10 U/mL and anti-Tg higher than 30 U/mL were interpreted as positive. Patients on levothyroxine treatment and/or patients with a serum TSH above reference ranges were defined to have hypothyroidism. Patients taking antithyroid medication and/or patients with a serum TSH below reference ranges were considered to have hyperthyroidism. Patients with TSH levels in normal ranges were accepted as euthyroid.

Imaging studies

Thyroid US was carried out with a high resolution ultrasound instrument (Esaote color Doppler US

(Taipei, Taiwan)) equipped with a 5.5-12.5 MHz linear probe. Isthmic nodule was defined when a center of nodule was localized lateral border of isthmus by drawing two imaginary lines perpendicular to skin surface from most lateral borders of trachea on the transverse scan compatible with Hahn and cols. (17).

US feaures of nodules such as diameters, presence of calcification (micro-, macrocalcification), taller than wider shape, echogenicity (isoechoic, hypoechoic, hyperechoic), texture (solid, cystic/mixed), presence of halo, presence of cystic degeneration, margins (irregular, regular), vascularity (peripheral, central) were noted. Large thyroid nodule was defined as one of three dimensions of the nodule is 40 mm or more in US evaluation. Furthermore, TIRADS categories of nodules were recorded. For TIRADS assessment, suspicious US features for malignant nodule (solid structure, low or very low echogenicity, irregular or microlobular borders, microcalcifications, and taller-than-wider) was scored as 1 for presence or 0 for absence. According to sum of each scores of suspicious features, TIRADS groups determined as 5 groups: Category 3, 4a, 4b, 4c, and 5 if sum of the scores were 0, 1, 2, 3 or 4, and 5; respectively (23).

Fine needle aspiration biopsy, cytopathology and histopathology

US-guided Fine Needle Aspiration Biopsy (FNAB) was performed by an experienced clinician by a 27 gauge needle and 20 mL volume syringe (Logic Pro 200 GE US machine and 7.5 MHz probe). FNAB was carried out on all nodules with a size of 1 cm or more. When there were clinical risk factors (family history of thyroid cancer) and/or suspicious US features, it was performed on nodules under 1 cm. Cytologic materials were categorized as nondiagnostic, benign, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm/suspicious for follicular neoplasm (FN/SFN), suspicious for malignancy and malignant according to Bethesda classification system (24).

Statistical analysis

The distributions of continuous variables such as age were examined by Shapiro-Wilk's test and normality plots. All continuous and discrete variables were reported as median (min-max), while categorical variables were expressed by frequency (%).

Patients with isthmic and non-isthmic nodules were compared by Mann-Whitney U test for continuous and discrete variables and by chi-square tests for categorical variables. In the case of more than 50% of cells have expected count less than 5, the p value of Monte-Carlo simulation based on 10000 samples were given for categorical variables.

The diagnostic accuracy measures of the cytology was examined by considering the Bethesda categories > II (AUS/FLUS, FN, SFN, suspicious for malignancy) as malignant in isthmic and non-isthmic nodules. Nondiagnostic cytologic results were excluded from the analysis. The diagnostic accuracy measures were provided with their 95% CIs determined by Wilson score method. Diagnostic accuracy measures were compared by Pearson chi-square, Yates chi-square or Fisher's exact test, where appropriate.

A p value < 0.05 was considered as statistically significant. Wilson's score CIs was obtained by using PropCIs package (25) in RStudio Software (Version 1.3.959) (26). All other analyses were performed via IBM SPSS Statistics 22.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

RESULTS

The age and gender distributions were similar between Group-1 (n = 251) and Group- 2 (n = 2076) (p > 0.05). Although TSH level was found as high significantly in Group-2 compared to Group-1 (p < 0.001), fT3 and fT4 levels were higher in Group-1 than Group-2 (p = 0.031 and p = 0.007, respectively). Anti-Tg positivity was found as more in Group-1 (p = 0.046). The presence of hyperthyroidism, the isthmus dimension, and the proportion of thyroidectomy decision because of having a large nodule were significantly higher in Group-1 compared to Group-2 (p < 0.05) (Table 1).

Totally 260 isthmic nodules from 251 patients in Group-1 and 4433 non-isthmic nodules from 2076 patients in Group-2 were compared according to US findings, cytology and histopathology results (Table 2). US findings, exception of presence of macrocalcification, were similar between groups (p > 0.05). Presence of macrocalcification was higher in non-isthmic nodules (p = 0.012). Furthermore, cytology findings were similar between groups (p > 0.05). Histopathology was determined as malignant in 16 isthmic nodules (6.2%) and 553 non-isthmic nodules (12.5%) (p = 0.002).

Table 1. Clinical and demographic features of patients in Group-1 and Group-2

	Group-1 (Isthmic) [n = 251]	Group-2 (Non-isthmic) [n = 2076]	p
	Median (min-max) n (%)	Median (min-max) n (%)	
Age, years	51 (23-85)	50 (18-84)	0.054
Gender, F	198 (78.9)	1612 (77.6)	0.657
TSH[a], µIU/mL	0.60 (0.001-11.00)	**1.10 (0.001-51.10)**	**<0.001**
fT3[b], pg/mL	**3.31 (1.40-9.16)**	3.28 (1.03-66.0)	**0.031**
fT4[c], ng/dL	**1.20 (0.48-4.20)**	1.15 (0.07-10.70)	**0.007**
Thyroid hormonal status			
Euthyroid	184 (73.3)	1614 (77.7)	0.113
Hypothyroid	3 (1.2)	**107 (5.2)**	**0.008**
Hyperthyroid	**64 (25.5)**	355 (17.1)	**0.001**
Anti-TPO positivity	45 (19.1)	396 (21.9)	0.332
Anti-Tg positivity	**60 (26.8)**	375 (21.0)	**0.046**
Isthmus dimension[d], mm	**14.5 (2-40)**	4.6 (0-40)	**<0.001**
Thyroidectomy indication			
Large nodule	**118 (47.0)**	640 (30.8)	**<0.001**
Hyperthyroidism	29 (11.6)	235 (11.3)	0.912
Cytology	81 (32.3)	**992 (47.9)**	**<0.001**
Parathyroid pathology	3 (1.1)	38 (1.8)	0.616
Other/unknown	20 (8.0)	171 (8.2)	0.980
Cytology subgroups causing thyroidectomy decision			0.375
Nondiagnostic	16 (19.8)	226 (22.8)	
AUS/FLUS and suspicious US findings	34 (42.0)	369 (37.2)	
FN/SFN	10 (12.3)	92 (9.3)	
Suspicious for malignancy	15 (18.5)	163 (16.4)	
Malignant	6 (7.4)	142 (14.3)	
Lymphocytic thyroiditis in histopathology	54 (21.5)	**573 (27.6)**	**0.040**

TSH: thyrotropin; fT3: free triiodothyronine; fT4: free thyroxine; Anti-TPO: anti-thyroid peroxidase antibody; Anti-Tg: anti-thyroglobulin antibody; AUS/FLUS: atypia of undetermined significance/ follicular lesion of undetermined significance; US: ultrasound; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm.

[a]n = 250 and n = 2069 for isthmic and non-isthmic, respectively. [b]n = 2062 for non-isthmic patients. [c]n = 2072 for non-isthmic patients. [d]n = 247 and n = 2042 for isthmic and non-isthmic, respectively.

The ratio of nodule detected as PTC histopathologically was higher in non-isthmic nodules compared to isthmic nodules (11.7% vs 5.8 %) (p = 0.003).

The demographic and clinical characteristics of the patients with malignant isthmic and non-isthmic nodules were given in Table 3. Groups had 16 and 503 malignant patients, respectively. Age, gender distributions and other laboratory findings except from anti-Tg positivity were similar (p > 0.05). It was significantly higher in isthmic group (p = 0.010). Furthermore, TNM classifications of malignant nodules and numbers of patients with RAI treatment were not different in two groups (p > 0.05). Stimulated Tg level was lower in patients with malignant isthmic nodules (p = 0.015).

US features, cytology and histopathology results of malignant isthmic and non-isthmic nodules were given in Table 4. The anti-Tg positivity and cytology of suspicious for malignancy were significantly higher in malignant isthmic nodules (p < 0.05). All of the ultrasonographic findings and TIRADS categories were similar between groups. Furthermore, tumor diameter, presence of lymphatic, vascular and capsular invasions and extrathyroidal extension were not different.

Clinical and ultrasonographic features of malignant and benign isthmic nodules were presented in Table 5. Antero-posterior and transverse diameters were significantly smaller in malignant isthmic nodules (p < 0.05). TIRADS categories and US features exception

Table 2. Ultrasonographic features, fine needle aspiration biopsy and histopathology results of nodules in Group-1 and Group-2

	Group-1 (Isthmic) [n = 260]	Group-2 (Non-isthmic) [n = 4433]	p
	Median (min-max) n (%)	Median (min-max) n (%)	
Antero-Posterior Diameter[a], mm	12.2 (3.0-58.5)	12.4 (0.1-70.2)	0.179
Transverse Diameter[b], mm	**17.0 (5.9-74.9)**	14.9 (2.9-256.0)	**0.046**
Longitudinal Diameter[c], mm	19.55 (4.0-74.1)	17.6 (3.9-113.0)	0.618
TIRADS categories			0.383
3	2 (0.7)	57 (1.3)	
4a	55 (21.2)	962 (21.7)	
4b	124 (47.7)	1891 (42.7)	
4c	79 (30.4)	1501 (33.8)	
5	0 (0.0)	22 (0.5)	
Taller than wider shape	33 (12.9)	756 (17.4)	0.064
Absence of peripheral halo	189 (72.7)	3162 (71.3)	0.636
Microcalcification	85 (32.7)	1590 (35.9)	0.299
Macrocalcification	49 (18.8)	**1146 (25.9)**	**0.012**
Echogenicity			0.109
Isoechoic	150 (58.1)	2248 (52.0)	
Hypoechoic	19 (7.4)	442 (10.2)	
Iso-hypoechoic	89 (34.5)	1634 (37.8)	
Texture, Solid	251 (96.5)	4219 (95.2)	0.392
Irregular margins	166 (63.8)	2641 (59.6)	0.172
Vascularity, peripheral	20 (76.9)	441 (73.3)	0.851
Cytology			0.097
Non-diagnostic	55 (21.2)	1173 (26.5)	
Benign	152 (58.3)	2407 (54.2)	
AUS/FLUS	29 (11.2)	459 (10.4)	
FN/SFN	8 (3.1)	83 (1.9)	
Suspicious for malignancy	13 (5.0)	172 (3.9)	
Malignant	3 (1.2)	139 (3.1)	
Histopathology, Malignant	16 (6.2)	**553 (12.5)**	**0.002**
Type of thyroid cancer			
Papillary cancer	15 (5.8)	**518 (11.7)**	**0.003**
Follicular cancer	0 (0.0)	20 (0.5)	0.624
Hurthle cell cancer	1 (0.4)	15 (0.3)	0.599
Benign	**244 (93.8)**	3880 (87.5)	**0.002**
PTC variants, classical	9 (60.0)	333 (64.3)	0.946

TIRADS: Thyroid Imaging Reporting and Data System; AUS/FLUS: atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm; PTC: papillary thyroid cancer [a]n = 250 and n = 2069 for isthmic and non-isthmic, respectively. [b]n = 2062 for non-isthmic patients. [c]n = 256 and n = 4392 for isthmic and non-isthmic, respectively.

of echogenicity were similar between two groups ($p > 0.05$). Hypoechoic feature was more prominent in malignant isthmic nodules compared to benign ones ($p = 0.003$). In cytology of malignant isthmic nodules, benign and ND results were not detected ($p < 0.05$). All isthmic nodules with non-diagnostic or benign cytology were diagnosed as benign histopathologically. AUS/FLUS and FN/SFN rates were similar between two groups ($p = 0.089$, $p = 0.081$; respectively). Lymphocytic thyroiditis in histopathology was significantly higher in the malignant isthmic nodules ($p < 0.05$).

Table 3. Clinicopathological features of patients with malignant isthmic and malignant non-isthmic nodules

Malignant nodules	Isthmic [n = 16]	Non-isthmic [n = 553]	p
	Median (min-max) n (%)	Median (min-max) n (%)	
Age, years	49.5 (31-68)	48 (19-84)	0.874
Gender, F	13 (81.3)	400 (79.5)	1.000
TSH[a], µIU/mL	1.96 (0.16-11.00)	1.60 (0.002-24.00)	0.391
fT3[b], pg/mL	3.12 (2.39-4.03)	3.20 (1.03-7.68)	0.949
fT4[c], ng/dL	1.29 (0.96-2.20)	1.17 (0.26-4.10)	0.127
Thyroid hormonal status			0.268
Euthyroid	11 (68.8)	427 (84.9)	
Hypothyroid	2 (12.5)	36 (7.2)	
Hyperthyroid	3 (18.7)	40 (7.9)	
Anti-TPO positivity	5 (33.3)	110 (25.8)	0.551
Anti-Tg positivity	**8 (57.1)**	104 (24.3)	**0.010**
Number of malignant focus[d]	1 (1-4)	1 (1-15)	0.378
Multifocality	4 (25.0)	197 (39.7)	0.354
Lymph node metastasis	1 (6.3)	52 (10.4)	1.000
T classification			0.053[e]
T1a	6 (37.5)	134 (30.8)	
T1b	8 (50.0)	122 (28.0)	
T2	2 (12.50)	76 (17.4)	
T3	0 (0.0)	99 (22.8)	
T4a	0 (0.0)	2 (0.5)	
T4b	0 (0.0)	2 (0.5)	
TNM stage			0.126[f]
Stage I	15 (93.8)	366 (73.4)	
Stage II	1 (6.2)	47 (9.4)	
Stage III	0 (0.0)	78 (15.6)	
Stage IVa	0 (0.0)	7 (1.4)	
Stage IVb	0 (0.0)	1 (0.2)	
RAI treatment	14 (93.3)	452 (97.8)	0.299
RAI dose, >100 mCi	5 (35.7)	238 (53.0)	0.315
Stimulated Tg level on 6th month[e], ng/mL	0.235 (0.02-6.06)	**2.170 (0.02-505.00)**	**0.015**

TSH: thyrotropin; fT3: free triiodothyronine; fT4: free thyroxine; Anti-TPO: anti-thyroid peroxidase antibody; Anti-Tg: anti-thyroglobulin antibody; TNM: tumour, node, metastasis, RAI: radioactive iodine. [a]n = 15 and n = 502 for isthmic and non-isthmic, respectively. [b]n = 499 for non-isthmic. [c]n = 502 for non-isthmic. [d]n = 496 for non-isthmic. [e]n = 12 and n = 437 for isthmic and non-isthmic, respectively. [f]Monte Carlo simulation results based on 10000 samples.

The distribution of cytology and histopathology results in isthmic and non-isthmic nodules was shown in Suplementary Table. Among the nodules with non-diagnostic cytology and benign cytology, benign histopathology were significantly more frequent than those in the non-isthmic nodules (100% vs 93%, p=0.044; 100% vs 96.4%, p = 96.4; respectively). Within other cytology groups, there were no significant difference between isthmic and non-isthmic nodules with respect to the histopathology results (p > 0.05).

After non-diagnostic cytology results were excluded, when defining as benign cytology was "benign" or "negative", and indeterminate (AUS/FLUS, FN/SFN, suspicious for malignancy) and malignant cytology results were "malignant" or "positive", the sensitivity and specificity were obtained as 1.000 (95% CI: 0.806-1.000) and 0.803 (95% CI: 0.741-0.854) in isthmic nodules and 0.817 (95% CI: 0.780-0.850) and 0.832 (95% CI: 0.818-0.845) in non-isthmic nodules, respectively (Table 6). There were no significant differences between

Table 4. Ultrasonography features, fine needle aspiration biopsy and histopathology results of nodules in malignant isthmic and malignant non-isthmic groups

Malignant nodules	Isthmic [n = 16]	Non-isthmic [n = 552]	p
	Median (min-max) n (%)	Median (min-max) n (%)	
Anti-TPO positivity	5 (33.3)	122 (26.1)	0.555
Anti-Tg positivity	**8 (57.1)**	115 (24.6)	**0.011**
Antero-posterior diameter[a], mm	7.45 (4.30-20.20)	10.50 (0.10-54.00)	0.071
Transverse diameter[b], mm	9.20 (6.40-32.00)	12.05 (3.90-67.90)	0.493
Longitudinal diameter[c], mm	10.05 (4.00-34.50)	13.50 (3.90-84.90)	0.122
TIRADS categories			0.943
3	0 (0.0)	2 (0.3)	
4a	3 (18.8)	96 (17.4)	
4b	5 (31.2)	176 (31.9)	
4c	8 (50.0)	267 (48.4)	
5	0 (0.0)	11 (2.0)	
Taller than wider shape	2 (13.3)	117 (22.4)	0.540
Absence of peripheral halo	13 (81.3)	405 (73.4)	0.579
Microcalcification	6 (37.5)	254 (46.0)	0.675
Macrocalcification	2 (12.5)	186 (33.7)	0.132
Echogenicity			0.161
Isoechoic	2 (12.4)	193 (35.6)	
Hypoechoic	3 (21.3)	125 (23.1)	
Iso-hypoechoic	9 (56.3)	224 (41.3)	
Texture, Solid	15 (93.8)	542 (98.2)	0.272
Irregular margins	10 (62.5)	352 (63.8)	1.000
Vascularity, peripheral	2 (100.0)	65 (61.9)	0.527
Cytology			
Non-diagnostic	0 (0.0)	79 (14.3)	0.146
Benign	0 (0.0)	87 (15.8)	0.149
AUS/FLUS	4 (25.0)	99 (17.9)	0.509
FN/SFN	2 (12.5)	27 (4.9)	0.196
Suspicious for malignancy	**8 (50.0)**	126 (22.8)	**0.031**
Malignant	2 (12.5)	134 (24.3)	0.381
Type of thyroid cancer			0.420
Papillary cancer	15 (93.8)	518 (93.9)	
Follicular cancer	0 (0.0)	20 (3.6)	
Hurthle cell cancer	1 (6.2)	14 (2.5)	
PTC variants, classical	9 (60.0)	333 (64.3)	0.946
Tumor diameter[d], mm	12.0 (5.0-35.0)	12.0 (1.0-90.0)	0.859
Microcarcinoma	6 (37.5)	219 (40.0)	1.000
Lymphatic extension	0 (0.0)	16 (2.9)	1.000
Vascular invasion	1 (6.3)	38 (6.9)	1.000
Capsular invasion	6 (37.5)	205 (37.5)	1.000
Extrathyroidal extension	0 (0.0)	96 (17.6)	0.087
Lymphocytic thyroiditis in histopathology	8 (50.0)	195 (35.3)	0.346

Anti-TPO: anti-thyroid peroxidase antibody; Anti-Tg: anti-thyroglobulin antibody; TIRADS: Thyroid Imaging Reporting and Data System; AUS/FLUS: atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm; PTC: papillary thyroid cancer.

[a]n = 548 for non-isthmic. [b]n = 15 and n = 524 for isthmic and non-isthmic, respectively. [c]n = 551 for non-isthmic. [d]n = 547 for non-isthmic.

isthmic and non-isthmic nodules with respect to the sensitivity, specificity, and overall accuracy of the cytology ($p > 0.05$). However, the NPV of the cytology was significantly higher in isthmic nodules, the PPV was found as significantly lower in the isthmic nodules compared to those in non-isthmic ones ($p < 0.05$) (Table 6).

Table 5. Clinical and ultrasonographic features of malignant and benign isthmic nodules

	Malignant [n = 16]	Benign [n = 244]	p
	Median (min-max) n (%)	Median (min-max) n (%)	
Antero-Posterior Diameter[a], mm	7.6 (4.3-20.2)	**12.35 (3.8-58.5)**	**0.011**
Transverse Diameter[b], mm	9.2 (6.4-32.0)	**17.1 (5.9-74.9)**	**0.010**
Longitudinal Diameter[c], mm	10.1 (7.5-34.5)	**19.95 (8.4-74.1)**	**0.002**
TIRADS categories			0.339[d]
3	0 (0.0)	2 (0.8)	
4a	3 (18.7)	52 (21.3)	
4b	5 (31.3)	119 (48.8)	
4c	8 (50.0)	71 (29.1)	
Taller than wider shape	2 (13.3)	31 (12.9)	1.000
Absence of peripheral halo	13 (81.3)	176 (72.1)	0.568
Microcalcification	6 (37.5)	79 (32.4)	0.882
Macrocalcification	2 (12.5)	47 (19.3)	0.774
Echogenicity			
Isoechoic	2 (12.4)	**148 (61.2)**	**<0.001**
Hypoechoic	**5 (31.3)**	14 (5.7)	**0.003**
Iso-hypoechoic	9 (56.3)	80 (33.1)	0.106
Texture, Solid	15 (93.8)	236 (96.7)	0.441
Irregular margins	10 (62.5)	156 (63.9)	1.000
Vascularity, peripheral	2 (100.0)	18 (75.0)	1.000
Cytology			
Non-diagnostic	0 (0.0)	**55 (22.5)**	**0.027**
Benign	0 (0.0)	152 (62.3)	**<0.001**
AUS/FLUS	4 (25.0)	25 (10.2)	0.089
FN/SFN	2 (12.5)	6 (2.5)	0.081
Suspicious for malignancy	**8 (50.0)**	5 (2.1)	**<0.001**
Malignant	**2 (12.5)**	1 (0.4)	**0.010**
Lymphocytic thyroiditis in histopathology	**8 (50.0)**	45 (18.4)	**0.006**

TIRADS: Thyroid Imaging Reporting and Data System; AUS/FLUS: atypia of undetermined significance/follicular lesion for undetermined significance; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm.

[a,b,c]n = 15 and n = 240 for malignant and benign, respectively. [d]Monte Carlo simulation results based on 10000 samples.

Table 6. Diagnostic accuracy measures of cytology in isthmic and non-isthmic nodules

Diagnostic accuracy measures	Isthmic nodules (n = 204)	Non-isthmic nodules (n = 3255)	p
Sensitivity (95% CI)	1.000 (0.806-1.000)	0.817 (0.780-0.850)	0.088
Specificity (95% CI)	0.803 (0.741-0.854)	0.832 (0.818-0.845)	0.310
Overall accuracy (95% CI)	0.819 (0.760-0.866)	0.830 (0.817-0.842)	0.681
PPV (95% CI)	0.302 (0.195-0.435)	0.451 (0.418-0.485)	**0.047**
NPV (95% CI)	1.000 (0.975-1.000)	0.964 (0.956-0.971)	**0.033**

PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval.

DISCUSSION

The clinical, ultrasonographical, and cyto-histopathological characteristics of isthmic and non-isthmic nodules were compared in the present study. Although isthmic nodules had lower malignancy rates compared to non-isthmic ones, US features (exception of macrocalcification), TIRADS categories and cytology results were similar between two groups. Furthermore, malignant isthmic nodules had similar US features, TIRADS categories and tumor characteristics as malignant non-isthmic nodules. Malignant isthmic nodules were smaller than benign ones. Isthmic nodules had higher NPV and lower PPV compared to non-isthmic nodules.

Prevalance of isthmic nodule was found as low compared to nodules located in lobes (7,11). US features associated with higher malignancy risk are primarily based on lober PTC (17) and there is limited data about US findings of isthmic nodules in the literature. In a study of small number of patients (16 benign and 12 malignant isthmic nodule), Goldfarb and cols. suggested that extend of operation could be more confidently predicted and planned when nodule evaluated with surgeon-performed US combined with FNAB findings in patients with isthmic nodule (14). Hahn and cols. studied with 48 patients with malignant isthmic nodule and 96 patients with malignant lober nodules. They found that isthmic nodules had more frequent circumscribed margin, a wider-than-tall shape, and ultrasonographical suspicion of extrathyroidal extension (tumor with capsular abutment of > 25% of its perimeter on ultrasound). First two are known as suggestive of benign features, but authors found them as predictors of malignancy in isthmic nodules. They suggested that these US characteristics appeared to have resulted from tumors growing in the thin isthmic space (17).

Nodule size was smaller in malignant isthmic nodules compared to benign isthmic nodules (14) although it was same in another study (27). The size of malignant isthmic nodules was found as significantly smaller than that of located left lobe in patients with metastasis (28). In a recent study with a large cohort, malignant nodules in isthmus were significantly smaller in size compared to that of middle, and lower, but not upper nodules of thyroid gland. They recommended that future consideration is given to adding a point to the guidelines for nodule location in the isthmus or using a lower size threshold for FNA or follow-up (7).

In the present study, 4.8% of nodules was located in isthmus and it was compatible with the literature. Almost all US features except for macrocalcification were similar in isthmic and non-isthmic nodules. It was noteworthy that when specifically isthmic nodules were evaluated, suspicious US features exception of hypoechogenicity, which was higher in malignant ones, were found as similar between malignant and benign ones, and malignant isthmic nodules had smaller size than benign isthmic nodules. The size data was compatible with the study of Goldfarb and cols. (14). These findings might support that the nodules in the isthmus, especially hipoechoic ones, should be evaluated with FNAB even if it is relatively small, as supported by the study of Jasim and cols. (7).

In our study, isthmic and non-isthmic nodules were found as similar regarding FNAB results. When malignant nodules were subdivided into two groups as isthmic and non-isthmic, any of the malignant isthmic nodules had non-diagnostic or benign results, but 14.3% and 15.8% of non-isthmic malignant nodules had non-diagnostic and benign results, respectively. Similarly, Goldfarb and cols. found that there was no nondiagnostic result in cytology of malignant isthmic nodule in their study in which isthmic nodules were evaluated in two groups as malignant and benign. Furthermore, they found that cytologies of all malignant isthmic nodules were malignant or suspicious for malignancy (14). Pastorello and cols. found that isthmic nodules had lower non-diagnostic results compared to lober nodules. They concluded that lower non-diagnostic results in isthmic nodules might be originated from easily accesible location of isthmus by biopsy due to the superficial location (10). Contrary to our result, Hahn and cols. did not find any significant difference between the two malignant groups (isthmic and non-isthmic) regarding the cytological findings. However, the rate of non-diagnostic result of isthmic nodule was higher in their study (2.1%) (17).

In the literature, malignancy rate in isthmus region was reported between 1% and 17.4% in different studies (7,9,10,12-21). In a study including 557 nodules with definitive cytologic diagnoses, although isthmic nodules had lower malignancy rate (2.5%), the rate was not significantly different in the isthmus, right (9.6%), or left lobe (7.2%) (8). Another study had shown higher malignancy rate in isthmic nodules (12.5%) compared to right (8%) and left (6.5%) lobes nodules but it did not also have statistical significance (9). On

the other hand, in a largely cytology-based study, the malignancy rate of isthmic nodules was significantly higher than the right and left lobes (8.1%, 3.6%, 3.1%; respectively) (10). Similarly, in an another study, thyroid nodule location was found an independent risk factor in predicting thyroid cancer. Isthmic nodules had the highest risk of malignancy compared to right and left lober ones (17.4%, 10.1%, 9.6%; respectively). Of all malignant nodules, the malignancy rate was 10% for isthmic nodules (7). In the present study, malignancy rate of isthmic nodules was significantly low compared to non-isthmic nodules (6.2 % vs 12.5%, p = 0.002). Considering all malignant nodules, the proportion of isthmic nodules was 2.8%. The different malignancy rates in different studies could be due to selection bias of study population. In these studies, definition of isthmic nodule, FNAB criteria, extend of thyroidectomy, nodule diameter, and dominant nodule originating from isthmus or not were different. Some studies included only patients with dominant nodules and others had carcinomas outside the nodule (in the parenchyma).

Classical variant of PTC in isthmic nodules was significantly more frequent than those in lober PTCs (29). However, tumor size was smaller in isthmic nodules compared to non-isthmic nodules in some studies (12), it was reported as similar in other studies (21,30,31). We found similar PTC variant distribution and tumor size in two groups.

PTCs arising in the isthmus had increased rate of multifocality in some studies. It ranges from 27.2% to 67% (12,14,18,20). Authors attributed the multifocality both to the midline position of tumors, which may be caused more easily tumor spread to both lobes and may be due to fact of common intraglandular metastasis of PTC (20). We found similar multifocality rate between patients with malignant isthmic and non-isthmic nodules as in other studies (17,30).

Extrathyroidal extension is one of prognostic factors which is placed in TNM classification system (29). It is associated with local recurrence, lymph node metastasis, distant metastasis and disease-specific mortality (17). Isthmic PTCs were found to have more frequent extrathyroidal extension compared to lober ones in some studies (17,30,32). They concluded that it might be resulted from tumors growing in the thin istmus (12). Although it was similar in other studies (21,31), Wang and cols. (16) found lower extrathyroidal extension. Also they did not find a relationship between extrathyroidal extension and central lymph node metastasis. Capsular and adjacent tissue invasions were also found as increased in isthmic PTCs in comparison to PTC located in lobes (12,17,18,21). In the present study, extrathyroidal extension, capsular and lymphovascular invasions were similar in two groups.

Lymph node metastasis had shown a risk factor for recurrence and further metastasis (30). The most common site of lymph node metastasis in PTC is central compartment (33). The location of the tumor is associated with neck netastasis (34). The rate of cervical lymph node metastasis is increased in isthmic PTC with regard to PTC arising from thyroid lobes (18,21,27,28,30,32,35). It was also reported that clinical stage was similar in isthmic and non-isthmic PTCs (12,17,31). We found similar lymph node involvement and TNM staging between groups.

A possible linkage between thyroid cancer and lymphocytic thyroiditis has been suggested. Karatzas and cols. found similar rate of histopathological lymphocytic thyroiditis in malignant and benign isthmic nodules, but in malignant isthmic nodules it was lower than in malignant non-isthmic nodules (27). There were some studies which reported similar lymphocytic thyroiditis rates in malignant isthmic and malignant non-isthmic nodules (21,32). Although, we found that patients with isthmic nodules had lower lymphocytic thyroiditis rate compared to patients with non-isthmic ones, it was significantly higher in malignant isthmic nodules than benign ones.

According to our knowledge, there has been no study about comparing diagnostic accuracy measures in isthmic and non-isthmic nodules in the literature. We found that the sensitivity, spesificity and overall accuracy of the cytology were similar in two groups. While NPV was significantly higher, PPV was significantly lower in isthmic group in comparison with non-isthmic nodules. These results can be interpreted as isthmic nodules with indeterminant cytology are more likely to be benign compared to non-isthmic nodules. Although sample size was small, Goldfard and cols. reported that all of the patients with indeterminate cytology (n = 5) had benign histopathology (14). These results support that the follow-up or limited surgery might be considered for the management of indeterminate isthmic nodules.

The strength of our study was that, to our knowledge, on this topic, it had the most nodules whose diagnoses were confirmed histopathologically in the literature. Additionally, our study extensively

examined demographic, ultrasonographic and cyto-histopathological features. However, retrospective design which could be a possible reason of selection bias for study population was a limitation of our study.

In conclusion, isthmic nodules have almost similar US and cytopathological features, and tumor characteristics to non-isthmic ones. They seem to be indolent because they had lower malignancy rate compared to non-isthmic nodules. Patients with isthmic nodule and indeterminate or nondiagnostic cytology might be candidates for follow-up. Careful examination of relatively small and hypoechoic nodules in the isthmus might be required due to the possibility of malignancy.

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Suplementary Table. The distribution of cytology and histopathology results in isthmic and non-isthmic nodules

Cytology	Isthmic nodules						Non-isthmic nodules						p[2]
	Malignant histopathology			Benign histopathology			Malignant histopathology			Benign histopathology			
	n	%*	%‡	n	%*	%‡	n	%*	%‡	n	%*	%‡	
Nondiagnostic	0	0.0	0.0	55	**22.5**	100.0	78	14.2	6.6	1095	**28.2**	93.4	**0.044**
Benign	0	0.0	0.0	152	**62.3**	100.0	86	15.7	3.6	2316	**59.7**	96.4	**0.032**
AUS/FLUS	4	25.0	13.8	25	10.2	86.2	99	**18.0**	21.6	360	9.3	78.4	0.447
FN/SFN	2	12.5	25.0	6	2.5	75.0	27	**4.9**	32.5	56	1.4	67.5	>0.999
Suspicious for malignancy	8	**50.0**	61.5	5	2.1	38.5	126	**23.0**	73.3	46	1.2	26.7	0.351
Malign	2	**12.5**	66.7	1	0.4	33.3	133	**24.2**	95.7	6	0.2	4.3	0.142
p[1]	**<0.001**						**<0.001**						

AUS/FLUS: atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm.
*: column proportion; ‡: row proportion.
[1]: test result for comparing the distribution of cytology between malign and benign nodules within isthmic and non-isthmic nodules.
[2]: test result for comparing the distribution of histopathology between isthmic and non-isthmic nodules within each cytology group.
Bold column proportions are significantly higher in the corresponding histopathology.

Active search of adult patients with persistently low serum alkaline phosphatase levels for the diagnosis of hypophosphatasia

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ABSTRACT

Objective: Alkaline phosphatase (ALP) is the main laboratory marker of hypophosphatasia (HPP), a rare disease unknown to most physicians. The prevalence of HPP has been widely discussed in the literature due to the diverse phenotypes of HPP. The purpose of this study was to search for patients with hypophosphatasemia based on previous biochemistry tests and reevaluate them to confirm the diagnosis of HPP. **Materials and methods:** A total of 289,247 biochemical tests for ALP in adults were performed from 2015 to 2019 in two tertiary hospitals in Rio de Janeiro were reviewed (Clementino Fraga Filho University Hospital – HUCFF – and Bonsucesso Federal Hospital – BFH). **Results:** A total of 1,049 patients were identified with ALP levels below 40 U/L, and 410 patients had hypophosphatasemia confirmed by at least two exams. After the active search of medical reports and/or interviews based on structured questionnaires, 398 subjects were excluded due to secondary causes of reduced ALP. The remaining 12 patients were invited to attend the medical consultation at HUCFF, accompanied by at least one first-degree relative. None of the patients or their relatives had a history or clinical manifestations consistent with HPP. Serum ALP was within reference values in all relatives, but persistently low in further laboratory evaluation in all the 12 patients, in whom secondary causes were ruled out. Thus, we cannot exclude the possibility that they might carry the mutations associated with HPP. **Conclusion:** Further image evaluations and genetic testing would be appropriate to confirm this asymptomatic adult form of HPP. Arch Endocrinol Metab. 2021;65(3):289-94

Keywords
Alkaline phosphatase; hypophosphatasia; bone

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IINTRODUCTION

Serum alkaline phosphatase (ALP) is one of the main tests requested in clinical practice, both in outpatients as well as in hospitalized patients. A low total ALP level is the main biochemical marker of hypophosphatasia (HPP). HPP is an autosomal dominant or autosomal recessive inborn error of metabolism with an extraordinary range of severity, and HPP is particularly caused by loss-of-function mutations within the gene that encodes the tissue-nonspecific isoenzyme of alkaline phosphatase (TNSALP) (1). There are North American, European and Canadian publications that estimate the prevalence of severe cases as 1/100,000 to 1/297,000 born alive (2,3).

The main problem in routine practice is that only high values of ALP are usually considered, as they may be associated with liver or bone diseases. In fact, low values should also be taken into account, mostly in patients with bone complaints. The identification of persistent hypophosphatasemia – which is a laboratory abnormality, not a disease – is very important because it may be an indicator of an asymptomatic adult or a carrier of a recessive mutation. Limited evidence exists

regarding the features that signal a potential association between hypophosphatasemia and HPP in adults (4).

Although HPP is a very rare disease, bone and dental disturbances are well documented in the literature in all classifications of the disease, since ALP is an important enzyme responsible for bone and teeth formation (5). The recognition of the disease in asymptomatic or oligosymptomatic elderly people with osteoporosis or fragility fractures would prevent the disastrous use of bisphosphonates, which is associated with worsening of bone mineralization (6). On the other hand, in young patients in the reproductive phase, genetic counseling would be important if hereditable recessive forms are identified (7). To date, there are no studies in Brazil regarding persistent hypophosphatasemia and its manifestations in adults. The main goal of this study was to identify patients with persistently low levels of ALP, after excluding secondary causes, in two tertiary hospitals in Brazil. As a secondary goal, we aimed to investigate family history, identifying ALP level reductions or possible signs and symptoms of HPP.

MATERIALS AND METHODS

A total of 289,247 biochemical tests of ALP were performed in adults (>18 years old) from January 2015 to December 2019 (n = 249,216) at the Clementino Fraga Filho Hospital (HUCFF) and from January to December 2015 (n = 30,031) at Bonsucesso Federal Hospital (BFH) were evaluated. The study was approved by the Reserch Ethics Committee of BFH and registered on "Plataforma Brasil" (CAAE number: 11466919.3.0000.5253). The screening led to 1,049 patients with at least one ALP result below 40 IU/l [cut-off point for adults according to most publications on this topic (8)], of whom 410 had at least two exams showing persistently low ALP levels at intervals of more than 30 days. The study evaluated all hospital specialties divisions, except the pediatrics department.

Data collection was performed through an active search and study of medical files as well as via telephone contact with patients and family members and interviews with patients with persistently low ALP levels. After the active search, 398 patients were excluded due to secondary causes of hypophosphatasemia. The ALP fluctuations (74%) could be justified by some diseases that can modify the bone turnover or may hinder the interpretation of ALP activity (pre-analytical or analytical interference). The remaining 12 patients (2.92% of 410 patients with at least two results < 40 U/L) were invited to come to HUCFF with their first-degree relatives for a clinical and laboratory study. All of them were subjected to investigative anamnesis addressing the signs and symptoms of HPP, such as bone abnormalities, early deciduous tooth loss, and family history of bone abnormalities based on a specific questionnaire, and the patients had blood samples drawn for repeat ALP examination by other biochemical methods.

During these five years (2015 to 2019), at least three different types of equipment for laboratory tests had been used by these hospitals. Tests for ALP were performed using Beckman Coulter model AU 5.800, Siemens dimension RXL, and Wiener BT-3000 equipment. The enzymatic method used to assess the activity consists of the colorimetric method recommended by the International Federation for Clinical Chemistry (IFCC) (9).

RESULTS

Our screening identified 12 adult patients with hypophosphatasemia, without any reason that could justify this laboratory alteration (Figure 1). The patients ranged in age from 23 to 70 years (median 43 years), three males and nine females. None reported any skeletal complaints, such as bone pain, childhood deformities or fragility fractures, but one woman complained of spontaneous muscle pain. They also did not report having chondrocalcinosis, nephrolithiasis, nephrocalcinosis, or early dental loss.

In male patients, ALP levels varied from 25 to 38 U/L (median 37 U/L), and in female patients, ALP levels varied from 18 to 37 U/L (median 32.5 U/L). We evaluated at least one first-degree family member of ten patients, and we did not find persistently low levels of ALP or clinical symptoms that suggested bone disease or early deciduous tooth loss. Only two patients did not have their families evaluated, both due to social issues, but there were no reports of bone deformities, and there were no health concerns. The relatives of ten patients were tested: children (n = 8), a mother (n = 1), and a sister (n = 1); however, all relative had normal ALP levels, as shown in Table 1.

The main causes of exclusion were fluctuations in ALP levels (74%). Despite the initial screening, we observed that subsequent tests results demonstrated in the medical files showed values higher than 40 IU/l during the five years of the retrospective study. The other causes

of exclusion have been well described in the literature (10), such as the use of glucocorticoids, chemotherapy medication in oncologic patients, immunosuppressants, fibrates, and bisphosphonates, cleidocranial dysplasia and Mseleni joint disease (Table 2).

DISCUSSION

HPP is a rare disease (RD) caused by a mutation in the *TNSALP* gene, which codifies non-tissue-specific ALP. It may present with recessive or dominant autosomal transmission, and more than 300 mutations have been identified (8). Patients with HPP have decreased serum ALP levels and elevations of the natural substrates pyridoxal-5'-phosphate (vitamin B6) and phosphoethanolamine. However, only the level of ALP is required for the diagnosis of HPP (10).

It was described in a previous study that 50% of adults with repeatedly low ALP levels had a mutation in the *TNSALP* gene (11). All mutations were predicted to impair the enzyme activity. In fact, haploinsufficiency was enough to reduce serum ALP activity, and in approximately one-half of the patients with identified mutations, there was biochemical evidence of the

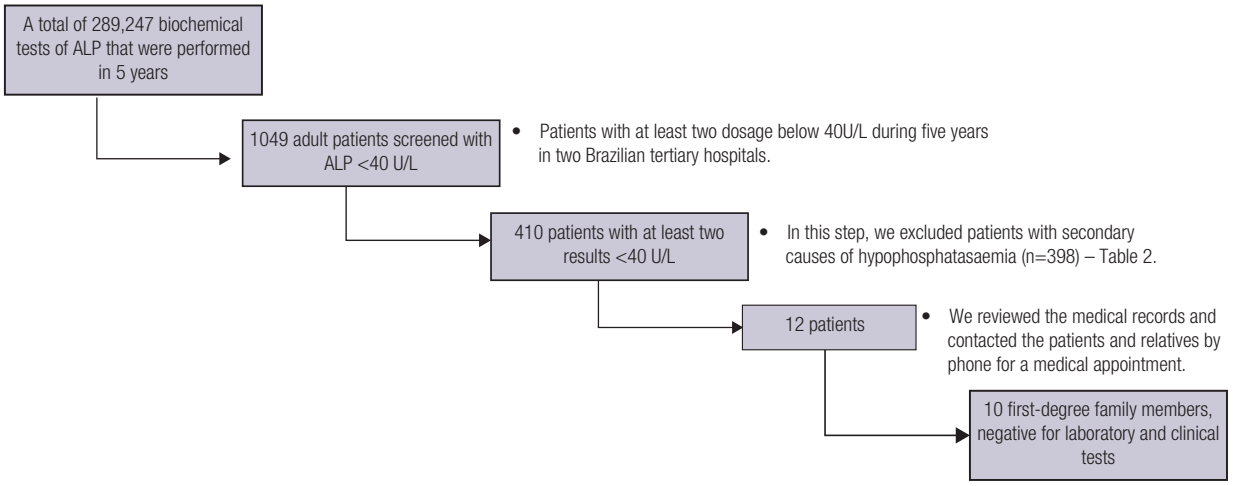

Figure 1. Study flowchart – patient selection.

Table 1. Clinical characteristics of patients with persistently low alkaline phosphatase levels

Patient	Age	Sex	Main diagnosis	Low ALP levels confirmed by three different methods/lower and higher values	Bone disease or fracture	Early tooth loss	Muscle complaints	Lithiasis, extraskeletal calcification	Number of investigated relatives affected/ yes or no
1	50	F	Cured hepatitis B	Yes/23-27	No	No	No	No	1/no
2	49	M	Hypertension Cured hepatitis C	Yes/25-33	No	No	No	No	0
3	38	F	Grade 3 obesity	Yes/18-29	No	No	No	No	1/No
4	39	F	Deep vein thrombosis	Yes/35-37	No	No	No	No	1/No
5	37	F	Grade 2 obesity	Yes/28-36	No	No	No	No	1/No
6	43	F	Subclinical hypothyroidism	Yes/32-33	No	No	No	No	1/No
7	43	M	Grade 1 obesity	Yes/28-30	No	No	No	No	1/No
8	46	F	Diabetes mellitus and hypertension	Yes/34-36	No	No	Yes	No	1/No
9	70	F	Resistant hypertension	Yes/27-37	No	No	No	No	1/No
10	44	F	Overweight	Yes/35-36	No	No	No	No	1/No
11	23	M	Low weight	Yes/25-38	No	No	No	No	0
12	32	F	Late postoperative cholecystectomy	Yes/30-33	No	No	No	No	1/No

ALP: alkaline phosphatase.

Table 2. Causes for patients exclusion

Reason for exclusion	Number of patients excluded	Percentage of patients excluded
Cardiac bypass	2	0.5%
Fibrates	5	1.25%
Multiple myeloma	2	0.5%
Malnutrition	1	0.25%
Cancer	21	5.27%
Glucocorticoids	36	9.04%
Chemotherapy	14	3.51%
Immunosuppressants	17	4.27%
Bisphosphonates	4	0.50%
Fluctuations in ALP levels	296	74%
Total	398	100%

ALP: alkaline phosphatase.

accumulation of phosphorylated substrates. Therefore, these adults may carry pathogenic, benign, or uncertain mutations that cause genetic variations. There is a positive correlation between the types of pathogenic mutations and high levels of substrates in blood and urine (8).

Despite its rarity, HPP should be fundamentally recognized in patients with persistently low ALP levels. Adult HPP presents after the age of 18 years and can include dentition loss, fractures, pain, and disability (12). The condition should be especially suspected in patients with recurrent metatarsal fractures that can heal poorly (13). Theoretically, dental anomalies may exist in every subtype of HPP (14). Comorbidities, such as renal failure, can affect the phenotype of HPP (15).

We detected 12 adult patients with this biochemistry profile who had no manifestations of HPP. These 12 patients may be within the spectrum of the adult form of HPP, which is generally oligosymptomatic or asymptomatic. ALP levels were normal in the 10 relatives tested, who were also completely asymptomatic. The genetic test can be used to confirm mutations associated with HPP, and this confirmation would allow for genetic counseling, as well as choosing adequate alternatives for osteoporosis or fracture treatment in adults, avoiding bisphosphonates (16). The molecules of these medications are very similar to one of the substrates that accumulates due to low ALP activity (inorganic pyrophosphate, an inhibitor of bone formation). Thus, the risk of excessive inhibition of bone turnover may lead to bone fragility and rare side effects such as atypical femoral fractures (11).

In the literature, the prevalence of persistently low ALP levels was 0.06% in the study of a large rural American population (17) (n = 885,165), constituted predominantly by individuals of northern European descent with no relevant ethnic diversity, with a mean age varying from 46 to 55 years. In this study, an important sex effect was observed, in which more women (n = 171; 64%) presented with low ALP levels than men (n = 98; 36%). These patients had more radiographic evidence of chondrocalcinosis, calcific periarthritis, enthesopathy, and diffuse idiopathic skeletal hyperostosis than the general adult patient population ($p < 0.001$). The exclusion criteria were the same as those used in the present study.

From a different perspective, a study conducted in a French tertiary hospital with fewer patients (2) (n = 48,755) showed the prevalence of persistently low ALP levels to be 0.13%. This study selected patients, especially from the departments of rheumatology, endocrinology, internal medicine and gastroenterology, but excluded patients from the emergency room. The mean age varied from 46.5 ± 17.7 years of age, and 73% were females, a gender predominance also found in our cohort and in other studies (2,17). Whether this predominance of females in the adult form of HPP is really true, more scientific researches are still needed to confirm. These patients showed radiographic evidence of chondrocalcinosis, a previous history of fractures and rickets in childhood, in addition to a healing delay of fractures.

One of the challenges of our study was to find the prevalence of HPP in our adult Brazilian population, because even in two tertiary healthy service no one severe case was identified. We highlight the fact that the population studied is very miscegenated, all from one state, with low socioeconomic levels, and with many undertreated comorbidities, which can delay the diagnosis of this rare disease. After our retrospective cohort study, we can hypothesize that the prevalence of syntomatic adult forms of HPP in Brazil may be lower than described in the literature. We can also speculate that this strategy was not good enough to identify potential patients, or that we would need to increase our sample size. Another possible explanation is that more severe forms in childhood can prevent individuals to reach the adult age due to high mortality.

Some mutations in the Brazilian population have been described. A 36-year-old male presented with multiple fractures, short stature, and early craniosyostosis. His

level of ALP was very low (6U/L). Genetic testing showed a homozygous missense mutation in *ALPL* gene c.443 C>T: p.Thr148Ile, also identified in his mother (18). Another case of a Brazilian child whose parents and sister had reduced ALP and increased PLP levels was published. His mother (36-year-old) was diagnosed with rickets in her childhood while his father had no history of fractures bone deformity or other clinical abnormalities observed. A homozygous c.98C>T (p.Ala33Val) missense mutation in the *TNSALP* gene was identified (19).

As mentioned above, HPP is a rare disease (RD). Currently, there are more than 6,000 RDs known in the world, of which 71.9% are of genetic origin (8), such as HPP. RDs are heterogeneous, numerous, and geographically very disparate. Few are curable, most are chronic, and many result in premature death as one of the possible manifestations of HPP. The challenges resulting from the low prevalence of RDs are knowledge scarcity and the chronic and life-threatening nature of RDs (20). Increasing numbers of patients and relatives are becoming involved in the management of their condition through the use of digital platforms, social media and tools to enable and increase their confidence as well as through the empowerment of families and the fundraising of research projects. The main responsibility lies with health professionals, who must keep themselves informed and updated to come to a correct diagnosis and treatment of these diseases.

The major limitation of the present study was not to further evaluate each patient of these 12 patients regarding some aspects like genetic testing, bone density and microarchitecture, and imaging diagnosis of chondrocalcinosis, nephrolithiasis and nephrocalcinosis. On the other hand, the great relevance of this study is to raise awareness among the medical community concerning a rare disease with a prevalence and an incidence not yet largely studied in the Brazilian population. Patients who have persistently low ALP levels could be recognized potentially as having an RD. These patients can be carriers of autosomal or recessive genetic mutations with low penetrance, and there is a risk that they could transmit this severe disease to their descendants. This important information enables genetic counseling and the prevention of a potentially catastrophic disease for family members.

Finally, we highlight the low prevalence of primary persistent hypophosphatasaemia in our sample and that our patients with persistently low ALP levels did not present any clinical manifestations of HPP, maybe because the adult form of HPP is generally less severe than the infantile types. Further studies are needed to confirm this prevalence and to investigate these asymptomatic patients in relation to image evaluations and genetic testing.

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Apoplexy in sporadic pituitary adenomas: a single referral center experience and *AIP* mutation analysis

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ABSTRACT

Objective: To analyze the clinical, laboratory, and radiological findings and management of patients with clinical pituitary apoplexy and to screen for aryl hydrocarbon receptor-interacting protein (*AIP*) mutations. **Subjects and methods:** The clinical findings were collected from the medical records of consecutive sporadic pituitary adenoma patients with clinical apoplexy. Possible precipitating factors, laboratory data, magnetic resonance imaging (MRI) findings and treatment were also analyzed. Peripheral blood samples were obtained for DNA extraction from leukocytes, and the entire *AIP* coding region was sequenced. **Results:** Thirty-five patients with pituitary adenoma were included, and 23 (67%) had non-functioning pituitary adenomas. Headache was observed in 31 (89%) patients. No clear precipitating factor was identified. Hypopituitarism was observed in 14 (40%) patients. MRI from 20 patients was analyzed, and 10 (50%) maintained a hyperintense signal in MRI performed more than three weeks after pituitary apoplexy (PA). Surgery was performed in ten (28%) patients, and 25 (72%) were treated conservatively with good outcomes. No *AIP* mutation was found in this cohort. **Conclusion:** Patients with stable neuroophthalmological impairments can be treated conservatively if no significant visual loss is present. Our radiological findings suggest that hematoma absorption lasts more than that observed in other parts of the brain. Additionally, our study suggests no benefits of *AIP* mutation screening in sporadic patients with apoplexy. Arch Endocrinol Metab. 2021;65(3):295-304

Keywords
Apoplexy; pituitary adenomas; *AIP*; familial isolated pituitary adenomas

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INTRODUCTION

Pituitary apoplexy (PA) is an acute event associated with hemorrhage or infarction and occurs in approximately 2% to 12% of those with a preexisting pituitary adenoma (1-4). Sudden, abrupt and intense headache, visual impairment, an altered level of consciousness and clinical manifestations of hypopituitarism are described in the acute onset of PA (2-6).

The pathophysiology of apoplexy involves changes in the pituitary blood supply and can be related to rapid tumor enlargement that increases metabolic demand and intrasellar pressure, leading to the compression of adjacent structures (7). Therefore, PA occurs mostly in macroadenomas (8-10). Similarly, nonfunctioning pituitary adenomas (NFPAs) are the most prevalent in a series of apoplexy, likely related to silent growth, identified only after the development of a mass effect (3,9,11).

Risk factors for PA have not been completely elucidated, and potential precipitating factors were identified in 10% to 40% of cases, including angiography

procedures, cardiac and other major surgeries, dynamic pituitary function tests, arterial hypertension, radiation therapy, head trauma, anticoagulation and antiplatelet therapy (2,9,12-14). Estrogen therapy, coagulopathies and intense exercise were also reported. Some case reports described PA during the treatment of acromegaly with octreotide (15).

Computed tomography (CT) is generally the first imaging tool available in the emergency room and can detect pituitary expansive masses in up to 80%-94% of cases, but a PA diagnosis is made in only 21% to 28% of cases (6,15,16). Magnetic resonance imaging (MRI) is the better choice for image analysis, confirming the diagnosis of PA in approximately 90% of patients (3,6,8,16).

No consensus exists concerning the best PA approach, and treatment can be conservative or surgical according to each case and condition (16,17). Some studies have demonstrated a good response in visual recovery with conservative management, considering selected patients with non-progressive neuroophthalmological deficits (6,18,19). During the acute phase, all the patients with clinical findings of suspected apoplexy must be tested for hypopituitarism, mainly because of the risk of severe hypocortisolism (2).

Interestingly, some series described PA as a feature of patients harboring germline mutations in the *aryl hydrocarbon receptor-interacting protein* (*AIP*) gene (20-22). Typically, patients with *AIP* mutations (*AIP*muts) have macroadenomas of young onset and aggressive behavior (20,21,23-25). *AIP*muts are present in approximately 20% of familial isolated pituitary adenoma (FIPA) kindreds and in 3.6% to 20% of apparently sporadic adenomas varying according to the age of the group analyzed, but mutations have low penetrance in both groups (21,22,26-28). To date, the frequency of *AIP*muts in patients with apparently sporadic pituitary adenomas presenting with PA is unknown.

Our study analyzed the clinical, radiological and therapeutic characteristics of patients who presented with clinical PA and were referred to a specialized neurosurgery center. We also evaluated the frequency of *AIP*muts in these apparently sporadic pituitary adenoma patients.

SUBJECTS AND METHODS

Patients

We retrospectively analyzed the files in our database from consecutive patients referred to a neurosurgery center [*Instituto Estadual do Cérebro Paulo Niemeyer* (IECPN)] with a clinical history and MRI report of PA from August 2013 to September 2017. We included patients who presented with sudden onset of severe headache and/or other neuroophthalmological symptoms (visual disturbance and/or ophthalmoplegia and/or altered consciousness) diagnosed with PA according to the UK Guidelines for the Management of Pituitary Apoplexy and who had undergone MRI to confirm PA (2). Headache was classified as thunderclap headache when it was described as bilateral and retroocular, with abrupt onset associated with progression to maximum intensity within a few minutes (29).

Blood samples were collected from the patients for genetic analysis (performed in our laboratory). At that time, they were interviewed to clarify all the symptoms presented at the acute phase. On admission, visual fields were assessed by confrontation tests and Goldmann manual campimetry.

Patients with clinical features and/or a history of X-linked acrogigantism (XLAG), multiple endocrine neoplasia type 1 (MEN-1) and 4 (MEN-4), Carney complex (CNC), association of pheochromocytoma/paraganglioma and pituitary adenoma syndrome (3PAs) and FIPA were excluded. All the subjects signed written informed consent, and the Ethics Committee of Medical School and *Hospital Universitário Clementino Fraga Filho* (HUCFF) of *Universidade Federal do Rio de Janeiro* (UFRJ) approved the study.

METHODS

Laboratory analyses

We collected the serum basal levels of growth hormone (GH), insulin-like growth factor type I (IGF-I), prolactin (PRL), thyroid-stimulating hormone (TSH), free thyroxine (FT4) and total testosterone (in males) from patient files. Women with regular menstrual cycles who did not use oral contraceptives were considered to have no gonadotropic axis deficiency. In post-menopausal women, the FSH levels were analyzed. We could not evaluate the presence of hypocortisolism in all the patients because some patients were transferred from other centers and were being treated with high doses of dexamethasone or hydrocortisone before admission to our center or were using oral corticosteroids. Hypopituitarism was defined as the presence of at least one endocrine axis.

We collected laboratory results reported at admission and at the last evaluation at IECPN until September 2017. The laboratory tests recorded at admission were performed, in cases, outside IECPN, at different laboratories.

Radiological evaluation

All the patients had at least one MRI described in the medical records confirming PA. Twenty of the 35 patients had undergone MRI at our center, and these MRI images were available in our database system. The MRIs of these 20 patients were reviewed by the same experienced neuroradiologist that analyzed the sagittal and coronal T1-weighted images (T1WI), with and without gadolinium contrast, and coronal T2-weighted images (T2WI).

Microadenomas were defined as those with a maximum diameter < 10 mm, and macroadenomas were defined as those with a maximum diameter ≥ 10 mm (30). The tumor volume was estimated using the DiChiro and Nelson formula (width × height × length × 0.5233) (31). Patients were grouped according to the MRI findings using the classification of typical stages of hematoma evolution in the brain, as shown in Table 1 (32).

Screening for AIP mutations

We used the PureGene Blood Kit (Gentra, Minneapolis, MN, USA) to obtain genomic DNA from 300 µL of whole blood following the manufacturer's instructions. DNA was resuspended in 100 µL of DNA Hydration Solution (Gentra). After extraction, PCR was performed using an Applied Biosystem ProFlex™ PCR System (Thermo Fisher Scientific, Foster City, CA, USA).

Genomic analyses included exons 1 to 6 from the *AIP* gene and flanking intronic sequences. Amplification and sequencing were performed using *AIP* PCR/Sanger Sequencing Primer pairs (Thermo Fisher Scientific™, Boston, MA). The reaction contained a mixture of 30 ng of genomic DNA, 2 U of Platinum® Taq DNA Polymerase (Invitrogen, Foster City, CA, USA), 1.5 mM $MgCl_2$ and 0.2 µM of each primer, with a total volume of 25 µL (Table 2).

PCRs followed an initial denaturation and enzyme activation at 94 °C/5 min and then 40 cycles of denaturation at 94 °C/45 sec, annealing at 94 °C/45

Table 1. Stages of hematoma as observed on MRI

Stage	Time since apoplexy	Hemoglobin	T1WI	T2WI
Acute	≤ 7 days	Deoxyhemoglobin	Isointense or Slightly Hyperintense	Very Hypointense
Subacute	> 7 days to ≤ 21 days	Methemoglobin	Hyperintense	Hyperintense
Chronic	> 21 days	Hemosiderin	Hypointense	Hypointense

MRI: magnetic resonance imaging; T1WI: T1-weighted imaging; T2WI: T2-weighted imaging. Radiological classification of hematoma evolution on MRI (32).

Table 2. Primers used for *AIP* gene sequencing

PRIMERS				
Cat N°	Lot n°	Description/Sequence	Exon	Product size
A15633 – Hs00394559	292760 G02	TGTAAAACGACGGCCAGTCCGAGACATTCCTAGGCTCCG	1	495
A15634 – Hs00394559	292788 B02	CAGGAAACAGCTATGACCGCCCGAATTCACCCCCTACTTAAA		
A15633 – Hs00394560	292760 G03	TGTAAAACGACGGCCAGTGGAAGCCCCGTCCCTTATGC	2	381
A15634 – Hs00394560	292788 B03	CAGGAAACAGCTATGACCAGTCTAGCAGAGGGTGGAGGGAG		
A15633 – Hs00394561	292760 G04	TGTAAAACGACGGCCAGTCGGAGTAGGGTCCCAGTTGTC	3	492
A15634 – Hs00394561	292760 G07	CAGGAAACAGCTATGACCGGAGACCCAGGGTACTGCCAA		
A15633 – Hs00394562	292760 G05	TGTAAAACGACGGCCAGTCCAGATGTGGGTCAGGTCTGC	4	501
A15634 – Hs00394562	292788 B04	CAGGAAACAGCTATGACCGTCGTACTTGTTGAGGATGGAAGA		
A15633 – Hs00394563	292788 B01	TGTAAAACGACGGCCAGTAAGGTACTGCCTGGAGGCTGAG	5	507
A15634 – Hs00394563	292788 B05	CAGGAAACAGCTATGACCTCATGTCTCCTGGCACCATGGG		
A15633 – Hs00394564	292760 G06	TGTAAAACGACGGCCAGTGTGGCATCCTCAGGTCAGGGA	6	509
A15634 – Hs00394564	292788 B06	CAGGAAACAGCTATGACCGTACCAGGAATGCCAGGTGATGAC		

AIP: aryl hydrocarbon receptor-interacting protein.

sec and extension at 72 °C/1 min. A final extension was performed at 72 °C for 7 min. PCR product clean-up was performed using the ExoSAP-IT® system (USB Corporation, Cleveland, OH, USA), and DNA sequencing using the Big Dye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific).

The products were sequenced in both directions on an ABI 3130xl Genetic Analyzer (Applied Biosystems), and electropherogram-derived sequences were aligned using Benchiling (https://benchiling.com/) and BioEditsoftware (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The reference sequences for the *AIP* gene used were as follows: ENSG00000110711 (https://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000110711;r=11:67483041-67491103), NG_008969.3 (https://www.ncbi.nlm.nih.gov/protein/NP_003968) and NM_003977.3 (https://www.ncbi.nlm.nih.gov/nuccore/NM_003977).

Statistical analysis

SPSS version 23.0 for Windows was used for statistical analysis, and the data were presented as percentages, means ± standard deviation (SD) or medians (min-max). Normal distribution was tested, and the Mann-Whitney test was used to compare numerical variables between groups. A p-value < 0.05 was considered significant.

RESULTS

Demographical and tumor characteristics

Thirty-five patients were included (20 males), with a mean age of 40.5 ± 17.1 years. Non-functioning pituitary adenomas were present in 23 patients (66%), seven (20%) harbored somatotropinomas, and five (14%) harbored prolactinomas. Only three patients (9%) had a previous diagnosis of pituitary adenoma before apoplexy: two patients harboring NFPA and one acromegaly patient. Only the acromegaly patient had started treatment before the PA episode (octreotide LAR four months before). The others were treatment naïve.

Clinical characteristics

Headache was the most common symptom, present in 31 patients (89%). Among these, 16 patients (52%) presented with a thunderclap headache, ophthalmoplegia was observed in 13 patients (37%), and six patients (17%) presented with ptosis. Ten (28%) patients presented with visual field defects, and changes in the level of consciousness were present in five (14%) patients. Five (14%) patients presented with ophthalmoplegia and visual loss concomitantly. At the last assessment, 9 (25%) patients persisted with some degree of visual field defects. No patient presented headache or a reduced level of consciousness at the last evaluation. The median period between admission and last evaluation was six months (ranging from 3 to 48 months). The clinical presentation data are summarized in Table 3.

Table 3. Clinical findings

Symptoms	At acute event N (%)	At last evaluation* N (%)
Headache	31 (89%)	0
Thunderclap headache	16 (46%)	0
Ophthalmological signs and symptoms[&]	18 (51%)	11 (31%)
Ophthalmoplegia	13 (37%)	03 (8%)
Ptosis	06 (17%)	02 (6%)
Visual field defect	10 (28%)	09 (25%)
Altered consciousness	05 (14%)	0

*Median follow-up: 6 months after the event (range: 3 to 48 months).
[&]Some patients presented more than one ophthalmological symptom.

Laboratory characteristics

At admission, 19 patients (54%) had deficiency of at least one pituitary axis. Hypothyroidism was present in nine patients (26%), and hypogonadism was also present in nine patients. None of them were using hormonal replacement before the acute event. We identified GH deficiency in six of 21 patients with IGF-I available at the first evaluation. Prolactin levels were available from 20 patients, and eight (20%) patients had hyperprolactinemia. We observed worsening of hypothyroidism and hypogonadism at the last evaluation, with 17 (48%) and 16 (45%) patients presenting these deficiencies, respectively. The time elapsed from the acute PA event to the first laboratory evaluation varied from 3 days to 5 months. Considering the group of 10 patients treated surgically, three (30%) improved pituitary function completely, two (20%) presented worsening of the pituitary axis, and five (50%) persisted with the same pre-operative hormonal deficits.

Predisposing factors

Potential predisposing factors were investigated, and no PA was found after cardiac surgery, radiotherapy,

endocrinological function testing, and the use of anticoagulant medication or antiplatelet agents. Nine patients had arterial hypertension, and eight patients were using oral contraceptives; in one patient, the onset of PA occurred during intense exercise (running); in another patient, PA occurred four months after starting octreotide LAR treatment.

Radiological characteristics

In our cohort, one microadenoma (9 × 8 × 7 mm) was found. The median tumor volume in the whole group was 5.4 cm³ (0.26-48.67 cm³), and the median larger tumor diameter was 2.9 cm (0.9-6.2 cm). Somatotropinomas exhibited larger tumor diameters and volumes than other tumor types. The median tumor volumes were 26.9 cm³ (5.64-48.67 cm³) and 4.5 cm³ (0.26-16.32) in acromegaly patients and other tumor types, respectively (p = 0.021). The median larger tumor diameters were 4.2 cm (1.0 to 6.2 cm) and 2.5 cm (0.9 to 4.5 cm) in somatotropinomas and other tumor types, respectively (p = 0.013). No significant difference was observed in the larger tumor diameter between groups with or without neuroophthalmological symptoms (p = 0.18).

Considering the 20 MRIs available in our database that were reviewed by our neuroradiologist, various signal intensities of the pituitary adenomas in both T1WI and T2WI were observed, regardless of the elapsed time since apoplexy (Table 4). The time elapsed from acute PA to MRI varied from 3 days to 5 months; most of the patients (17 patients) were in the chronic phase. No clear pattern of evolution of the hemorrhagic image was observed after the episode of PA. Seventeen patients in this group presented with acute PA events more than three weeks before the MRI scan had been performed; in two of them, PA occurred five months before. However, in eight patients, a hyperintense signal in T1WI was still present; in six patients, a heterogeneous pattern (some areas of hyperintense

Table 4. Radiological findings

Stages of Hematoma*	ΔT symptoms and first MRI at IECPN	Tumor Type	T1	T2	Optic chiasma compression	High SI in T1	Surgery during the follow-up	ΔT from first to last MRI at IECPN	MRI findings at last MRI
≤ 7 days									
Acute	3 d	ACRO	Hyperintense	Hyperintense	Yes	Yes	Yes		
Acute phase	7d	NFPA	Heterogenous	Heterogenous	Yes	Yes	No		
> 7 days and ≤ 21 days									
Subacute phase	20d	NFPA	Hyperintense	Hyperintense	Yes	Yes	Yes		
> 21 days	28 d	NFPA	Hyperintense	Hyperintense	No	Yes	No		
Chronic	28 d	NFPA	Hyperintense	Isointense	Yes	Yes	Yes		
	28 d	NFPA	Heterogeneous	Heterogeneous	Yes	Yes	No	3 m	Empty sella
	28 d	NFPA	Heterogeneous	Heterogeneous	Yes	Yes	Yes		
	30 d	NFPA	Isointense	Isointense	No	No	No	2 m	Heterogeneous
	30 d	NFPA	Hyperintense	Isointense	Yes	Yes	Yes		
	2 m	PRL	Hyperintense	Heterogenous	No	Yes	No		
	2 m	NFPA	Isointense	Hypointense	No	No	Yes		
	2 m	NFPA	Heterogeneous	Hyperintense	Yes	Yes	Yes		
	2 m	NFPA	Isointense	Heterogeneous	No	No	No	5 m	Heterogenous
	2 m	NFPA	Heterogeneous	Heterogeneous	Yes	No	No		
	3 m	ACRO	Isointense	Heterogeneous	No	No	Yes		
	3 m	PRL	Isointense	Isointense	No	No	No	7 m	Empty sella
	3 m	PRL	Hyperintense	Heterogeneous	No	Yes	Yes		
	3 m	PRL	heterogeneous	Heterogeneous	Yes	Yes	Yes		
	5 m	NFPA	Isointense	Heterogeneous	No	No	No	8 m	Empty sella
	5 m	NFPA	Hyperintense	Hypointense	No	Yes	No	8 m	Empty sella

MRI: magnetic resonance imaging; SI: signal intensity; ACRO: somatotropinoma; NFPA: non-functioning pituitary adenoma; PRL: prolactinoma; d: days; m: months; T1 and T2 classification ΔT: time elapsed from PA to examination.

signals and other areas of hypointense signals in T1WI at the same time) was observed.

We compared the first and last MRI scans of six patients who were treated conservatively. Progression to an empty sella was verified in four patients, and two patients maintained the heterogeneous pattern in the T1WI described above. The interval from the first to last MRI of these six patients was 5 months on average, ranging from three to eight months. No re-bleeding was observed. These radiological findings are summarized in Table 4. Figure 1 illustrates the MRI of a patient in the acute phase of PA.

Treatment characteristics

Seventeen (48%) patients did not present any visual field impairment, ophthalmoplegia or ptosis and were treated conservatively. Eight (23%) patients presented with only ophthalmoplegia and/or ptosis with no visual loss and received conservative treatment. All 10 (28%) patients who presented visual loss had undergone surgery. In this group visual recovery was complete in one patient (10%) and partial in three patients (30%), and no improvement was observed in the remaining six (60%) patients.

AIP mutation screening

No *AIP* mutation was identified. We found four different single-nucleotide polymorphism (SNP) variants: two intronic variants [c.132C>T p.Asp44= in exon 2, c.516C>T p.Asp172= in exon 4] and two nonsynonymous variants [c.682 C>A and c.920 A>G] in exons 5 and exon 6 in 31 and 9 patients, respectively.

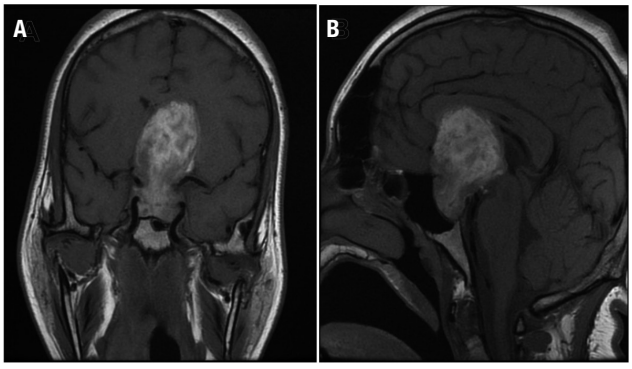

Figure 1. Coronal T1 (A) and sagittal T1-weighted imaging (B) sequences of the magnetic resonance imaging of a 16-year-old patient performed three days after the pituitary apoplexy event and showing a mass in the sellar region with important suprasellar and infrasellar extension, presenting hyperintense areas.

The most frequent allelic variant (31 patients) was present in exon 5, c.682C>A, encoding Q228K. In exon 6, we found other nonsynonymous SNP variants, c.920A>G, encoding Q370R (nine patients) and one intron variant insertion (c.788-30_788-29ins-/TGCCCAC). All the variants observed were considered benign in the general population (33,34).

DISCUSSION

In our cohort, acute onset of headache was the most common symptom of PA and was managed conservatively in patients without visual loss. Radiological evaluation showed that persistent high signal intensity in T1WI lasted longer than usually described in other areas of the brain. Additionally, we showed that *AIP*mut screening is likely not useful in these patients.

Male predominance was observed to be similar to most series. However, in our cohort, the mean age was 40.5 years, younger than that reported in the literature, in which patients presented most frequently in the 5th or 6th decade of life (3,9,35-40).

Headache was the most frequent symptom, and these results agreed with the literature, in which headache was present in 63%-100% of cases (29,37,40,41). Thunderclap headache was described in approximately 46% of patients with PA, and we observed similar results (51%), with no worsening evolution results in this group (29,37,40,41).

Neuroophthalmological symptoms and visual field defects were present in 51% (n = 18) of patients, including ptosis and ophthalmoplegia. These results were similar to the literature, in which a frequency of visual disturbance varying from 23% to 81% (3,42-44) and impairment of III, IV and VI cranial nerves of 52% was described (3,45).

Hypopituitarism is described in 50%-86% of all investigated patients (3,6,45). In our study, we observed a frequency of 54% at admission that progressed to 60% at the last evaluation. Similarly, other studies showed that improvement in pituitary function after PA, independent of treatment should not be expected (6,37,42,46). One limitation of our study was that we could not precisely estimate the frequency of hypocortisolism in our patients because many patients were admitted to other centers before being transferred to ours and were already using high doses of dexamethasone/hydrocortisone or oral corticosteroids at admission in our center. Additionally, the real

frequency of GH deficiency could not be determined because we did not perform functional tests, such as the insulin tolerance test, in patients with normal IGF-I levels to ensure that they were not deficient.

The initiation or withdrawal of dopamine agonist; octreotide withdrawal; thrombolytic, anticoagulation and antiplatelet therapy; estrogen therapy; coagulopathies, dengue hemorrhagic fever; cardiac and other major surgeries; dynamic pituitary function tests; radiation therapy; pregnancy and postpartum state have been listed as potential apoplexy precipitating factors (1,9,12-14,43,47,48). In our study, nine patients (25%) had arterial hypertension, consistent with the results of other publications, indicating that this condition is a common feature in patients with PA (3,6,9,13,18). However, Möller-Goede and cols. (9) published a review with 574 patients and observed that arterial hypertension and diabetes mellitus did not increase the risk of PA, similar to that in a previous study by Biousse and cols. (13). One patient had syncope during intense exercise that might be related to an abrupt change in tumor vascular pressure, previously described in other conditions associated with an increase in blood pressure (23).

Magnetic resonance imaging is the most important radiological tool to study apoplexy, with sensitivity ranging from 80% to 90% (16). Typical MRI descriptions during the acute phase include areas of hyperintense signal on the pituitary region on T1WI (49,50). However, many descriptions of hematoma evolution after apoplexy are derived from what is observed in other parts of the brain, and a lack of specific studies exists to describe hematoma evolution in PA (30,49). Some single-center studies of PA have been reported, but imaging features were, in general, not detailed (2,36,37). Generally, PA imaging studies describe acute events without MRI at follow-up (15,44,50).

The most frequent radiological feature of PA in the literature is hyperintense signals on T1WI, but other conditions can present the same characteristics, such as aneurysms, lipomas and Rathke cleft cysts (RCCs) (32). T2WI can help in the differentiation of an intracystic hypointense nodule, a typical sign of RCC related to proteinaceous fluid (32,51).

The two most specific image patterns of PA, sphenoid sinus mucosal thickening and fluid debris or fluid-fluid level (hyperintense signal on T1WI in upper fluid), were not found in our series (15,52). The first pattern can appear even before the vascular event, suggesting an engorgement caused by large adenomas or large collections of blood and likely related to severity and generally observed in the acute phase (52). The second pattern is the fluid-fluid level due to free extracellular methemoglobin in the upper fluid layer and with blood residue in the lower layer and is mostly described in the subacute phase (49-52).

A persistent hyperintense signal on T1WI was observed in our series even when MRI was performed in a later period, suggesting slower pituitary hematoma absorption. In vascularized areas of the central nervous system (subdural and epidural areas), oxygen tension remains high and slower from one stage to the next than in the brain itself (30,49). This condition can interfere with the evolution of hematoma and may explain our MRI results in patients outside the acute or subacute phase. Piotin and cols. (50) published an analysis of MRI patterns in PA and showed that, in general, high signal intensity, particularly hyperintensity in T1WI, suggests the presence of blood, but pituitary hemorrhage may present with a persistent hyperintense signal. We found a pituitary ring sign, typically described in the acute phase in a patient who had 30 days of evolution since the occurrence of PA symptoms (Figure 2 A; B) (52).

The risk of re-bleed can occur in a range from 6% to 11% of cases described in different studies, and the results are similar regardless of surgical or conservative approach (15,18,41,45,53). We found no apparent re-bleeding, and an empty sella was observed in four of six patients who had undergone a second MRI at IECPN. It took three to eight months after PA for the emergence of an empty sella, suggesting a longer time for hematoma absorption on pituitary topography.

The management of patients with PA can be surgical or conservative, and several retrospective studies

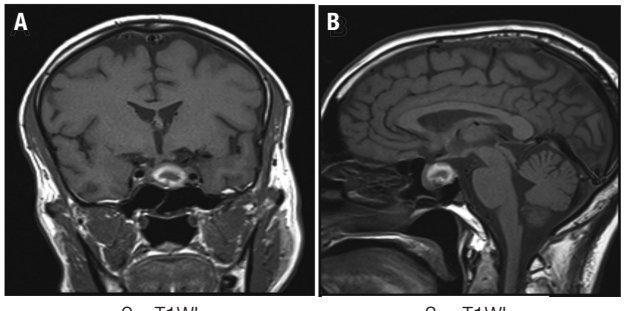

Figure 2. Coronal (A) and sagittal T1-weighted imaging (B) sequences of magnetic resonance imaging performed in a patient with classical pituitary apoplexy, 30 days after the acute event and showing a ring area of hyperintense signals in the sellar lesion.

have shown that the results are similar. In particular, conservative management is performed in patients with mild symptoms and stable ophthalmological deficits (6,37,40,42). Many studies have demonstrated spontaneous resolution of visual and neurological symptoms with expectant management (54-57). However, until now, no randomized trial has compared both strategies (35,36,39). The severity of symptoms at presentation and presence and progression of visual impairment should be considered parameters to guide better treatment for these patients (6,18,37,40). We initially treated 25 patients without visual impairment conservatively despite other neuroophthalmological symptoms (ptosis and/or ophthalmoplegia) with good outcomes.

Some series suggest that apoplexy may be a clinical feature of patients with *AIP* mutations, as noted by Igreja and cols. (20), who described apoplexy in 8% of familial series. This mutation is associated with larger tumors with aggressive behavior and a young onset (21,22,58). In our series, *AIP* screening revealed no mutation (33,59).

Two nonsynonymous SNP variants that promote amino acid substitutions (Q228K and Q307R) were described as missense variants with increased prevalence in FIPA patients (34). In the same study, sporadic forms of Cushing's disease were also associated with a variant of Q307R, and sporadic acromegaly was associated with a variant of Q228K (34). Both allelic variants have already been described in some subpopulations with moderate frequency (59). However, until now, no functional study was performed to confirm the relevance or pathogenicity of these allelic forms, and no other study found an association with FIPA or any other familial disease (59).

Recently, a large study with 2,227 patients analyzed variables that could help in *AIP* screening and, indeed, apoplexy was more frequent in *AIP*mut patients. The mechanisms by which *AIP* mutations may lead to apoplexy may be related to rapid cell growth and proliferation (3,24). However, after multivariate analysis, it was not one of the variables that helped predict patients with an *AIP*mut (60). Most of the patients included in this study had a family history of pituitary adenomas. In our study, which included only patients with sporadic pituitary adenomas, we also found no *AIP* mutations, indicating that it is likely not valuable to perform *AIP* mutation screening in patients with PA.

In conclusion, apoplexy is associated with neuroophthalmological symptoms in a great proportion of patients but can be managed conservatively in selected cases. Images of PA on MRI may not present the classic evolution described for hemorrhagic events in other areas of the central nervous system, with the persistence of areas of hyperintense signals on T1WI after the acute and subacute phases. *AIP* mutations are not common, and *AIP* screening should not be performed in the absence of other features suggesting the presence of this mutation.

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Genetic variants in the *SLC16A11* gene are associated with increased BMI and insulin levels in nondiabetic Chilean population

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ABSTRACT

Objective: To study the association of *SLC16A11* gene variants with obesity and metabolic markers in nondiabetic Chilean adults. **Materials and methods:** This cross-sectional study included 263 non-diabetic adults. The genotype of the rs75493593 polymorphism of SLC16A11 gene was performed by real-time PCR. It's association with adiposity markers (body weight, BMI, waist circumference and fat mass percentage), metabolic markers (glucose, insulin, $HOMA_{IR}$, leptin, total cholesterol, LDLc, HDLc, triglycerides, ALT, GGT and hsCRP) and blood pressure was analyzed by linear regression. **Results:** The minor allele (T) of the *SLC16A11* gene (rs75493593) has a frequency of 29.7% among Chileans. Risk genotypes (GT and TT) were associated with a significant 1.49 mU/l increase in plasmatic insulin for each copy of the minor allele (95% CI: 0.12, 2.87, $p < 0.05$). This association remained significant after adjusting for socio-demographic variables, physical activity and smoking (1.36 mU/l, 95% CI: 0.16, 2.58 $p < 0.05$), but was lost when BMI was included as a confounding factor. Higher BMI was also significantly associated with polymorphic genotypes in *SLC16A11*, independent of socio-demographic variables. **Conclusion:** The minor allele of the *SLC16A11* gene (T) is highly prevalent among Chileans and is associated with increased insulin and BMI in nondiabetic individuals. These findings suggest that the genetic variant in *SLC16A11* is not only associated with type 2 diabetes as previously shown in Mexicans, but is also related to early metabolic alterations in healthy subjects that may lead to type 2 diabetes. Arch Endocrinol Metab. 2021;65(3):305-14

Keywords

SLC16A11; diabetes mellitus type 2; obesity; monocarboxylate transporter; hyperinsulinemia

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INTRODUCTION

Obesity has been identified as a major modifiable risk factor for type 2 diabetes (T2D). Pathophysiological conditions that occur with obesity, like low-grade inflammation, increased plasmatic-free fatty acids and insulin resistance, are directly related to the pathogenesis of T2D (1). As a reflection of the close interrelationship between these two conditions, it has been reported that more than 80% of people with T2D are overweight or obese (2). Furthermore, worldwide trends in the prevalence of T2D have closely mirrored those of obesity, doubling from 1980 to 2014 (3).

There are important differences in the prevalence of T2D among populations. Mexico and some Caribbean nations have over a 14.5% prevalence, which are the highest in the North American continent (4). In South America, Chile leads in T2D prevalence at 12.3%, according to the latest national health survey (5). Culturally-based lifestyle differences are a major contributor to the different prevalence of T2D among populations, including nutrition, physical activity and sedentarism. However, genetic variability related to ethnicity is also likely because the heritability of T2D and obesity has been estimated to be between 40% and 70% (6,7).

Genome-wide association studies (GWAS) for diabetes and obesity have been conducted mainly with European populations, revealing that both pathologies are highly polygenic and share some genetic determinants (6,7). For instance, the single nucleotide polymorphism (SNP) rs9939609 in the *FTO* gene has been identified as a common risk factor for obesity and T2D in several populations, including Chileans (8,9). Subsequent studies with non-European groups have discovered additional genetic variants with low prevalence among Europeans, but that are highly associated with T2D in other populations (10,11). For example, a haplotype of 5 SNPs in the *SLC16A11* gene was found in association with a 22% increase in T2D incidence in a Mexican population (12). Interestingly, this haplotype has a frequency of 50% in Mexican Native Americans but less than 1% in Europeans and Africans, therefore it was suggested that the haplotype may represent a common genetic T2D-susceptibility variant for Latin Americans (12). Although the association was later confirmed for Mexicans in the HCHS/SOL cohort, it was not replicated for other Latin American groups like Caribbeans, Central Americans or South Americans, even after the exclusion of young controls and adjustment for BMI (13). Subsequent in vitro studies have shown that the haplotype affects the aminoacidic sequence of the gene product, the monocarboxylate transporter type 11, which is most abundantly expressed in the thyroid gland and liver (14). In the latter tissue, these gene variants provoke reduced expression levels and impaired translocation of the transporter to plasma membrane, leading to intracellular accumulation of triglycerides (14).

Due to the high prevalence of obesity and T2D among Chileans and the heterogenic effect of the haplotype on T2D in different Hispanic groups, we studied the association of *SLC16A11* with adiposity and metabolic markers, using the rs75493593 SNP as a proxy for the 5 SNP haplotype in healthy Chilean adults.

MATERIALS AND METHODS

The complete sample was composed of 472 individuals from the GENADIO study, but only 263 of them had information regarding the rs75493593 genotype in the *SLC16A11* gene. The GENADIO project was approved by the ethics committees of University of Concepcion, University of Chile and University of Glasgow; and took place between 2009 and 2011. The objective was to evaluate the prevalence of risk factors for cardiovascular diseases in Chile (15). The studied population included individuals of Mapuche and European descent living in the Biobío and Los Ríos regions. The Mapuche are the most populous indigenous group in Chile, accounting for a 79.8% of the indigenous people in the country (16). Individuals were selected who had no history of metabolic or cardiovascular disease or use of prescribed drugs (15).

Allelic variant determination of *SLC16A11* gene

Allelic variants of the SNP rs75493593 in the *SLC16A11* gene were determined in genomic DNA isolated from blood leukocytes through QIAamp DNA Blood Midi Kit (QUIAGEN, Ltd, UK). Alleles were identified through real time PCR on an ABI 7900-HT thermocycler, using TaqMan pre-designed SNP genotyping assay with specific probes. All of the analyses were performed in duplicate, with a 98% of reproducibility.

Adiposity markers

The anthropometric measurements were taken by trained personnel using standardized protocols (17). Body weight and height were determined with an

electronic scale (TANITA TBF 300A, USA) and height rod (SECA A800, USA) with an accuracy of 100 g and 1 mm, respectively. Waist circumference (WC) and hip perimeter were measured with a non-distensible tape measure (SECA Model 201, US) using the anthropometric technique (17). Nutritional status was classified based on the World Health Organization's body mass index (BMI) cut-off points for adults: underweight: <18.5 kg/m^2; normal weight: 18.5-24.9 kg/m^2; overweight: 25.0-29.9 kg/m^2 and obese: ≥30.0 kg/m^2. The values used to define central obesity in men and women were WC ≥ 102 and 88 cm, respectively. Body composition was determined by measuring four skinfolds (bicipital, sub-scapular, supra-iliac and triceps) and the algorithm of Durnin and Womersley was applied to estimate the percentage of fat mass (18).

Metabolic markers and blood pressure

Blood samples were obtained by venous puncture after 10 to 12 hours of fasting. Basal glycemia, total cholesterol (TC), HDL-cholesterol (HDLc) and triglycerides (TG) were measured using enzymatic end-point methods (Roche Diagnostics GmbH, Mannheim, Germany); and the enzymes gamma-glutamyltransferase (GGT) and alanine aminotransferase (ALT) were determined through kinetic assays (Randox Laboratories Ltd., Co. Antrim, Ireland). LDL-cholesterol (LDLc) was estimated using the Friedewald equation (19). Insulin and leptin were determined by ELISA (Diagnostic System Labs, TX, USA and Linco Research Inc., St. Louis MO, USA) and HOMA-IR (Homeostasis Model Assessment of Insulin Resistance) was determined through the following formula: insulinemia in fasting (mU/mL) x fasting glycemia (mg/dL)/405 (19). High sensitivity C-reactive protein (hsCPR) was measured by immunoturbidimetry (Kamiya Biomedical, Seattle, WA, USA). The average of two determinations was considered for each sample. Systolic (SBP) and diastolic (DBP) blood pressure were taken in supine position with an automatic tensiometer (OMRON M10-IT Healthcare UK Limited, Milton Keynes, UK) after a period of 10 minutes of rest.

Sociodemographic and lifestyle variables

Sociodemographic data (age, gender, area of residence, educational level, income and ethnicity) and data associated with lifestyles were collected through validated surveys (15). Cardio-respiratory fitness was measured using the Chester Step Test and the results were registered in METs (Metabolic equivalents for task), according to Buckley and Cols. recommendations (20). Physical activity levels (PA) and sitting time were estimated by accelerometry of movement (Actigraph GTM1, USA). The intensity of PA and energy expenditure were determined by the Freedson algorithm (21).

Statistical analysis

The characterization data of the studied population are presented as averages and standard deviations (SD) for continuous variables, and as a percentages for categorical variables. A linear regression analysis was applied to determine the association between rs75493593 polymorphism and adiposity makers (body weight, BMI, WC and fat mass %). The same analysis was applied to investigate the association with metabolic markers (glycemia, insulin, $HOMA_{IR}$, TC, HDLc, LDLc, TG, ALT, GGT, hsCRP and leptin) and blood pressure (SBP and DBP).

The genotype of SNP rs75493593 was coded following an additive genetic model (0 = GG – homozygous for the protective allele, 1 = GT – heterozygous for the risk allele, 2 = TT – homozygous for the risk allele), and subsequently the increase in the health outcome (adiposity or metabolic marker) was estimated for each additional copy of the risk variant (T allele) by linear regression analysis. These results are presented as averages or beta coefficients along with their respective 95% confidence interval (95% CI).

The adiposity marker data were adjusted for confounding variables by using three statistical models: Model 0 – unadjusted; Model 1 – adjusted for age, gender, ethnicity, educational level, income, socioeconomic status and area of residence (urban/rural); Model 2 – adjusted for model 1 but also for PA, sitting time and smoking. An additional statistical model was included for the data on metabolic markers and blood pressure: Model 3, which incorporated BMI as a confounder. The distribution of the Hardy-Weinberg equilibrium of the alleles of the *SLC16A11* gene was evaluated by the Chi-square test. The STATA SE v14 program was used for all of the analyses. The level of significance was defined as $p < 0.05$.

RESULTS

The cohort characteristics according to *SLC16A11* genotype (GG, TG or TT) are presented in Table 1.

In general, carriers and non-carriers of risk allele (T) showed only minor differences in sociodemographic, physical activity and adiposity markers among (Table 1). However, the prevalence of the protective genotype (GG) is higher in Europeans than in Mapuches (65% versus 34%; Table 1). The allele frequency in the *SLC16A11* locus was 0.703 for the protective allele (G) and 0.297 for the risk allele (T), which is distributed according to the Hardy-Weinberg equilibrium (X^2= 0.738, Table 2).

Table 1. Cohort characteristics according to *SLC16A11* genotype (rs75493593)

Variable	*SLC16A11* genotype (rs75493593)		
	GG	GT	TT
n	129	112	22
Age (years)	36.1 ± 13.8	37.8 ± 12.3	34.1 ± 11.9
Gender (% women)	57	58	50
Place of residency (% urban)	64	54	64
Ethnia (%)			
European	65	55	54
Mapuche	34	44	45
Education (%)			
Elementary	12.4	22.5	13.6
Secondary	53.4	32.4	72.7
Higher	34.1	40.1	13.6
Income (%)			
Low	28.9	34.5	31.8
Medium	14.8	15.5	9.9
High	56.2	50.0	59.1
Smoking (%)			
Yes	58	50	59
No	41	50	41
Physical activity & fitness			
Physical activity (MET/min/week)	872.9 ± 287.9	912.6 ± 279.0	825.5 ± 329.7
Sitting time (min/day)	525.3 ± 92.6	514.5 ± 87.7	555.4 ± 103.4
Adiposity			
Body weight (kg)	70.2 ± 10.5	70.9 ± 10.6	72.5 ± 9.3
BMI (kg/m^2)	27.2 ± 3.8	27.9 ± 3.6	28.6 ± 3.9
Nutritional status (%)			
Underweight	0.8	0	0
Normal	25.6	25	18.2
Overweight	46.5	50.9	45.4
Obese	27.1	24.1	36.4
Waist circumference (cm)	94.7 ± 12.0	96.4 ± 9.9	98.4 ± 10.3
Central obesity (%)	59.7	56.2	63.6
Fat mass (%)	29.3 ± 4.6	29.3 ± 4.7	29.2 ± 4.6

Data presented as mean and standard deviation for continuous variables and as % for categorical variables.

Table 2. Allele frequency of rs75493593 in *SLC16A11* gene

rs75493593	n	Genotype frequency (%)	Allele frequency (%)	p value for HWE
GG	129	49.1	70.3	0.738
GT	112	42.6		
TT	22	8.4	29.7	

HWE: Hardy Weinberg Equilibrium.

The association between genotype in the *SLC16A11* locus and adiposity markers are presented in Table 3 and Figure 1A. Although we found higher body weight, BMI and waist circumference in carriers of the risk haplotype, only BMI showed a statistically significant increase for each copy of the risk allele. In the unadjusted model, BMI increased by 0.7 kg/m² for each copy of the risk allele but the change was not significant (p = 0.052). When the association was adjusted by socio demographic variables in Model 1, it remained not significant (p = 0.053) and the increase in BMI was reduced to 0.65 kg/m² for each copy of the risk allele. Only when physical activity variables were included in the fully adjusted model did the association reached significance (p = 0.033). The strength of the association remained practically unchanged in the model 3, with an increase of 0.7 kg/m² (95% CI: 0.05; 1.33) in BMI for each copy of the risk allele.

The association between genotype in the *SLC16A11* locus and metabolic markers are presented in Table 4 and Figure 1B. Among glucidic metabolic markers, we found a significant association between the risk allele of *SLC16A11* with higher insulin levels but not with glycemia, $HOMA_{IR}$ or leptin levels. In the unadjusted

Table 3. Association of *SLC16A11* genotype (rs75493593) with adiposity markers

Variables	*SLC16A11* genotype (rs75493593)			Effect of the additive genetic model	p value
	GG	GT	TT		
Body weight (kg)					
Model 0	70.2 (68.4; 72.0)	70.9 (67.0; 72.9)	72.6 (68.2; 77.0)	1.00 (-0.99; 2.98)	0.324
Model 1	70.3 (68.6; 72.0)	70.9 (69.1; 72.7)	72.4 (68.2; 76.6)	0.87 (-1.02; 2.77)	0.366
Model 2	70.2 (68.5; 70.9)	71.0 (69.2; 72.9)	72.3 (68.1; 76.4)	0.96 (-0.91; 2.84)	0.312
BMI (kg/m²)					
Model 0	27.2 (26.6; 27.9)	28.0 (27.2; 28.6)	28.6 (27.0; 30.2)	0.70 (-0.01; 1.40)	0.052
Model 1	27.3 (26.7; 27.9)	27.8 (27.1; 28.4)	28.9 (27.4; 30.3)	0.65 (-0.01; 1.31)	0.053
Model 2	27.3 (26.7; 27.9)	27.9 (27.2; 28.5)	28.8 (27.4; 30.2)	0.70 (0.05; 1.33)	0.033
Waist circumference (cm)					
Model 0	94.7 (92.8; 96.6)	96.4 (94.4; 98.5)	98.4 (93.8; 103.0)	1.8 (-0.28; 3.89)	0.090
Model 1	94.4 (93.0; 96.8)	96.1 (94.1; 98.1)	98.9 (94.3; 103.4)	1.63 (-0.43; 3.69)	0.122
Model 2	94.8 (93.0; 96.7)	96.2 (94.2; 98.2)	98.7 (94.2; 103.3)	1.71 (-0.35; 3.76)	0.103
Fat mass (%)					
Model 0	29.3 (28.5; 30.2)	29.4 (28.5; 30.2)	29.2 (27.2; 31.2)	-0.02 (-0.91; 0.86)	0.959
Model 1	29.3 (28.6; 30.1)	29.4 (28.6; 30.2)	294 (27.6; 30.2)	0.02 (-0.80; -0.85)	0.952
Model 2	29.3 (28.5; 30.0)	29.4 (28.6; 30.2)	29.3 (27.5; 31.1)	0.06 (-0.75; 0.88)	0.879

Data presented as means and their 95% CI. Analysis were adjusted as described in methods section. The effect of the additive genetic model represent the change in the outcome per 1 additional copy of the risk allele.

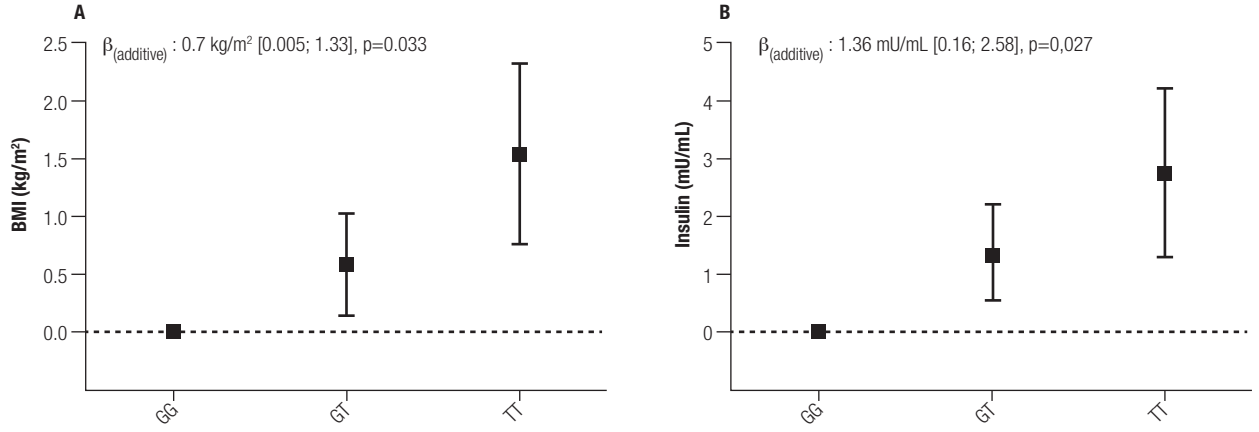

Figure 1. Association between *SLC16A11* genotype with BMI (A) and insulin (B). Data presented as differences between the reference allele (G) and the genotypes bearing the risk allele (T) and their respective standard errors. The analysis was adjusted by age, gender, ethnicity, educational level, income, place of residence, physical activity and smoking (Model 2).

Table 4. Association of *SLC16A11* (rs75493593) with metabolic markers

Variables	*SLC16A11* genotype (rs75493593)			Effect of the additive genetic model	p value
	GG	GT	TT		
Glycemia (mg/dL)					
Model 0	101.5 (98.9; 105.1)	96.9 (93.0; 100.7)	105.5 (96.9; 114.1)	-0.81 (-4.74; 3.13)	0.687
Model 1	101.5 (98.2; 104.8)	96.7 (93.1; 100.3)	106.4 (98.4; 114.4)	-0.56 (-4.26; 3.13)	0.765
Model 2	101.3 (98.0; 104.6)	97.0 (93.5; 100.6)	106.0 (98.1; 113.9)	-0.36 (-3.98; 3.24)	0.841
Model 3	101.4 (98.1; 104.7)	97.0 (93.4; 100.5)	105.8 (97.8; 113.7)	-0.54 (-4.20; 3.11)	0.770
Insulin (mU/mL)					
Model 0	6.0 (4.8; 7.3)	7.4 (6.1; 8.8)	9.2 (6.1; 12.2)	1.49 (0.12; 2.87)	0.034
Model 1	6.2 (5.0; 7.3)	7.3 (6.0; 8.5)	9.0 (6.2; 11.8)	1.28 (0.02; 2.54)	0.046
Model 2	6.1 (5.0; 7.2)	7.4 (6.2; 8.6)	8.8 (6.2; 11.5)	1.36 (0.16; 2.58)	0.027
Model 3	6.4 (5.4; 7.4)	7.2 (6.1; 8.4)	8.0 (5.6; 10.5)	0.83 (-0.30; 1.96)	0.150
Leptin (ng/mL)					
Model 0	13.0 (10.7; 15.3)	13.8 (11.2; 16.2)	11.6 (6.1; 17.2)	-0.83 (-2.62; 2.45)	0.949
Model 1	13.0 (10.8; 15.3)	13.8 (11.3; 16.2)	11.5 (6.1; 16.8)	-0.17 (-2.62; 2.27)	0.891
Model 2	13.0 (10.8; 15.2)	13.8 (11.4; 16.2)	11.4 (6.0; 16.8)	-0.14 (-2.59; 2.30)	0.909
Model 3	13.5 (11.3; 15.6)	13.5 (11.2; 15.8)	10.2 (5.0; 15.4)	-0.95 (-3.33; 1.43)	0.435
$HOMA_{IR}$					
Model 0	1.59 (1.27; 1.92)	1.80 (1.45; 2.15)	2.23 (1.45; 3.01)	0.27 (-0.08; 0.63)	0.130
Model 1	1.63 (1.34; 1.92)	1.76 (1.45; 2.08)	2.21 (1.51; 2.91)	0.22 (-0.10; 0.54)	0.173
Model 2	1.60 (1.32; 1.88)	1.81 (1.50; 2.10)	2.16 (1.49; 2.82)	0.25 (-0.56; 0.55)	0.331
Model 3	1.67 (1.42; 1.93)	1.76 (1.48; 2.04)	1.97 (1.34; 2.59)	0.12 (-0.17; 0.40)	0.418
TC (mg/dL)					
Model 0	181.2 (172.8; 189.5)	180.6 (171.5; 189.6)	189.5 (169.4; 209.7)	-0.62 (12.92;11.68)	0.921
Model 1	181.4 (173.4; 189.3)	179.7 (171.1; 188.3)	192.7 (171.5; 211.9)	2.63 (-6.11; 11.36)	0.554
Model 2	180.8 (173.0; 188.5)	180.6 (172.1; 189.0)	191.7 (173.0; 210.5)	3.10 (-5.43; 11.63)	0.475
Model 3	183.6 (177.7; 190.4)	178.8 (171.4; 186.2)	184.6 (168.0; 201.1)	-1.67 (-9.26; 5.91)	0.664
HDLc (mg/dL)					
Model 0	36.1 (33.4; 38.7)	38.2 (35.3; 41.0)	31.4 (25.0; 37.8)	-0.46 (-3.38; 2.47)	0.758
Model 1	36.2 (33.6; 38.8)	38.2 (35.4; 40.9)	30.8 (24.6; 36.9)	-0.73 (-3.56; 2.10	0.612
Model 2	36.2 (33.7; 38.8)	38.0 (35.3; 40.8)	31.0 (24.8; 37.1)	-0.81 (-3.63; 2.00)	0.572
Model 3	35.4 (33.1; 37.8)	38.6 (36.0; 41.1)	33.1 (27.5; 38.6)	-0.60 (-1.98; 3.18)	0.650
LDLc (mg/dL)					
Model 0	122.8 (113.8; 131.8)	121.6 (111.9; 131.3)	135.9 (114.3; 157.6)	3.32 (-6.52; 13.16)	0.507
Model 1	122.8 (114.2; 131.3)	120.9 (11.7; 130.2)	139.3 (118.7; 160.0)	4.08 (-5.33; 13.50)	0.394
Model 2	122.2 (113.8; 130.6)	121.8 (112.7; 130.9)	138.3 (118.2; 158.6)	4.57 (-4.65; 13.78)	0.330
Model 3	125.2 (117.8; 132.6)	119.8 (111.9; 127.8)	130.4 (112.7; 148.2)	-0.71 (-8.85; 7.44)	0.865
TG (mg/dL)					
Model 0	112.6 (102.5; 122.8)	104.9 (94.0; 115.8)	111.8 (87.3; 136.2)	-3.51 (-14.59; 7.58)	0.536
Model 1	113.2 (103.7; 122.6)	103.9 (93.7; 114.1)	113.7 (91.0; 136.5)	-3.68 (-14.04; 6.68)	0.485
Model 2	112.8 (103.4; 122.1)	104.5 (94.4; 114.7)	113.0 (90.4; 135.6)	-3.32 (-13.60; 6.96)	0.525
Model 3	115.4 (106.6; 124.1)	102.8 (93.4; 112.3)	106.2 (85.1; 127.2)	-7.89 (-17.52; 1.74)	0.108
ALT (U/L)					
Model 0	37.0 (33.1; 41.0)	36.8 (32.5; 41.0)	40.1 (30.5; 49.7)	0.77 (-3.58; 5.12)	0.727
Model 1	37.5 (33.6; 41.4)	30.1 (31.9; 40.3)	41.0 (31.6; 50.4)	0.44 (-3.82; 4.71)	0.837
Model 2	37.2 (33.4; 41.0)	36.5 (32.4; 40.6)	40.5 (31.4; 49.6)	0.70 (-3.43; 4.84)	0.738
Model 3	37.9 (34.3; 41.6)	36.0 (32.1; 40.0)	38.5 (29.7; 47.3)	-0.63 (-4.66; 3.39)	0.756

Variables	*SLC16A11* genotype (rs75493593)			Effect of the additive genetic model	p value
	GG	GT	TT		
GGT (U/L)					
Model 0	32.7 (27.8; 37.6)	33.3 (28.0; 38.6)	39.4 (27.5; 51.2)	2.18 (-3.20; 7.57)	0.426
Model 1	33.1 (28.5; 37.7)	32.8 (27.8; 37.8)	39.2 (28.1; 50.3)	1.63 (-3.41; 6.68)	0.525
Model 2	32.9 (28.4; 37.5)	33.1 (28.1; 38.0)	38.9 (27.8; 49.9)	1.79 (-3.22; 6.80)	0.482
Model 3	33.9 (29.5; 38.3)	32.4 (27.7; 37.2)	36.3 (25.7; 46.9)	0.08 (-4.78; 4.94)	0.974
hsCRP (mg/L)					
Model 0	1.41 (1.17; 1.64)	1.24 (0.98; 1.49)	1.31 (0.73; 1.88)	-1.01 (-0.36; 0.15)	0.441
Model 1	1.40 (1.17; 1.62)	1.23 (0.99; 1.48)	1.38 (0.83; 1.93)	-0.07 (-0.32; 0.18)	0.559
Model 2	1.39 (1.16; 1.61)	1.26 (1.01; 1.50)	1.36 (0.82; 1.90)	-0.06 (-0.31; 0.18)	0.618
Model 3	1.46 (1.26; 1.66)	1.21 (0.99; 1.42)	1.16 (0.67; 1.63)	-0.20 (-0.42; 0.02)	0.081
SBP (mmHg)					
Model 0	124.2 (121.3; 127.1)	121.2 (118.1; 124.3)	122.9 (115.8; 129.9)	-1.66 (-4.82; 1.51)	0.304
Model 1	124.4 (121.8; 127.1)	120.8 (117.9; 123.6)	123.7 (117.2; 130.2)	-1.76 (-4.17; 1.19)	0.242
Model 2	124.4 (121.7; 127.1)	120.8 (117.9; 130.2)	123.7 (117.2; 130.2)	-1.73 (-4.68; 1.23)	0.252
Model 3	124.7 (122.0; 127.4)	120.6 (117.8; 129.5)	122.8 (116.3; 129.3)	-2.26 (-5.21; 0.69)	0.132
DBP (mmHg)					
Model 0	75.8 (73.6; 78.0)	75.3 (72.9; 77.6)	75.9 (70.6; 81.2)	-0.23 (-2.64; 2.16)	0.844
Model 1	6.0 (73.9; 78.2)	75.0 (72.6; 77.3)	76.5 (71.3; 81.7)	-0.30 (-2.66; 2.06)	0.803
Model 2	75.9 (73.8; 70.0)	75.1 (72.8; 77.4)	76.3 (71.2; 81.5)	-0.21 (-2.56; 2.13)	0.857
Model 3	76.1 (74.0; 78.3)	75.0 (72.7; 77.2)	75.6 (70.5; 80.8)	-0.63 (-2.97; 1.71)	0.597

Data presented as means and their 95% CI. Analysis were adjusted as described in methods section. The effect of the additive genetic model represent the change in the outcome per 1 additional copy of the risk allele.

model, the mean insulin levels was 1.49 mU/mL higher (95% CI: 0.12; 2.87; p = 0.034) for each copy of the risk allele. This association remained significant after adjusting for sociodemographic variables in Model 1 (p = 0.046) and for physical activity variables in Model 2 (p = 0.027). However, the association lost significance (p = 0.150) when we included BMI as a confounder in Model 3. Regarding lipidic markers, we did not find any significant association between the risk allele and changes in total cholesterol, HDLc, LDLc or triglyceride levels. Finally, no significant association with liver enzymes ALT or GGT, the inflammatory marker hsCPR or blood pressure was found in our population.

DISCUSSION

The GWAS developed by the SIGMA Consortium for the Mexican population first revealed the association of five exonic variants of the *SLC16A11* gene with the development of T2D (12). The five polymorphisms of this haplotype respectively generate a silent mutation (L187L) and four missense mutations in the gene product (V137I, D127G, G340S and P443T) (14). Interestingly, these five SNPs segregate together, enabling us to use the SNP rs75493593 corresponding to P443T, as a proxy for this haplotype (12). The prevalence of the 5-SNP haplotype has been estimated at 50% among Mexicans of indigenous origin, 28% among Mexicans of mixed indigenous-European descent, 12% in Asians and less than 2% in the European population (12,22). Interestingly, African populations present a different haplotype that encompasses only two of the 5 SNPs, specifically to D127G and L187L. This haplotype has a prevalence of 35% among Africans, but it is not associated with T2D (12). A recent study with the HCHS/SOL cohort confirmed the association of between the 5-SNP haplotype and T2D among Mexicans, but not among other Latin American groups like South Americans, Central Americans and Caribbeans (13). The lack of association in the latter populations could be related directly to their high African ancestry (23). However the lack of association among South American populations is puzzling because their genetic background and the haplotype prevalence are similar to those of Mexican mestizos. Although our study did not investigate the association between the haplotype and T2D, our data show higher

insulin levels among Chilean haplotype carriers, and therefore supports an association with T2D among South Americans.

Mapuches are the main ancestral population in the central and southern regions of Chile and account for 79.8% of the total indigenous people in the country (16). An estimated 44% of Chileans have an Amerindian genetic component, which is similar to the proportion found in Mexican demographics (24). The 29.7% prevalence of the *SLC16A11* risk haplotype among Chileans that we report, is consistent with the similar genetic background of Mexicans. Despite our small sample size, we can observe that the prevalence of the risk haplotype is more prevalent among people of Mapuche descent than among those of Hispanic descent. Previous studies have shown that rural Mapuches have a very low prevalence of T2D (4.1%), while the prevalence is twice as high among urban Mapuches (25). In addition, Mapuches present a high prevalence of particular SNPs that are not shared with other indigenous populations in the Chilean territory, but these previous studies did not analyzed the *SLC16A11* locus (26). Given to the strong effect of rural/urban environments on T2D risk of among Mapuches it would be interesting to investigate whether the association between *SLC16A11* genetic variants and T2D interacts with environmental factors like place of residency, educational level or variables related to physical activity.

Our study reveals an association between the risk haplotype of the *SLC16A11* gene and higher levels of BMI and insulin among Chilean nondiabetic population. The higher insulin in carriers of the risk haplotype does not represent a true hyperinsulinemic state (e.g. > 15 mIU/L) (27); rather, it is related directly to the nutritional state because the association was lost when it was adjusted for BMI in Model 3. Nevertheless, it is interesting to note that the increase BMI and insulin were not accompanied by significant increases in $HOMA_{IR}$, indicating a lack of association with insulin resistance. The absence of association with waist circumference and percentage of body fat, also indicates that the body fat distribution is not consistent with insulin-resistance state, as revealed by the null association with markers of central obesity. An increase in insulin levels not connected to insulin resistance could result counterintuitive since the hyperinsulinemic state is traditionally viewed as a compensatory response to insulin resistance (28). However, new evidence is challenging this paradigm and postulating that the hyperinsulinemia would be a primary exacerbated response of beta cells to chronic overnutrition further followed by insulin resistance as a protective response by periferic tissues. This possibility is consistent with the fact that our sample comprised young subjects (36 years old, on average) without overt metabolic disturbances who could be going through an early phase of hyperinsulineamia. Whatever the reason for this dilemma, it is also possible that the modest sample size of our study may also have failed to show association with markers of insulin resistance due to the low statistical power. Other study in Mexican population have reported decreased insulin action, together with higher ALT and GGT levels in risk haplotype carriers with T2D (29). Since both studies support the involvement of the risk haplotype in the development of insulin resistance, this genetic variant could represent an early marker of T2D. In agreement with this proposal, the GWAS developed by the SIGMA Consortium, reported that the risk haplotype advances the development of T2D by 2.1 years and that its association with T2D was stronger in younger people (12). Furthermore, a case-control and case-parent trio study found an association between *SLC16A11* and the risk of pediatric-onset T2D in Mexican families (30). The association between the risk haplotype and increased BMI that we report is striking because previous studies have shown an association in the opposite direction but only among diabetics (30). Analysis of longitudinal data suggests that risk haplotype carriers lose more weight than noncarriers do after diabetes onset (31). As our data were obtained from a young nondiabetic population which could represent an early stage in the progression of diabetes, we believe that it is possible that the participants bearing the risk allele may have experienced weight loss after the establishment of T2D. This hypothesis will require further testing in a longitudinal study starting several years before the onset of diabetes.

Over 90% of SNPs associated with obesity are located in noncoding regions or even in intergenic regions of the genome (32). These locations make it difficult to define causal relationships between the SNP and disease-related functional alterations. This is the case for the obesity-susceptibility alleles in the first intron of the *FTO* gene, whose connection to obesity has been linked to their role as a cis-regulatory element of the IRX3 transcription factor (33). In

contrast, the SNP studied here directly causes a missense mutation, which initially was reported to be associated with reduced expression and abnormal subcellular localization, causing functional impairment of the monocarboxylate 11 transporter in the liver (14). Recently, Zhao and cols. reported conflicting data showing that *SLC16A11* ablation in the knockout mice did not provoke metabolic alterations related to T2D. Only the reincorporation of the mutated *SLC16A11* gene into the knockout rendered a mouse that developed excessive lipid accumulation and insulin resistance when fed a high fat diet (34). In line with the latest evidence, Zhang and cols. reported that reducing hepatic *SLC16A11* expression prevented triglyceride accumulation in the liver and maintained glucose tolerance in mice fed a high-fat diet (35). Interestingly, expression of the wildtype *SLC16A11* was induced in the liver of mice by a high fat diet and reduced by endurance exercise, which suggests that the gene is deeply involved in the sensing of lifestyle changes (35). In this sense, it is possible that the association between the haplotype of *SLC16A11* and T2D may depend largely on environmental conditions, which would explain why the association with T2D is heterogeneous among Latin American populations with very similar genetic backgrounds but living in diverse environments.

Limitations: A limitation of our study was the selection of a population with no history of metabolic diseases and with an average age of under 40 years, which prevents establishing an association of the polymorphism rs75493593 with obesity and T2D. Nonetheless, a positive association between *SLC16A11* risk genotypes with BMI and insulin was found in nondiabetic individuals, which indicates that the SNP can be considered an early risk marker for obesity and T2D. Another limitation of our study was the small sample size, which precluded association analysis for specific groups of the population, such as obese versus normal subjects. However, the statistical power was sufficient to find an association between the haplotype and both increased BMI and insulin. An eQTL analysis conducted to explore whether the association of the SNP rs75493593 are mediated by changes in the expression of the gene product would have been informative, which also constitutes a limitation of our study.

In conclusion, the data presented here shows that the SNP rs75493593 in the *SLC16A11* gene has an allele frequency of 29.7% and is associated with an increased BMI and insulin levels in the Chilean population. It will be important to perform new studies in order to estimate the contribution of different genetic variants to the development of highly prevalent diseases, such as obesity and T2D, in order to facilitate the timely identification of at risk individuals for preventive interventions. Recent mechanistic studies have attempted to elucidate the link between the functional alteration of *SLC16A11* and the pathogenesis of T2D (34-36). This information will pave the way for targeting this solute carrier, for personalized T2D treatment and prevention.

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Dynamic risk allows us to adequately select patients with differentiated thyroid cancer who do not require radioiodine treatment

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ABSTRACT

Objective: The treatment of patients with differentiated thyroid cancer (DTC) was modified in the last decade towards a more individualized approach according to the risk of recurrence (RR). We compared the outcomes of patients with low and intermediate RR (LRR and IRR) who received or did not receive radioiodine remnant ablation (RRA) after assessing the dynamic risk. **Materials and methods:** We included 307 DTC patients with LRR and IRR submitted to total thyroidectomy. All patients were reclassified according to the dynamic risk stratification (low or high). Patients with high dynamic risk received RRA (141 patients). **Results:** LRR patients who received RRA presented a frequency of structural incomplete response (SIR) of 5% at the end of the follow-up, compared to 2% in those who did not receive it (p=0.353). IRR patients treated with RRA had a frequency of SIR of 22%, compared to 5% in patients without RRA (p=0.008). **Conclusions:** This study demonstrates the usefulness of dynamic risk assessment to decide RRA in a cohort with a long-term follow-up. The lower prevalence of SIR at the end of the follow-up in patients who did not receive RRA highlights the adequate selection of those who would not benefit from RRA, even with an intermediate risk of recurrence. Arch Endocrinol Metab. 2021;65(3):315-21

Keywords

Thyroid cancer; dynamic risk; without remnant ablation; structural incomplete response

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INTRODUCTION

Differentiated thyroid carcinoma (DTC) has an excellent prognosis, with 10-year overall survival above 95% (1). Total thyroidectomy (TT) followed by radioiodine remnant ablation (RRA) was the usual treatment for DTC (2). Currently, DTC therapy is decided according to the risk of recurrence (RR) of the disease (3-5). An individualized approach is recommended by the American Thyroid Association (ATA) guidelines (6) – among those of other societies (7-9) – in which postoperative assessing is suggested to determine the RR. These stratification systems consider the histopathological report, intraoperative findings, preoperative immediate and postoperative relevant data. The static classification of the initial RR could be improved by using the dynamic risk to decide RRA. The dynamic risk implies the re-stratification of the initial RR of DTC patients considering the different responses to the treatment – excellent, indeterminate (IR), biochemical incomplete (BIR), and structural incomplete (SIR) – using specific data obtained during the follow-up: thyroglobulin (Tg) and anti-thyroglobulin antibodies (TgAb) levels and results of imaging studies, including neck ultrasound (US), computed tomography (CT), and so forth, guided by the initial RR assessment. This strategy would provide a more accurate prediction of the RR and a more individualized approach (3).

Our hypothesis was that the correct selection of patients with a low dynamic risk in the first 12 months after surgical treatment would be associated with a lower frequency of structural incomplete response in the long-term follow-up, independently of the initial RR, and probably be associated with a higher prevalence of excellent response to treatment, so the aim of this study was to compare the outcomes of patients with an initial low and intermediate RR (LRR and IRR) who received or did not receive RRA after assessing the dynamic risk.

MATERIALS AND METHODS

Data source and study population

We retrospectively reviewed our database containing 551 files records of patients with DTC who were followed up on from January 2011 to June 2018 in the Division of Endocrinology, Hospital de Clínicas-University of of Buenos Aires after implementing the decision of remnant ablation based on the dynamic risk assessment. Inclusion criteria were the following: (i) age older than 18 years, (ii) adequate clinical and pathological data to allow an accurate determination of the initial RR, (iii) a low or intermediate RR, (iv) at least two measurements of thyroglobulin (Tg) and anti-thyroglobulin antibodies (TgAb) levels, and (v) a minimum follow-up of 12 months after initial treatment to enable defining the initial response to the therapy.

Of 419 patients with LRR and IRR, 50 were excluded because they were treated with lobectomy, 40 were excluded due to lack of follow-up (less than 12 months) and 22 were excluded due to insufficient data in the follow-up. With these criteria, 307 DTC patients were included in this study.

Each patient was stratified by using the eighth edition of the American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) staging system, and the risk of recurrence was assessed by using the modified risk stratification system from the 2009 ATA guidelines proposed by the American Thyroid Association (ATA) (low, intermediate or high) (6,10).

Dynamic risk classification

After the initial response to treatment was determined, patients with initial LRR and IRR were reclassified into a low dynamic or high dynamic risk group according to the variables shown in Table 1 (3,5,11). They were divided into two groups – Group 1 (G1): n=141 patients who received RRA (patients with a high dynamic risk), and Group 2 (G2): n=166 patients who did not receive RRA (patients with a low dynamic risk) (Figure 1).

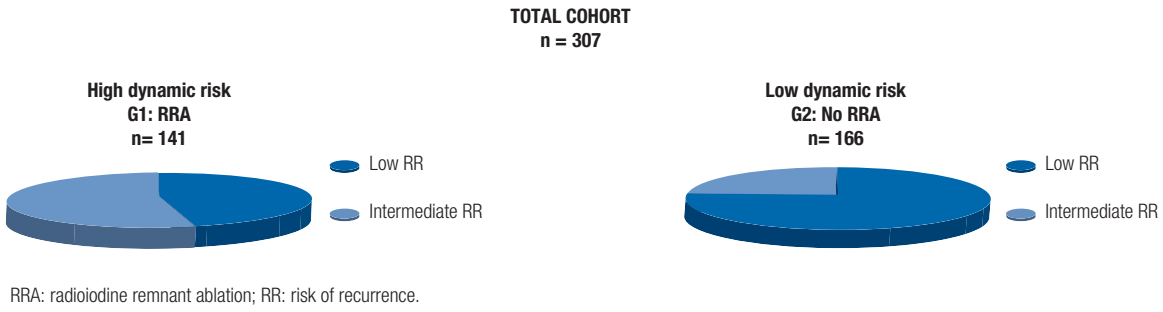
Response to therapy assessment definitions based on initial therapy

The responses to therapy (initial response to treatment and the response at the end of the follow-up) were assessed according to the American Thyroid Association guidelines (6) and the classification proposed by Momesso and cols. (12) in G1 and G2, respectively.

Table 1. Dynamic risk classification

Low dynamic risk	Initial excellent response Tg levels < 5 ng/mL under hormonal therapy without any suspicious ultrasonographic findings Indeterminate response with stable or declining TgAb levels
High dynamic risk	Initial structural incomplete response Indeterminate response with ultrasonographic suspicious findings Biochemical incomplete response with Tg > 5 ng/mL levels under hormonal therapy or increasing Tg levels during follow-up Biochemical incomplete response with increasing TgAb levels

Tg: thyroglobulin; TgAb: anti-thyroglobulin antibodies.



RRA: radioiodine remnant ablation; RR: risk of recurrence.

Figure 1. Dynamic risk classification.

Clinical management during the follow-up

Each patient was assessed with Tg and TgAb measurements under hormonal therapy (at 30, 90, and 180 days after surgery) and a neck ultrasound every 6 months after initial treatment. Neck ultrasonography was performed using a 13-MHz linear transducer. Central and bilateral neck lymph node compartments and the superior mediastinum were inspected. Suspected lesions were evaluated by US-guided fine needle aspiration cytology (FNAC) and measurement of Tg and TgAb in washing fluid.

Serum thyroglobulin and anti-thyroglobulin antibodies measurement

Serum Tg and TgAb were assessed using one commercial immunometric assay, and the same assay was used throughout a patient's follow-up. Serum Tg level was measured by Tg Electrochemiluminescence (EQLIA) Cobas e 411 (Roche) with analytical and functional sensitivities of 0.04 ng/mL and 0.1 ng/mL, respectively. A TgAb assay comprised the TgAb Electrochemiluminescence Cobas e411 (Roche). The serum TgAb level was considered negative when it was 20 IU/mL or lower, according to the manufacturer's recommendations.

Ablation protocol

Our ablation protocol used fixed radioiodine activities based on the extent of the initial disease. Therapeutic doses of ^{131}I ranged from 2.75 to 3.7 GBq (75-100 mCi ^{131}I). A low-iodine diet was prescribed from one week before radioiodine administration through two days afterwards. Radioiodine was administered following that interval in all cases with thyroid hormonal withdrawal (THW) for at least 3 weeks, starting from thyroidectomy and TSH levels above 50 mIU/L. A post-therapy whole body scan (WBS) was performed 5–7 days after therapeutic radioiodine administration.

Statistical analysis

Epidemiological data are presented as the mean ± standard deviation (SD) or as the median and range. Categorical variables are presented as percentages and absolute numbers. Categorical variables were compared using a chi-squared test or Fisher's exact test, and continuous variables using the Student's *t* test. The normal distribution of continuous data was confirmed using the Kolmogorov-Smirnov test. A p value < 0.05 was considered statistically significant. Hazard ratios and confidence intervals using log-rank analysis were also calculated. Univariate and multivariate Cox proportional hazards models were used with time to no evidence of disease as the outcome variable; the results were expressed as the hazard ratio (HR) with a 95% confidence interval. The initial risk was considered as a covariate. All statistical operations were performed using Stata 14.1 (Stata Corp, Texas, and USA).

RESULTS

Patient's characteristics

The demographic and clinical features of the included patients can be observed in Table 2. One hundred and

Table 2. Baseline characteristics of 307 patients with differentiated thyroid cancer included in the study

Sex (%, n)	
Female	83.4 (256)
Male	16.6 (51)
Age (years)	
Mean (SD)	46.1 (± 14.4)
Tumor size (cm)	
Mean (SD)	1.4 (± 1.38)
Histology (%, n)	
PTC classic	73 (224)
PTC follicular	17 (53)
PTC hidden sclerosing	2 (6)
PTC oncocytic	3.3 (10)
PTC tall cell <40%	2.7 (8)
Follicular thyroid cancer	2 (6)
Primary tumor (T) (%, n)	
T1a	39.4 (121)
T1b	33.2 (102)
T2	8 (25)
T3a	6 (18)
T3b	13.4 (41)
Regional Lymph Nodes (N) (%, n)	
N0/Nx	72.6 (223)
N1a	13.7 (42)
N1b	13.7 (42)
AJCC/UICC Stage (%, n)	
I	83.7 (257)
II	16.3 (50)
Risk of recurrence (%, n)	
Low	62.2 (191)
Intermediate	37.8 (116)
Ablative radioiodine dose (mCi)	
Mean (SD)	80.89 (± 17.86)
Follow-up (months)	
Mean ±SD	59.5 (± 22.31)

SD: standard deviation; PTC: papillary thyroid cancer; AJCC/UICC: American Joint Committee on Cancer/International Union against Cancer.

forty-one patients received RRA after surgery, and one hundred and sixty-six patients did not. According to the ATA RR classification, those patients treated with RRA, 47% and 53% were considered to have low and intermediate RR, respectively, and among patients treated without RRA, these percentages were 75% and 25%.

Initial response to treatment and status at final follow-up in patients with initial low risk of recurrence with and without radioiodine remnant ablation (low and high dynamic risk, respectively)

The frequency of initial structural incomplete response (SIR) was 9% in patients treated with RRA and 0% in patients who did not receive RRA (p=0.002). The prevalence of SIR at the final follow-up was 5% and 2% in patients with and without RRA, respectively.

Patients who received RRA had lower frequency of excellent response at the initial evaluation. The frequency of no evidence of disease (NED) at the end of the follow-up was 52% in patients treated with RRA and 73% in those who did not receive RRA (p=0.004) (Table 3 and Figure 2).

Initial response to treatment and status at final follow-up in patients with initial intermediate risk of recurrence with and without RRA (low and high dynamic risk, respectively)

The frequency of initial SIR was 24% in patients treated with RRA and 0% in patients who did not receive RRA (p=0.001). The percentage of SIR at the end of the follow-up was 22% in patients treated with RRA and 5% in those who did not receive RRA (p=0.008).

At the initial evaluation, the percentage of excellent response was similar in patients treated with and without RRA. Patients who received RRA had similar frequency of "no evidence of disease status" at the end of the follow-up to that among patients who did not receive RRA (Table 4 and Figure 3).

DISCUSSION

The dynamic risk approach was initially proposed by Michael Tuttle (3). This stratification system constitutes a paradigm shift in the management of patients with DTC, mostly in those initially classified as harboring an intermediate RR or, less frequently, in patients with a high RR. In these patients treated with total thyroidectomy and RRA, an excellent response to therapy results in a significant decrease in the likelihood of having persistent or recurrent disease (from 18% to 2% in intermediate-risk and 66% to 14% in high-risk patients). Likewise, SIR is associated with an increased likelihood of having persistent structural disease or recurrence in each of the initial risk categories (3% to 13% in low RR, 18% to 41% in intermediate RR, and 66% to 79% in high RR) (3). Several observational and prospective studies validated the risk stratification system in patients treated with total thyroidectomy and RRA, showing similar results (5,13-22).

In 2014, Momesso and Tuttle (23) proposed the definition for the responses to treatment for DTC patients treated with lobectomy or total thyroidectomy without RRA. The first validation of the dynamic risk assessment in DTC patients without RRA was

Table 3. Response to therapy in patients with initial low risk of recurrence with and without radioiodine remnant ablation

Patients with initial low RR	RRA (n = 67)	No RRA (n = 124)	P
Initial response to treatment			
Excellent response (%, n)	34 (23)	49 (61)	0.034
Indeterminate response (%, n)	46 (31)	49 (61)	0.408
Biochemical incomplete (%, n)	11 (7)	2 (2)	0.010
Structural incomplete	9 (6)	0 (0)	0.002
Clinical status at the end of follow-up			
NED (%, n)	52 (35)	73 (90)	0.004
Indeterminate (%, n)	31 (21)	23 (29)	0.154
Biochemical incomplete (%, n)	12 (8)	2 (2)	0.004
Structural incomplete (%, n)	5 (3)	2 (3)	0.353

RRA: radioiodine remnant ablation; NED: no evidence of disease.

Table 4. Response to therapy in patients with initial intermediate risk of recurrence with and without radioiodine remnant ablation

Patients with initial intermediate RR	RRA (n = 74)	No RRA (n = 42)	P
Initial response to treatment			
Excellent response (%, n)	26 (19)	38 (16)	0.117
Indeterminate response (%, n)	31 (23)	52 (22)	0.020
Biochemical incomplete (%, n)	19 (14)	10 (4)	0.140
Structural incomplete	24 (18)	0 (0)	0.001
Clinical status at the end of the follow-up			
NED (%, n)	45 (33)	64 (27)	0.032
Indeterminate (%, n)	24 (18)	29 (12)	0.386
Biochemical incomplete (%, n)	9 (7)	2 (1)	0.143
Structural incomplete (%, n)	22 (16)	5 (2)	0.008

RRA: radioiodine remnant ablation; NED: no evidence of disease.

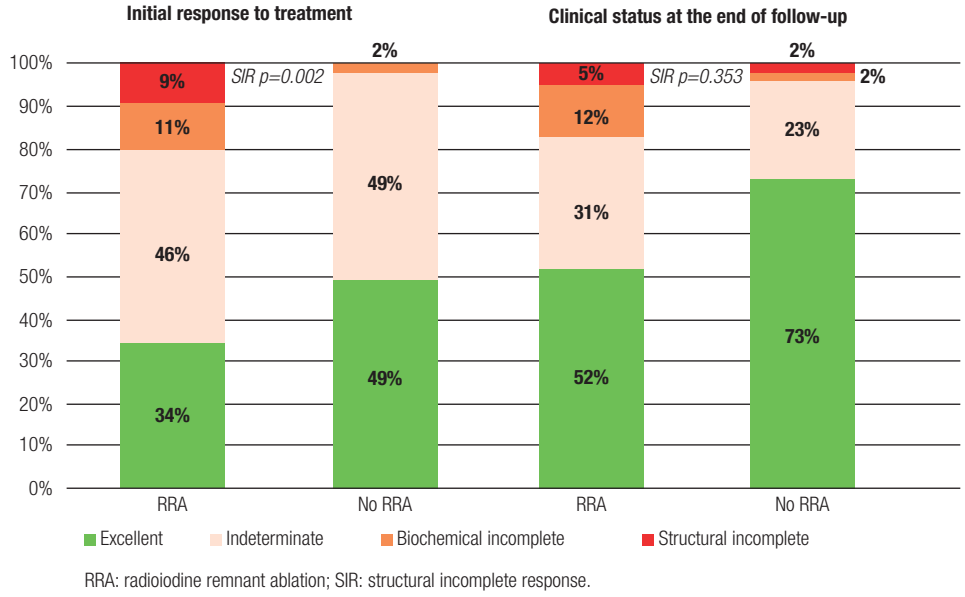


Figure 2. Response to therapy in patients with initial low risk of recurrence with and without radioiodine remnant ablation

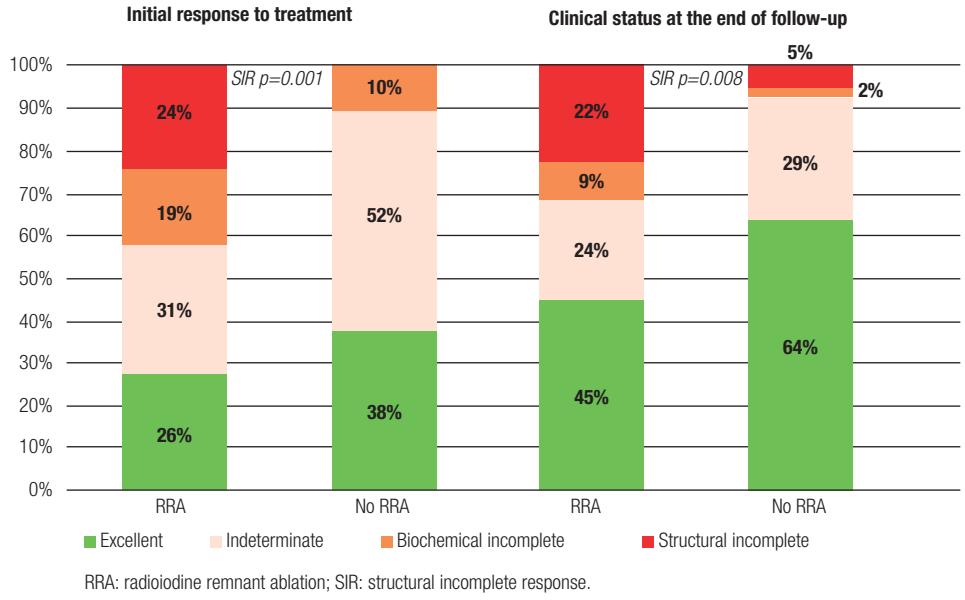


Figure 3. Response to therapy in patients with initial intermediate risk of recurrence with and without radioiodine remnant ablation

performed in 2016, showing similar results to those observed in patients who were treated with total thyroidectomy and RRA (12). Other studies reported a percentage of SIR between 1 and 2.9% at final follow-up in patients with low RR and intermediate RR who did not receive RRA (12,17,24-26).

Recently, we reported the responses to treatment in patients with low and intermediate RR in whom the decision for RRA was made immediately after surgery in comparison to the responses of patients who did not receive RRA due to the use of dynamic risk assessment (low dynamic risk). The frequency of SIR was 11.3% in patients treated with RRA and 0.9% in patients who did not receive remnant ablation, with a statistically significant difference (27). Our hypothesis regarding the high frequency of SIR in patients treated with RRA can be related to the percentage of subjects with a high probability of lymph node recurrence in the group

treated with radioiodine that probably was not changed radically by the radioiodine administration.

In most patients treated without RRA included in a previous study, Tg levels evolved spontaneously to undetectable levels, demonstrating the benefit and usefulness of the dynamic assessment to decide RRA (27).

All the published studies addressing this topic are based on retrospective observational data; nevertheless, three prospective randomized, multicenter clinical trials that are currently ongoing will surely clarify this issue (28-30).

Our current study compared the outcome of DTC patients with low RR and intermediate RR who received RRA with the outcome of those who did not receive RRA in the dynamic risk. The frequency of SIR was higher in patients who did not receive RRA without a statistically significant difference when it was compared with those patients who received RRA. However, we found statistically significant differences in the dynamic risk when we compared the initial and final SIR in patients with initial low and intermediate RR who received RRA with those in patients who did not receive RRA. Five patients who did not receive RRA evolved with locoregional recurrence (cervical lymph nodes) during the follow-up, and all of them underwent a new cervical surgery. On the other hand, five patients with initial SIR who received RRA evolved without structural disease, but surgical treatment was necessary to achieve this status. Our hypothesis was that the RRA did not impact the frequency of SIR during the follow-up of patients who received RRA. One of the strengths of our study was the applicability of dynamic risk for RRA in a cohort with a long-term follow-up. The main limitation of our study was its retrospective design.

We observed that patients with intermediate RR who did not receive RRA considering the dynamic risk had a good prognosis, having a higher frequency of excellent response and lower prevalence of SIR at the end of the follow-up compared to those with initial intermediate RR. This clearly demonstrates that the dynamic risk stratification also allows for predicting the risk of structural incomplete response during the long-term follow-up in patients with initial intermediate RR who do not receive RRA.

In conclusion, this study demonstrates the usefulness of the dynamic risk assessment in the decision on radioiodine remnant ablation in a cohort with a long-term follow-up. The higher frequencies of excellent responses associated with a lower prevalence of structural incomplete response at the end of the follow-up in patients who did not receive remnant ablation highlights the adequate selection of those who would not benefit with this approach.

Ethical approval: the study was approved by the Institutional Review Board.

Informed consent: informed consent was obtained from all participants included in the study.

Disclosure: no potential conflict of interest relevant to this article was reported.

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Percutaneous injection of ethanol for thyroid nodule treatment: a comparative study

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ABSTRACT

Objective: Percutaneous ethanol injection (PEI) is an alternative to surgery for the treatment of thyroid nodules (TNs). However, size reductions of treated (TTNs) and untreated TN (UTNs) have not been compared. Volumetric reductions in TTNs with PEI were evaluated by comparing TTNs and UTNs in the same patient, and independent variables predicting good post-PEI outcomes were analyzed. **Materials and methods:** Overall, 282 patients with multinodular goiters were selected. Two nodules located in different lobes were compared for common disease behaviors. Overall, 150 nodules were selected from 75 patients (6 M: 69 F) with a mean age of 50.1 ± 17.4 years. This prospective nonrandomized intervention study prioritized treating TNs of greater volume or single hyperfunctioning TNs. A single observer experienced in PEI and an ultrasound specialist performed the interventions. **Results and discussion:** TTNs (mean volume: 14.8 ± 16.2 mL) were reduced by 72.6 ± 27.3% of their initial volume, while UTNs increased by a mean of 365.7 ± 1.403.8% ($p < 0.00001$). The patients underwent a mean of 4.0 ± 3.1 outpatient PEI sessions without relevant complications. Logistic regression analysis showed that the magnitude of the PEI induced reduction was associated with the number of treatment sessions ($p = 0.03$, CI [1.1-38.2]) and not with ultrasonographic characteristics of the nodules. Each PEI session increased the rate of TN reduction by a factor of 6.7. **Conclusions:** PEI is a well-tolerated outpatient procedure that effectively reduces the volume of TNs and is noticeably superior to conservative treatment for all ultrasonographic classifications. Arch Endocrinol Metab. 2021;65(3):322-7

Keywords
Thyroid nodule; nodular goiter; ethanol; ablation techniques

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INTRODUCTION

Many studies have reported a high frequency of thyroid nodules (TNs) in autopsies of people without thyroid dysfunction (1-3). More than 50% of women aged over 50 years have a TN (2). More than 90% of TNs are benign (3), and few will grow to compress cervical structures, causing esthetic concerns or hyperfunctioning nodules. Thus, the ideal treatment for such a prevalent pathology should be easy to perform, free of complications, and cost effective.

Benign TNs are managed through observation, leading to good outcomes (4). Surgical TN treatment, the main therapeutic option for malignant TNs and large benign TNs, is cautiously considered, even in certain malignant TN cases (5,6). Surgery often results in definitive hypothyroidism, and rarely, recurrent laryngeal nerve lesions and hypoparathyroidism are reported to produce dysphonia and hypocalcemia associated with changes in bone quality (7). Sclerotherapy by percutaneous ethanol injection (PEI), endorsed as a

safe and effective alternative for cystic TNs (8,9), is also an effective therapy to reduce solid TNs of different sizes (10), whether hyperfunctioning or not. PEI is a safe outpatient procedure without complications and with short- and long-term TN-reducing effects. Thus, the objective of the present study is to evaluate PEI for multinodular goiter treatment.

MATERIALS AND METHODS

This prospective nonrandomized intervention study used convenience sampling and a nonrandomized control group due to ethical considerations. The largest (dominant) or hyperfunctioning nodule was treated to respect this clinical interest.

The multinodular goiter patients had a PEI-treated (case) and a PEI-untreated (control) nodule. These were in different lobes to avoid confounding treated and untreated TNs. A volume reduction below 0.7% between sessions represented no reduction (10).

In total, 282 patients were treated using PEI between May 2001 and August 2017, initially at Professor Edgar Santos University Hospital and subsequently at São Rafael Hospital (Salvador, BA, Brazil). Of these initial 282, 75 patients with multinodular goiter (two TNs) were selected, totaling 150 nodules evaluated. The inclusion criteria were older than 16 years of age, nodules diagnosed as benign by two puncture aspirations, and no family history of thyroid cancer. Thyroid hormone treatment for the sole purpose of reducing TN volume, a protocol no longer recommended by the American Thyroid Association (8), was discontinued before alcohol sclerotherapy.

The patients underwent clinical and laboratory evaluation, including 99mTechnetium-sestamibi thyroid scintigraphy and a radioactive iodine uptake test (provided by *Instituto de Pesquisas Energéticas e Nucleares* – IPEN, São Paulo, Brazil), when excess thyroid hormone and absent antithyroid antibodies suggested a hyperfunctioning TN. Patients with a heterogeneous pattern on thyroid ultrasound, characteristic of thyroiditis, were excluded to avoid confounding TN and thyroiditis pseudonodules.

The ALOKA SSD 1700 software DYNAVIEW II® Doppler ultrasound scanner with a 7.5-MHz transducer was used for the ultrasonographic study to classify the TNs as follows: 1) solid, 2) predominantly solid with cysts, 3) mixed, when it was not possible to measure cysts dispersed in solid areas, 4) predominantly cystic, and 5) cystic.

Both treated (TTNs) and untreated (UTNs) TNs were measured at their largest diameter, and their volume was calculated using height, width, and anteroposterior diameter multiplied by a constant (0.52). Absolute (99.6 GL) and sterile ethanol (v/v) (both ethanol products from Health Tech, Alto da Mooca, São Paulo, Brazil) were added to 10 mL ampoules using 5 mL and 10 mL disposable syringes. The ethanol dose injected percutaneously during each PEI session was not previously defined, as it was based on the level of patient acceptance and specific TN characteristics. In cystic lesions, the volume of ethanol injected was approximately the same as the volume of liquid content aspirated. The procedure was performed by the same professional (Daysi Alcântara-Jones) experienced with this technique since 2001.

The degree of TN volume reduction was calculated using the equation: $Degree\ of\ reduction\ (\%) = 100 - \left(\frac{100\ x\ Vf}{Vi}\right)$, where *Vf* is the final volume (after treatment) and *Vi* is the initial volume (before treatment). The degree of TN reduction in the largest diameter was calculated by $Degree\ of\ reduction\ (\%) = 100 - \left(\frac{100\ x\ Mf}{Mi}\right)$, where *Mf* is the largest diameter of the nodule after PEI and *Mi* is the largest diameter before PEI. The TNs that grew were registered with negative values to indicate a negative reduction. To understand the relationship between the number of PEI sessions and TN reduction, the variable "number of sessions" was stratified into "one session", "two to four sessions" and "five sessions or more".

The criteria for evaluating completion of treatment were as follows: nodule size smaller than 0.5 cm^3 or 1.3 cm in its largest diameter; increased consistency, which made ethanol injection difficult; and absence of vascularization in a previously solid TN under treatment. The treatment was also discontinued when the needle was obstructed in solid nodules with calcification foci.

Statistical analysis

Demographic data are expressed as the mean, median, and standard deviation. Thyroid-stimulating hormone (TSH) levels were stratified as hyperthyroidism, subclinical hyperthyroidism, and euthyroidism. The normality of the measurement distributions was assessed using the Shapiro-Wilk test. The paired t-test was used to compare the volume, largest diameter, and degree of reduction in TTNs and UTNs, and the Wilcoxon test was used to compare nonparametric variables. The Kruskal-Wallis test was used to assess the degree

of TTN reduction by ultrasound classification. The statistical software R version 3.6.3 was used for logistic regression analysis, which assessed which independent variables influenced TN reduction. A p-value < 0.05 was considered significant for all analyses. All volunteers signed the informed consent form after full explanation of the purpose and nature of all procedures used. The authors ensured that research involving human subjects complied with the Declaration of Helsinki, and the study was approved by the Research Ethics Committee (REC) of São Rafael and of Edgard Santos University Hospital (Salvador, BA, Brazil) in 2003, which was updated in the Plataforma Brasil database in 2017 (registration number: 2.597.674).

RESULTS

One hundred fifty patients (mean age, 50.1 ± 17.4; median, 46.5; 92.0%, female), with ages ranging between 16 and 84 years, participated in the study. No hypothyroidism cases were diagnosed during selection or after treatment (TSH mean ± SD (μIU/mL): 1.3 ± 1.1, $T4_L$ mean ± SD (ng/dL): 1.3 ± 1.0). There were 12 hyperfunctioning TNs: 4 (6.5%) hyperthyroidism and 8 (13.1%) subclinical hyperthyroidism. Ultrasonographic standards of TTNs were as follows: solid: 29 (39.1%), predominantly solid: 12 (16.2%), predominantly cystic: 11 (14.9%), cystic: 9 (12.2%), and mixed: 13 (17.5%). Ultrasonographic standards of UTNs were as follows: solid: 32 (47.0%), predominantly solid: 10 (14.7%), predominantly cystic: 4 (5.9%), cystic: 13 (19.1%), and mixed: 9 (13.2%). There was loss of information on the ultrasound pattern of one TTN and seven UTNs. TTN localization was as follows: right side: 44 (58.7%), left side: 30 (40.0%), and isthmus: 1 (1.3%). UTN localization was as follows: right side: 33 (44.0%), left side: 35 (46.7%), and isthmus: 7 (9.3%).

Table 1 shows that the difference between the measurements of the TTNs before and after PEI was highly significant with regard to TNs volume and largest diameter, while the table shows that there were no significant reductions in UTNs with regard to TNs volume and largest diameter. The difference in the degree of reduction in the volume of the TTNs (72.6 ± 27.3% [mean ± SD]) and UTNs (-365.7 ± 1403.8% [mean ± SD]) was very significant (p-value: 5.0×10^{-12}) and similar to the degree of reduction in the largest diameter of TTNs (43.2 ± 28.3 [mean ± SD]) compared to UTNs (-85.4 ± 251.6 [mean ± SD]) (p-value: 1.1×10^{-12}). The UTNs, unlike the TTNs, grew during the observation period but developed no clinical signs due to their small size (mean initial largest diameter of 1.1 cm ± 0.8 SD and mean initial volume of 0.7 ± 1.4 SD mL). The percentages of the mean reduction in TTNs distributed by each ultrasound standard are shown in Figure 1.

The number of sessions was heterogeneous, ranging from one to eight sessions. The patients underwent 4.0 ± 3.0 (median, 3) PEI sessions, and the duration of treatment was 22.6 ± 27.6 (median, 10) months. Figure 2 shows the mean reduction percentage achieved in TTNs stratified by the number of sessions.

Table 1. Metric indices of untreated and treated TNs using PEI

	Before	After	P-value
Treated TN			
Volume in cm3 (mean ± SD) (N = 75)	14.8 ± 16.2	3.5 ± 5.5	2.5×10^{-8}
Largest diameter in cm (mean ± SD) (N = 75)	3.9 ± 2.1	2.1 ± 1.2	10.8×10^{-8}
Untreated TN			
Volume in cm3 (mean ± SD) (N = 71)	0.73 ± 1.4	1.3 ± 2.6	0.32
Largest diameter in cm (mean ± SD) (N = 73)	1.1 ± 0.8	1.4 ± 1.0	0.3

Abbreviations: PEI, percutaneous ethanol injection; TN, thyroid nodule

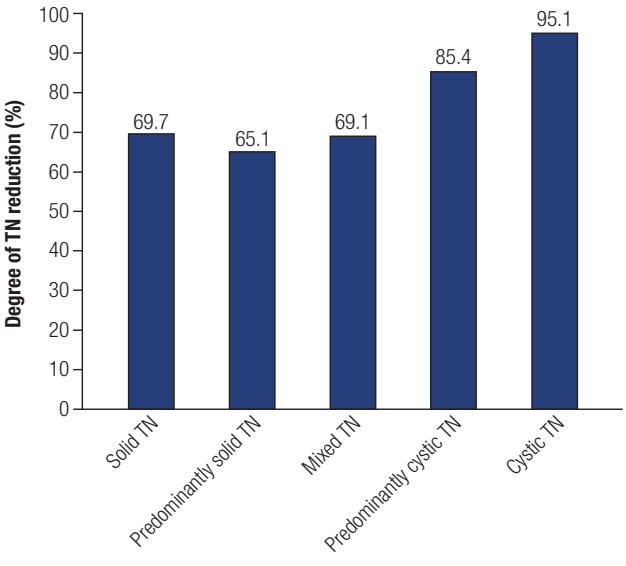

Figure 1. Degrees of volume reduction in TTNs by ultrasonographic standard.

The degrees of largest diameter reduction in the TTNs by ultrasonographic standards were as follows: (solid TN: 40.7%, predominantly solid TN: 34.8%, mixed TN: 35.2%, predominantly cystic TN: 53.1%, cystic TN: 63.8%).

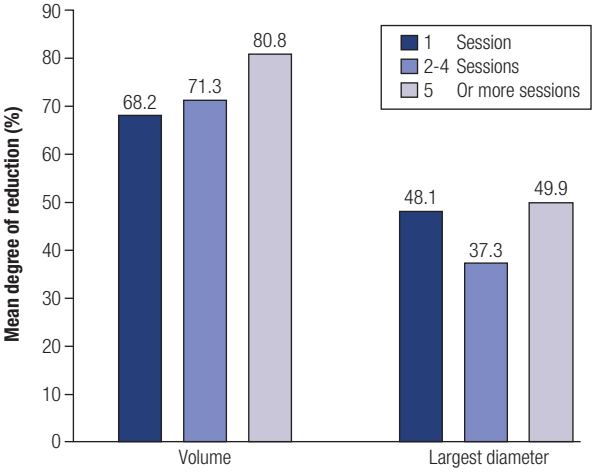

Figure 2. Degree of reduction in TTNs stratified by number of sessions.

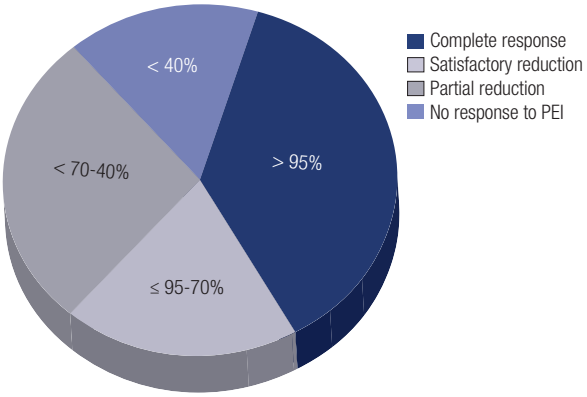

Figure 3. Treated thyroid nodule volume reduction (%).

Only 43 (57.3%) patients met the criteria to complete the treatment and were observed in the long term. The results of the other 32 patients presented here refer to the measurements of the nodules one to two weeks after the second-to-last PEI session.

No response to treatment was considered when the reduction was <40% of the initial volume (Figure 3). Partial reduction was considered when the reduction was ≥40%; at this level, the patient usually reports that the TN has "disappeared", being able to sleep in lateral decubitus position homolateral to the nodule and/or no longer complaining of obstruction such as coughing and hoarseness. Considering a ≤ 40% volume reduction (no response) as the dependent variable in logistic regression analysis, the PEI reduction power was associated with the number of treatment sessions (p = 0.03, CI [1.1-38.2]) and not with the ultrasonographic characteristics of the nodules and that each PEI session increased the rate of TN reduction by a factor of 6.7 times (p = 0.03).

Pain in the jaw or tooth homolateral to the nodule being treated was a rare self-reported complaint at the time of the injection, that ceased in seconds. Younger patients often said, "it hurts like a puncture", referring to puncture aspiration of the thyroid, or "it hurts like a tattoo". A local burning sensation, discomfort, and eventually facial redness were reported. Sometimes, patients reported feeling nothing in one session and pain in another. They classified pain as grade 6 to 7 on the visual analog scale while smiling. Older patients may have complained of pain from the hyperextended neck position necessary during PEI. A patient who presented dysphonia in a PEI session reestablished her voice one week later.

DISCUSSION

This work compared the degree of reduction in TTNs and UTNs in the same patient, which is considered an ideal comparison model since genetic and environmental patterns were the same for treatment and control. This model to assess this pathology has hitherto not been found in the literature with regard to comparing two usual behaviors in TN monitoring: observation (11,12), which was used for smaller and/or nonfunctioning TNs, and PEI, the technique to be tested. It is a weakness of the study that the volumes of NTs, TTNs and UTNs are not comparable at the beginning, respecting ethical principles. The strong point was having the same observer performing all ultrasounds and a single observer performing all PEI sessions.

This study reported that while some UTNs may have grown, there was a statistically significant reduction in TTNs, similar to the percentages previously reported (13-15). It is possible that, due to the small volume of the UTNs, a slight change (1-2 mm) in the position of the USG probe outside the nodule may have resulted in a measurement bias and a significant increase in the small volume of these UTNs. This possible measurement bias in large TTNs would be insignificant. A similar experimental study with 49 patients undergoing PEI and a control group receiving conservative treatment reported a volume reduction rate of 78.2% in the TTNs, with statistically significant differences between the pre- and postintervention volumes (16). However, the control group showed no statistically significant difference between the pre- and post-observation volumes.

Solid TNs developing over a long period often have gross calcification foci that can obstruct the needle during a PEI session. Immediate repuncture is not prudent, and interruption of the session is recommended to avoid ethanol leakage through the initial puncture. This condition can increase the number of total sessions during treatment. Solid TN should be frequently monitored, and early treatment is indicated once it may result in a substantial reduction with fewer PEI sessions, thereby improving PEI outcomes.

PEI is recommended as a first-line treatment for cystic nodules (8). The comparison between TN reduction by volume and largest diameter showed that the more cystic the nodule was, the greater the response to PEI. Studies exclusively analyzing cysts and/or pseudocysts (9,14,17) reported degrees of reduction greater than or equal to 70%. Better PEI results (fewer sessions) for cystic TNs are due to the aspiration of the liquid inside the cystic or predominantly cystic TNs, which reduces the volume of the nodule. Ethanol avoids liquid recurrence when compared with simple USG-guided fine needle aspiration (FNA) of cystic TNs (8).

There was a more uniform TN reduction response in relation to the number of sessions (Figure 2) considering TN volume rather than the largest diameter. TNs have many different forms, and the formula used to measure spherical structures in different directions, such as TNs, is not ideal. There is a tendency to start alcohol sclerotherapy by infiltrating the areas distant from the carotid, jugular, trachea and esophagus to avoid complications. An oval TN (longer than round) tends to be very close to these structures, slightly reducing its largest diameter and showing less response to PEI when compared to a round TN, which is more uniformly reduced in length, width and depth. The tumor effect is the most important cause of cervical and thoracic compression, which is based on the TN volume and not its diameter. Figure 2 compares TN volume reduction with the number of PEI sessions, showing a positive association between the number of sessions and volume reduction. This relationship was previously described when TN treatment was limited to one or two PEI sessions, which resulted in a lower reduction rate mainly in solid TNs (10).

The results of the present study were underestimated because in 42.7% of cases, the treatment was discontinued, not due to technical criteria but for abandonment. Since the strength of the PEI session to reduce TNs has been proven, it is assumed that if the data from the last session were known, the results obtained in this study would be even better.

Although solid and mixed TNs require more sessions, their response to PEI has been quite satisfactory (10,18,19). In addition, TN reduction occurs even after the end of treatment (20-24). Cystic and predominantly cystic TNs treated with PEI were reported to exhibit a volume reduction of 93% after a seven-year follow-up (23). PEI is safe and effective for treating toxic adenoma and pre-toxic adenoma, and it is widely available in Europe (25).

Additionally, PEI sessions and puncture aspiration have similar costs (23,25). PEI is recognized as an effective low-cost treatment without significant side effects (26).

A lower cutoff point (currently 4 cm) (8) for benign TN would better define treatment, as smaller TNs can be reduced with fewer sessions.

In conclusion, ethanol ablation is a proven method that significantly reduces TN volume and largest diameter both in solid and cystic nodules. It is a well-tolerated procedure, noticeably superior to conservative treatment for all ultrasonographic classifications and can be indicated as a first-line treatment for benign TN.

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Third trimester HbA1c and the association with large-for-gestational-age neonates in women with gestational diabetes

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ABSTRACT

Objective: To evaluate the association between HbA1c levels measured in the third trimester and the risk for large for gestational age (LGA) in neonates of mothers affected by gestational diabetes mellitus (GDM). Secondarily, we aimed to identify an ideal cut-off for increased risk of LGA amongst pregnant women with GDM. **Materials and methods:** Observational retrospective review of singleton pregnant women with GDM evaluated in a diabetes and pregnancy clinic of a tertiary and academic hospital. From January/2011 to December/2017, 1,085 pregnant women underwent evaluation due to GDM, of which 665 had an HbA1c test in the third trimester. A logistic regression model was performed to evaluate predictors of LGA. A receiver-operating-characteristic (ROC) curve was used to evaluate the predictive ability of third trimester HbA1c for LGA identification. **Results:** A total of 1,085 singleton pregnant women were evaluated during the study period, with a mean age of 32.9 ± 5.3 years. In the multivariate analysis, OGTT at 0 minutes (OR: 1.040; CI 95% 1.006-1.076, p = 0.022) and third trimester HbA1c (OR: 4.680; CI 95% 1.210-18.107, p = 0.025) were associated with LGA newborns. Using a ROC curve to evaluate the predictive ability of third trimester HbA1c for LGA identification, the optimal HbA1c cut-off point was 5.4% where the sensitivity was 77.4% and the specificity was 71.7% (AUC 0.782; p < 0.001). **Conclusions:** Few studies in the Mediterranean population have evaluated the role of HbA1c in predicting neonatal complications in women with GDM. A third trimester HbA1c > 5.4% was found to have good sensitivity and specificity for identifying the risk of LGA. Arch Endocrinol Metab. 2021;65(3):328-35

Keywords
Third trimester HbA1c; large for gestational age; gestational diabetes mellitus; neonatal complications

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INTRODUCTION

The worldwide prevalence of gestational diabetes mellitus (GDM) has increased (1) and it is nowadays the commonest endocrine pregnancy complication.

In 2008, the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study was the first large-scale multinational study to show that maternal hyperglycemia between 24-28 weeks was linearly and positively correlated with large-for-gestational-age (LGA) infants, caesarian rate, cord-blood serum C-peptide level, and neonatal hypoglycemia. No glycemic threshold for a greater risk was identified for most outcomes. In fact, GDM is associated with several adverse maternal and fetal outcomes; one of the most

worrying is the increased risk for macrosomia and later obesity in the offspring (2-6).

Pregnancies complicated by GDM result in maternal and fetal hyperglycemia. When maternal hyperglycemia occurs, glucose in excess crosses the placenta and reaches the fetal circulation, stimulating fetal insulin secretion. Hyperinsulinemia and glucose excess *in utero* cause insulin-sensitive tissue hypertrophy, promoting accelerated growth that can lead to macrosomia and/or large-for-gestational-age (LGA) neonates (1,4,7,8). GDM severity during pregnancy has been clearly linked with fetal overgrowth (2,5,6,9,10,11), while conversely, HbA1c improvement has been associated with a lower risk of LGA (12). HbA1c, a measure of glycated hemoglobin that serves as an indicator of blood glucose control in the three months prior, can also provide information about metabolic control in pregnancy. During pregnancy, interpretation of HbA1c should take into consideration not only the hemodilution phenomenon but also the existence of a reduced erythrocyte life span, especially in late pregnancy (13,14). Third trimester HbA1c target is not yet defined in women with GDM, nor it is currently used to screen for GDM complications. Given the potentially serious consequences for the mother and the child, there is significant interest in predicting the occurrence of LGA, and its accurate identification holds potential for guiding appropriate management and intervention.

The aim of this study was to evaluate the association between HbA1c levels measured in the third trimester and the risk for LGA in neonates of mothers affected by GDM. Secondarily, we aimed to potentially identify an ideal cut-off for increased risk of LGA newborns amongst pregnant women with GDM.

MATERIALS AND METHODS

We performed an observational retrospective review of singleton pregnant women with GDM in a Diabetes and Pregnancy Clinic of a tertiary and academic hospital, *Centro Materno-Infantil do Norte, Centro Hospitalar e Universitário do Porto*, Portugal. From January/2011 to December/2017, 1,085 pregnant women underwent evaluation due to GDM, 661 of whom had a measurement of third trimester HbA1c.

Diagnosis and classification of GDM were performed according to the International Association for Diabetes in Pregnancy Study Group (IADPSG) 2010 recommendations: fasting plasma glucose (FPG) ≥ 92 mg/dL (5.1 mmol/L) but < 126 mg/dL (7.0 mmol/L) in first trimester or FPG ≥ 92 mg/dL (5.1 mmol/L) and/or glucose ≥ 180 mg/dL (10.0 mmol/L) and/or ≥ 153 mg/dL (8.5 mmol/L), at 1 h and 2 h, respectively after the ingestion of glucose in the 75 g oral glucose tolerance test (OGTT) performed between weeks 24 and 28 of pregnancy (7). Medical nutrition therapy alone or combined with hypoglycemic drugs was the treatment given to achieve the following therapeutic goals: glucose before meals ≤ 95 mg/dL (≤5.3 mmol/L); glucose 1 h after meals: ≤140 mg/dL (≤7.8 mmol/L), according to national standards (15).

Gestational age was estimated by the last menstrual period and was confirmed or corrected by ultrasonography. Pre-pregnancy BMI was calculated from self-reported pre-pregnancy weight and height. Excess gestational weight gain was defined by the IOM guidelines (16). An infant was classified as LGA if its birth weight was ≥ 90th percentile for gestational age, or small-for-gestational-age (SGA) if its birthweight was < 10^{th} percentile for gestational age, based on the Fenton chart (17). Macrosomia was defined as a newborn weight greater than 4,000 g. Prematurity was defined as delivery occurring before the gestational age of 37 weeks. A composite outcome of neonatal complications was created and included at least one of the following: neonatal respiratory distress, neonatal hypoglycemia, neonatal jaundice (requiring phototherapy), shoulder dystocia, clavicle fracture, Erb's palsy or admission to the neonatal intensive care unit.

Relevant demographic, maternal, and infant data, such as maternal age, obstetric history (parity and previous macrosomia), treatment of GDM, Apgar scores at 1 and 5 minutes, adverse perinatal events and congenital malformations were also recorded. Third trimester HbA1c values were recorded at a median of 34 (IQR: 31-37) weeks of gestation. HbA1c was evaluated using an affinity chromatography method (Variant II turbo, BioRad Laboratories, CA, USA), with intra- and inter-assay coefficients of variation of < 0.78% and < 0.66%.

Statistical analysis

Statistical analysis was performed using IBM SPSS® version 25.0 and MedCalc®, p-values < 0.05 were considered significant. For continuous quantitative variables, distribution normality was tested through

histogram observation and kurtosis and skewness analysis. The results are presented as mean values ± standard-deviation and median values (25-75 percentiles). The chi-square test was used to analyze differences between groups in categorical variables. The Student t-test for independent variables and the Mann-Whitney test were used to compare continuous variables with normal and non-normal distribution between groups, respectively. A logistic regression model was performed to evaluate predictors of LGA, adjusting for potential confounders using a stepwise regression with a forward inclusion approach. A receiver-operating-characteristic (ROC) curve was used to evaluate the predictive ability of third trimester HbA1c for LGA identification.

This study was approved by the local Ethics committee (157-DEFI/156-CES). Due to the retrospective nature of the study, consent to participate was waived by the Ethics Committee.

RESULTS

A total of 1,085 singleton pregnant women were evaluated during the study period; 85% (n = 922) were Portuguese Caucasian women with a mean age of 32.9 ± 5.3 years. Regarding pre-pregnancy BMI, 34.5% (374/1,085), 34.6% (375/1,085) and 29.5% (320/1,085) of the women had normal weight, overweight, and obesity, respectively. Considering the IOM recommendations for weight gain during pregnancy, 31.5% had an excessive gain. Mean gestational age at delivery was 38.5 ± 1.5 weeks and 7.8% (85/1,085) were preterm. Concerning the 1,085 newborns, mean neonatal birth weight was 3,188.5 ± 49.5 g, 4.5% (49) of them were LGA, 12.4% (134) were SGA using the Fenton chart, but using the International Standards for Size at Birth, the percentage of LGA increases to 6.9% (75) and SGA reduces to 8.9% (97); 4.8% (52) were macrosomic and 19.2% (208) had at least one adverse neonatal outcome. The clinical characteristics of the pregnant women with GDM are presented in Table 1.

When comparing pregnant women with and without LGA offspring, pregnant women with LGA offspring had a greater prevalence of a previous macrosomic newborn (24.5% vs. 4.4%; p < 0.001), a higher prevalence of pre-pregnancy obesity (49.0% vs. 28.6%, p < 0.002) and a higher excessive gestational weight gain (52.1% vs. 30.6%; p = 0.002). Woman with LGA newborns also presented higher fasting blood glucose in the first trimester [92.0 (83.3-102.8) vs. 85.0 (78.0-94.0), p < 0.001), higher glucose at time 0 in the OGTT [93.5 (86.8-105.0) vs. 85.0 (77.0-92.0); p < 0.001], a higher third trimester HbA1c [5.70 (5.35-5.80) vs. 5.30 (5.00-5.50); p < 0.001] and a higher rate insulin therapy (59.2 vs. 53.3%; p = 0.001). A comparison of the clinical characteristics of pregnant women with and without LGA offspring is presented in Table 2.

Table 1. Clinical characteristics of pregnant women with gestational diabetes mellitus

	N = 1085
Age (years)	32.9 ± 5.3
Body mass index (kg/m²)	26.5 ± 5.6
Pre-pregnancy BMI category (kg/m²)	
Low weight (<18)	1.5% (16/1085)
Normal weight (18-24.9)	34.5% (374/1085)
Overweight (25-29.9)	34.6% (375/1085)
Obese (≥30)	29.5% (320/1085)
Multiparous	13.9% (151/1085)
Gestation at GDM diagnosis (weeks)*	25 (19-27)
Metformin therapy	5.2% (56/1085)
Insulin therapy	36.3% (395/1085)
Timing of insulin initiation (weeks)*	30 (23-32)
Total daily insulin dose (units)*	23 (14-27)
Gestational weight gain (IOM)	
Insufficient	35.5% (380/1069)
Adequate	32.9% (352/1069)
Excessive	31.5% (337/1069)
Gestational age at delivery (weeks)	38.5 ± 1.5
Prematurity	7.8% (85/1085)
Neonatal birth weight (g)	3188.5 ± 49.5
Small for gestational age (SGA)[1]	12.4% (134/1085)
Large for gestational age (LGA)[1]	4.5% (49/1085)
Macrosomia	4.8% (52/1085)
Apgar score at 1 minutes*	9 (8-9)
Apgar score at 5 minutes*	10 (10-10)
At least one adverse neonatal outcome	19.2% (208/1067)
Neonatal respiratory distress	2.7% (18/671)
Neonatal hypoglycaemia	4.8% (51/1065)
Neonatal jaundice	9.0% (98/1085)
Shoulder dystocia, fractures and Erb's palsy	1.3% (14/1085)
Admission to neonatal intensive care unit	4.5% (49/1085)
Congenital malformations	2.0% (22/1085)
Perinatal death	0.2% (2/1085)

Data are presented as mean ± standard deviation, unless otherwise indicated by * corresponding to data presented as median, 25th and 75th percentiles. BMI: body mass index; SGA: SMALL for gestational age; LGA: large for gestational age. [1]Fenton chart.

A logistic regression was performed to identify variables that could predict LGA risk (Table 3). In the univariate analysis, previous macrosomia history, pre-pregnancy obesity, excessive gestational weight gain, higher fasting blood glucose in the 1st trimester and at 0' in the OGTT, need for insulin therapy, and higher third trimester HbA1c were factors associated with LGA. In the multivariate analysis, the following factors were associated with LGA newborns: OGTT at 0 minutes (OR: 1.040; CI 95% 1.006-1.076, p = 0.022) and third trimester HbA1c (OR: 4.680; CI 95% 1.210-18.107, p = 0.025). Excessive gestational weight gain, pre-pregnancy obesity and previous macrosomia history lost statistical significance in the multivariate analysis; nevertheless, they presented a *p* value close to 0.05.

Using a ROC curve to evaluate the predictive ability of third trimester HbA1c for LGA identification, the optimal HbA1c cut-off point was at 5.4% (36 mmol/mol) where the sensitivity was 77.4% and the specificity

Table 2. Comparison of the clinical characteristics of pregnant women with and without large for gestational age offspring

	Women with LGA newborns	Women without LGA newborns	p
Maternal age (years)*	35.0 (31.0-37.5)	33 (29.0-37.0)	0.154
Initial BMI (kg/m^2) *	27.1 (22.5-30.7)	26.6 (22.9-30.4)	<0.001
Pre-pregnancy BMI category			
Low weight	0%	1.5% (16/1036)	0.785
Normal weight	20.4% (10/49)	35.1% (364/1036)	0.034
Overweight	30.6% (15/49)	34.7% (360/1036)	0.552
Obese	49.0% (24/49)	28.6% (296/1036)	0.002
Gestational weight gain (IOM)			
Insufficient	18.8% (9/48)	36.3% (371/1021)	0.013
Adequate	29.2% (14/48)	33.1% (338/1021)	0.570
Excessive	52.1% (25/48)	30.6% (312/1021)	0.002
Previous macrosomia history	24.5% (12/49)	4.4% (46/1036)	<0.001
First trimester fasting blood glucose (mg/dL)*	92.0 (83.3-102.8)	85.0 (78.0-94.0)	<0.001
OGTT (minutes)*			
0	93.5 (86.8-105.0)	85.0 (77.0-92.0)	<0.001
60	190.5 (138.5-214.5)	180.0 (157.0-193.0)	0.174
120	160.5 (135.5-180.8)	156.0 (129.0-169.0)	0.642
Insulin therapy	59.2% (29/49)	35.3% (366/1036)	0.001
Third trimester HbA1c*	5.70 (5.35-5.80)	5.30 (5.00-5.50)	<0.001

Data are presented as mean ± standard deviation, unless otherwise indicated by * corresponding to data presented as median, 25th and 75th percentiles. BMI: body mass index; IOM: Institute of Medicine; OGTT: 75 g oral glucose tolerance test.

Table 3. Predictors of large for gestational age

	Univariate analysis		Multivariate analysis	
	Crude OR (CI 95%)	p	Adjusted OR (CI 95%)	p
Pre-pregnancy BMI category				
Normal weight	Reference		Reference	
Overweight	1.500 (0.665-3.383)	0.329	1.306 (0.272-6.264)	0.738
Obese	2.778 (1.308-5.900)	0.008	4.733 (0.905-24.765)	0.066
Excessive gestational weight gain (IOM)	2.464 (1.377-4.409)	0.002	3.028 (0.862-10.640)	0.084
Previous macrosomia history	6.180(2.968-12.866)	<0.001	6.130 (0.986-38.123)	0.052
Fasting blood glucose (mg/dL)	1.057 (1.032-1.083)	<0.001	1.021 (0.955-1.090)	0.535
OGTT (0 minutes)	1.030 (1.14-1.047)	<0.001	1.040(1.006-1.076)	0.022
Insulin therapy	2.657 (1.482-4.764)	0.001	1.319 (0.387-4.492)	0.658
Third trimester HbA1c	13.606 (6.061-30.544)	<0.001	4.680 (1.210-18.107)	0.033

IOM: Institute of Medicine; OGTT: 75 g oral glucose tolerance test.

was 71.7% (AUC 0.782; p < 0.001) using the Fenton Chart to define LGA (Figure 1). Using International Standards for Size at Birth to define LGA, the optimal HbA1c cut-off point remains at 5.4% (36 mmol/mol) where the sensitivity was 50.7% and the specificity was 72.2% (AUC 0.638; p < 0.001) (Figure 2).

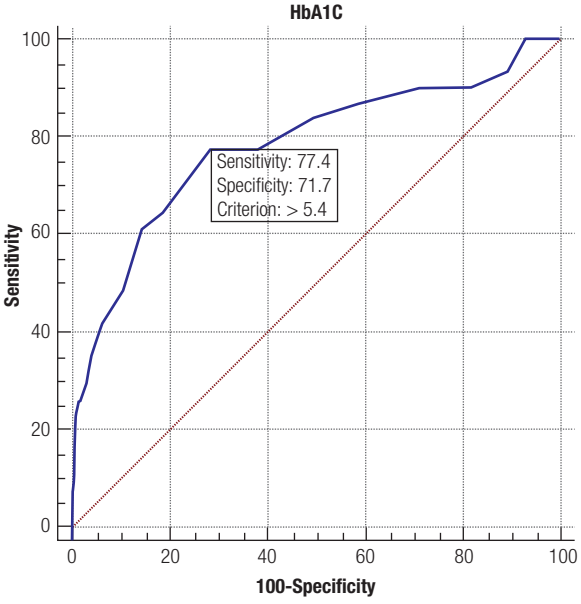

Figure 1. ROC curve analysis of third trimester HbA1c values for LGA prediction using the Fenton Chart.

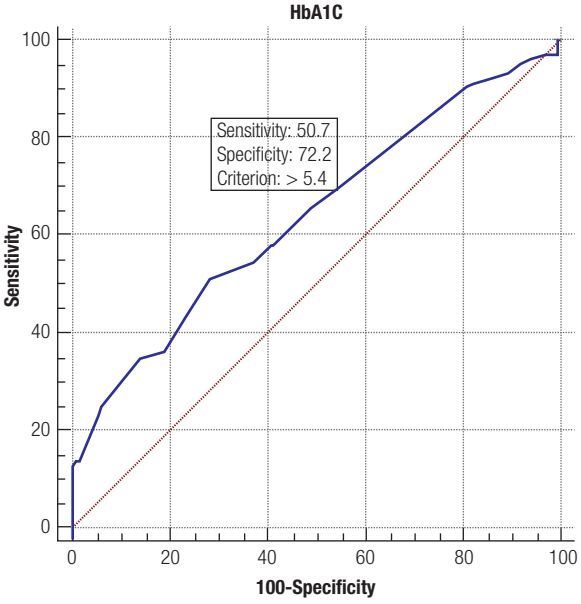

Figure 2. ROC curve analysis of third trimester HbA1c values for LGA prediction using the International Standards for Size at Birth.

DISCUSSION

In our study, in a seven-year cohort of 1085 pregnancies, the incidence of LGA newborns was 4.5% using the Fenton chart and 6.9% using the International Standards for Size at Birth. In the literature, the overall LGA incidence varies from 5%-20% in developed countries (18). It is a known fact that poorly controlled GDM, pre-pregnancy obesity and excessive weight gain during pregnancy increase the risk of LGA and several other neonatal complications (2,11,19-21). Several studies have reported that previous macrossomia history, pre-pregnancy obesity and weight gain during pregnancy above the recommended guidelines are associated with an increased incidence of LGA (22-26). Dong and cols. (2018) described that the LGA offspring is characterized by decreased fetal insulin sensitivity and impaired β-cell function (27). Moreover, in this study, pregnant women with characteristics that favored the development of insulin resistance, such as pre-pregnancy obesity, higher weight gain and need for insulin therapy, were associated with a higher risk of having LGA newborns. Interestingly, the prevalence of macrossomia was slightly higher than that of LGA newborns (three more cases). A possible explanation for this finding is that gestational age at birth in our study was later, with a mean of 38.5 weeks, and macrossomia is defined as birth weight > 4,000 g, irrespective of gestational age. This is contrary to the definition of LGA that correlates birth weight with gestational age. Moll and cols., in their study, also describes a higher prevalence of macrosomia compared to LGA (28).

In our logistic regression model, pre-pregnancy obesity, excessive gestational weight gain (IOM) and previous macrosomia history lost significance as LGA predictors, probably because of insufficient power due to sample size. Nevertheless, glucose levels at the beginning of the OGTT and third trimester HbA1c remained as independent factors for predicting the risk of having LGA offspring.

Tavares and cols. (26) described that LGA newborns were significantly more common in the group of women with combined change in the OGTT (hyperglycemia both in fasting and after a dextrose load), even after adjustment for potential confounders. Brankica and cols. (29) and Ouzilleau and cols. (30) found that high levels of fasting blood glucose in the OGTT were better predictors of LGA. Mello and cols. (31) reported fasting [1.04 (CI 95% 1.01-1.06)] and 1 h

[1.03 (CI 95% 1.02-1.03)] and 2 h [1.03 (1.02-1.04)] glucose values in the OGTT performed between 26-30 weeks of gestation as independent risks factors for LGA newborns. In our study, only fasting blood glucose in the OGTT was found to be an independent risk factor for LGA newborns, with an odds ratio similar to that reported by Mello and cols. (31). Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) represent two different types of glucose metabolism disorder. In fact, Tripathy and cols. (32) showed that not only do IGT and IFG have poor concordance between them, but also that they have different underlying pathophysiological mechanisms. The major determinant of IFG seems to be defective insulin action rather than an impaired β-cell function: the fasting glucose level is largely determined by endogenous glucose production, which depends on hepatic insulin sensitivity. As such, subjects with defective hepatic insulin sensitivity are more prone to developing IFG. Moreover, IFG seems to be more closely related to other features of metabolic syndrome (elevated triglyceride and total cholesterol concentrations, lower HDL cholesterol concentrations and higher waist-to-hip ratio) than IGT; thus, patients with IFG are at higher risk of developing diabetes (32). On the other hand, IGT seems to be mainly determined by impaired insulin secretion (β-cell dysfunction) in relation to glycemia and the degree of insulin resistance (32).

HbA1c has been proposed as a useful marker for predicting LGA and other neonatal complications. During pregnancy, the life span of red blood cells decreases from about 120 days to about 90 days, together with increased erythropoietin production. HbA1c values also decrease by 12-16 weeks of gestation, with a further decrease that plateaus by gestational weeks 20-24. HbA1c levels may start to rise again in the third trimester (33-36). The findings of this study identify third trimester HbA1c level as a determinant of LGA in pregnancies affected with GDM. We found that for every 1%-unit increase in third trimester HbA1c, the odds of having an LGA infant are increased by a factor of 4.7 (CI 95% 1.2-18.1, p = 0.025).

Shushan and cols. found that early control of GDM (before 34 weeks) resulted in a an 18% lower rate of LGA infants, compared to late control of GDM (after 34 weeks) (37). This is a reminder that GDM needs to be well controlled to ensure better fetal outcomes, identifying the risk of a large-for-gestational age fetus before the 34th week, as this will enable measures to be implemented for better glycemic control and decrease the risk for a LGA fetus.

Mañé and cols. (2019) have shown that in Latin-Americans, a first trimester HbA1c ≥ 5.8% (40 mmol/mol) and HbA1c ≥ 5.9% (41 mmol/mol) were associated with a higher risk of macrosomia and LGA, respectively. On the other hand, in a South-Central Asian population, the Hb1Ac cut-off associated with macrosomia and LGA was a first trimester HbA1c ≥ 5.7% (39 mmol/mol) and ≥ 5.4% (36 mmol/mol), respectively. Curiously, no association was found between first trimester HbA1c and obstetric outcomes among Caucasians (38). Sweeting and cols. suggested that a single HbA1c measurement during the time of universal screening for GDM at 24-28 weeks of gestation can be useful in clinical practice, to classify women with GDM with high or low risk for a LGA infant. They found that a HbA1c > 5.9% (41 mmol/mol) at 24-28 weeks of gestation appears to be associated with a higher risk of adverse outcomes, which included macrosomia, LGA, cesarean section and hypertensive disorders (39). Wong and cols. (2017) showed that elevated HbA1c, measured at diagnosis of GDM or at 36 weeks of gestation, were both independent predictors of LGA offspring and neonatal hypoglycaemia. They reported HbA1c cut-offs of 5.4% (36 mmol/mol) at diagnosis and 5.5% (37 mmol/mol) at 36 weeks (40). Our results also evidence that women with GDM who had a third trimester HbA1c, between 31-37 weeks of gestation (median of 34 weeks), above 5.4% (36 mmol/mol) were more likely to have an LGA infant. Our results are in accordance with those of other studies that found that HbA1c could be helpful in predicting LGA. For example, Barquiel and cols. found that a third trimester HbA1c level > 5% (31 mmol/mol) is a modifiable risk factor–that influences neonatal overgrowth and neonatal complications in mothers with GDM (22). There are few reports about the definition of a cut-off value of HbA1c in gestational diabetes, and those that do exist vary in relation to mother's ethnicity, trimester of evaluation and method of definition.

This study has some limitations that deserve comment. First, it was a retrospective study with an associated bias that cannot be ruled out. Second, the majority of our population is Caucasian and from the Mediterranean area; therefore, our results are not generalizable to other populations. This study also has some strengths; we have a large sample size of women with GDM, all evaluated by the same protocol.

Moreover, the identification of a third trimester A1c cut-off for LGA could help physicians in clinical practice.

In conclusion, in this study, a third trimester HbA1c > 5.4% (36 mmol/mol) was found to have good sensitivity and specificity for the identification of an increased risk of LGA offspring among women with GDM. This highlights the importance and value of a HbA1c measurement in the third trimester, as it can alert physicians to this risk and help them to better manage these pregnant women. Few studies have been published in the Mediterranean population concerning the definition of a third trimester HbA1c value from which the risk of LGA increases, and further studies are needed, to validate the HbA1c cut-off point suggested by our study.

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Ethical approval: all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the local Ethics committee (157-DEFI/156-CES). Due to the retrospective nature of this study, consent to participate was waived by the Ethics Committee. This article does not contain any studies with animals performed by any of the authors.

Availability of data and materials: the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: L.F., J.V. and J.D. designed the study; L.F. and A.A. acquired the data; M.S. reviewed the database; L.F. and S.P. performed the data analysis; L.F., S.P., J.V. and J.D. interpreted the data; L.F. and M.S. wrote the article. All authors revised it critically for important intellectual content. All authors approved the final version submitted and are accountable for all aspects of the work. All authors read and approved the final manuscript.

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Ultrasonographic differentiation and Ultrasound-based management of partially cystic thyroid nodules

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ABSTRACT

Objective: To determine sonographic features of malignancy in partially cystic thyroid nodules and assess the diagnostic efficacy of these features for differentiating between benign and malignant lesions in the nodules with indeterminate cytology. **Subjects and methods:** From January 2016 to December 2017, a total of 91 patients with 94 partially cystic thyroid nodules who had undergone ultrasound-guided fine-needle aspiration biopsy and thyroid surgery in our hospital were included in this study. The sonographic features of the thyroid nodules were analyzed to identify the predictive features of malignancy and assess the diagnostic efficacy of these features. **Results:** The features of hypoechogenicity, microcalcification, composition, and an eccentric solid component with an acute angle had statistically significant associations with malignant nodule (p<005) by univariable analysis. Binary logistic regression analysis showed that microcalcification and hypoechogenicity were significantly associated with malignancy. Using the combination of microcalcification, hypoechogenicity, and a solid component comprising of greater than or equal to 50% of the total volume, the diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 97.6%, 32.7%, 53.9%, and 94.4%, respectively. In these nodules with indeterminate cytology, this combination also exhibited a high sensitivity of 92.3% and an NPV of 83.3%. **Conclusion:** This study demonstrated that microcalcification and hypoechogenicity were independently associated with malignancy in partially cystic thyroid nodules. The combination of microcalcification, hypoechogenicity, and a solid portion that is greater than or equal to 50% of the total volume will help guide clinical decisions in mixed cystic solid nodules. Arch Endocrinol Metab. 2021;65(3):336-41

Keywords

Partially cystic thyroid nodules; ultrasonographic features; fine-needle aspiration biopsy

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INTRODUCTION

Thyroid nodules are a common clinical problem, occurring in 19-68% of the healthy population (1,2). Ultrasonography (US) is the primary imaging modality used to estimate the risk of malignancy in thyroid nodules. It also plays a crucial role in decisions regarding fine-needle aspiration (FNA) and a complementary role in the decisions involving medical management after an FNA is performed (3-6).

According to their composition, thyroid nodules can be classified as solid, mixed, or cystic based on a US evaluation. Partially cystic thyroid nodules (PCTNs) have both solid and cystic portions as a result of cystic degeneration of neoplastic or non-neoplastic nodules. They account for approximately 15% to 53.8% of all sonographically detected nodules (7-9). Most of them were considered benign lesions and hence managed conservatively. However, the percentage of malignancy in PCTNs varies from about 2% to 18% (7,8,10).

US features including hypoechogenicity, a taller-than-wide shape, the presence of microcalcifications, and lobulated/irregular margin are widely applied to differentiate benign from malignant nodules in solid thyroid nodules (6,11-14). However, sonographic features as predictors for risk of malignancy in PCTNs are different from that of solid nodules (7). Lee and cols. reported that a predominantly solid component, eccentric location, and microcalcification were associated with an increased risk of malignancy in PCTNs (8). In contrast, Na and cols. reported that only microcalcification was

independently predictive of malignancy in PCTNs (7). In summary, US features predictive of malignancy in PCTNs have not been well established.

The purpose of the present study was to determine thyroid sonography features related to malignancy in PCTNs and assess the diagnostic efficacy of these features for differentiating between malignant and benign nodules with indeterminate cytology.

SUBJECTS AND METHODS

Patients

This retrospective study analyzed a total of 94 partially cystic thyroid nodules from 91 patients in the First Affiliated Hospital of Xi'an Jiaotong University between January 2016 to December 2017. Patients who met the following criteria were included in this study: (1) each nodule consisted of a solid and cystic component; (2) the patients underwent US-guided fine-needle aspiration biopsy (FNAB) based on the 2015 American Thyroid Association (ATA) guidelines or at the patients' request, and (3) the histopathologic result of each nodule was confirmed after surgery. US-guided FNAB was performed by a clinician with more than ten years of experience (>2,000 cases/year). Thyroid US records were reviewed by a radiologist (Yanjun, Liu) with four years of thyroid sonography experience (approximately 10,000 cases/year). The composition of the nodules based on US findings was classified as solid, mixed, or cystic. Purely cystic nodules indicated a completely anechoic nodule with or without a comet-tail artifact. PCTNs were characterized by the presence of both solid and cystic components. Only PCTNs where thyroid surgery was done were included in this study. The indications for thyroid surgery were a malignant or suspicious malignant cytology, or by the patients' choice. All patients signed informed consent concerning the future use of their clinical-pathological data for research purposes. This study was approved by the Ethics Committee of our hospital.

Sonographic evaluation

Thyroid sonography was performed by a high-resolution sonographic instrument (NEMZ017, Toshiba, Japan) equipped with a 6- to 15-MHz linear probe. The sonographic images of PCTNs were retrospectively evaluated by an experienced radiologist blinded to the pathological results. The US features of all nodules were evaluated according to (1) composition, (2) percent of the solid component, (3) margin, (4) echogenicity of the solid portion, (5) presence of echogenic foci, (6) shape, and (7) halo.

The nodules were divided into the following three groups based on composition: group 1, a spongiform defined as the aggregation of multiple microcystic components in more than 50% of the nodule's volume; group 2, had a solid portion of less than 50% (not a pure cyst); and group 3, had a solid portion of greater than or equal to 50%.

According to the position of the solid portion, the nodules were classified as either eccentric or not. An eccentric nodule was defined as one with the solid portion not located in the center and abutted only on the side of the cyst wall. The eccentric configuration was subdivided into either an acute or blunt angle depending on the angle between the solid and adjacent cyst walls.

"Large comet-tail artifacts" are echogenic foci with V-shaped echoes that are greater than 1 mm deep. Macrocalcifications are coarse echogenic foci greater than 1 mm in diameter accompanied by acoustic shadowing. Microcalcifications are punctate echogenic foci less than or equal to 1mm in diameter with non-shadowing. Peripheral calcifications lie along all or part of a nodule's margin.

The solid component's echogenicity was classified as iso-echogenicity, hyperechogenicity, hypoechogenicity and marked (or very) hypoechogenicity by comparing the echogenicity of the solid component with thyroid parenchyma or strap muscles. The margins were further divided into well-circumscribed (smooth), ill-defined, lobulated, irregular, and extra-thyroid extension. The shape was assessed by the ratio of anteroposterior (A) and transverse (T) diameter ($A/T \geq 1$ or $A/T < 1$) of the nodule. A regular, hypoechoic halo presented with a regular smooth profile corresponding to the pericapsular arrangement of nodule vascularity.

FNA was performed under US guidance, using a 23-gauge needle by an experienced endocrinologist. If a nodule was predominantly cystic, FNA for the solid portion was done after the fluid was aspirated. Afterward, the cytology was interpreted according to the Bethesda System for Reporting Thyroid Cytopathology (BSRTC).

Statistical analysis

The statistical analysis was performed using the SPSS Statistics 20.0 for Windows (IBM SPSS statistics). Values

are expressed as mean ± SD for continuous variables and as proportions for categorical variables (%). Groups were compared using independent-sample t-test, chi-square, and Fisher's exact test depending on the distribution. We performed binary logistic regression after univariable analysis to determine independent predictors for malignancy in PCTNs. Sensitivity, specificity, positive, and negative predictive values for malignancy and accuracy were calculated for US features. Statistical significance was set for P-value < 0.05.

RESULTS

A total of 91 patients (68 females, 23 males) with 94 nodules were included in this study and ages ranged from 14 to 78 (mean age, 43.4 ± 13.1 years). There were solitary nodules in 88 patients and two nodules in three patients. The mean nodule size was 2.9 ± 1.2 cm (range, 1.1-8.6 cm).

The cytologic and histopathologic correlation is summarized in Table 1. Based on cytology, all the nodules were categorized as follows: nondiagnostic (cyst fluid only) (n=5), benign (n=29), atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) (n=7), follicular neoplasm/suspicious for a follicular neoplasm (FN/SFN) (n=2), suspicious for malignancy (n=21), malignancy (n=30). The four categories nondiagnostic, AUS/FLUS, FN/SFN, and suspicious for malignancy, were considered indeterminate cytology. None of the nodules with nondiagnostic and benign cytology were malignant after thyroid surgery. The frequency of malignancy for AUS/FLUS, FN/SFN, suspicious for malignancy, and malignancy were 28.6%, 50.0%, 47.6%, and 96.7%, respectively.

US features and histopathologic results are summarized in Table 2. Univariable analysis was performed to determine sonographic features associated with a malignant nodule. The hypoechogenicity and microcalcification showed a statistically significant association with malignant nodules (P < 0.05). The prevalence of malignancy was significantly higher in the nodules with a solid portion greater than or equal to 50% (P = 0.001). An eccentric solid component with an acute angle showed a slight increase in malignant nodules (P = 0.048). However, neither overall analysis nor stratified analysis based on the percent of solid portion (≥50% or <50%, data not shown) showed a significant association of an eccentric configuration with malignancy. The size of the nodule, shape, margin, and halo presence were not significantly associated with a malignant nodule. Binary logistic regression analysis demonstrated that microcalcification and hypoechogenicity were significantly associated with malignancy. There was no significant association of age and sex with either benign or malignant nodules.

We also evaluated the diagnostic values of three sonographic features, which were significantly associated with malignant nodules in partially cystic thyroid nodules. Among them, microcalcification revealed the highest PPV (75.0%), accuracy (72.3%), but low sensitivity (57.1 %) as shown in Table 3. A combination of any two of the three sonographic features showed that microcalcification and hypoechogenicity exhibited the highest specificity (65.4 %), PPV (65.4 %), NPV (81.0 %), and accuracy (72.3%). When compared with the combination of all three features, there was an increased sensitivity (97.6%) and NPV (94.4%), but slightly decreased accuracy (61.7 %). Moreover, we evaluated these three features' diagnostic value in the nodules with indeterminate cytology (Table 4) and the results were like that of all nodules. The combination of the three features showed high sensitivity (92.3%) and NPV (83.3%). These results imply that there is no need for a repeat FNA for PCTNs with indeterminate cytology in the absence of these three features.

Table 1. Cytological and histopathological results of all thyroid nodules

Cytology	Benign (n = 52)	Malignancy (n = 42)	Malignancy %
ND/UNS	5 (9.6%)	0 (0.0%)	0.0%
Benign	29 (55.7%)	0 (0.0%)	0.0%
AUS/FLUS	5 (9.6%)	2 (4.8%)	28.6%
FN/SFN	1 (1.9%)	1 (2.4%)	50.0%
Suspicious for malignancy	11 (21.2%)	10 (23.8%)	47.6%
Malignancy	1 (1.9%)	29 (69.0%)	96.7%

ND: nondiagnostic; UNS: unsatisfactory; AUS/FLUS: atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: follicular neoplasm/suspicious for a follicular neoplasm.

Table 2. US features of benign and malignant partially cystic thyroid nodules

US features	Benign (n = 52)	Malignancy (n = 42)	P-value	
			Uni-	Multi-
Mean size (cm)	3.18	2.62	0.181	0.275
Age (years)	47.02	38.93	0.909	0.080
Sex (female/male)	40/10	28/13	0.279	0.816
Composition				
Solid portion ≥ 50%	30 (44.8%)	37 (55.2%)	**0.001**	0.081
Solid portion < 50%	22 (81.5%)	5 (18.5%)		
Position of the solid portion				
Eccentric	18 (62.1%)	11 (37.9%)	0.501	0.324
Non-eccentric	34 (52.3%)	31 (47.7%)		
Eccentric configuration (n = 29)				
With an acute angle	4 (36.4%)	7 (63.6%)	**0.048**	–
With a blunt angle	14 (77.8%)	4 (22.2%)		
Shape				
Taller than wide	0 (0.0%)	1 (100%)	0.447	1.000
Wider than tall	52 (55.9%)	41 (44.1%)		
Margin				
Smooth	46 (60.5%)	30 (39.5%)		
Ill-defined	5 (38.5%)	8 (61.55)	0.084	0.445
Lobulated or irregular	1 (20.0%)	4 (80.0%)		
Echogenicity				
Hiper- or isoechoic	39 (68.4%)	18 (31.6%)	**0.002**	**0.034**
Hypoechoic	13 (35.1%)	24 (64.9%)		
Containing calcification				
None	41 (73.2%)	15 (26.8%)		
Macro-	2 (40.0%)	3 (60.0%)		
Peripheral (rim)	1 (100.0%)	0 (0.0%)	**< 0.001**	**0.027**
Micro	8 (25.0%)	24 (75.0%)		
The presence of halo				
Yes	5 (62.5%)	3 (37.5%)	0.728	0.830
No	47 (54.7%)	39 (45.3%)		

Uni-: univariable analysis; Multi-: binary logistic regression analysis.

Table 3. Diagnostic efficacy of US features in partial cystic thyroid nodules

US features	Sensitivity	Specificity	PPV	NPV	Accuracy
a. Microcalcification	57.1%	84.6%	**75.0%**	71.0%	**72.3%**
b. Solid portion ≥ 50%	88.1%	42.3%	55.2%	81.5%	62.8%
c. Echogenicity	57.1%	75.0%	64.9%	68.4%	67.0%
a or b	92.9%	36.5%	54.2%	86.4%	61.7%
b or c	92.9%	38.5%	54.9%	87.0%	62.8%
a or c	81.0%	65.4%	65.4%	81.0%	72.3%
a or b or c	**97.6%**	32.7%	53.9%	**94.4%**	61.7%

Table 4. Diagnostic efficacy of US features in these nodules with indeterminate nodules

US features	Sensitivity	Specificity	PPV	NPV	Accuracy
a. Microcalcification	38.5%	81.8%	**55.5%**	69.2%	**65.7%**
b. Solid portion ≥ 50%	69.2%	27.3%	36.0%	60.0%	42.9%
c. Echogenicity	53.8%	59.1%	43.8%	68.4%	57.1%
a or b	84.6%	27.3%	40.7%	75.0%	48.6%
b or c	76.9%	22.7%	37.0%	62.5%	42.9%
a or c	76.9%	50.0%	47.6%	78.6%	60.0%
a or b or c	**92.3%**	22.7%	41.4%	**83.3%**	48.6%

DISCUSSION

Partially cystic thyroid nodules are common findings in ultrasonographic examination (7,15). The findings from previous studies suggest the suspicious US features of mixed cystic solid thyroid nodules were different from that of solid nodules. The 2015 American Thyroid Association (ATA) guidelines have addressed the clinical management of partially cystic thyroid nodules based on a small number of studies (6). According to the 2015 ATA guidelines, PCTNs were recommended for FNA if greater than or equal to 1cm, or if the solid hypoechoic component included one or more features such as irregular margins, microcalcifications, a taller than wide shape, rim calcifications with small extrusive soft tissue components, and evidence of extrathyroidal extension (ETE). Most of these suspicious sonographic features were more common in solid malignant nodules and less frequent in partially cystic thyroid nodules. Our results revealed a marked increase in hypoechogenicity and microcalcification in the solid component of malignant nodules. Combining these two features showed 72.3% accuracy and 81.0% NPV for PCTNs. The Mayo clinics reported that nodules with the solid portion occupying less than 50 % of the volume occurred in only 2.5% of 360 consecutively surgically removed thyroid cancers (16). Our findings also showed that malignancy was less frequent in nodules with a solid portion of less than 50%. Also, using the combination of hypoechogenicity, microcalcification and composition showed an NPV of 94.4%. This indicates that the absence of these three features is a low risk for malignancy and only observation without an FNA is needed.

Eccentric configuration with an acute angle has been associated with malignancy in PCTNs (7,8,15,17). In this study, eccentric configuration with an acute angle rather than a blunt angle was slightly more frequent in the malignant nodules. In the 2015 ATA guidelines, PCTNs with an eccentric solid portion had an estimated 5-10% risk; they were categorized as having low suspicion if there were no microcalcification, irregular margin, and a taller than wide shape. In contrast, this sonographic feature was not considered in other ultrasonographic risk stratification systems including the 2016 Korean Society of Thyroid Radiology (18), the 2017 American College of Radiology (19), and the 2017 European Thyroid Association Guidelines (20). Hence, it was not independently predictive of malignancy in these nodules.

A taller than wide shape and lobulated/irregular margins were less frequently seen in PCTNs, although they were well-established for predicting malignancy in solid thyroid nodules. In concordance with previous studies, they were not significantly correlated with the malignancy of mixed cystic solid nodules. These sonographic features may have a limited role in differentiating malignant from benign mixed thyroid nodules.

It is also worth mentioning that microcalcification had the highest accuracy but the lowest sensitivity than the other two US features in PCTNs with indeterminate cytology. Combined with echogenicity and a solid portion greater than or equal to 50%, the sensitivity and NPV were increased to 92.3% and 83.3%, respectively. A repeat FNA might not be considered for nodules with indeterminate cytology if these three features in the solid component were absent. In summary, these suspicious sonographic features will help guide clinical decision-making when managing PCTNs with indeterminate cytology.

There were a few limitations of this study. First, this retrospective study did not include PCTNs without histopathologic results; second, there was a small sample size; and third, the FNA decision-making criteria were not all based on the 2015 ATA guidelines (some FNAs done were based on the patients' choice). Finally, most patients with PCTNs were diagnosed with

conventional PTC and only one patient had a follicular variant of PTC.

In conclusion, this study demonstrated that microcalcification and hypoechogenicity were independently associated with malignancy in partially cystic thyroid nodules. The combination of microcalcification, hypoechogenicity, and a solid portion greater than or equal to 50% will be helpful to make clinical decisions in mixed cystic solid nodules.

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Adipose tissue-derived stromal/stem cells + cholecalciferol: a pilot study in recent-onset type 1 diabetes patients

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ABSTRACT

Objective: Adipose tissue-derived stromal/stem cells (ASCs) and vitamin D have immunomodulatory actions that could be useful for type 1 diabetes (T1D). We aimed in this study to investigate the safety and efficacy of ASCs + daily cholecalciferol (VIT D) for 6 months in patients with recent-onset T1D. **Materials and methods:** In this prospective, dual-center, open trial, patients with recent onset T1D received one dose of allogenic ASC (1 x 10^6 cells/kg) and cholecalciferol 2,000 UI/day for 6 months (group 1). They were compared to patients who received chol-ecalciferol (group 2) and standard treatment (group 3). Adverse events were recorded; C-peptide (CP), insulin dose and HbA1c were measured at baseline (T0), after 3 (T3) and 6 months (T6). **Results:** In group 1 (n = 7), adverse events included transient headache (all), mild local reactions (all), tachycardia (n = 4), abdominal cramps (n = 1), thrombophlebitis (n = 4), scotomas (n = 2), and central retinal vein occlusion at T3 (n = 1, resolution at T6). Group 1 had an increase in basal CP (p = 0.018; mean: 40.41+/-40.79 %), without changes in stimulated CP after mixed meal (p = 0.62), from T0 to T6. Basal CP remained stable in groups 2 and 3 (p = 0.58 and p = 0.116, respectively). Group 1 had small insulin requirements (0.31+/- 0.26 UI/kg) without changes at T6 (p = 0.44) and HbA1c decline (p = 0.01). At T6, all patients (100%; n = 7) in group 1 were in honeymoon vs 75% (n = 3/4) and 50% (n = 3/6) in groups 2 and 3, p = 0.01. **Conclusions:** Allogenic ASC + VIT D without immunosuppression was safe and might have a role in the preservation of β-cells in patients with recent-onset T1D. ClinicalTrials.gov: NCT03920397. Arch Endocrinol Metab. 2021;65(3):342-51

Keywords

Type 1 diabetes; pancreatic function; adipose tissue-derived stromal/stem cells

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease characterized by immune-mediated destruction of pancreatic β-cells requiring life-long insulin treatment. Preservation or recovery of residual β-cells could cure the disease (1-4) and avoid insulin requirements. Even a partial response, unable to induce cure, appears to be beneficial, leading to lower insulin doses and reduced frequency of both severe hypoglycemia and chronic complications (5).

Immunomodulatory and immunosuppressive agents have been tested to preserve β-cell function (6-8). Non-myeloablative transplantation of autologous hematopoietic stem cells (HSC) showed favorable results and exogenous insulin withdrawal (at least for a short period) in individuals with T1D, but it requires immunosuppression (9). Mesenchymal stem cells (MSCs) are potential alternatives due to their immunomodulatory properties without the need for immunosuppression (10,11). MSCs decrease proliferation and activation of natural killers (NK), dendritic and T cells, reduce secretion of inflammatory cytokines and may have antiapoptotic properties (12). Therefore, they may have a protective role in the autoimmune destruction of β-cells. Adipose tissue represents an abundant and easily accessible source of MSCs (13), which may be of great clinical interest.

Vitamin D is another potential immunomodulatory agent. In vitro and in vivo studies suggest that 25(OH) vitamin D inhibits lymphocyte proliferation and modifies the Th1/Th2 cytokine profile, which may reduce damage associated with the Th1 immune response (14,15). Gabbay and cols. have shown preservation of C-peptide (CP) secretion with 2,000 UI/day of vitamin D (VIT D) (16) in patients with recent-onset T1D. However, the benefits of VIT D supplementation are still controversial (17-19).

T1D has a complex pathophysiology that involves multiple immune pathways. Thus, it is probable that the ideal intervention for its cure would include a combination of drugs with different mechanisms of actions. The aim of this study was to investigate the safety and efficacy of adipose tissue-derived stromal/stem cells (ASCs) infusion + daily cholecalciferol (VIT D) for 6 months in patients with recent-onset T1D. We also performed a pilot analysis comparing these results with those obtained from a previous case control study that investigated the effect of solely VIT D supplementation in patients with T1D (16).

RESEARCH DESIGN AND METHODS

Patient selection and study design

This was a prospective, dual-center, open trial in which patients with recent onset T1D received one dose of allogenic ASC and VIT D 2,000 UI/day for 6 months. The sample was selected by convenience. Participants signed an informed consent. The study was approved by the Institutional Review Board (17488313.1.0000.5257, University Hospital Clementino Fraga Filho [HUCFF]) and registered at ClinicalTrial.gov (NCT03920397). Inclusion criteria were diagnosis of T1D according to American Diabetes Association (ADA) criteria for < 4 months; ages between 16 and 35 years, and positive glutamic acid decarboxylase antibody (GADA). Malignancy, infections, pregnancy, breastfeeding, renal dysfunction and diabetic ketoacidosis were exclusion criteria.

Lipoaspirate human samples and ASC culture

Adipose tissue samples were obtained through liposuction of three healthy females. Donor's serology testing was negative for syphilis, Chagas disease, Hepatitis B and C, HIV and HTLV. Donors had Cytomegalovirus IgG+ with negative polymerase chain reaction (PCR) in blood samples and ASCs.

ASCs were isolated, cultured and characterized as previously described (13). Samples were processed at the Core Cell Technology facility of Pontifícia Universidade Católica do Paraná. Briefly, 100 mL of adipose tissue was washed in sterile phosphate-buffered saline (PBS) (Gibco Invitrogen). A one-step digestion by 1 mg/mL collagenase type I (Invitrogen) was performed for 30 minutes at 37 °C during permanent shaking, followed by a filtration step through a 100 µm mesh filter (BD FALCON, BD Biosciences Discovery Labware). The cell suspension was centrifuged at 800 g for 10 minutes, and erythrocytes were removed through a lysis buffer with pH 7.3. The remaining cells were washed at 400 g for 10 minutes and then cultured at a density of 1×10^5 cells/cm^2 in T75 culture flasks and DMEM-F12 (Gibco Invitrogen) supplemented with 10% of fetal calf serum, penicillin (100 units/mL), and streptomycin (100 µg/mL). The culture medium was replaced three days after seeding, and then twice a week. ASCs were subcultured after reaching 80% confluence, with 0.5% trypsin/EDTA (Invitrogen) solution. Cells were related at a density of $4x10^3$ cells/cm^2 for expansion (13).

Quality control of cell suspension sterility was evaluated by tests to detect bacteria and fungi (Bact/Alert 3D, Biomerieux), endotoxins (Endosafe™ PTS, Charles River) and Mycoplasma (KIT MycoAlert™ PLUS Mycoplasma Detection, Lonza). Cell viability was performed by flow cytometry using the vital dye 7-AAD (7-Aminoactinomycin D – BD#559925) to determine the percentage of viable cells and Annexin V protein (BD#51-65875X) to determine the percentage of cells in apoptosis. Cytogenetic analysis was performed using the GTG-banding method.

Cells were phenotypically characterized by flow cytometry before the clinical application, using the following monoclonal antibodies: FITC-labeled CD14 (BD#555397), CD45 (BD#555482), CD19 (BD#555412), CD44 (BD#555478); PE-labeled CD73 (BD#550257), CD90 (BD#555596), CD166 (BD#559263), PerCP-labeled HLA-DR (BD#551375); APC-labeled CD34 (BD#555824), CD105 (BD#562408), CD29 (BD#559883) all purchased from BD (Pharmingen). At least 100,000 events were acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo 10 (TreeStar) software (13) (Supplementary Material – Supplementary Table S1).

ASC infusion

On the day of infusion, the ASC monolayer were dissociated as described above, and 1 x 10^6 cells/kg of the recipient patient were resuspended in 5 mL of saline solution with 50% albumin and 5% ACD (Anticoagulant Citrate Dextrose Solution). The Cell suspension was sent to the hospital in a cooler with recycled ice.

Patients that received ASCs were admitted to the hospital on the day of the infusion and discharged 24 hours after infusion. A single dose of ASCs was infused in a peripheral upper arm vein for 15-20 minutes. Patients started taking oral cholecalciferol 2,000 UI one day after the infusion of ASCs.

Safety tests

Adverse events were recorded during hospitalization and at each follow-up outpatient visit (T1, T3, and T6), with clinical and laboratory exams (blood count, lipids, renal and hepatic function, TSH, free tyroxine, anti-TPO, calcium, phosphorus and 25(OH) vitamin D, performed with automated biochemical equipment CMD 800 IX1).

Clinical and pancreatic function evaluation

Participants were followed for 6 months. In the first visit (T0), all patients were interviewed and had a physical exam. Weight, height, body mass index (BMI), blood pressure, heart frequency, frequency of hypoglycemia and insulin dose/kg of body weight were evaluated at T0 and after 1 (T1), 3 (T3) and 6 (T6) months. Insulin dose adjustments were performed at each visit as necessary. Patients received nutritional guidance according to ADA recommendations (20). Blood samples were drawn at T0, T1, T3 and T6 for the following measurements: HbA1c (High Performance Liquid Chromatography by boronate affinity), blood count and biochemistry analysis, 25(OH) vitamin D (automated CMD 800 IX1), GADA (ELISA assay, Euroimmun brand and Molecular Devices Spectra max reader) and CP (Microparticle Chemiluminescent Immunoassay, Architect Abbott) before and 30, 60, 90 and 120 minutes after liquid mixed meal (Glucerna®). The area under the curve (AUC) for CP was calculated. Adverse events were recorded during hospitalization and at each follow-up visit.

Comparison with previous case-control study using only VIT D supplementation as intervention

We compared our results with patients previously included in a case-control study that investigated the effects of a daily dose of 2,000 UI VIT D without ASC in individuals with recent onset T1D and similar age (>15 y/o), from a different population (São Paulo) in the same region of the country, Southeastern Brazil (16). Therefore, we established three patient groups for comparison: 1) ASCs + VIT D supplementation; 2) VIT D supplementation; 3) Conventional treatment. Group 3 comprised individuals from both centers: Rio de Janeiro (n = 2) and São Paulo (n = 4). Insulin therapy was prescribed for all patients. Dose adjustments were performed according to glycemic control. Changes in HbA1c, CP and insulin dose/kg were compared between groups. CP was analyzed by immunofluorometric assay (AutoDelfia) at T0 and T6, considering basal and peak stimulated CP after a mixed-meal test (MMT). AUC was not available for comparison.

Statistical analysis

Data are expressed as mean ± standard deviation. Descriptive statistics have been used to summarize patients' characteristics. Comparisons of categorical

variables were performed with Chi square test. A Wilcoxon test was used to compare results at baseline and after follow-up in each group. Continuous variables were compared using Kruskal-Wallis for multiple-group comparison and Mann-Whitney for two-by-two comparisons. A Spearman test was used to investigate correlation between continuous variables. Statistical tests are based on a 2-sided significance level of 0.05. For multiple comparisons, Bonferroni correction was applied and the significance level of 0.017 was considered. SPSS software, version 21.0 was used for statistical analyses.

RESULTS

Clinical characteristics of the study group

Eleven patients were interviewed, and two were excluded (one used glucocorticoid, and another had renal dysfunction). Nine patients were evaluated: seven received ASCs + VIT D and two were included as controls. All completed 6 months of follow-ups.

The mean age of patients that underwent intervention was 27.14 ± 6.49 years old; 3 were males, and 2 were non-whites. The T1D duration at T0 was 2.6 ± 1.03 months. Mean initial serum 25(OH) vitamin D was 33.06 ± 13.55 ng/mL, patients were not taking any vitamin supplementation prior to the study. The control patients were 16 and 20 years old. Clinical characteristics of the study group are described in Table 1.

ASC infusion and adverse events

For ASC infusion, the mean number of cells was 67.71 x 10^6, with 95.10% cell viability. Tests for a microorganism's growth control were negative. ASCs were immunophenotypically characterized as follows: CD105: 94.18%; CD73: 96.46%; CD90: 99.80%; CD29: 99.15%; CD166: 94.04%; CD44: 89.13%; CD14:1.94%; CD34: 0.59%; CD45: 0.87%; CD19: 0.71%; HLA-DR: 0.64%. No clonal chromosomal rearrangements were detected. Samples were approved by cytogenetic quality control for therapeutic use.

All patients had transient headache and mild local infusion reactions. Other immediate adverse events were tachycardia (n = 4) and abdominal cramps (n = 1). Four patients developed local thrombophlebitis within the first week and two had transient mild eye floaters during infusion, with no subsequent visual abnormalities. One patient developed central retinal vein occlusion at T3, with complete resolution at T6.

Insulin dose, glycemic control and GADA status in ASC + VIT D group

In those who received ASC + VIT D, the mean insulin dose at baseline was 0.31 ± 0.26 UI/kg. Insulin dose/kg remained stable at T6 compared to T0 (p = 0.44), except for one patient who became insulin free for 4 months. Insulin doses/kg at T1, T3 and T6 were 0.26 ± 0.21 UI/kg, 0.25 ± 0.17 UI/kg and 0.28 ± 0.14 UI/kg, respectively. After intervention, there was a decrease in HbA1c (7.77 ± 1.14% at T0, 6.21 ± 0.49% at T3 and 6.56 ± 0.66% at T6; T0 vs. T6 p = 0.018). Mean GADA titers remained stable throughout the study (227.65 ± 107.94 units/mL at T0 and 228.51 ± 125.96 units/mL at T6; p = 0.91).

Table 1. Clinical Characteristics of each patient in the ASC + VIT D group

	Age	Gender	Ethnicity	Body mass index	Disease duration	ASC concentration
Patient 1	26	Male	White	26.06 kg/m^2	4 months	78 x 10^6 cells
Patient 2	35	Male	Non-white	25.91 kg/m^2	4 months	74 x 10^6 cells
Patient 3	28	Male	White	23.38 kg/m^2	2 months	65 x 10^6 cells
Patient 4	34	Female	White	23.56 kg/m^2	2 months	73 x 10^6 cells
Patient 5	16	Female	White	20.96 kg/m^2	3.5 months	55 x 10^6 cells
Patient 6	23	Female	Non-white	20.76 kg/m^2	1.7 months	60 x 10^6 cells
Patient 7	28	Female	White	23.71 kg/m^2	2 months	69 x 10^6 cells
Patient 8	16	Male	White	18.25 kg/m^2	2 months	Control
Patient 9	20	Female	Non-white	23,71 kg/m^2	4 months	Control

kg: kilograms; m: meters; ASC: adipose tissue-derived stem/stromal cells.

Evaluation of pancreatic function in ASC + VITD group

All patients who received ASC + VITD had an increase in basal CP 6 months after intervention (T0 = 0.80 ± 0.38 ng/dL; T1 = 0.86 ± 0.48 ng/dL; T3 = 0.74 ± 0.28 ng/dL; T6: 1.04 ± 0.47 ng/dL; T0 vs. T6 p = 0.018), as shown in Figure 1. The mean increase was 40.41 ± 40.79%.

Both peak CP and AUC after MMT remained stable 6 months after ASC + VIT D (p = 1.0 and p = 0.62). Peak CP before intervention, at T3 and at T6 were 2.83 ± 1.24 ng/dL, 2.65+/-1.46 ng/dL and 2.82 ± 1.24 ng/dL, respectively (Figure 2). AUC before intervention and at T1, T3 and T6 were 237.08 ± 95.18 ng/mL, 256.82 ± 138.4 ng/mL, 225.92 ± 98.30 ng/mL and 250.26 ± 92.38 ng/mL, respectively. Four patients had increases in peak CP (57.1%), and 5 (71.4%) had an increase in AUC at T6.

There was an inverse correlation between HbA1c and either peak CP (r = -0.821, p = 0.023) or CP AUC (r = -0.929, p = 0.003) at T6, but not with basal CP (p = 0.25). There was no correlation between insulin dose/kg and basal CP, peak CP or CP AUC at T6 (p = 0.7, p = 0.33, and p = 0.64, respectively).

Comparison of ASC + VIT D (group 1) with a previous study with VIT D (group 2) and controls (group 3)

Data from patients who received ASCs + VIT D (group 1) were compared with data from a previous study (16) where a group received 2,000 UI VIT D (group 2) and another remained on standard insulin treatment (group 3). Clinical data from all 3 groups are described in Table 2.

There was a similar age and gender distribution among groups (Table 2). Basal and peak CP at T0 and T6 in each group are described in Figures 1 and 2. Group 1 had an increase in basal CP over 6 months (p = 0.018), but basal CP remained stable in groups 2 and 3 (p = 0.58 and p = 0.116, respectively), as shown in Figure 1. In Table 2, there was a difference in basal CP at T6 (p = 0.028) comparing all three groups. For two-by-two comparisons, the difference was seen only between groups 1 and 3, as shown in supplementary Figure 1 (Figure S1). An increase in basal CP was observed in all patients in group 1 vs. 3/4 (75%) and 2/6 (33.3%) patients in groups 2 and 3, respectively (p = 0.011). The percentage of increase in basal CP was higher in patients who received any intervention (ASCs + VIT D or VIT D) than in others (+48.41 ± 77.24 vs. -33.15

Table 2. Age, insulin dose, Hba1c and basal and peak C peptide in group 1 (ASC + VIT D), group 2 (VIT D) and group 3 (controls)

	ASC + VIT D Group 1 (n = 7)	VIT D Group 2 (n = 4)	Controls Group 3 (n = 6)	p value *
Age (years)	27.14 ± 6.49	20.75 ± 6.18	19.17 ± 4.02	p = 0.09
Female/male	4 (57.1%)/3 (42.9%)	2 (50%)/2 (50%)	3 (50%)/3 (50%)	p = 0.79
Insulin dose/kg T0	0.31 ± 0.26 UI/kg	0.46 ± 0.25 UI/kg	0.57 ± 0.31 UI/kg	p = 0.9
Insulin dose/kg T6	0.28 ± 1.44 UI/kg	0.55 ± 0.31 UI/kg	0.63 ± 0.28 UI/kg	p = 0.89
Absolute insulin alteration	-0,028 ± 0,21 UI/kg	0.08 ± 0.15 UI/kg	0.06 ± 0.25 UI/kg	p = 0.9
% of insulin dose alteration	15.39 ± 51.67%	20.26 ± 30.45%	23.53 ± 63.80%	p = 0.89
HbA1c T0	7.77 ± 1.14%	9.15 ± 3.05%	8.08 ± 2.4%	p = 0.89
HbA1c T3	6.2 ± 0.49%	6.65 ± 1.03%	8,33 ± 3.25%	p = 0.38
HbA1c T6	6.55 ± 0.66%	6.37 ± 0.62%	7.87 ± 2.01%	p = 0.379
Basal CP T0 (ng/dL)	0.80 ± 0.38	0.52 ± 0.33	0.59 ± 0.39	p = 0.473
Basal CP T6 (ng/dL)	1.04 ± 0.47	0.60 ± 0.29	0.31 ± 0.22	**p = 0.028**
Absolute basal CP modification (ng/dL)	0.23 ± 0.14	0.07 ± 0.57	- 0.28 ± 0.52	**p = 0.045**
% basal CP modification	40.41 ± 40.79	62.41 ± 127.08	33.15 ± 51.6	p = 0.107
Peak CP T0	2.83 ± 1,24	1.27 ± 1.02	1.29 ± 0.58	**p = 0.031**
Peak CP T6	2.82 ± 1,24	1.95 ± 1.38	0.90 ± 0.48	**p = 0.011**
Absolute peak CP modification (ng/dL)	-0.007 ± 1.08	0.67 ± 0.36	-0.39 ± 0.89	p = 0.16
% peak CP modification	8.69 ± 53.1	59.66 ± 11.38	-17.72 ± 55.65	p = 0.079

ASC: adipose tissue-derived stem/stromal cells; VIT D: cholecalciferol; Hba1c: glycated hemoglobin; CP: C peptide; T0: before intervention; T6: 6 months after intervention; group 1: ASC + VIT D; group 2: VIT D; group 3: Controls. Basal CP, Peak CP and insulin dose in two by two comparisons are described in supplementary Figures 2 and 3 (Figures S2 and S3).

± 51.60%; p = 0.035). No differences were found in percentage of basal CP increase from T0 to T6 between the three groups, as shown in Table 2 (p = 0.107). Two-by-two comparisons did not show differences between groups in the percentage of CP alteration from T0 to T6 after Bonferroni correction (p = 0.035, p = 0.257 and p = 1.0 for ASC + VIT D vs controls, VIT D vs controls and ASC + VIT D vs. VIT D, respectively).

Peak CP remained stable after 6 months in all 3 groups as described in Figure 2. In Table 2, there was a difference in peak CP at T0 and T6, comparing the three groups (p = 0.031 and p = 0.011, respectively). In two-by-two comparisons, the difference was significant only between groups 1 and 3 at T6 (p = 0.01) (Supplementary Figure 1; Figure S1). The percentage of increase in peak CP did not differ between groups, p = 0.079 (Table 2). There was no difference between groups in the frequency of individuals who had an increase in peak CP after 6 months (p = 0.44).

HbA1c improved in group 1 after 6 months (7.77 ± 1.14% vs. 6.55 ± 0.66, p = 0.018), but had no difference in group 2 (p = 0.14) and group 3 (p = 0.67), as shown in Figure 3. Insulin dose/kg was similar before and after intervention in all groups (group 1 T0 = 0.31 ± 0.26 UI/kg vs. T6 = 0.28 ± 1.44 UI/kg, p = 0.44; group 2 T0 = 0.46 ± 0.25 UI/kg vs. T6 = 0.55 ± 0.31 UI/kg, p = 0.28; group 3 T0 = 0.57 ± 0.31 UI/kg vs. T 6= 0.63 ± 0.28 UI/kg, p = 0.46). Two-by-two comparisons between groups are described in supplementary Figure 2 (Figure S2). Percentage of insulin dose increase after 6 months was similar between groups (p = 0.89).

At T6, all patients (100%/n = 7) in group 1 were in a honeymoon period (insulin dose ≤ 0.5 UI/kg with HbA1c < 7.5%) vs. 75% (n = 3/4) in group 2 and 50% in group 3 (n = 3/6), p = 0.01.

At T0, vitamin D levels did not differ between groups (33.06 ± 13.55 ng/mL,27.85 ± 11.17 ng/mL, 24.1 ± 8.51 ng/mL for groups 1, 2 and 3, respectively, p = 0.26). At T6, groups 1 and 2 had similar vitamin D levels (45.0 ± 14.63 vs. 55.89 ± 15.78; p = 0.41), which were higher than in group 3 (28.33 ± 5.44; p = 0.002). There was no correlation between basal or peak CP and vitamin D levels in those patients who received oral VIT D (p = 0.39 and p = 0.86, respectively).

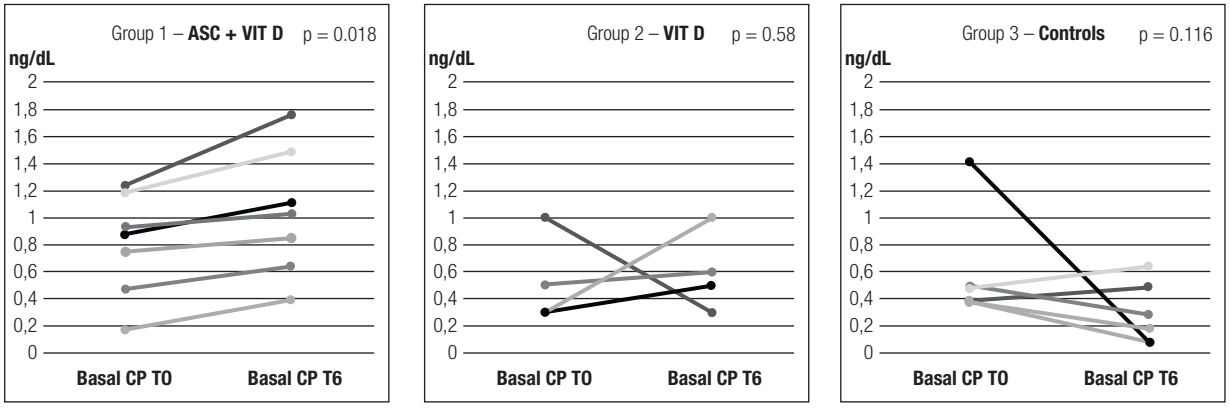

Figure 1. Basal C peptide distribution at T0 and T6 in each group.

Individual patient basal C-peptide before and 6 months after intervention for each group (group 1: ASC + VIT D; group 2: VIT D; group 3: Controls). CP: c peptide; T0: basal; T6: after 6 months; VIT D: vitamin D; ASC: adipose tissue-derived stem/stromal cells.

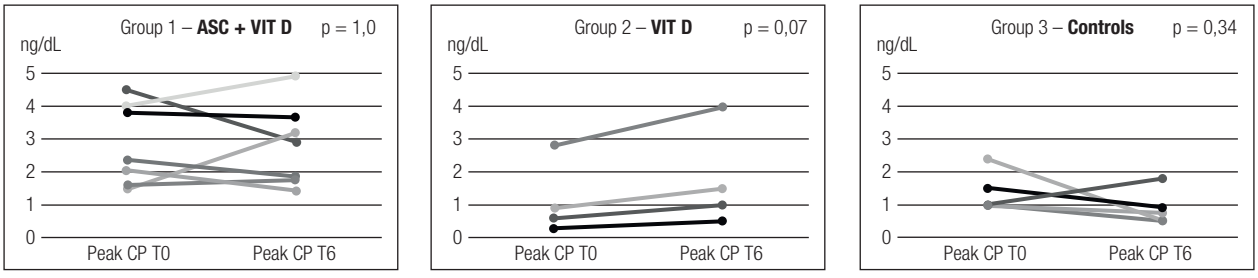

Figure 2. Peak C peptide distribution at T0 and T6 in each group.

Individual patient peak C peptide before and 6 months after intervention for each group (group 1: ASC + VIT D; group 2:VIT D; group 3: Controls). CP: c peptide; T0: basal; T6: after 6 months; VIT D: vitamin D; ASC: adipose tissue-derived stem/stromal cells.

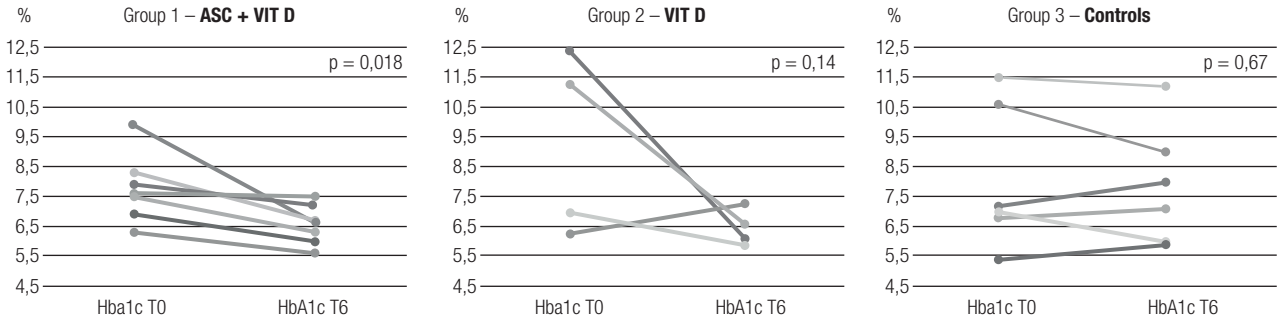

Figure 3. Hba1c at T0 and T6 in each group.

Individual patient basal C-peptide before and 6 months after intervention for each group (group 1: ASC + VIT D; group 2: VIT D; group 3: Controls). CP: c peptide; T0: basal; T6: after 6 months; VIT D: vitamin D; ASC: adipose tissue-derived stem/stromal cells.

DISCUSSION

This study evaluated the safety and efficacy of allogenic ASCs without immunosuppression associated with 2,000 UI VIT D supplementation for 6 months in patients with recent-onset T1D. After intervention, all patients had a good glycemic control and low insulin requirements, without significant decline in β-cell function, with few or transient complications.

This was the first trial to use allogenic ASCs without immunosuppression in patients with T1D of short duration. Other authors have investigated the role of adult stem cells from different origins in the preservation of β-cells for patients with T1D. HSC transplantation with cyclophosphamide and anti-thymoglobulin led to significant increase in CP and transient insulin independence, but the potential toxicity of immunosuppressive agents limits their widespread clinical use (9). Carlsson and cols. evaluated the efficacy of autologous bone marrow MSC in recent onset T1D patients (21). Both AUC and peak CP improved after 1 year when compared to controls with no adverse events. As bone marrow is not easily accessed, subcutaneous adipose tissue might be a more interesting source of MSC (13). Although Thakkar and cols. have tested allogenic and autologous ASC for patients with T1D, the study population comprised individuals with long-standing disease, concomitant use of immunosuppressive agents, bone marrow transplantation and ASC culture to generate insulin-secreting cells in vivo and intraportal infusion (22).

Allogenic ASC was used in this trial. Differently from autologous cells, they could potentially replace the autoreactive host immune system with a more tolerant donor profile (23). There is concern whether the immune properties of the MSC are preserved in individuals with diabetes, which favors the use of allogenic cells for transplantation in this scenario. The safety and promising outcome of this clinical trial using allogenic cells encourages the generation of master banks (cryopreserved cells) for cell therapy. ASC can be isolated from young, healthy donors, fully characterized, expanded in vitro and cryopreserved for their use in a range of diseases, including T1D.

Therapy with ASC was safe and led to few or transient adverse events. Most patients presented tachycardia during infusion, which resolved soon after its suspension. Transient thrombophlebitis was also frequent. These events might have been associated with high cellularity concentration, high viscosity, or other cell stabilizing products. One patient presented central vein occlusion 3 months after infusion, with complete resolution. This was probably not associated with the therapy since it occurred months after the intervention.

Six months after intervention, there was an absolute increase in basal CP in the group that received ASC + VIT D. As basal endogenous insulin secretion has a micropulsatile pattern and may have paracrine effects in the regulation of glucagon secretion, it is possible that this improvement might influence glycemic control during a fasting state (24,25). Peak CP and CP AUC remained stable in this period after the intervention with HbA1c within target and low insulin requirements. Although these findings could represent a honeymoon state, previous data from the Trialnet and others indicate that there is a decrease in CP AUC and peak CP in patients with similar ages in the first 6-12 months of T1D (26,27). However, most patients included in Trialnet were Caucasian, and there is scarce information about the progression of the β-cell function in T1D from multiethnic populations.

In order to determine whether the favorable evolution of β-cell function was related to the treatment, we compared data from patients who underwent ASC infusion + VIT D with individuals who received standard insulin treatment in this study or in a previous analysis of a similar population, as well as with individuals that received only VIT D as intervention (16). The improvement in basal CP observed in patients that received ASC + VIT D was not seen in the other groups. A difference in basal CP at T6 was observed among the 3 groups, but in two-by-two comparisons the difference was seen only between group 1 (ASC infusion + VIT D) and 3 (controls). Although this suggests that treatment with ASC + VIT D is associated with better basal CP evolution, this information should still be interpreted cautiously, due to the limited number of patients in each group, the absence of differences in the absolute or percentage of basal CP difference between groups as well as the lack of peak CP alterations or differences in the whole sample, which is a more traditional and reliable marker for β-cell function.

Patients in group 1 had an improvement in HbA1c without a significant increase in insulin dose/kg, which might be secondary to the increase in basal insulin secretion observed in those patients. Moreover, all patients in ASC + VIT D therapy group were in honeymoon phase after 6 months, which was superior to that observed in groups that used vitamin D without ASC (3/4) and conventional therapy (3/6).

This study indicates a potential benefit of the combination of peripheral ASC infusion + oral VIT D supplementation for patients with the recent onset of T1D. The infusion of these cells in the peripancreatic area might have more pronounced effects, as it has been previously shown that part of the stem cells that are infused in peripheral veins migrates to the lungs, which could compromise their immunomodulatory action (28,29). Moreover, it is possible that the slightly beneficial effects of stem cells for patients with the recent onset of T1D observed in our group, when compared to more striking results observed in former studies with hematopoietic stem cells (9), might have been due to concomitant immunosuppression used in those. In this study, we administered ASC without immunosuppressive agents to avoid their toxicity (9).

This study has some limitations. First, the limited number of patients might have influenced the results. However, this was a pilot study with the priority of establishing the safety of ASC. As therapy has proven to be safe, further, larger studies may be developed. Second, it is not possible to determine whether the beneficial effect of ASC in pancreatic function was due to immune modulation or secondary to their differentiation in beta cells. Moreover, this was an open study, and most participants accepted entrance only in the intervention arm. Therefore, comparison between individuals that received ASC + VIT D with patients in standard insulin treatment depended mostly on a previous study performed on a similar population. Finally, a longer follow-up is necessary to investigate the long-term safety and efficacy of ASC + VIT D. However, this was the first study to show the safety of allogenic ASC without immunosuppression in patients with T1D of short duration, which opens a new possibility for clinical trials.

To conclude, therapy with allogenic ASC + VIT D without immunosuppression was safe and might have a role in the preservation of β-cells in patients with recent-onset T1D.

Authorship: D.B.A., M.F.C.P, C.C.S and D.L.S. researched data. J.R.D. K.R.S.P and L.S.B. wrote the manuscript and researched data. M.R, C.L.K.R., L.Z. reviewed/edited the manuscript and contributed to the discussion. R.R contributed to the supervision of statistical analysis. C.E.B.C., M.G., S.D., J.E.P.O. and A.M. contributed to the discussion and reviewed the manuscript. P.R.S.B, A.C.S and D.R.D processed the ASC cells. D.L.S is the corresponding author. The manuscript has been read and approved by all authors.

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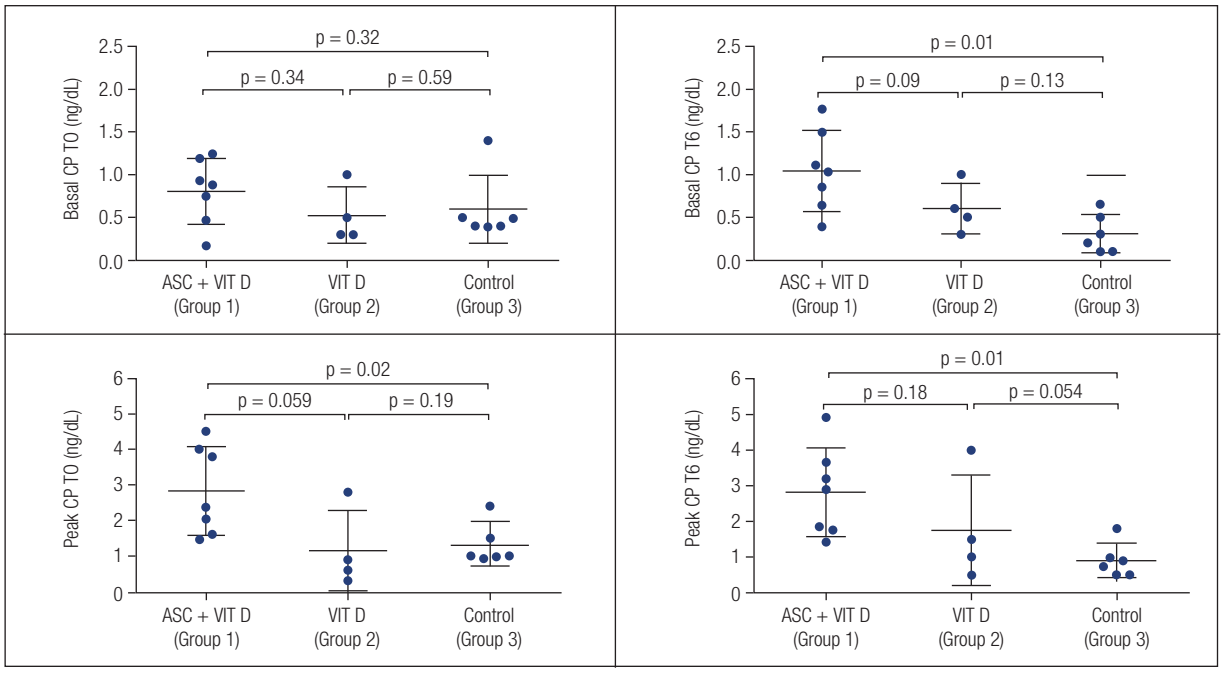
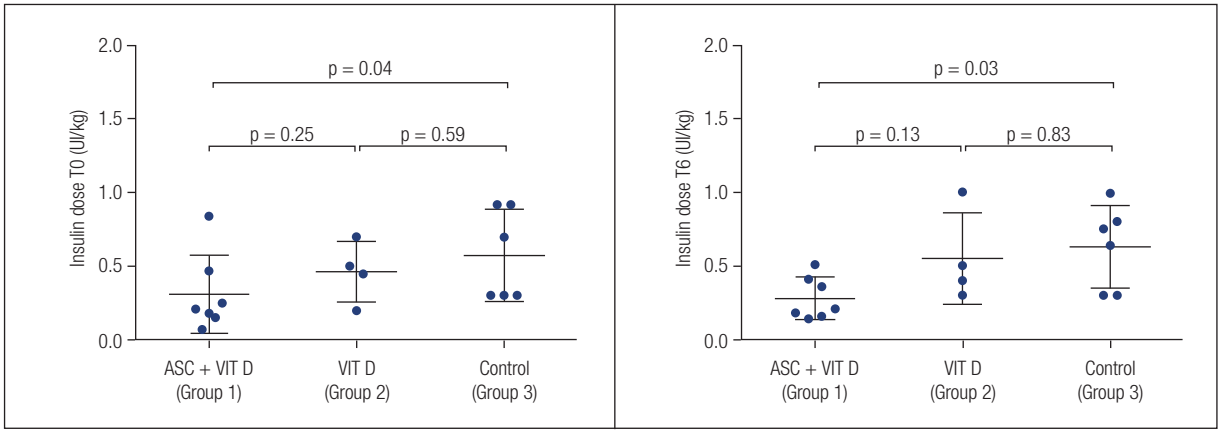


Supplementary figure 1 (figure S1). Basal and peak CP before (T0) and 6 months (T6) after ASC infusion and VIT D supplementation.

*P value with Bonferroni correction, level of significance of 0.017.



Supplementary figure 2 (figure S2) 2. Insulin dose/kg before (T0) and 6 months after ASC infusion and VIT D supplementation.

Figure 2 describes insulin dose/kg before (T0) and 6 months (T6) after intervention for each group (group 1: ASC + VIT D; group 2: VIT D; group 3: Controls); T0: basal; T6: after 6 months; VIT D: vitamin D; ASC: adipose tissue-derived stem/stromal cells. P values represent comparisons two-by-two.

*P value with Bonferroni correction, level of significance of 0.017.

Supplementary table 1. Flow cytometer

Marker	CD 105	CD 73	CD 90	CD 29	CD 166	CD 44	CD 36	CD 14	CD 34	CD 45	CD 19	HLA-DR	CD 31	CD 106
Means	50,42%	51,16%	49,66%	47,45%	46,09%	12,80%	5,92%	0,71%	0,67%	0,71%	0,50%	0,57%	2,39%	3,77%
DP	0,055	0,415	0,434	0,442	0,467	0,395	0,131	0,038	0,005	0,007	0,003	0,005	0,002	0,039

Data are means ± SD (95% CI).

FITC-labeled CD14 (BD#555397), CD45 (BD#555482), CD19 (BD#555412), CD44 (BD#555478); PE-labeled CD73 (BD#550257), CD90 (BD#555596), CD166 (BD#559263), PerCP-labeled HLA-DR (BD#551375); APC-labeled CD34 (BD#555824), CD105 (BD#562408), CD29 (BD#559883) all purchased from BD (Pharmingen). At least 100.000 events were acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo 10 (TreeStar) software11A.

Effect of iodine supplementation in pregnancy on neurocognitive development on offspring in iodine deficiency areas: a systematic review

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ABSTRACT

Objective: To investigate the effect of iodine supplementation during gestation on the neurocognitive development of children in areas where iodine deficiency is common. **Materials and methods:** Based on the PRISMA methodology, we conducted the search for articles in the PubMed, LILACS and Scopus databases, between March and April 2020, without limitation of dates. We used descriptors in English, Portuguese, and Spanish, without filters. Four clinical trials and four cohort articles were included in the review. **Results:** The maximum supplementation was 300 µg of potassium iodide per day. The Bayley scale and Children's Communication Checklist-Short were used to assess neurodevelopment in children. There was no significant improvement in the children's mental development index and behavioural development index in the supplemented group; however, the psychomotor development index (PDI) showed improvement in the poorer gross motor skills. We found differences in the response time to sound in the supplemented group living in mild deficiency areas. **Conclusion:** Daily supplementation with iodine can improve poor psychomotor development of children living in mild to moderate iodine deficiency areas. Thus, it is necessary to perform further studies to assess the effect of supplementation on neurodevelopment before, during and after gestation in mild to moderate iodine deficiency areas. Arch Endocrinol Metab. 2021;65(3):352-67

Keywords

Potassium iodide; cognition; child; pregnant woman

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INTRODUCTION

Iodine deficiency affects almost 2 billion people worldwide (1). In 2017, 18 countries were identified in which women of reproductive age were iodine-deficient, whereas for pregnant women, this was found in 39 countries (2). At this stage, deficiency induces the occurrence of irreversible brain damage in children (1). In fact, inadequate iodine intake in the foetal period may cause dwarfism, cretinism, mental retardation, deafness, psychomotor defects, or congenital anomalies, and may lead to miscarriage or stillbirth (3). Throughout growth, it negatively affects physical and neurocognitive development, especially hippocampal development and memory functions, and in adult life, causes goiter and hypothyroidism (4).

The recommended daily intake of iodine is 90 µg in the age group 0-59 months, 120 µg in 6-12-year-olds, 150 µg in adolescents and adults, and 250 µg during gestation and lactation (5). To ensure sufficient iodine intake, women who are planning pregnancy, pregnant or lactating should be recommended by the American Thyroid Association and European Thyroid Association to ingest daily oral supplements containing 150 µg of iodine (6,7). The World Health Organization (WHO) affirm that this supplementation should be

undertaken when iodized salt does not reach over 90% of households (5).

Recent findings in mild iodine deficiency areas in Israel and Iceland report the improvement of iodine intake in pregnant women supplemented with iodine compared with those not taking iodine supplements (8,9). Other studies in mild iodine deficiency areas in Brazil showed that supplementation corrects maternal thyroid indices and avoids impairment of the neuropsychological development in the offspring (10).

However, the effectiveness of iodine supplementation in pregnant women at improving children's cognitive development is poorly explored and uncertain (11-13). Therefore, this review aimed to investigate the effect of iodine supplementation during gestation on children's neurocognitive development in iodine deficiency areas.

MATERIALS AND METHODS

This systematic review sought to answer the following question: "What is the effect of iodine supplementation during gestation on children's cognitive development?". The review protocol was registered in PROSPERO (International Prospective Register of Ongoing Systematic Reviews) with the identification number CRD42019116962.

We used the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (14) methodology to select articles. To identify the articles, we conducted the search in the PubMed, LILACS (Health Sciences in Latin America and the Caribbean) and Scopus databases, from March 1st to April 1st 2020, without limitations of dates. We used the descriptors: "iodine AND supplementation AND child AND development AND cognitive", provided by DeCS (Health Science Descriptors) (15), in English, Portuguese, and Spanish, without filters (Supplement Appendix 1).

After the searches and elimination of duplicates by database and between databases, we registered all articles in a spreadsheet in Microsoft Excel®. Then, we recorded data from the articles, detailing the year, authorship, place of origin, type of study, target population, sample size, dose and time of supplementation, tests to assess neurocognitive development, and main results observed.

The inclusion criteria were that the studies should be randomized or non-randomized controlled trials or cohorts that evaluated the effect of iodine supplementation during gestation on the neurocognitive development of children living in moderate to severe, mild to moderate, severe, moderate, or mild iodine deficiency regions. We included all children in this study, without any age limit, provided that the study presented some scale of measurement of their neurodevelopment. Studies on the effect of intake of fortified foods, as well as literature reviews, cross-sectional studies, animal model studies and studies that assessed supplementation in pregnant women with thyroid disease were discarded (Supplemental Table 1).

The PICO was defined, namely: Population – pregnant women; Intervention – iodine supplements (iodine supplement use, iodine supplement coverage, iodine content in supplements); Comparator – other children of mothers without iodine supplement use; and Outcomes – development index (mental, psychomotor and verbal), sound response time, IQ (Intelligence Quotient) score (verbal, performance, and reasoning), skills score (language, reading, and writing), mapping test, reading, mathematics and special education.

The scale used to assess neurodevelopment in children selected from the included articles was the Bayley and Children's Communication Checklist-Short (CCC-S).

The Bayley scale has three indices: mental, psychomotor, and behavioural development. The mental development index assesses the visual perceptual acuity, discrimination between objects, problem solving skills, language, and memory (16-18). The psychomotor development index (PDI) is assessed through postural control and appendicular motricity (16-18). The behavioural development index (BDI) assesses the follow-up of instructions, attitudes, and energy during the test, among other social behaviors (16-18). The Bayley score includes cognition and psychomotor skills with mental index (MDI), with a mean score of 100 (SD 15, range 55-155). The mean language (BDI) score was 100 (SD 15; range 45-155). Severe to moderate neurodevelopmental issues were defined as a mean MDI < 85 or BDI < 85, or both < 70; mild to moderate issues were defined as 85–100, and adequate function was defined as ≥ 100 (19).

However, the CCC-S is effective as a standardized assessment at identifying children with clinically-significant language impairment (20), containing 13 items that best discriminate typically-developing children from peers with language impairment in the

validation study (21), with a high degree of internal consistency. Each item provides an example of language behaviour in everyday contexts and covers speech, vocabulary, grammar, and discourse. The items are scored as 0 – absent response, or 1 – present response, with final analysis using statistical methods.

The quality of the studies was assessed according to the checklist of Joanna Briggs Institute (JBI) Critical Appraisal Tools of the Faculty of Health and Medical Sciences at the University of Adelaide, South Australia (22,23). The checklist consider each question should be answered through four options: Yes (Y), No (N), Unclear (U) and Not Applicable (NA). The bias risk percentage calculation is done by the amount of "Y" that has been selected in the checklist. When "NA" was selected, this question was not considered in the calculation, according to the guidelines of JBI. This tool classifies the studies in: up to 49% is considered a high risk of bias. From 50% to 70% is moderate and above 70% is low risk of bias.

RESULTS

The search resulted in 136 articles, of which eight were included in the review (Figure 1 and Supplement Appendix 1). The studies dated from the year 2009 (24) to 2019 (28), four of which were performed in Spain (24,26,27,29), two in Norway (25,28), one in India or Thailand (30), and the other in Australia or New Zealand (31). Two studies were performed in mild to moderate iodine deficiency areas (24,31), five in mild iodine deficiency areas (25-29), and one in a severe iodine deficiency area (29).

Regarding the design, four studies were randomized clinical trials (RTC) (24,26,30,31) and four were cohorts (25,27,28,29). Seven of eight authors used the Bayley scales to assess development of children under 36 months old (24-27,29-31), Gowachirapan and cols. also assessed the IQ of children above 60 months old (30), whereas Abel and cols. used the Children's Communication Checklist-Short for children between 36 and 96 months old (28) (Table 1).

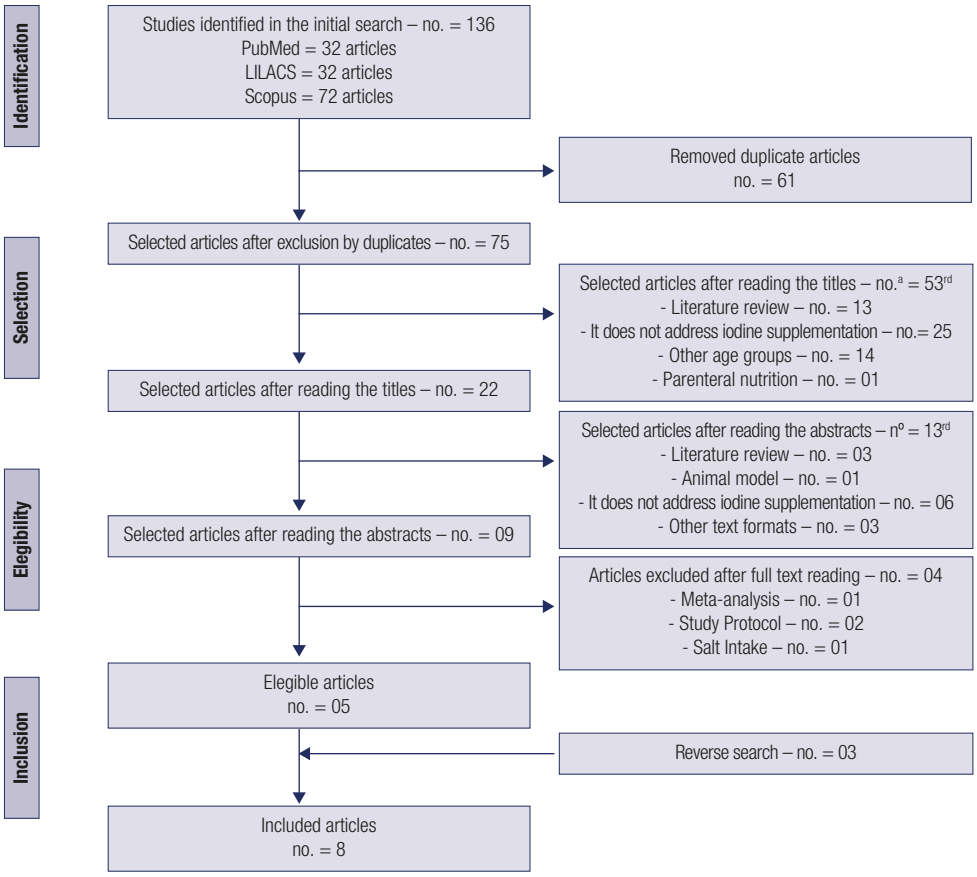


Figure 1. Identification and selection of articles. a. Supplemental Table 1.

Table 1. Supplementation results in neurocognitive development of children in iodine deficiency areas

Author/Year	Methodology			Main results
	Children by type of mother's supplementation	Study design	Skills assessed	
Murcia *et al.*, 2011 (27)	Spain <100 µg/day of KI (n = 169) 100-149 µg/day (n = 298) ≥150 µg/day (n = 222) Maternal MUIC: NA	Study: Cohort CA: 11-16 months GA: < 12th weeks NA	**Bayley Scales 1st ed. (16)** Mental development Psychomotor development	**Mild iodine deficiency areas** ↑ in the KI group (≥150 µg), compared to the KI group (<100 and between 100-149 µg) ↑ in the KI group (≥150 µg), compared to the KI group (<100 and between 100-149 µg) ↓ 5,2 scores and ↑ 1,8 odds of a PDI < 85 in the KI group (≥150 µg/day).*
Rebagliato *et al.*, 2013 (29)	Spain <100 µg/day of KI 100–149 µg/day ≥150 µg/day Maternal UIC in both group: 102 (71-169) µg/L	Study: Cohort CA: 12-30 months GA: NA	**Bayley Scales 1st ed. (16)** Mental development Psychomotor development	**Mild iodine deficiency areas** ↑ odds in the KI group (≥150 µg), compared to KI groups (<100 and 100-149 µg) ↓ score in KI group (≥150 µg). ↑ odds in the KI group (≥150 µg), compared to KI groups (<100 and 100-149 µg) ↓ score in KI group (≥150 µg).
Markhus *et al.*, 2018 (25)	Norway 175 µg/day of KI (n = 155) Placebo (n = 658) 851 pregnant women Maternal UIC: 92 (56-200) µg/L in supplemented group, 77 (50-120) µg/L in control.	Study: Cohort CA: 6 and 18 months GA: 16-26th week	**Bayley Scales 3rd ed. (18)** Mental development Psychomotor Development Behavior Verbal IQ (WPPSI – III)	**Mild iodine deficiency areas** ↑ in the treated group compared to the placebo group ↑ in the treated group compared to the placebo group ↓ in the treated group compared to the placebo group ↑ in the treated group compared to the placebo group
Abel *et al.*, 2019 (28)	Oslo, Norway 175 µg/day of KI (n = 14,665) Placebo (n = 24,806) 39,471 pregnant women Maternal UIC: 83 (43-138) µg/L L in supplemented group, 59 (32-101) µg/L in control	Study: Cohort CA: 36 and 96 months GA: 1-22th week	**CCC-S and CCC-2 (20,21)** Language skills[a] Reading skills[a] Writing skills[a] Mapping test Reading[b] Mapping test mathematics[b] Special education[b]	**Mild iodine deficiency areas** ↑ in the treated group compared to the placebo group ↓ in the treated group compared to the placebo group ↓ in the treated group compared to the placebo group* ↓ in the treated group compared to the placebo group* ↓ in the treated group compared to the placebo group* ↓ in the treated group compared to the placebo group*
Velasco *et al.*, 2009 (24)	Spain 300 µg/day of KI (n = 133) Placebo (n = 61) Maternal MUIC: 263.0 ± 120.8 µg/L in supplementation, in control: 87.6 ± 62.1 µg/L	Study: Non-randomized controlled trial CA: 3-18 months GA: 8th to 12th week until lactation	**Bayley Scales 2nd ed. (17)** Mental development Psychomotor Development Behavior	**Mild to Moderate iodine deficiency areas** ↑ in the treated group, compared to the control group. ↑ in the treated group, compared to the control group.* ↑ in the treated group, compared to the control group.*
Santiago *et al.*, 2013 (26)	Spain Iodized salt (n = 38) 200 µg of KI (n = 55) 300 µg (n = 38) Maternal MUIC: NA	Study: Randomized controlled trial CA: 6-18 months GA: 10th week	**Bayley Scales 3rd ed. (18)** Mental development Psychomotor Development	**Mild iodine deficiency areas** ↑ in the control group, compared to the KI group (200 µg), compared to 300 ↑ in the control group, compared to the KI group (200 µg), compared to 300
Zhou *et al*, 2015 (31)	New Zealand and Australia 150 µg/day KI (n = 27) Placebo (n = 26) Maternal MUIC: 200 µg/L in supplementation and 150 µg/L in control	Study: Randomized controlled trial CA: 18 months GA: 20th week	**Bayley Scales 3rd ed. (18)** Mental development Psychomotor Development Behavior	**Mild to Moderate iodine deficiency areas** ↑ in the placebo group, compared to the treated group. ↑ in the placebo group, compared to the treated group. ↑ in the placebo group, compared to the treated group.
Gowachirapant *et al.*, 2017 (30)	Thailand and India 200 µg/day of KI (n = 303) Placebo (n = 312) 832 pregnant women (T0) Maternal MUIC: NA	Study: Randomized controlled trial CA: 12 and 24 months GA: 14th week CA: 60 and 72 months GA: 14th week	**Bayley Scales 3rd ed. (18)** Mental development Psychomotor Development Behavior Sound response time (T). Verbal IQ (WPPSI – III) IQ performance (WPPSI – III) IQ reasoning (WPPSI – III)	**Mild iodine deficiency areas** ↑ in the placebo group, compared to the treated group* ↔ between groups ↔ between groups ↑ in the treated group compared to the placebo group* ↑ in the treated group compared to the placebo group ↑ in the treated group compared to the placebo group ↑ in the treated group compared to the placebo group

MUIC: median urinary iodine concentration; NA: not available; GA: gestational age at the beginning of supplementation; Ed.: edition; KI: potassium iodide; n: sample number; T0: initial time; PDI: Psychomotor Development Index; T.: test; WPPSI-III: 3rd ed Primary Intelligence Scale; IQ: intelligence quotient; CA: Child's age in the test application; NA: not applicable; a. standardized beta; b. odds ratio. * Results with statistical significance. ↑ - increased; ↓ - reduced; ↔ - no difference.

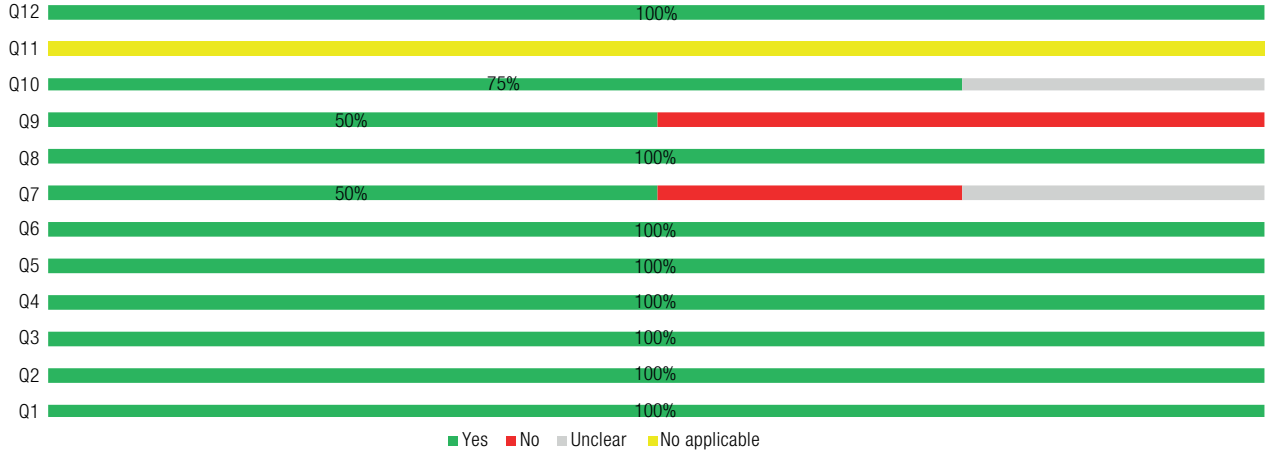
The maximum supplementation was 300 µg of potassium iodide (KI) per day (24,26) and one study did not specify supplementation dosages (28). Among the reviewed studies, five started supplementation in the first trimester (24,26,27,29), one in the 14th week (30), another between the 16th and 26th week (25), and one used four different start time categories (28). Only one study continued the supplementation in the lactation period (24); the others finished at the child's birth (Table 1). Most studies used KI (24,26,30,31); however, some studies did not specify the source of the supplementation (Table 1 and Supplemental Table 2).

The results found an association between supplementation with 150 µg of KI/day and poorer gross motor skills of the PDI standardized beta 0.18 (95% CI: -0.33, - 0.03, p = 0.02) in one study (25), but in another four studies (24,26,27,29) supplementation with ≥ 150 µg of KI/day was associated with a 5.2-point decrease in PDI (95% confidence interval: -8.1, -2.2), decrease in PDI with < 85, odds ratio: 1.7 (95% confidence interval: 1.1, 2.6). The supplementation with 200 or 300 µg of KI/day was related to lower PDI than the iodized salt group. However, another outcome of our study showed that intake of 300 µg of KI/day in breastfeeding was associated with a mean 6.1 ± 0.9 -point increase in PDI compared to the control. Three other studies (28,30,31) did not find an association between iodine supplementation and neurodevelopment in children (Table 1 and Supplemental Table 3).

Regarding the quality analysis of the studies, the authors observed some limitations in reporting the methods of all trials, leaving some uncertainty in the assessment of several bias criteria, because in two point assessed in RCT studies were high risk of bias (<50%) but, as the studies in many points were moderate or above low risk bias and evidenced a clear delineation of the intervention, as well as were published in good journals we assumed to use all studies include in our review (Figures 2 and 3).

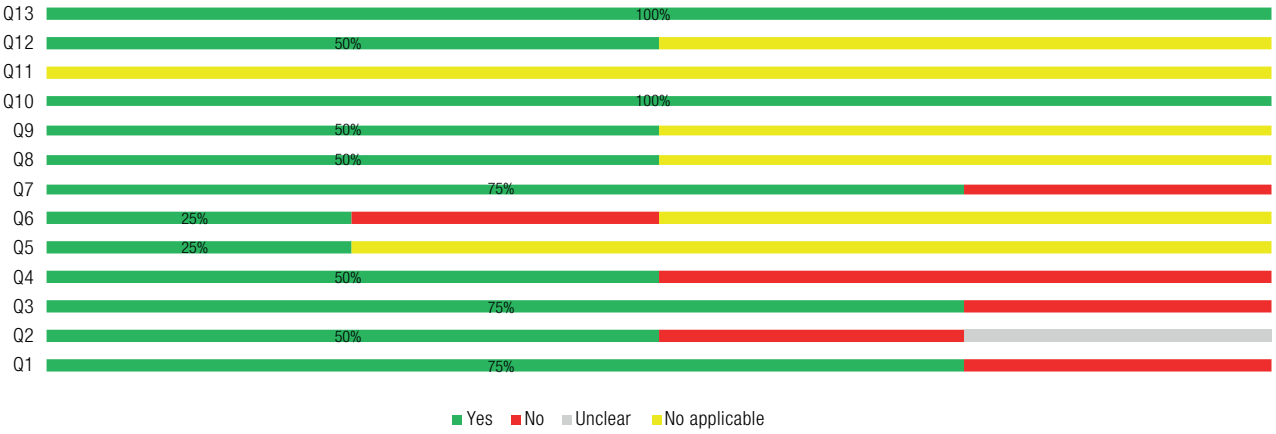
DISCUSSION

The findings showed an association between iodine supplementation and poor psychomotor development of children aged between 3 and 18 months, living in mild to moderate iodine deficiency areas.



Q1. Were the two groups similar and recruited from the same population?
Q2. Were the exposures measured similarly to assign people?
Q3. Were to both exposed and unexposed groups?
Q4. Was the exposure measured in a valid and reliable way?
Q5. Were confounding factors identified?
Q6. Were strategies to deal with confounding factors stated?
Q7. Were the groups/participants free of the outcome at the start of the study (or at the moment of exposure)?
Q8. Were the outcomes measured in a valid and reliable way?
Q9. Was the follow up time reported and sufficient to be long enough for outcomes to occur?
Q10. Was follow up complete, and if not, were the reasons to loss to follow up described and explored?
Q11. Were strategies to address incomplete follow up utilized?
Q12. Was appropriate statistical analysis used?

Figure 2. Methodological assessment quality of included studies using Joanna Briggs Institute's standardized critical appraisal instrument for cohort studies.



Q1. Was true randomization used for assignment of participants to treatment groups?
Q2. Was allocation to treatment groups concealed?
Q3. Were treatment groups similar at the baseline?
Q4. Were participants blind to treatment assignment?
Q5. Were those delivering treatment blind to treatment assignment?
Q6. Were outcomes assessors blind to treatment assignment?
Q7. Were treatment groups treated identically other than the intervention of interest?
Q8. Was follow up complete and if not, were differences between groups in terms of their follow up adequately described and analyzed?
Q9. Were participants analyzed in the groups to which they were randomized?
Q10. Were outcomes measured in the same way for treatment groups?
Q11. Were outcomes measured in a reliable way?
Q12. Was appropriate statistical analysis used?
Q13. Was the trial design appropriate, and any deviations from the standard RCT design (individual randomization, parallel groups) accounted for in the conduct and analysis of the trial?

Figure 3. Methodological assessment quality of included studies using Joanna Briggs Institute's standardized critical appraisal instrument for RCT studies.

Although not significant, other studies have shown positive results, in which children of supplemented mothers presented higher values of the psychomotor development index (25,27,29,31), behavioural (25,27,29), mental (24,25,30) and communication skills (28), when compared to those who were not supplemented. On the other hand, supplementation of mothers with between 150 and 200 µg of KI per day had no positive effect on the neurocognitive development of their children, as much as in those living in mild as well as moderate iodine deficiency areas, and in some studies the scores were low PDI point and your chance assessed were worse in the treated group (Supplemental Table 2).

None of the RCTs show an association between supplementation and child neurodevelopment, except for a negative association between iodine supplementation and expressive language (BSID) at 1 year in a single trial. The non-RCT studies show mixed results: with a positive association in one case and a negative association in the second. Children in the treatment group were associated with a lower PDI score than in the control group, with a better speaker skills score, poorer skills in the languages domain, lower mapping test results in reading in school, and suboptimal or low scores in mathematics.

Recent evidence has demonstrated these outcomes presented above, showing that 18-month-old children of mothers supplemented with 220-390 µg of KI per day had lower cognitive, language and motor scores (32).

In addition, Gowachirapan and cols. (2017) identified all development scale in primary results with placebo group had higher scores than the treatment group (30) in children aged 12 to 24 months in mild iodine deficiency areas.

Our findings mostly covered children under 24 months old, and the poor psychomotor effect on the children of supplemented mothers was demonstrated in this age group. In our results, the mothers supplemented from the 14th gestational week had a negative association between supplementation and child neurodevelopment, at ages from 14th months.

However, the start of supplementation at the 14th gestational week how showed our findings seem to be

late to start supplementation, since the development of the nervous system occurs mainly between the 5-6th gestational weeks and birth, and between birth and 2 years, for infants and children (33,34).

Most of the mothers were supplemented from the 1st trimester of gestation, and in one study, the treatment continued during lactation. Through the results of this study, it was possible to verify that the psychomotor and behavioral development differed significantly among children of mothers supplemented with 300 µg of KI per day, living in areas with mild to moderate iodine deficiency (24). Recommendations from the American Thyroid Association and European Thyroid Association indicate that supplementation started in the pre-gestational period is more effective (6,7).

Supplementation with ≥ 150 µg of KI per day in pregnancy can be improve poor psychomotor development in children. This outcome is observable in lactation if supplementation dose is doubled (300 µg of KI per day).

In another study, children of mothers living in mild iodine deficiency areas and supplemented with 200 µg of KI per day during gestation showed a better response time to sound at 60 to 72 months than their is not supplemented group, but there was no difference between the groups (Supplemental Table 2) (30). This was the only study that used other methods to assess child development beyond the Bayley scale (30), and was the only one that assessed children over two years old, showing that this may be a more interesting time to assess the children's development. However, use of the Children's Communication Checklist-Short showed to be better for the assessment of skills and knowledge, including the domains of writing, speaking, reading, mathematical calculations and all languages in older children (>3 years old). This method uses the mental and behavioural skills applicable to the Bayley scale (mental and behaviour development index), and we did not find an association between iodine supplementation and this score in our results (28). These findings were reported for other authors that used the CCC-S to assess older children and used the Bayley score to assess the infant group; they did not find clear differences between these groups (35).

Although the findings showed poor psychomotor development in the children of the supplemented mothers, it seems that this effect is more pronounced in younger children compared to older children using the Bayley scale. However, we observed a high score of the sound response time in children from 60 months, open in this age the children are keen senses.

The use of developmental scales requires caution, since they depend on the evaluator's observation (36). Despite this, the use of these scales seems to have good results for those living in areas with mild to moderate iodine deficiency. However other factors that may interfere in test results are family income, mother's education, inadequate urinary iodine concentration (UIC) of the mother, and the presence of siblings, since they directly influence the family stimulus that the child receives (7,11,30,36).

The lack of similarity between initial time, duration, dosage of supplementation, and the time of application of neurocognitive development tests were limiting factors. In addition, three of the seven studies did not assess behavioural development.

The authors observed that supplementation during lactation brings interesting results, which may be the starting point for future research. In areas with mild to moderate iodine deficiency, changes are more likely to develop in children's psychomotor, behavioural, and mental capabilities. The authors questioned whether the duration of supplementation may have a greater influence than the dose administered, since we did not find any studies with a longer time of supplementation with a lower dose of iodine content, nor did we obtain further assessments of lactation.

The best neurodevelopmental can be good in children with mother living in iodine adequate areas. However, in these results, the mothers in the control group had below adequate UIC, showing iodine deficiency for maternal group in region, which can affect the outcomes in their offspring. Additionally, according to Mao and cols. (2018), the supplementation of pregnant women living in areas of mild iodine deficiency did not have any effect on their children's neurocognitive development (35).

Improving some factors, such as the start and end times of supplementation, iodine sufficiency of the mothers and the iodine deficiency in the areas where the mothers live, as well as the age of the children and the type of scale used in the tests, can contribute to better results. Therefore, iodine supplementation, if well implemented, can reduce risks to the population and, consequently, reduce public health expenditure.

Final remarks: In general, in this study we did not find an association between iodine supplementation in pregnant women and the neurodevelopment of their children in mild to

moderate iodine deficiency areas. Despite this, supplementation in pregnancy and lactation can be improve poor psychomotor development in children. However, in older children, it seems to have a greater effect on the sound response time. Supplementation in pregnant women also improved urinary iodine concentration of the mother and her children, as well as leading to a high PDI score in young children. Thus, it is necessary to perform further studies using the Bayley scale or another scale alongside the Children's Communication Checklist-Short (CCC-S) or CCC-2 to assess the effect of iodine supplementation in pregnant women in iodine deficiency areas on the neurodevelopment of children before, during and after pregnancy.

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Supplement Appendix 1

The full list of search terms used in the literature search

In March 1st to April first week of 2020 The PubMed, LILACS and Scopus databases were searched for relevant articles.

For PubMed (01 RCT and 02 cohort article), LILACS (01 cohort articles) and Scopus database (03 RCT and 01 cohort articles) the following search terms were used; "iodine AND supplementation AND child And development AND cognition ".

******** ********

For PubMed (03 articles), the following search terms were used: ((iodine) AND (supplementation) AND (child) AND (development) AND (cognition)).

((((“iodine”[MeSH Terms] OR “iodine”[All Fields] OR “iodides”[MeSH Terms] OR “iodides”[All Fields]) AND Supplementation[All Fields]) AND (“child”[MeSH Terms] OR “child”[All Fields])) AND (“growth and development”[Subheading] OR (“growth”[All Fields] AND “development”[All Fields]) OR “growth and development”[All Fields] OR “development”[All Fields])) AND (“Cogn Int Conf Adv Cogn Technol Appl”[Journal] OR “cognitive”[All Fields])

******** ********

For LILACS (01 article), the following search terms were used: “iodine AND supplementation AND child And development AND cognition”.

tw:((tw:(iodine)) AND (tw:(supplementation)) AND (tw:(child)) AND (tw:(development)) AND (tw:(cognitive))))

******** ********

For Scopus database (04 articles), the following search terms were used: “iodine AND supplementation AND child And development AND cognition”

(TITLE-ABS-KEY (iodine) AND TITLE-ABS-KEY (supplementation) AND TITLE-ABS-KEY (child) AND TITLE-ABS-KEY (development) AND TITLE-ABS-KEY (cognition))

Total: 08 articles included

LILACS: 01.

Scopus: 03.

PubMed: 04.

Supplemental Table 1. Excluded studies and reasons for exclusion

Reference	Reason for exclusion
A review of the iodine status of UK pregnant women and its implications for the offspring	Is revision
Assessing infant cognitive development after prenatal iodine supplementation	Is revision
Benefit-Cost Analysis in Disease Control Priorities, Third Edition.	Not iodine
Can multi-micronutrient food fortification improve the micronutrient status, growth, health, and cognition of schoolchildren? A systematic review	Is revision
Consequences of iodine deficiency and excess in pregnant women: an overview of current knowns and unknowns	Not iodine
Dietary micronutrients are associated with higher cognitive function gains among primary school children in rural Kenya	Not iodine
Does prenatal micronutrient supplementation improve children's mental development? A systematic review	Is revision
Driving Policy Change to Improve Micronutrient Status in Women of Reproductive Age and Children in Southeast Asia: The SMILING Project.	Is revision
Effect of iron-, iodine-, and β-carotene-fortified biscuits on the micronutrient status of primary school children: A randomized controlled trial	Not pregnant
Effect of micronutrient supplement on health and nutritional status of schoolchildren: Study design	Not pregnant
Effects of iodine supplementation during pregnancy on child growth and development at school age	Supplementation in child
Effects of maternal iodine nutrition and thyroid status on cognitive development in offspring: A pilot study	Not supplementation
Effects of nutrients (in food) on the structure and function of the nervous system: Update on dietary requirements for brain. Part 1: Micronutrients	Not iodine
Effects of nutritional interventions during pregnancy on infant and child cognitive outcomes: A systematic review and meta-analysis	Is revision
Feeding the brain – The effects of micronutrient interventions on cognitive performance among school-aged children: A systematic review of randomized controlled trials	Is revision
Food ingredients and cognitive performance	Not iodine
Growth, development and differentiation: A functional food science approach	Not iodine
Hypothyroxinemia and pregnancy	Not child neurodevelopment
Impact of iodine supplementation in mild-to-moderate iodine deficiency: Systematic review and meta-analysis	Is revision
Iodine as essential nutrient during the first 1000 days of life	Not pregnant
Iodine deficiency and iodine prophylaxis in pregnancy	Is revision

Reference	Reason for exclusion
Iodine deficiency in pregnancy and the effects of maternal iodine supplementation on the offspring: a review.,	Is revision
Iodine deficiency in pregnancy, infancy and childhood and its consequences for brain development,	Is revision
Iodine fortification of foods and condiments, other than salt, for preventing iodine deficiency disorders.	Not supplementation
Iodine intake from supplements and diet during pregnancy and child cognitive and motor development: The INMA Mother and Child Cohort Study.	Not intervention
Iodine Nutrition During Pregnancy: Past, Present, and Future.	Is revision
Iodine nutrition in pregnancy and lactation.	Is revision
Iodine plus n-3 fatty acid supplementation augments rescue of postnatal neuronal abnormalities in iodine-deficient rat cerebellum,	Not human
Iodine supplementation improves cognition in iodine-deficient schoolchildren in Albania: A randomized, controlled, double-blind study	Supplementation in child
Iodine supplementation improves cognition in mildly iodine-deficient children	Supplementation in child
Iodine supplementation in pregnancy and its effect on child cognition	Not child neurodevelopment
Iodine: it's important in patients that require parenteral nutrition.	Not supplementation
Iron deficiency and cognitive functions	Not iodine
Malnutrition, brain development, learning, and behavior,	Not iodine
Maternal iodine status is associated with offspring language skills in infancy and toddlerhood.	Not iodine
Micronutrient adequacy and morbidity: Paucity of information in children with cerebral palsy.	Not iodine
Micronutrient interventions on cognitive performance of children aged 5-15 years in developing countries.	Is revision
Micronutrient supply and health outcomes in children.	Not iodine
Micronutrients in pregnancy in low- and middle-income countries.	Not iodine
Mild iodine deficiency in pregnancy in Europe and its consequences for cognitive and psychomotor development of children: A review,	Is revision
Multiple micronutrient supplementation for improving cognitive performance in children: Systematic review of randomized controlled trials,	Is revision
Neurocognitive outcomes of children secondary to mild iodine deficiency in pregnant women,	Not child neurodevelopment
Nutrient supplementation and neurodevelopment timing is the key,	Not iodine
Nutrition and brain development in early life,	Not iodine
Nutrition and development: Other micronutrients' effect on growth and cognition,	Not iodine
Nutrition and neurodevelopment in children: Focus on NUTRIMENTHE project,	Not iodine
Nutritional deficiencies and later behavioral development,	Not supplementation
Overall child development: Beyond pharmacological iodine supplementation	Is revision
Prevention and control of iron deficiency anemia amongst young children,	Not iodine
Promoting early child development with interventions in health and nutrition: A systematic review	Is revision
Role of iodine-containing multivitamins during pregnancy for children and brain function: protocol of an ongoing Randomized controlled trial: the SWIDDICH study.,	Not child neurodevelopment
Suggested use of sensitive measures of memory to detect functional effects of maternal iodine supplementation on hippocampal development,	Not in human
Summary of the public affairs committee symposium at the teratology society 2011 annual meeting: The thyroid and iodine: Impacts on pregnancy and child health	Not article
Systemic endocrinopathies (thyroid conditions and diabetes): impact on postnatal life of the offspring.	Not supplementation
Teratology public affairs committee position paper: Iodine deficiency in pregnancy	Not supplementation
The adverse effects of mild-to-moderate iodine deficiency during pregnancy and childhood: A review,	Is revision
The Assessment of Cognitive Performance in Children: Considerations for Detecting Nutritional Influences,	Not iodine
The Effect of Intermittent Antenatal Iron Supplementation on Maternal and Infant Outcomes in Rural Viet Nam: A Cluster Randomized Trial	Not iodine
The effect of iodine supplementation in pregnancy on early childhood neurodevelopment and clinical outcomes: Results of an aborted randomized placebo-controlled trial,	Not child neurodevelopment
The effects of iodine deficiency in pregnancy and infancy,	Not supplementation
The importance of adequate iodine during pregnancy and infancy,	Not iodine
The influence of dietary status on the cognitive performance of children,	Not iodine

Reference	Reason for exclusion
The role of nutrition in children's neurocognitive development, from pregnancy through childhood,	Is revision
Therapy of endocrine disease: Impact of iodine supplementation in mild-to-moderate iodine deficiency: systematic review and meta-analysis.	Is revision
Thyroglobulin level at week 16 of pregnancy is superior to urinary iodine concentration in revealing preconceptual and first trimester iodine supply.	Is revision
Thyroid and iodine nutritional status: a UK perspective.	Is revision
Thyroid-stimulating hormone (TSH) concentration at birth in Belgian neonates and cognitive development at preschool age.	Not supplementation
What do we know about iodine supplementation in pregnancy?	Is revision
Biomarkers of Nutrition for Development (BOND): Vitamin B-12 Review",2018	Review and not iodine
Iodine as essential nutrient during the first 1000 days of life",2018	Not pregnant
Iodine intake from supplements and diet during pregnancy and child cognitive and motor development: The INMA Mother and Child Cohort Study",2018	Not iodine intake
Iodine supplementation for the prevention of mortality and adverse neurodevelopmental outcomes in preterm infants. 2019	Review
Mild-to-moderate gestational iodine deficiency processing disorder",2019	Review
Role of iodine-containing multivitamins during pregnancy for children's brain function: Protocol of an ongoing randomized controlled trial: The SWIDDICH study",2018	Protocol
Supplementing mothers and their offspring with long-chain ω-3 PUFAs offers no benefit compared with placebo in infant development",2019	Not iodine
The effect of iodine deficiency during pregnancy on child development",2019	Review
Thyroid function in preterm infants and neurodevelopment at 2 years. 2020	Direct supplementation in infant
WITHDRAWN: Iodine supplementation for preventing iodine deficiency disorders in children. Nov. 2018	Review
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Supplemental Table 2. Description of the interventions applied in the evaluated studies

Author/ Year	Dosage	Supplementation interval	Form of supplementation
Velasco *et al.,* 2009[24]	300 µg/day of iodine	≤13th week of pregnancy to lactation	Potassium iodide (KI)
Murcia *et al.*, 2011[27]	<100 µg/day 100–149 µg/day ≥150 µg/day	≤13th week of pregnancy to delivery	Different supplementary sources of iodine
Santiago *et al.*, 2013[26]	200 µg/day 300 µg/day	≤10th week of pregnancy to delivery	KI
Rebagliato *et al.*, 2013[29]	<100 µg/day of KI 100– 149 µg/day ≥150 µg/day	≤13th week of pregnancy to delivery	KI or vitamin/ mineral preparations containing iodine
Zhou *et al.,* 2015[31]	150 µg/day	≤20th week of pregnancy to delivery	KI
Gowachirapant *et al.,* 2017[30]	200 µg/day	≤14th week of pregnancy to delivery	KI
Markhus *et al.*, 2018[25]	150 – 200 µg/day	≤26th week of pregnancy to delivery	Different supplementary sources of iodine
Abel *et al.,* 2019[28]	NA	Week 0-26 before pregnancy GW: 0–12 GW: 12–22	Different supplementary sources of iodine

KI- Potassium iodide; GW: Gestational Week; NA- Not available.

Supplemental Table 3. Effects of iodine supplementation in pregnancy on neurocognitive development on offspring

Trial	Iodine Status of region	Mental development (MDI)	Psychomotor development (PDI)	Behaviour development (BDI)	Sound response time (T).	Verbal IQ (WPPSI – III)	IQ performance (WPPSI – III)	IQ reasoning (WPPSI – III)
Velasco *et al.*, 2009 (24)	Maternal MUIC during pregnancy (last third trimester): MUIC 263.0 ±120.8 µg/L iodine (n=133) > control 87.6 ± 62.1 µg/L (n=31, Iodine in breast milk: within 3.6 ± 2.9 months of lactation, intake 1.8 ± 1.2 µg/100 mL, in 300 µg/day of supplemented group (n = 67), vs. 1.4 ± 0.6 µg/100 mL, without supplement in control group (n=21). Infant MUIC: 203.5 ± 150.6 µg/L iodine, in 300 µg/day supplemented group (n= 75) > control 114.6 ± 60.9 µg/L iodine (n= 30) Southern of Spain (Malaga and Osuna in Andaluzia state) is areas of mild to moderate iodine deficiency and iodine deficiency in pregnant	Infant: NA	Maternal intake of 300 µg/day, that were breastfeeding compared with without group (n= 19) associated with a 6.1+0,9 point high in PDI with control. High PDI in child was associated also with lower FT4 in third trimester of pregnancy in mother in supplemented group (r: 0.50; P <0.0001).	Mother intake 300 µg/day of iodine in supplements as compared with the control group was seems more in agreement with for the following items: To reaction to the mother, Odds reaction: 4.3 (CI 95%; 1.69 –10.86, P <0.05), Cooperation, Odds reaction: 17.29 (CI 95%; 2.00 –148.86) 0.009 22.45, P<0.008), activity, Odds reaction: 8.55 (CI 95%; 1.64–44.44, P<0.01), and producing sounds by banging, Odds reaction: 9.00 (CI 95%; 2.66 –30.44) (P <0.001).	NA	NA	NA	NA
Murcia *et al.*, 2011 (27)	Maternal MUIC during pregnancy: NA. Infant MUIC.: NA Valencia, is north of Spain,there does not know of iodine situation. But recently evidence (INNA Project) showed an increased risk of raised thyroid stimulating hormone (TSH) during the first half of pregnancy for women who consumed iodine supplements, because in some years ago there were iodine deficiency	Infant: NA	Maternal intake of ≥150 mcg/day, compared with <100 mcg/day, of iodine from supplements was associated with a 5.2- point decrease in PDI (95% confidence interval: -8.1, -2.2) and a 1.8-fold increase in the odds of a PDI <85 (95% confidence interval: 1.0, 3.3)	NA	NA	NA	NA	NA	NA

Trial	Iodine Status of region	Mental development (MDI)	Psychomotor development (PDI)	Behaviour development (BDI)	Sound response time (T).	Verbal IQ (WPPSI – III)	IQ performance (WPPSI – III)	IQ reasoning (WPPSI – III)
Santiago *et al.*, 2013 (26)	Maternal MUIC during pregnancy: NA MUIC least 1 year before becoming pregnancy: 177.1 + 82.3 µg/L in 200 µg group (n=55) and 222.0 +85.5 µg/L in 300 µg/L in supplemented group (n=38), with control 130.2 + 64.8 µg/L (n=38) Iodine in breast milk: Not diff. in groups but all is >100 µg/L: in treatment groups is high intake: 2.4·+1.2 µg/100ml in 200 µg group, 2.2+1.5 µg/100ml in 300 µg group, that in control group: 1.4+0.9µg/100ml Infant MUIC: NA Objective of this study was assessment iodine intake and their consequences in health in area with iodine deficiency	Maternal supplementation with 200 or 300 µg/day was related with lower PDI than the iodized salt group (NA). The results showed that the age at which this psychometric test was performed correlated significantly with a scales MDS (r 0·93,P,0·001) and the PDS (r 0·90, P,0·0001), but not with indices (MDI and DPI). The showing a age to be important factor of measured its testes.		NA	NA	NA	NA	NA
Rebagliato *et al.*, 2013 (29)	Maternal MUIC during pregnancy: Median in three municipality of Valencia, in both groups is Asturias 102 (71–169) µg/L (n = 412) Gipuzkoa 169 (108–284) µg/L (n = 548) Sabadell 90 (52–148) µg/L (n = 559) Infant MUIC: NA In Asturias and Gipuzkoa is iodine sufficiency areas and Sabadell is several. But the supplementation iodine program is implemented in both because the author wanted to see effect of neurodevelopment on infant in sufficiency and deficiency iodine consumer.	A negative association of iodine supplementation with low development. No difference with groups, site of study and user of iodized salt or supplement. But consumption during pregnancy of 150 µg/day or more of iodine was related to a decrease in Psychomotor development score, although only significantly so in Asturias less than 85 (OR = 1.7, 95% CI: 1.1, 2.6).		NA	NA	NA	NA	NA

Trial	Iodine Status of region	Mental development (MDI)	Psychomotor development (PDI)	Behaviour development (BDI)	Sound response time (T).	Verbal IQ (WPPSI – III)	IQ performance (WPPSI – III)	IQ reasoning (WPPSI – III)
Zhou *et al*., 2015 (31)	Maternal MUIC during pregnancy 3rd trimester: 200 µg/L in supplemented group > (n=27), with 150 µg/L (n=26) in control Iodine in breast milk: 6 weeks after birth was 1.1 (0.8–1.5) µg/100ml in both groups. 150 µg/day of KI (n=29) for supplemented group, with without iodine supplements in control (n=30) Infant MUIC: NA Australia and New Zeland are moderate iodine deficiency areas.	Infant: NA	Infant: NA	Infant: NA	NA	NA	NA	NA
Gowachirapan *et al*., 2017 (30)	Maternal MUIC during pregnancy: NA 200 µg/day of KI group (n=303) Placebo (n=312) Infant MUIC: NA In INNA Study, the authors found in a sample of women from the Valencia region, an iodine-sufficient areas, that maternal consumption of multivitamins containing iodine was related to lower psychomotor achievement of their infants at 1 year of age.	All the scores in the primary outcomes were higher in mean in the placebo than in the intervention group (not significant).			Infant: no diff iodine vs. control (n=unclear, means not presented)	Infant: A negative association of iodine supplementation with expressive language (BSID) at 1 year in the first one	Infant: NA	Infant: no congenital goitre was found in either group
Markhus, M. W., 2018 (25)	Maternal MUIC during pregnancy 1st trimester: Median 92 (56,200) µg/L in supplemented group (n=658) > and 77 (50,120) µg/L in control (n=155). In total: 79% of women had a MUIC < 150 µg/L and 28% < 50 µg/L. 175 µg/day of KI (n=155) for supplemented group, with without iodine supplements in control (n=658). Infant MUIC: NA	Infant: NA	Supplementation with 150 µg/day was associated with poorer gross motor skills, standardized beta = - 0.18 (95% CI = -0.33, - 0.03, p = 0.02).	NA	NA	Infant: NA But women having a low MUIC in pregnancy (50> but <100 µg/L) was significantly associated with poorer skills in language domains (receptive and expressive) in infancy and toddlerhood.	NA	NA

Trial	Iodine Status of region	Mental development (MDI)	Psychomotor development (PDI)	Behaviour development (BDI)	Sound response time (T).	Verbal IQ (WPPSI – III)	IQ performance (WPPSI – III)	IQ reasoning (WPPSI – III)
Abel M.H. al., 2018 (28)	Maternal MUIC during pregnancy 1st and 2nd trimester: Median 83 (43, 138) µg/L in supplemented group (n= 14,665) > and 59 (32, 101) µg/L in control (n= 24,806). 175 µg/day of KI (n=14,665) for supplemented group, with without iodine supplements in control (n=24,806). Infant MUIC: NA	NA The results are consistent in demonstrating no beneficial effects of iodine supplement use in pregnancy. Not have any associations between maternal use of iodine-containing supplements and child outcomes. But in sample, 6.9% of the 8year old were granted special education at school. According to the mother, 28% had suboptimal or low score on the mandatory mapping test in school in reading, and 18% had suboptimal or low score in mathematics.			NA	NA But in control: Materna iodine intake was associated with child language skills at age 3 years. children with mild to moderate language delay at 3 years (3.1%) scored on average 1.2 SD higher on the language score (CCC-S) at 8 years (95% CI 1.1, 1.3), and those with severe language delay at 3 years (0.7%) scored 2.8 SD higher (95% CI 2.3, 3.2). Higher scores at 8 years indicated poorer language skills.	NA	NA

NA, Not available; MUIC, median urinary iodine concentration; n, number; KI, iodide potassium; No diff, no difference; UI, urinary iodine; CCC-S: Children's Communication Checklist-Short.

Management of thyroid disorders during the COVID-19 outbreak: a position statement from the Thyroid Department of the Brazilian Society of Endocrinology and Metabolism (SBEM)

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ABSTRACT

This position statement was prepared to guide endocrinologists on the best approach to managing thyroid disorders during the coronavirus disease (COVID-19) pandemic. The most frequent thyroid hormonal findings in patients with COVID-19, particularly in individuals with severe disease, are similar to those present in the non-thyroidal illness syndrome and require no intervention. Subacute thyroiditis has also been reported during COVID-19 infection. Diagnosis and treatment of hypothyroidism during the COVID-19 pandemic may follow usual practice; however, should avoid frequent laboratory tests in patients with previous controlled disease. Well-controlled hypo and hyperthyroidism are not associated with an increased risk of COVID-19 infection or severity. Newly diagnosed hyperthyroidism during the pandemic should be preferably treated with antithyroid drugs (ATDs), bearing in mind the possibility of rare side effects with these medications, particularly agranulocytosis, which requires immediate intervention. Definitive treatment of hyperthyroidism (radioiodine therapy or surgery) may be considered in those cases that protective protocols can be followed to avoid COVID-19 contamination or once the pandemic is over. In patients with moderate Graves' ophthalmopathy (GO) not at risk of visual loss, glucocorticoids at immunosuppressive doses should be avoided, while in those with severe GO without COVID-19 and at risk of vision loss, intravenous glucocorticoid is the therapeutic choice. Considering that most of the thyroid cancer cases are low risk and associated with an excellent prognosis, surgical procedures could and should be postponed safely during the pandemic period. Additionally, when indicated, radioiodine therapy could also be safely postponed as long as it is possible. Arch Endocrinol Metab. 2021;65(3):368-75

Keywords

Thyroid disorders; hypothyroidism; hyperthyroidism, subacute thyroiditis; COVID-19

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INTRODUCTION

The novel coronavirus disease (COVID-19) caused by the acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has affected millions of people worldwide since the first reported case in December 2019 (1). In early 2020, Brazil became the epicenter of the outbreak in Latin America (2) and the second country with the highest infection rate worldwide, behind only the United States (3).

The COVID-19 pandemic requires a joint effort from health care professionals of all areas of knowledge, including endocrinologists, to help fight the progress

and consequences of the infection within their areas of expertise (4-6). Increasing evidence suggests that patients with prior endocrine diseases are at increased risk of developing severe COVID-19 (7-9), especially individuals with type 2 diabetes mellitus (10,11) or obesity (12), while data on thyroid involvement in SARS-CoV-2 infection are still scarce (13-16). A few studies that have addressed this area of concern have found associations of COVID-19 infection with abnormalities of the pituitary-thyroid axis (17), subacute thyroiditis (18-21), Hashimoto's thyroiditis (22), and thyrotoxicosis (23). Thus, the challenges for endocrinologists and clinicians caring for patients with COVID-19 are the recognition of potential thyroid abnormalities in patients with no preexisting thyroid disease and the management of patients with previously diagnosed thyroid disorder. Based on these considerations, the Thyroid Department of the Brazilian Society of Endocrinology and Metabolism has prepared the present position statement to guide endocrinologists on delivering the best care to patients with thyroid disorders during the pandemic. For preparation of this statement, the authors performed a search of the English language literature on PubMed, Google Scholar, SciELO, and LILACS using the keywords "COVID-19" plus "thyroid", "hypothyroidism", "hyperthyroidism", "Graves' disease", Graves' ophthalmopathy", or "thyroiditis".

PATIENTS WITHOUT PREEXISTING THYROID DISEASE

Thyroid diseases are among the most common endocrine disorders in the general population and are highly prevalent in Brazil, one of the countries with the highest rates of thyroid diseases worldwide (24). About 12.3–17.5% of the adult population (25-27) has some type of thyroid disorder, many of whom are undiagnosed. Therefore, many individuals who become infected with COVID-19 may present with an unknown thyroid disorder.

Both SARS-CoV-1 and now SARS-CoV-2 have been associated with abnormal thyroid function (9). A retrospective Chinese study (17) identified lower serum TSH and total T3 levels in hospitalized patients with COVID-19 compared with controls not infected with the virus. These hormonal abnormalities affected 18% of 50 patients with laboratory-confirmed COVID-19 and worsened with the severity of the disease. Similar findings are generally observed in critically ill patients during the acute phase of several diseases in the absence of hypothalamic-pituitary-thyroid primary dysfunction, a condition known as "euthyroid sick syndrome" or "non-thyroidal illness syndrome" (28), and have also been reported in patients with nonsevere COVID-19 (29). However, the Chinese study reported that 34% of the patients with COVID-19 infection presented low serum TSH levels only (and normal T3), which is an uncommon finding in non-thyroidal illness syndrome (17).

In another retrospective study (23), including 287 consecutive patients hospitalized for COVID-19 in non-intensive care units, 20.2% had thyrotoxicosis, 5.2% had hypothyroidism, and 74.6% were euthyroid. In that study, Lania and cols. did not clarify the exact mechanisms responsible for thyroid dysfunctions. However, they hypothesized that thyrotoxicosis was caused by destructive thyroiditis since thyrotoxicosis was often mild, improved spontaneously, and antithyroid antibodies were negative in the nine patients in whom these antibodies were evaluated. More recently, Muller and cols. also showed a substantial number of thyrotoxicosis cases (n=13) among 85 patients with COVID-19 (30).

Subacute thyroiditis after COVID-19 has also been reported. The first case was reported in an 18-year-old woman in Italy, who presented typical manifestations of subacute thyroiditis 15 days after the identification of SARS-CoV-2 on oropharyngeal swab (20). The patient presented mild elevation in serum free T4 and free T3 levels, undetectable serum TSH level, and multiple diffuse hypoechoic areas on thyroid ultrasound. She was treated with prednisone 25 mg/day, and her thyroid function and inflammatory markers normalized within 40 days. After the publication of this case, other similar cases have been reported (18,19,21,31). Another recent report was the first description of a patient developing Hashimoto's thyroiditis after (7 days) mild COVID-19 infection (22).

Multiple mechanisms could be involved and partially explain the thyroid abnormalities observed during COVID-19 infection, including direct effects of the virus on the thyroid and pituitary cells, and indirect systemic effects by inflammatory cytokines (9,17,23,32). Of note, the angiotensin-converting enzyme 2 receptor, considered the gateway of SARS-CoV-2 entry into cells, is highly expressed in the thyroid gland (33), but detection of SARS-CoV-2 specifically in the thyroid tissue has not been reported to date.

The finding of low T3 levels in the context of non-thyroidal illness syndrome has been associated with increased mortality (28), but there is currently no consistent evidence showing a benefit of T3 administration in patients with the syndrome (34). Still, an ongoing phase II randomized, double-blind, placebo-controlled trial is investigating the potential impact of high-dose intravenous T3 on the recovery of critically ill patients with COVID-19 infection (35).

Regarding patients without preexisting thyroid disorders, this statement conclude that clinicians and endocrinologists should keep in mind the occurrence of possible thyroid disorders during and after COVID-19 infection. The most frequent findings related to thyroid hormone levels in patients with COVID-19, particularly in individuals with severe disease, are similar to those present in the non-thyroidal illness syndrome and require no intervention. As these manifestations are generally transient and do not reflect an actual thyroid abnormality, investigation of thyroid function during acute COVID-19 infection in critically ill patients should be avoided and only performed when a thyroid disorder is strongly suspected. Thyroid function could be assessed in patients recovering from COVID-19 infection depending on the context, *e.g.*, severe COVID-19 infection, personal or family history of autoimmune thyroid disease, and presence of thyroid-related symptoms. Subacute thyroiditis may develop simultaneously with COVID-19 or up to 6 weeks after the symptoms of COVID-19 have disappeared (18-21,31), and nonsteroidal antiinflammatory agents should be favored in this setting (36). If required, low-dose prednisone, preferably up to 20 mg/day (37) or even 25 mg/day (21), could be safely administered when patients fail to respond to nonsteroidal antiinflammatory agents (36).

PATIENTS WITH PREEXISTING THYROID DISEASE

Hypothyroidism

Hypothyroidism is the most common thyroid disorder, ranging in prevalence from 5–15% of the general population. Hypothyroidism may be caused by Hashimoto's thyroiditis and may occur after radioiodine therapy or thyroidectomy. Independent from the etiology, hypothyroidism treatment consists of levothyroxine replacement to maintain normal serum TSH levels (38).

One study showed no association between hypothyroidism and a higher risk of infection and increased morbidity and mortality with COVID-19 (39). However, poorly controlled hypothyroidism may increase a patient's risk of viral infection and complications (40).

Importantly, ongoing recommendations for diagnosing and treating hypothyroidism should be maintained during the COVID-19 pandemic (39). The usual levothyroxine dose should be maintained if a patient develops COVID-19 infection (41,42), and frequent blood test monitoring should be avoided, especially in patients receiving regular treatment. The same applies for patients recently diagnosed with hypothyroidism or in whom treatment has not been initiated yet. An exception should be made for patients who manifest symptoms of uncontrolled hypothyroidism, in whom serum TSH and free T4 levels should be measured (39,40); telemedicine is an option for adjustment of levothyroxine dose in these patients (43).

Levothyroxine is available at different commercial presentations and generic forms, and no shortage of levothyroxine has been reported during the pandemic (39,41,42). The patients should be instructed not to stock up on levothyroxine, so this medication remains available for everyone (40).

Graves' disease

Management of Graves' disease (GD), the leading cause of hyperthyroidism (44,45), includes antithyroid drugs (ATDs), radioiodine therapy, or surgery. Radioiodine therapy and total thyroidectomy (TT) are considered definitive therapies and intend to render the patient hypothyroid, requiring lifelong levothyroxine replacement, thus preventing recurrence of hyperthyroidism (44,45).

The diagnosis of GD is based on the occurrence of clinical findings of hyperthyroidism associated with the presence of diffuse goiter, Graves' ophthalmopathy (GO) (20–50% of the cases), and laboratory tests compatible with thyrotoxicosis (44,45). Finding of positive serum thyroid receptor antibodies (TRAb) helps to define an autoimmune involvement. Thyroid scintigraphy should be reserved for patients with an equivocal diagnosis and in those with nodular goiter and undetectable TRAb (44,45).

The treatment of hyperthyroidism caused by GD in the current phase of the COVID-19 pandemic can

be divided into two scenarios: 1) treatment of patients with a prior diagnosis of GD and on regular treatment with ATD, and 2) treatment of patients with recently diagnosed GD who have not started therapy yet.

In the first scenario, and especially at the current stage of the pandemic when face-to-face consultations can be difficult, treatment with ATD should not be interrupted, as any relapse would require an urgent medical appointment and increase the risk of complications (*e.g.*, thyroid storm), which can be triggered by infections, including COVID-19 infection (45). Treatment with ATD is generally maintained for 12–24 months; after this period, the medication can be suspended. Alternatively, prolonged use of low-dose ATD may be considered, as it is safe and may increase the chance of GD remission (46,47). During the pandemic, telemedicine may be an alternative to manage patients with hyperthyroidism (43). If possible, definitive treatment of GD (radioiodine therapy or TT) may be carried out after the pandemic is over.

In the second scenario (patients with a recent diagnosis of GD), ATD should be the first therapeutic option due to possible restrictions regarding nuclear medicine or surgical treatment at this time. The rare but potential side effects of ATDs should be kept in mind, especially agranulocytosis (< 500 neutrophils/mL). This complication affects 0.3–0.5% of the patients using ATD and has a mortality rate of about 5%. It occurs more frequently in older patients, at the beginning of treatment, and with high doses of ATD, especially methimazole (45). Patients should be advised about the possibility of leukopenia and to seek immediate help for measurement of white blood cell count in the presence of fever, odynophagia, and flu-like symptoms. In patients with severe side effects (agranulocytosis, drug-induced hepatitis), ATDs should be suspended, and definitive treatment should be performed (45,47).

Periods of increased stress (*e.g.*, war, pandemic) tend to be associated with increased rates of autoimmune diseases (48,49). Considering that, patients currently experiencing GD remission may present recurrence of the disease, and an eventual increase in the number of new GD cases may occur. Clinicians must be attentive to this fact to promptly identify new cases and carry out early diagnosis and treatment.

Thyroid storm

Thyroid storm is a life-threatening condition characterized by excessive thyroid hormone secretion or release that can lead to the collapse of several organs and eventual death (50,51). Treatment of thyroid storm consists of supportive measures, beta-blockers, ATDs, and glucocorticoids. Some issues related to thyroid storm must be highlighted, considering the current pandemic. The treatment of thyroid storm should be carried out in a hospital setting, which increases the risk of the patient becoming infected with SARS-CoV-2. Additionally, the use of glucocorticoids during thyroid storm, which had been initially avoided in patients with COVID-19 infection, may be incorporated in the therapeutic arsenal of this endocrine emergency, since recent studies has demonstrated a beneficial effect of corticosteroids on outcome of patients infected with coronavirus after hospitalization (52,53).

In theory, it is possible for COVID-19 infection to trigger thyroid storm in patients with poorly controlled hyperthyroidism or in those with undiagnosed hyperthyroidism. Furthermore, the challenges in obtaining adequate medical follow-up in the context of the COVID-19 pandemic may, eventually, favor the onset of thyroid storm.

Concerning ATDs, the American Thyroid Association (ATA) favors the use of propylthiouracil over methimazole during thyroid storm, since propylthiouracil inhibits T4 to T3 conversion in peripheral tissues, consequently reducing the effects of T3 on target tissues and leading to faster clinical improvement (36). However, reduced T4 to T3 conversion can also be achieved effectively with other measures, such as administration of glucocorticoids (300 mg hydrocortisone intravenous load, followed by 100 mg every 8 hours), high doses of propranolol, and solutions containing inorganic iodine (36). Importantly, iodine-containing solutions, if chosen, must be administered at least 1 hour after the first dose of the chosen ATD.

Surgical treatment should be performed in the rare circumstance of a patient not responding satisfactorily to ATDs, developing severe side effects associated with these agents, or being unable to undergo radioiodine therapy (*e.g.*, unavailability of radioiodine, pregnancy, lactation). In preparation for thyroidectomy in these situations, it is recommended the administration of solutions containing inorganic iodine (potassium iodide, Lugol's solution, and iodinated contrasts) in addition to corticosteroids and beta-blockers (as previously described) (54).

Graves' ophthalmopathy

Up to 50% of the patients with GD have some degree of clinically manifested GO, the main extrathyroidal manifestation of GD (44,45,50,55). However, most cases of GO are classified as mild and present remission either spontaneously or with general measures such as control of hyperthyroidism, use of eye drops and lubricating gels, and cessation of smoking. However, about 3–5% of the cases of GO progress to moderate/severe and severe forms, requiring more aggressive therapies such as retroorbital radiotherapy, use of systemic corticosteroids, and even urgent orbital decompression (55).

Glucocorticoids on immunosuppressive doses are the treatment of choice in patients with moderate/severe GO without risk of visual loss but should be avoided during the COVID-19 pandemic; during this time, we recommend other alternative measures, such as retroorbital radiotherapy. A cumulative radiotherapy dose of 20 Gy over 10 days may result in antiinflammatory effects and improve diplopia (55).

In patients with moderate/severe GO using glucocorticoids at nonimmunosuppressive doses (*e.g.*, prednisone < 20 mg daily), the glucocorticoid may be maintained. Reinforcement of measures to prevent SARS-CoV-2 infection is essential in these patients. In case these patients become infected with the virus, the glucocorticoid should be reconsidered, depending on the progression of the patient's clinical condition.

In patients with severe GO who are not infected with the SARS-CoV-2 and are at risk of visual loss (due to optic neuritis or corneal ulcer), the therapeutic choice is intravenous glucocorticoid despite a potential increased risk of COVID-19 infection resulting from the therapy. In cases defined as medical emergencies (*e.g.*, severe GO with a risk of visual loss), once treatment with intravenous glucocorticoid starts, personal protection, hygiene, and social distancing must be escalated to reduce the risk of SARS-CoV-2 infection. However, if these patients at risk of visual loss are infected with COVID-19 while using systemic glucocorticoids, the treatment should be reassessed continuously depending on the progression of the ophthalmopathy and the COVID-19 infection. Surgical procedures such as eyelid occlusion (for corneal ulcers) and orbital decompression could be considered (55).

Hospitalization rates for COVID-19 seem to be unaffected by the use of certain types of immunomodulators by patients with rheumatic diseases (56-58). However, recent reports have indicated that the use of rituximab (an anti-CD20 monoclonal antibody) could increase the risk of severe secondary infection in patients with COVID-19 infection (57,58). Based on that, we recommend against the use of rituximab for the treatment of GO during the COVID-19 pandemic.

Toxic nodular goiter

Toxic nodular goiter is the second most common cause of hyperthyroidism in the general population (36,50). Unlike GD, which may progress with remission with drug therapy alone, definitive treatment is usually required in patients with toxic nodular goiter (36,50). For those situations in which nuclear medicine or surgical procedures are not available due to the pandemic, ATDs may be used to control hyperthyroidism until definitive treatment can be performed (59). If ATDs are used, the same precautions must be taken regarding attention to side effects, as described above.

Amiodarone-induced thyrotoxicosis

Thyrotoxicosis secondary to use of amiodarone can occur either by increased production of thyroid hormones (type 1) or by destruction of the thyroid parenchyma (type 2). Correct identification of the type of thyrotoxicosis is essential since the treatment of each type is different: ATDs should be used in type 1, while glucocorticoids are used in type 2 (60,61).

Concerning type 2 thyrotoxicosis, initial use of prednisone 30 mg/day is recommended, followed by a gradual dose decrease over approximately 3 months (61). This dose of prednisone may be immunosuppressive, thus exposing the patient to an increased risk of COVID-19 infection, particularly to severe forms of this disease (62). If the administration of glucocorticoids becomes necessary, the patients should be instructed to avoid any situations or habits that may increase their risk of becoming infected. The glucocorticoid should be administered for the shortest possible time and be gradually tapered.

Thyroid cancer

Issues regarding malignant disease management during the COVID-19 pandemic is still controversial. As known, the majority of thyroid cancer cases, especially those found incidentally, are low risk and associated with an excellent prognosis (63). Nickel and cols., recently suggested that

those surgical procedures could and should be postponed safely during the pandemic period (64).

In more aggressive cases in which surgery cannot be postponed, such as anaplastic thyroid cancer or invasive tumors and bulky lymph node metastasis, surgery should be performed under a safety protocol, usually regarding pre-operative SARS-CoV-2 testing and avoiding visitors during the hospitalization period. Patients should also respect social distancing pre and postoperatively (65). Aguiar Junior and cols., from AC Camargo Cancer Center, São Paulo, showed that using a preventing protocol not only with RT-PCR test 2-3 days before, but also questionnaire with any suggestive COVID-19 symptoms and social isolation, oncology patients operated during the pandemic period had a low rate of newly diagnosed COVID-19 infection and complications. Of 540 patients included, 41 (7,6%) tested positive and surgery was postponed. Among the remaining 454 patients with a negative test that operated, no COVID-19-related symptoms or complications were observed during the in-hospital postoperative period, and no readmissions due to COVID-19 were identified (65).

In the follow-up of patients with thyroid cancer, those with excellent, indeterminate, and biochemical incomplete response to therapy should keep taking their levothyroxine pills regularly. Subclinical hyperthyroidism due to suppressive therapy, in the meantime, is not considered a risk factor for complications due to COVID-19 (63).

Patients with advanced thyroid cancer, with distant metastases (especially the lungs), or using specific cancer drugs, such as sorafenib, lenvatinib, or vandetanib, may be at greater risk for severe COVID-19, both by the extent of the disease and by possible adverse effects of medicines. These patients must be more careful, maintain social isolation, and follow all other measures already disclosed by the competent authorities to high-risk people.

Nowadays, radioiodine therapy (RAI) is not recommended for all differentiated thyroid cancer cases (63). Those low to intermediate-risk cases and with low postoperative thyroglobulin should be spared from RAI (63), which is even more important when the risk getting COVID-19 infection is enhanced by going out to get treated.

In cases in which RAI is indicated, especially for metastatic disease, RAI could be safely postponed with no impact on recurrence rates (66,67). So, the recommendation is to postpone as long as it is possible. On the other hand, the Brazilian Committee for Nuclear Energy has changed RAI recommendation during the COVID-19 pandemic. They have published a document that allows physicists to release patients with a higher radiation activity (>50 mCi) than was previously allowed, which made it possible to give up 100 mCi of RAI in some cases, with no need for hospitalization (68).

CONCLUSION

To the best of our knowledge, controlled hypothyroidism and hyperthyroidism are not associated with an increased risk of COVID-19 infection, nor these conditions predispose the patient to more severe forms of the disease. Patients with hyperthyroidism should be treated with ATDs, although clinicians must be aware of the possibility of rare side effects with these medications, particularly agranulocytosis. Definitive treatment of hyperthyroidism (with radioiodine therapy or TT) should be postponed until the pandemic is over.

When necessary, corticosteroids should be used for the shortest period possible and preferably at low doses (*i.e.*, prednisone 20 mg daily). In situations in which this therapy is required (*e.g.*, severe GO requiring glucocorticoids at immunosuppressive doses), the clinician must consider the risks and benefits of the treatment.

Considering that most of thyroid cancer cases are low risk and associated with an excellent prognosis, surgical procedures could and should be postponed safely during the pandemic period. When indicated, RAI could also be safely postponed as long as it is possible.

The Thyroid Department of the Brazilian Society of Endocrinology and Metabolism supports the development of research for a better understanding of the role of the thyroid in the context of the risk and severity of COVID-19 infection, and the progression and recovery from this disease.

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A novel mutation in *PRKAR1A* gene in a patient with Carney complex presenting with pituitary macroadenoma, acromegaly, Cushing's syndrome and recurrent atrial myxoma

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SUMMARY

Carney complex (CNC) is a rare syndrome of multiple endocrine and non-endocrine tumors. In this paper we present a 23-year-old Iranian woman with CNC who harbored a novel mutation (c.642dupT) in *PRKAR1A* gene. This patient presented with pituitary macroadenoma, acromegaly, recurrent atrial myxoma, Cushing's syndrome secondary to primary pigmented nodular adrenocortical disease and pigmented schwanoma of the skin. *PRKAR1A* gene was PCR amplified using genomic DNA and analyzed for sequence variants which revealed the novel mutation resulting in substitution of amino acid cysteine instead of the naturally occurring valine in the peptide chain and a premature stop codon at position 18 (V215CfsX18). This change leads to development of tumors in different organs due to lack of tumor suppressive activity secondary to failure of synthesis of the related protein. Arch Endocrinol Metab. 2021;65(3):376-80

INTRODUCTION

Carney complex (CNC) is a rare form of multiple endocrine and non-endocrine tumor syndrome that affects both males and females. Major clinical manifestations are pigmented mucocutaneous nevi, acromegaly secondary to pituitary somatotroph adenoma, Cushing's syndrome (CS) secondary to primary pigmented nodular adrenocortical disease (PPNAD), atrial myxoma and tumors of other organs such as thyroid, testes and ovaries. The disease is rarely encountered and less than 1,000 cases have been so far reported (1,2).

CNC is genetically heterogenous. In approximately 70% of cases the syndrome results from mutations in *PRKAR1A* gene which codes for regulatory subunit type 1 alpha of protein kinase A. The disease is transmitted through autosomal dominant pattern in 2/3 of cases. De novo mutations seem to be the cause of disease in the remaining 1/3. More than 125 pathogenic mutations have so far been reported in patients with CNC (3,4).

Majority of cases have been reported from North America and also European countries and there are limited information on clinical and genetic characteristics of CNC in Middle East region. In this paper, we present an Iranian patient with CNC who harbored a novel mutation (c.642dupT) in *PRKAR1A* gene and presented with acromegaly, recurrent atrial myxoma and CS.

CLINICAL REPORT

A 23-year-old Iranian woman was admitted to the ward of thoracic surgery at Kasra General Hospital, Tehran, Iran because of recurrence of cardiac myxoma. She was well until 4 years ago when symptoms began gradually with palpitation, dyspnea and exercise intolerance. Cardiovascular evaluation at that time revealed a 7 x 5 x 3 cm left atrial myxoma that was operated at another hospital. After surgery she gradually developed edema of the face, enlargement of hands and feet and recurrent headaches. Two years ago, evaluations by an endocrinologist revealed that serum growth hormone was 112 mIU/L (normal values 0-55) and was not suppressed by oral glucose tolerance test. Pituitary MRI revealed a pituitary macroadenoma and one year ago she underwent transsphenoidal pituitary surgery in 2 sessions at another hospital. Histopathologic evaluation revealed a pituitary adenoma. The patient felt well until 6 months ago when she developed palpitation and exertional dyspnea. She also complained of facial puffiness and edema of hands and feet. Hormonal evaluation showed high GH [112 mIU/L (normal 0-50)] and high IGF1 799 ng/mL [(normal values for age 115-340)]. Sandostatin LAR 20 mg every month was started for the patient. She had regular menstrual cycles and personal history was otherwise negative. Family history was also negative for similar disorder.

Physical examination revealed a tall young woman with a height of 186 cm and weight of 100 kg. Her face was coarse and edematous. Careful examination of the skin and mucosa revealed a pigmented macule in palpebral conjunctiva (Figure 1) and a soft tissue lesion in the back (Figure 2). Rest of physical examination was negative. Echocardiography showed a 2.1 x 1.8 cm myxoma in base of left atrium (Figure 3) and pituitary MRI revealed a macroadenoma (Figure 4). Routine laboratory evaluation was normal**.** Possibility of CS was proposed based on values of serum and urine cortisol; however, further evaluation of the adrenal axis was postponed because of cardiac surgery.

Figure 1. Pigmented macule in palpebral conjunctiva.

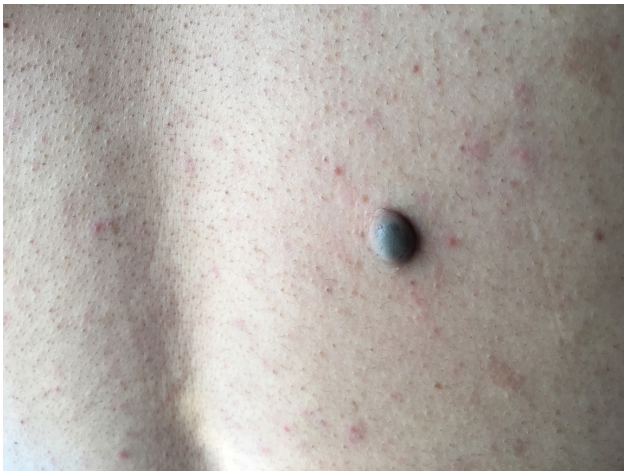

Figure 2. Skin lesion in the back diagnosed as pigmented schwanoma.

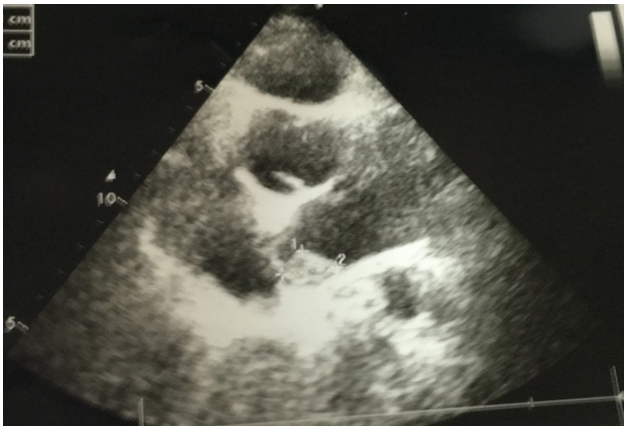

Figure 3. Echocardiography showed a 2.1 by 1.8 cm myxoma in base of left atrium.

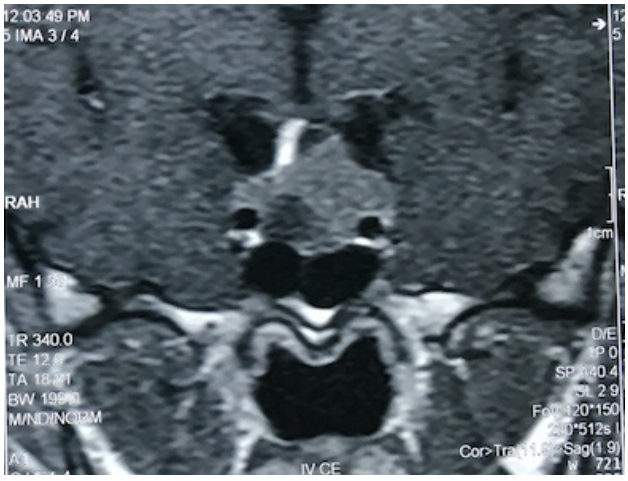

Figure 4. Pituitary MRI revealed a macroadenoma.

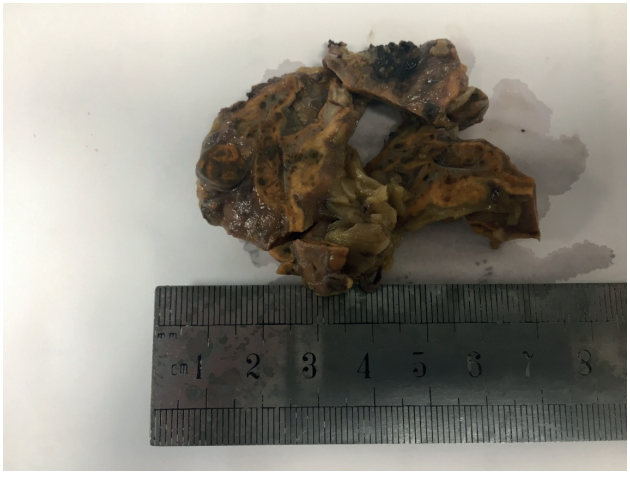

Figure 5. Pigmented areas could be seen dispersed in both resected adrenals.

Cardiac surgery was uneventful and the patient recovered without complications. The soft tissue lesion of the back was also excised at same session. Histopathologic evaluation revealed atrial myxoma. The skin lesion of the patient was diagnosed as pigmented schwannoma.

Two months after surgery she was readmitted for evaluation of CS. Computerized tomographic scanning (CT) of adrenal glands revealed minimal enlargement of the glands. CT scan of abdominal and pelvic cavities for evaluation of kidneys, uterus and ovaries were negative. Ultrasound evaluation of thyroid gland and breasts were also negative. She underwent low dose and high dose dexamethasone tests; the results are shown in Table 1.

Unsuppressed serum and urine cortisol and low plasma ACTH were in favor of diagnosis of CS and the patient was referred for laparoscopic bilateral adrenalectomy. Right adrenal was 6 cm and left adrenal was 7 cm (normal < 5 cm). Combined weight of adrenals was 25.5 grams (normal 7-10 grams). Multiple nodules could be seen overlying both adrenals. On macroscopic evaluation of the resected adrenals, pigmented areas could be seen dispersed in both adrenals (Figure 5). Histopathologic characteristics of the adrenal glands were in favor of PPNAD.

METHODS

To evaluate the genetic characteristics of the disease, DNAs of the patient and her brother, who was clinically asymptomatic, were extracted from peripheral white blood cells and sent to the Unit on Genetics and Endocrinology, Developmental Endocrinology Branch, National Institute of Child Health for sequencing the *PRKAR1A* gene. Using genomic DNA from the submitted specimen, the coding regions and splice junctions of the requested gene were PCR amplified and capillary sequencing was performed. Bi-directional sequence was assembled, aligned to reference gene sequences based on human genome build GRCh37/UCSC hg19, and analyzed for sequence variants. This work has been conducted with the informed written consent of the patient and in accordance to the ethics committee of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. All work also conforms to the provisions of the Declaration of Helsinki.

RESULTS

The study revealed a novel mutation, c.642dupT in the patient. Gene sequencing for *PRKAR1A* in his brother

Table 1. Values of serum ACTH and serum and urine cortisol at baseline and after DST

Test	Baseline	LDDST	HDDST
Serum cortisol (µg/dL)	17 (14-20)	**13** (<5)	**14** (<5)
24 h UFC (µg/day)	**419** (<190)	**358** (<20)	**350** (20)
ACTH (pg/mL)	**5** (7-63)	**0.1** (<10)	**0.3** (<10)

LDDST: Low dose dexamethasone suppression test; HDDST: high dose dexamethasone suppression test. Abnormal values in bold. Reference values in brackets.

failed to show any mutation. This duplication causes a frameshift starting with codon Valine 215, changes this amino acid to a cysteine residue and creates a premature stop codon at position 18 of the new reading frame, denoted p.Val215CysfsX18(V215CfsX18).

DISCUSSION

In this paper, we present an Iranian patient with CNC who harbored a novel pathogenic mutation in *PRKAR1A* gene and presented clinically with acromegaly, recurrent atrial myxoma and CS.

Studies on patients who presented with constellation of apparently unrelated disorders such as CS, acromegaly, cardiac myxoma and mucocutaneous brown-black macules led Carney JA and his colleagues present a new form of multiple tumor syndrome (5). With increasing knowledge on this rare disorder, CNC is being diagnosed more frequently.

From clinical point of view, mucocutaneous spotty lesions are the most frequent presenting symptoms which are seen in 70%-80% of patients (1,3,6-8). The lesions are seen as brown-black macules around the lips, genitalia or in palpebral conjunctiva. Schwannoma of the skin is a rare manifestation of the disease which was seen in our patient. Atrial myxoma is seen in 20%-53% of the patients and is the most worrisome component of the syndrome that imposes major complications such as cerebral emboli, cardiac arrhythmias and congestive heart failure. It is also the leading cause of death in CNC (1,3,4,7,9,10).

CS secondary to PPNAD and acromegaly secondary to pituitary somatotroph adenoma are seen in 25%-60% (3,6-8,10) and 10%-12% of cases respectively (1,3,4,6-8). Thyroid nodules, breast ductal tumor and large cell calcifying Sertoli cell tumor (LCCSCT) of the testes are seen with lower frequency in these patients.

Diagnosis of CS secondary to PPNAD can be challenging. Occasionally, patients present with atypical CS; UFC may be normal or near-normal but cortisol diurnal rhythm is consistently abnormal (2,11). During 6-day Liddle test, there is progressive paradoxical increase in the UFC in the 6th day (2,11,12). In our patient such a pattern was not seen but there was no suppression by LDDST and HDDST and pathology clearly identified PPNAD.

Biochemical acromegaly (elevation of growth hormone and IGF-I levels) can be found in 75% of patients (10) but clinically evident acromegaly is seen less frequently in patients with CNC (13).

The mutation in our patient, duplication of T in coding sequence 642 (c.642dupT), is reported for the first time. Based on the fact that the mutation was found only in the affected member of the family, our conclusion is that the mutation is pathogenic and lack of the disease in the family of the patient points to the de novo nature of the mutation. The mutation resulted in substitution of amino acid cysteine instead of the naturally occurring valine in the peptide chain leading to a premature stop codon at position 18 (V215CfsX18). Synthesis of messenger RNA is impaired due to premature termination of the gene and the truncated mRNA is rapidly decayed without translation to protein. Failure to develop the tumor suppressor protein in those with premature stop codon renders them more susceptible to tumorigenesis. Development of multiple components of the syndrome in our patient (recurrent myxoma, acromegaly, cutaneous nevi, cutaneous schwanoma and CS) may be due to complete lack of tumor suppressive activity secondary to complete lack of the related protein.

Initially it was assumed that since mutations in CNC result in premature stop codon and subsequently non-sense mediated mRNA decay (NMD) and lack of protein production, there is no correlation between genotype and phenotype and no significant differences can be identified between CNC patients (6); however in a study on 353 patients with CNC, some genotype-phenotype correlations were reported (8). The *PRKAR1A* pathogenic mutations include missense, nonsense, frameshift, splice site mutations and sometimes large deletions which usually result in NMD but except in nonsense mutations that always result in NMD, there is also possibility of altered protein expression (1) and phenotypic diversity cannot be predicted by type of detected mutation. Overall, those mutations resulting in altered protein production are associated with higher number of CNC manifestations (8). Acromegaly, cardiac myxoma, lentigines and psammomatous melanotic schwannoma (PMS) were more often associated with exonic mutations (2,8). In 25 patients with CNC and acromegaly, one third of the mutations were in exon 3 (14). One fifth of the mutations resulted in altered protein production and in 17 patients mutations resulted in premature stop codons. In 3 patients no mutations could be defined (14).

Tumour-suppressor genes generally act in a recessive way, requiring loss of both copies to induce tumorigenesis (15); it has been proposed that tumorigenesis in CNC may be caused by second hit in different tissues (16).Unfortunately, it was not possible for us to examine surgical tissues for assessing the second hit, but at least adrenocortical tumorigenesis in CNC seems to occur apart from the second hit (15) although more studies are needed.

Management of the patient is our major concern at present. Left atrial myxoma has been successfully removed at this session, but recurrence of atrial myxoma 4 years after the first cardiac surgery is a real concern. Growth hormone hypersecretion has not yet been controlled despite 2 times of pituitary surgery and monthly injection of 20 mg sandostatin LAR. Unfortunately, pegvisomant is not available to us. Lowering serum growth hormone in this case is crucial because studies by Bandettini and cols. have shown that lowering serum GH in patients with CNC reduces the recurrence rate of cardiac myxomas (17).

In conclusion, Herein, we presented a new case of CNC who harbored a novel mutation in *PRKAR1A* gene and presented with recurrent atrial myxoma, acromegaly, CS, pigmented schwanoma of the skin and multiple cutaneous nevi.

Compliance with ethical standards: this work has been conducted with the consent of the patient and in accordance to the standards of institutional/national Ethics Committee. All work also conforms to the provisions of the Declaration of Helsinki and Tokyo.

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No association between vitamin D status and COVID-19 infection in São Paulo, Brazil

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ABSTRACT

In recent years the immunomodulatory actions of vitamin D, a steroid hormone, have been extensively studied. In 2020, due to the COVID-19 pandemic, the question arose as to 25(OH)D status would be related to susceptibility to SARS-CoV-2 infection, since several studies pointed out a higher prevalence and severity of the disease in populations with low levels of 25(OH)D. Thus, we investigated the 25(OH)D levels in adults "Detected" positive for SARS CoV-2 by RT-PCR (reverse transcriptase polymerase chain reaction) test, and in negative controls, "not Detected", using the Fleury Group's examination database, in Sao Paulo, Brazil. Of a total of 14.692 people with recent assessments of 25(OH)D and RT-PCR tests for COVID-19, 2.345 were positive and 11.585 were negative for the infection. The groups did not differ in the percentage of men and women, or in the age distribution. There were no differences in the distribution of 25(OH)D between the two groups (p = 0.08); mean 25(OH)D of 28.8 ± 21.4 ng/mL and 29.6 ± 18.1 ng/mL, respectively. In the specific population studied, clinical, environmental, socioeconomic and cultural factors should have greater relevance than 25(OH)D in determining the susceptibility to COVID-19. Arch Endocrinol Metab. 2021;65(3):381-5

Keywords
Vitamin D; coronavirus; COVID-19

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INTRODUCTION

The musculoskeletal effects of vitamin D are widely studied, as well as its endocrine actions in regulating the homeostasis of calcium and phosphorus. Cholecalciferol or "vitamin D", synthesized in the skin, is metabolized in the liver to 25-hydroxycholecalciferol (25(OH)D) and then in the kidney to its biologically active form, 1,25-dihydroxycholecalciferol (1,25(OH)2D). The metabolite 25(OH)D is the major circulating form of vitamin D in humans, and it is used to reflect person's vitamin D status.

Vitamin D deficiency causes secondary hyperparathyroidism, osteomalacia, osteopenia and an increased risk of fractures. In addition to its classic

functions in osteomineral metabolism, the extensive distribution of vitamin D (VDR) receptors in human tissues and the action of the active hormone, 1,25(OH)2D (calcitriol), in regulating the transcription and expression of countless genes, indicate the importance of nonskeletal actions of this hormone. Experimental and clinical studies have revealed the intracrine action of vitamin D in the immune system, particularly in monocytes and macrophages, with a modulating role for both innate and adaptive immune responses against a number of microorganisms, including viruses (1,2).

Autophagic encapsulation of viral particles is also a cellular process enhanced by both 25(OH)D and 1,25(OH)2D (3), with a fundamental role in reducing viral infectivity, for example for human immunodeficiency virus type 1 (4). In addition, other data revealed a role of vitamin D in pulmonary protection against acute respiratory infections, in particular its action on capillary permeability, which plays a fundamental role in the pathophysiology of many diseases with pulmonary involvement (5,6). Respiratory epithelial cells constitutively express 1α-hydroxylase resulting in local activation of vitamin D. Vitamin D-dependent genes including cathelicidin and CD14 are upregulated after the exposure of airway epithelial cells to the inactive vitamin D precursor (7).

In 2020, during the coronavirus disease (COVID-19) pandemic, several retrospective studies were published, showing an association between low 25(OH)D status and increased susceptibility to and severity of SARS-CoV-2 infection, suggesting a deleterious effect of hypovitaminosis D on the incidence and clinical evolution of COVID-19 (8-12). Experimental research has shown that 1,25(OH)2D modulates the expression of angiotensin-converting enzyme 2, which is the receptor for the entry of SARS CoV-2 into cells. VDR-null mice showed more severe acute lung injury in a sepsis model than their wild-type counterparts (13). Thus, it became essential to study the relationship between 25(OH)D and SARS CoV-2 incidence, with the aim of identifying an easily modifiable factor that can play a preventive role in all populations susceptible to infection.

OBJECTIVE

To compare 25(OH)D levels between individuals infected with SARS-CoV-2, with diagnostic confirmation by RT-PCR (reverse-transcriptase polymerase chain reaction), and individuals negative for SARS-CoV-2, using the Fleury Group's database.

SUBJECTS AND METHODS

Data source

Fleury Group is a medical organization that provides supplemental health services in Brazil. Data were collected from the Fleury Group's Caché database, of 14692 individuals who underwent RT-PCR tests for the diagnosis of COVID-19, from March to July 2020, who also had 25(OH)D measured; participants were identified by a unique register number. The study protocol was approved by the research and ethics committee of Fleury Group (protocol number 4.409.445, CAAE 39961120.7.00005474). Informed consent was not required since the data were anonymized.

Study design

This was a retrospective study that collected records from individuals of both genders, between 18 and 90 years old, with RT-PCR results for SARS CoV-2 and who simultaneously had their 25(OH)D measured over a period of 30 days before or after the collection of the sample for COVID-19 RT-PCR test. In cases of patients with more than one vitamin D test, the most recent in relation to the RT-PCR date, was selected. Records with 25(OH)D above 100 ng/mL were excluded to avoid distortions in the analysis of vitamin D averages. After removing entries with missing data and inconclusive diagnostic tests for COVID-19, the new dataset (n = 13930) was divided into two groups: "Detected" or positive for COVID-19 (2345 patients) and "Not Detected" or negative for COVID-19 (11585 patients).

Biochemical analysis

25OH vitamin D – Liason, CLIA, DiaSorin, Saluggia, Italy, reference range: 20-60 ng/mL, intra and inter-assay coefficient of variation are 6.0% and 8.0%, respectively; RT-PCR – molecular test developed entirely in house according to the Charité protocol and a confirmatory test by the CDC protocol when necessary for confirmation, using clinical samples from the respiratory tract.

Statistical analysis

To assess the significant differences between the groups, the normality of the two groups was confirmed (Kolmogorov-Smirnov Normality Test) and then, the difference between the means of the groups was verified using the Welch T test (Software R, www.r-project.org). This is a parametric test, adapted from the Student's t-test, whose objective is to compare two independent groups, without the hypothesis of equal population variance. The test considers the difference between the number of patients in each group when calculating the real difference between the means (14). Statistical significance was defined as $p < 0,05$.

RESULTS

There was no difference between groups regarding the percentage of men and women, or regarding the age distribution. There was no significant difference for the mean 25(OH)D between men and women, or between adults and the elderly (over 60 years), (multiple T tests with Bonferroni correction). The Detected Group had a mean 25(OH)D of 28.8 ± 21.4 ng/mL, with a median of 26.0 ng/mL. The not Detected Group had a mean 25(OH)D of 29.6 ± 18.1 ng/mL, with a median of 27.0 ng/mL. Table 1 shows the percentage of individuals and the means of 25(OH)D, separated by the ranges of the values, < 12 ng/mL, 12-20 ng/mL, 20-30 ng/mL and > 30 ng/mL, in both Detected and not Detected groups (15,16).

There was no difference in the distribution of 25(OH)D between the "Detected" and "not Detected" groups ($p = 0.0811$). Figure 1 shows the dispersion of the values of 25(OH)D between the Detected and not Detected groups.

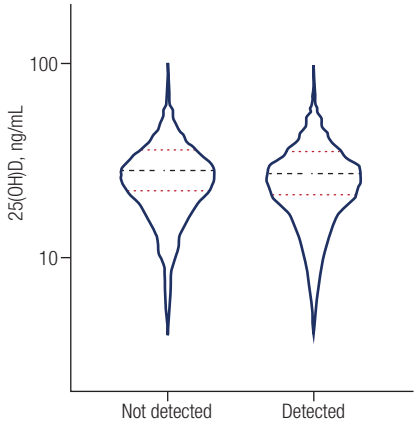

Figure 1. The image shows the dispersion of 25(OH)D, in patients Detected and Not Detected for COVID-19; means are indicated by the black line and two standard deviations (SD) by the red line.

DISCUSSION AND CONCLUSIONS

Studies prior to the COVID-19 pandemic period have demonstrated the role of vitamin D in innate and adaptive immunity, particularly in protection against viral and bacterial infections. Martineau et al demonstrated, in a large meta-analysis of randomized controlled trials, that vitamin D supplementation reduced the risk of experiencing at least one acute respiratory tract infection (17). The initial spread of COVID-19 occurred in countries that were going through the winter, had a high prevalence of hypovitaminosis D. Together, these data raised the question of the role of vitamin D to susceptibility and the severity of the disease.

Numerous studies initially linked 25(OH)D status to susceptibility and mortality from SARS-CoV-2, although causality cannot be demonstrated (8-12). The clinical evolution and severity of COVID-19 respiratory

Table 1. Number of patients, mean and percentage of patients by ranges of 25(OH)D, for the "Detected" and "not Detected" groups. Multiple T tests with Bonferroni correction were applied; t-test were performed using the log-transformed values of the means

25(OH)D	Group	n	%	Means 25(OH)D ng/mL	Group	n	%	Means 25(OH)D ng/mL	p value
< 12 ng/mL	Detected	137	5.84	8.68	Not detected	579	5.0	9.01	0.10
12-20 ng/mL	Detected	448	19.1	15.83	Not detected	2066	17.8	16.08	0.13
20-30 ng/mL	Detected	911	38.8	24.51	Not detected	4306	37.2	24.6	0.42
> 30 ng/mL	Detected	849	36.2	43.4	Not detected	4634	40.0	42.84	0.73

disease has enormous complexity and competition from numerous other confounding factors, such as obesity, hypertension, socioeconomic level, quality of medical care, comorbidities and probably, the degree of exposure and genetic susceptibility. However, individuals with inadequate 25(OH)D could have an additional risk of contracting a viral infection such as COVID-19, and possibly a greater risk of an unfavorable clinical course (3,6,18). Additionally the social isolation, imposed to control the pandemic, could be a predisposing factor to less sun exposure.

However, our study showed no difference in 25(OH)D status in a large group of Brazilian infected individuals with SARS CoV-2 and non infected controls. The same conclusion was reached by Hastie and cols. (19) and Raisi-Estabragh and cols. (20), using UK Biobank data. Neither study supports the hypothesis of a link between vitamin D levels and the risk of SARS CoV-2 infection, nor does 25(OH)D explain the ethnic differences in COVID-19 prevalence.

The population sample evaluated in this study has a high socioeconomic level, has access to private medical services, and is predominantly of Caucasian origin; therefore, we were unable to assess socioeconomic or ethnic-racial factors that could affect infectivity. Another aspect to be considered is that the pandemic spread in Brazil during late summer and early fall, periods characterized by higher levels of solar irradiation; therefore, low 25(OH)D is less prevalent. Unfortunately, we were also unable to control for other clinical risk parameters for COVID-19, such as weight, diabetes and other comorbidities.

Despite all of the evidence described in the literature on the immunological action of vitamin D, we did not observe differences between 25(OH)D status and COVID-19 susceptibility in a large Brazilian population sample. The strength of this study is the number of participants, mostly Sao Paulo residents, the largest city in Brazil located in the southeastern region of the country. The study population, both with and without SARS CoV-2 infection, has a lower prevalence of hypovitaminosis D, compared to that described in the European or American populations, or even within specific population subgroups living in Sao Paulo, such as the elderly over 80, institutionalized or chronically ill patients (16).

In conclusion, clinical, environmental, socio-economic and cultural factors have greater relevance than vitamin D status in determining the susceptibility to SARS-CoV-2 infections in the population studied.

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Correlation of overweight condition and obesity with mortality by COVID-19 in Brazil's state capitals

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ABSTRACT

Objective: To evaluate the correlation between the prevalence of overweight condition and obesity with mortality rates due to COVID-19 in Brazil's state capitals. **Materials and methods:** This is an ecological study, whose units of analysis were the 26 state capitals and the Federal District of Brazil. Prevalence was estimated by the results of the *Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico* 2019 (VIGITEL). The general mortality rates due to COVID-19 were collected on the official website of the Brazilian Ministry of Health (MH) and stratified by the same Brazilian capitals evaluated in the VIGITEL survey. The rates included the period between the 1st and 29th Epidemiological Weeks of 2020. The Partial Correlation Test (r) was used, controlled for confounding factors, to evaluate the correlation between the prevalence of overweight/obesity and the overall mortality rates due to COVID-19. **Results:** The mean mortality rate for COVID-19 in the period was 65.1 deaths per 100,000 inhabitants. Regarding the prevalence of obesity and overweight, 20.2% and 54.7% were the mean values observed in the state capitals, respectively. The prevalence of obesity was positively correlated with the overall mortality rate due to COVID-19, with mean positive correlation (r=0.380) and statistically significant correlation (p=0.034). **Conclusion:** This study pointed out that, at the aggregate level, there is a concomitant and correlated increase in mortality rates due to COVID-19 and prevalence of obesity in Brazilian capitals. The data found may contribute to actions to cope with the pandemic aimed at this population. Arch Endocrinol Metab. 2021;65(3):386-91

Keywords
Mortality; COVID-19; SARS-CoV-2; obesity; overweight

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INTRODUCTION

The first cases of pneumonia of unknown cause occurred at the end of 2019 in China (1). In January 2020, the etiological agent was identified and classified as Sars-CoV-2, a new species of Coronavirus (2) and, at the end of the same month, the World Health Organization (WHO) declared Coronavirus disease (COVID-19) a Public Health Emergency of International Importance (3). Brazil presented a mortality rate of 50.7 per 100,000 inhabitants until August 14, 2020, with important regional differences (4).

As research developed, more has been understood about the disease and its risk factors. COVID-19 was indicated to have worse prognosis and a higher risk of death when associated with obesity (5) and this condition has also been pointed out by several observational hospital-based studies (6-8) as an important factor associated with a worse prognosis for the infection. This association was also described for the SARS/MERS virus in previous publications, with indications of strong correlation between obesity and complications due to infection by other genetically similar to Sars-CoV-2 coronaviruses (5). Correlation with overweight condition was scarcely reported in the literature (6). To the day of this study, population-based research in the matter was also scarce.

Additionally, the situation in Brazil regarding obesity and overweight condition rates and the pandemic require special attention, due to the fact that the prevalence of obesity and overweight are high in the country and have increased significantly over the years (9). Accordingly, if these findings are extended to a populational level, the healthcare system will have another challenge in face of the pandemic: a

significant and increasing portion of the population at higher risk of worse COVID-19 infection outcomes. Better understanding of the matter could be crucial to guide public health policies and avoid an even heavier healthcare system burden.

Considering the scarcity of population-based studies, the fact that hospital-based studies are susceptible to selection bias, that overweight condition and obesity rates are of high magnitude in Brazil and the possible relationship between these factors and higher mortality due to COVID-19, the necessity of a populational analysis becomes clear to better understand this relation at an aggregate level, helping to delineate health policies.

Therefore, our research aimed to evaluate the correlation between the prevalence of overweight condition and obesity with mortality rates due to COVID-19 in Brazil's state capitals at an aggregate level.

MATERIALS AND METHODS

Design and sample

This is an ecological study in which the units of analysis were the 26 state capitals of Brazil and the Federal District, aiming to investigate the existence of correlation of overweight and obesity prevalence, in adults (≥ 18), with general COVID-19 mortality rates. Data on prevalence, aggregate to capital level, for overweight condition, obesity and covariates were extracted from the *Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico* 2019 (VIGITEL – Surveillance of Risk and Protective Factors for Chronic Diseases) survey (10). The general mortality rates due to COVID-19 were collected from the official website of the Brazilian Ministry of Health (MH) (4) and stratified by the same Brazilian capitals evaluated in the VIGITEL survey.

Overweight and obesity data

Both overweight and obesity prevalence were extracted from the results of the *Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico* 2019 (VIGITEL – Surveillance of Risk and Protective Factors for Chronic Diseases) survey. The VIGITEL is a population-based study which interviews adults (≥ 18) who live in residences with landline telephones in all 26 state capitals and the Federal District of Brazil and aims to understand this population's health in order to guide programs and actions that reduce the occurrence of chronic diseases. The VIGITEL's sampling methodology allows to compare capital cities risk factor and protection estimates, between adult inhabitants. Detailed information on the sampling and data collection process was previously described (10). The Body Mass Index (BMI) is the parameter used by VIGITEL to determine overweight condition and obesity cutoffs and is calculated by dividing weight in kilograms by the height in square meters – both weight and height are self-reported within the survey. An individual with a BMI ≥ 25 kg/m^2 was considered to be overweight and individuals with BMI ≥ 30 kg/m^2 were considered to have obesity. It is worth noting that overweight condition, within VIGITEL, is a broader measure which also includes obesity (BMI ≥ 30 kg/m^2), whereas obesity is a narrower concept.

COVID-19 mortality data

General mortality rates due to COVID-19, during the period between the 1st and 29th Epidemiological Week of 2020 (11), were collected from the official website of the Brazilian Ministry of Health (MH). Mortality rates in the website were calculated by multiplying the number of confirmed COVID-19 deaths of resident population – TCU's (*Tribunal de Contas da União*) 2019 estimated population for FPM's (*Fundo de Participação dos Municípios*) quotas (12) – by 100.000 (4). The process of updating data on cases and deaths confirmed by COVID-19 in Brazil was carried out daily by the Ministry of Health, through official information provided by the State Health Secretariats of the 27 Brazilian Federative Units (4). Data was stratified by Brazilian capital cities, but they are not discriminated by age, socioeconomic condition or sex.

Covariates

The prevalence of diabetes mellitus (DM), systemic arterial hypertension (SAH) and smoking were selected as covariates, since they were previously described as risk factors for COVID-19 infection (13). All prevalence was obtained from the VIGITEL 2019 survey as well. We considered, in this study, the VIGITEL's estimated prevalence of diabetes mellitus and systemic arterial hypertension assessed by personal statement of whether a diagnosis was already established by a doctor, regardless of treatment. These variables were collected through the questions: "Has any doctor ever told you

that you have diabetes?" and "Has any doctor ever told you that you have high blood pressure?". The individual who answered positively to the question "Do you currently smoke?" was considered a smoker, regardless of number of cigarettes, frequency and duration of the smoking habit.

Analysis

The data were represented by means with standard deviation (SD), medians, minimum and maximum values. The graphical representation was made using a scatter plot. The variables obesity, overweight and mortality due to COVID-19 were also stratified by Brazilian capitals. We used the Partial Correlation Test (r), controlled for confounding factors, to evaluate the correlation between the prevalence of overweight/obesity and the overall mortality rates due to COVID-19. The use of correlation tests between similar measures was previously described (14). This technique was chosen because it allows the evaluation of the pure relationship between two variables, after statistically eliminating the influence of other independent variables. Cohen's parameters (15) were used for the interpretation of correlation values (r): between 0.10 and 0.29 to indicate a non-existent or small correlation, between 0.30 and 0.49 to indicate that there is a mean correlation and between 0.50 and 1 to indicate a large correlation. Data was analyzed with the program IBM (SPSS®) version 25. The variables DM, SAH and smoking were used as control variables. The significance level adopted was 5% ($p < 0.05$).

Ethical aspects

The VIGITEL was approved by the National Committee of Ethics in Research. (CAAE: 65610017.1.0000.0008). The mortality rates due to COVID-19 are available for public access, without the identification of participants and its use exempt approval of the Ethics Committee, according to resolution no. 510, of April 7, 2016, of the National Health Council.

RESULTS

The mean mortality rate due to COVID-19 was 65.1 deaths per 100,000 inhabitants in Brazil's state capitals, with a minimum coefficient of 6.9/100,000 in Campo Grande (MS) and a maximum of 135.5/100,000 in Belém (PA). Regarding the prevalence of obesity and overweight, averages of 20.2% and 54.7% were observed in these state capitals, respectively (Table 1).

Table 1. Description of mortality rates, prevalence of obesity and overweight according to Brazilian capitals and description of the mean, median, minimum and maximum values of the study's covariates. Capital cities of Brazil

Brazilian capitals	COVID-19 mortality rate*	Obesity	Overweight
Aracaju	69.1	20.6	53.6
Belém	135.5	19.6	53.3
Belo Horizonte	13.6	19.9	52.5
Boa Vista	85.9	21.2	54.3
Campo Grande	6.9	22.5	58
Cuiabá	69.5	22.5	55.8
Curitiba	18.4	19.4	53.7
Florianópolis	7.6	17.8	53.6
Fortaleza	134.3	19.9	55.6
Goiânia	20.4	19.5	52.7
João Pessoa	64.0	20.4	54.7
Macapá	64.4	22.9	53.3
Maceió	65.9	20	54.4
Manaus	89.9	23.4	60.9
Natal	75.0	22.5	56.6
Palmas	9.4	15.4	49.9
Porto Alegre	13.7	21.6	59.2
Porto Velho	85.9	19.9	56.6
Recife	123.7	21.7	59.5
Rio Branco	75.9	23.3	56.6
Rio de Janeiro	114.6	21.7	57.1
Salvador	53.6	18.1	51.8
São Luís	96.1	17.2	50.3
São Paulo	71.6	19.9	55.8
Teresina	68.6	17.6	52.7
Vitória	88.7	17.6	49.1
Distrito Federal	36.0	19.6	55
Mean (SD)	**65.1 (38.8)**	**20.2 (2.0)**	**54.7 (2.8)**
Median	69.1	19.9	54.4
Minimum	6.9	15.4	49.1
Maximum	135.5	23.4	60.9
	Diabetes Mellitus	**Systemic Arterial Hypertension**	**Smoking**
Mean (SD)	6.8 (1.1)	23.3 (3.4)	8.3 (2.7)
Median	6.8	24.3	7.9
Minimum	4.6	16.9	4.4
Maximum	8.6	28.5	14.6

Weighted prevalence (VIGITEL. 2019).
* Estimated for every 100.000 inhabitants.

Figure 1 shows the dispersion graph between the prevalence of obesity (a), overweight (b) and the mortality rates due to COVID-19 in Brazilian capitals. Visually, a greater slope of the line is observed between the prevalence of obesity and mortality due to COVID-19.

The prevalence of obesity was positively correlated with the overall mortality rate due to COVID-19, with statistically significant (p=0.034) and mean positive correlation (r=0.380). Although no statistical significance was evidenced, we also observed a mean positive correlation (r=0.367) between COVID-19 mortality rate and overweight (Table 2).

Table 2. Correlation between overweight and obesity (2019) and mortality rates due to COVID-19 (2020) in the capital cities of Brazil

Variables	COVID-19 mortality rate	
	r*	p-value
Overweight	0.367	0.078
Obesity	0.380	0.034

r: Partial Correlation coefficient.
*Correlation adjusted for diabetes, hypertension and smoking variables.

DISCUSSION

Corroborating our findings, prospective and hospital-based articles found an association between obesity and progression to the severe form of COVID-19 infection (6,7), obesity as a risk factor for mortality (8,16) and longer hospital stay for these patients (17). Moreover, Palaiodimos and cols. (18) reported that severe obesity is independently associated with a worse prognosis and mortality. Although the underlying mechanisms are still unclear, literature has indicated potential reasons why obesity may be a risk factor for severe COVID-19 infection and higher mortality: the presence of uncontrolled chronic obesity-related comorbidities (19), impaired pulmonary function, elevated angiotensin-converting enzyme 2 (ACE2) expression (20), chronic inflammation (13), oxidative stress and lipotoxicity (5). Cardiac damage, aggravated inflammatory response and increased coagulation activity were correlated to mortality amongst patients with obesity (16). Furthermore, evidence has shown that obesity has been linked to increased susceptibility to infections in general (5).

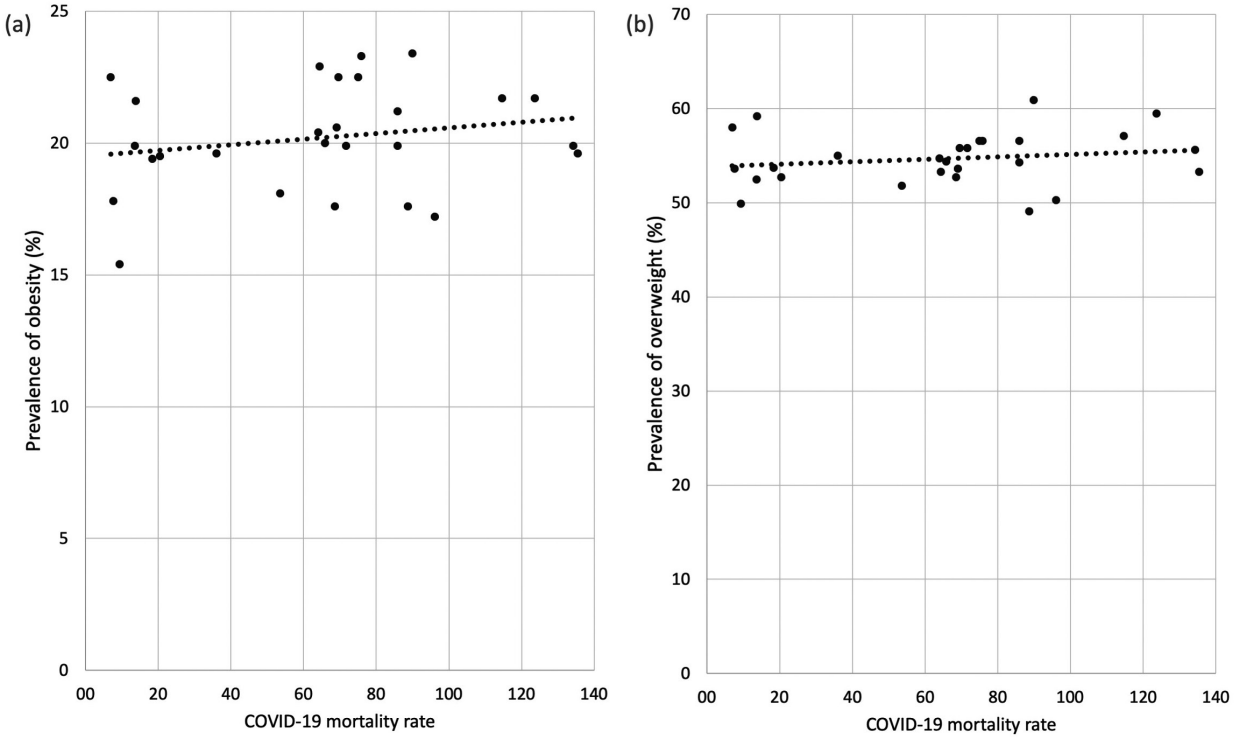

Figure 1. (**A**) Scatter plot between prevalence of obesity and mortality rates by COVID-19; (**B**) Scatter plot between prevalence of overweight and mortality rates by COVID-19. Capital cities of Brazil.

Regarding overweight condition, this study did not verify a population-level correlation between overweight and mortality due to COVID-19 and literature on the matter is scarce (6). In Brazil general overweight and obesity rates have increased with time and the situation is challenging. The country has a national strategic plan that aims to combat non transmissible chronic diseases (*Plano de Ações Estratégicas para o Enfrentamento de Doenças Crônicas Não Transmissíveis*) (21), and one of its purposes is to halt the growth of both overweight and obesity rates. The VIGITEL survey – which supplies the aforementioned plan – shows that, from 2006 to 2019, overweight and obesity rates increased from 42.6% to 55.4% and 11.8% to 20.3%, respectively (10). In face of a pandemic this data is particularly concerning because of the possible impacts to the National Healthcare System (SUS – *Sistema Único de Saúde*) in terms of occupation, expenditure and availability – since an expressive portion of brazilian population may be at higher risk of worse prognosis. Greater understanding of how COVID-19 may impact people with overweight condition and obesity at a populational level – the aim of this study – is of great importance to delineate Healthcare strategies and public policies, preventing and preparing for potential worsened scenarios.

Limitations of this study must be considered when interpreting the results. Two secondary data sources were included in this study, one (VIGITEL) is a population-based survey and the other is a mortality rate information system (Brazilian Ministry of Health (MH) website). Vigitel (10) is based on the interviewees' report, which may have led to information bias. However, researchers attest to the validity of this strategy, demonstrating high sensitivity (> 91%) and specificity (> 83%) values for BMI, calculated via self-reported height and weight (22). Also, the source of information used to verify deaths due to COVID-19 is influenced by the testing capacity of the capital cities observed, as well as by the ability to notify and monitor epidemiological surveillance of each site, with recognized underreporting of cases and deaths (23) and important regional differences (4). It is important to highlight though that the mortality rates information system in Brazil evolves in terms of quality and coverage, generating reliable data for research (24).

In addition, an important limitation concerns variables such as age, socioeconomic condition and sex. The prevalence measures used here refer to resident adults aged 18 years or older, whereas the mortality rate refers to all resident individuals, since the database does not discriminate rates by age. Therefore, although deaths due to COVID-19 mostly occur among adults (25), this fact may have influenced the observed results. Equally important, socioeconomic condition and sex variables couldn't be evaluated due to the same database restrictions. In order to evaluate these factors, other sources would have to be included in the analysis, compromising comparability and interfering in the results' heterogeneity. Therefore, VIGITEL and the HM website were added as the only sources.

Moreover, the epidemiological design itself is susceptible to aggregation bias or ecological fallacy, therefore, the relationship observed between variables at the aggregate level may not be valid at the individual level – although, as mentioned before, hospital-based prospective studies results were consistent with our findings.

We were able to conduct a population based study that, to the best of our knowledge, is the first investigation addressing the relationship between prevalence of overweight condition and obesity with mortality rates due to COVID-19 nationwide. In conclusion, our results showed that, at aggregate level, there is a concomitant and correlated increase in mortality rates due to COVID-19 and prevalence of obesity in Brazilian capitals. Additional studies are needed to confirm and expand our results. Better understanding in this matter, especially at a population level, is crucial in order to support focused public policies.

Individual collaboration of each author in the preparation of the manuscript: Raquel Alencastro Veiga Domingues Carneiro contributed to the study design, writing and revision of the final article. Danúbia Hillesheim contributed to the study design, data analysis and interpretation, writing and reviewing the final article. Ana Luiza Curi Hallal contributed to the conception and design of the study, writing and final review of the article.

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T3 therapy in hypothyroidism. Still more questions than answers

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DEAR EDITOR,

We have read with interest the article published by Dora J, which recommends against prescribing liothyronine (L-T3) alone or in combination with levothyroxine (LT4) for hypothyroidism, because the short half-life of triiodothyronine (T3) means multiple daily doses are required, and there is no clear evidence in humans that combination therapy is superior to L-T4 alone (1). No guidelines have recommended thyroid hormone replacement with L-T3 alone, but the question remains whether L-T3 + L-T4 combination therapy may be better than levothyroxine alone in a selected group of patients with hypothyroidism (2).

Deiodinases are enzymes that mediate the activation and inactivation of thyroid hormones. Genetic variation in these enzymes is associated with altered thyroid function and adverse health outcomes. Studies have shown that individuals with genetic variations in deiodinase type 2 and the thyroid hormone transporter protein MCT10 experience additional benefits with combined LT3 + LT4 therapy, though the sample size was small (3). On the other hand, single nucleotide polymorphisms may have little discriminatory power to determine which patients could benefit from combination therapy (3).

The 2012 ETA guideline suggests that combined therapy could be considered experimentally in hypothyroid patients treated with L-T4, who have persistent complaints despite having target TSH values, provided they have previously given support to deal with the chronic nature of their disease and associated autoimmune diseases have been ruled out (4). The NICE guideline does not give a clear recommendation on the use of liothyronine, but states that, although long-term safety is uncertain, it may play a role in patients with symptoms of hypothyroidism despite adequate replacement with levothyroxine (5).

Recently, the 2021 ETA Consensus established established evidence-based recommendations and patient criteria for LT3 + LT4 combination therapy in hypothyroidism. After excluding other causes of persistent symptoms, patients who do not report clinical improvement with a dose of at least 1.2 ug/kg/day of levothyroxine should be considered for combination therapy. Likewise, those with low baseline serum total T3 levels while taking LT4 monotherapy should also be include. Noteworthy, they suggest that future combination therapy trials should consider including polymorphism genotyping, and should be adequately powered to study the effect of this polymorphism on trial outcomes (6).

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The recommended initial dose of LT4:LT3 is 13:1 to 20:1, a ratio that mimics the ratio of physiological thyroxine (T4) and T3 secretion by the human thyroid gland, representing a dose of 5 or 10 μg LT3 for patients taking 100 to 200 μg LT4. If no improvement is seen after 3-6 months of treatment, assessed by questionnaires such as ThyPRO, treatment should be discontinued (6).

We emphasize that more studies are needed to identify the subgroup of hypothyroid patients that may benefit from the use of liothyronine, in addition to levothyroxine, probably through the identification of new biomarkers or genetic polymorphisms.

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T3 therapy for hypothyroidism: choosing wisely still requires careful bench to bedside translation

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TO THE EDITOR,

We thank Zavaleta and cols. (1) for their interest in our work and the opportunity to expand the discussion on Choosing Wisely recommendations for managing thyroid-related conditions (2).

In an average adult, the thyroid gland produces around 80 mcg of thyroxine (T4) and 20 mcg of triiodothyronine (T3) per day to match the body's metabolic demands. Thus, the activation of the prohormone T4 to the active hormone T3 mainly occurs at peripheral organs, a process fine-tuned by the deiodinases type 1 (D1) and 2 (D2) enzymes accordingly to specific tissue needs. The physiological background and the fact that some patients remain symptomatic regardless of normal thyrotropin (TSH) levels under levothyroxine (LT4) replacement has fuelled the interest in the potential theoretical advantages of adding liothyronine (LT3) to LT4 as a therapeutic strategy. Given that D2 activates approximately 60% of circulating T3 and that some genetic variants of D2, like the D2-Thr92Ala polymorphism, are associated with impaired enzyme activation of T4 into T3, patients homozygotic for the D2-Ala92 genotype might comprise a group that could benefit from combined LT4 + LT3 therapy (3-5). Notwithstanding, despite some preliminary studies had indicated that this might be the case, these findings remain to be confirmed in larger studies before being routinely incorporated in clinical practice.

One should exercise caution when translating bench concepts and preliminary clinical studies to widespread clinical practice, balancing evidence of benefits and harms. In this regard, several studies have attempted to clarify the combined LT4 + LT3 therapy effects. A systematic review that included 9 randomized trials reported beneficial effects of combination LT4 + LT3 therapy only in one trial (6). Subsequently, a meta-analysis of 11 published randomized trials totalizing 1,216 patients showed no benefit of combined LT4 + LT3 therapy on fatigue, bodily pain, anxiety, depression, or general quality of life (7). Of interest, a systematic review that evaluated the pooled prevalence rate for preference of combination therapy over LT4 among hypothyroid patients aware of the combined LT4 + LT3 therapy was 46.2% (95% confidence interval 40.2%, 52.4%) with no difference from chance (P = 0.231) (8).

On the other hand, the potential harms associated with LT3 supplementation can not be underestimated. Since T3 is the active hormone, supraphysiological doses may directly induce thyrotoxicosis, posing risks for serious cardiovascular events

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(arrhythmias and embolism) and other related adverse effects. Notably, many countries (like Brazil) do not have LT3 formulations commercially available. Thus, in this context, the options to acquire LT3 would rely on formulating LT3 at local pharmacies, a practice that should be highly discouraged given the embodied risks of overdose.

We recognize that knowledge is dynamic and that in the future, we could learn that some subgroups of patients may benefit from LT4 + LT3 therapy for hypothyroidism. If that is the case, we will be glad to revise our position. Until there, we understand that LT4 monotherapy is the most convenient, effective, and safe choice to treat hypothyroidism, maximizing value to our patients.

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Transoral thyroidectomy: A reflexive opinion on the technique

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DEAR EDITORS AND COLLEAGUES

When analyzing the literature published on thyroidectomy using a "timeline" we can observe that we have notoriously progressed from a procedure that was almost prohibited in the mid-nineteenth century due to complications to a point where it is considered safe, resolutive, and highly efficient. This progress is especially due to the advances in surgical techniques. Theodore Kocher (Nobel Prize in 1909 for his contribution to thyroidectomies) flawlessly described the anatomical basis for the success of this surgical technique 130 years ago. Besides this, advances in anesthetic procedures, surgical materials, and medications were fundamental to intraoperative and postoperative advances and patient management. With time, the knowledge on the different thyroid diseases has exponentially increased and serves as indicators for the extension of treatment and surgical procedures, all efforts should be directed to apply them into clinical practice (1).

In the past twenty years, the introduction of new technologies such as the application of energy in surgical instruments and neuromonitoring brought advances to the procedure. These technologies helped providers to achieve a shorter surgical time, a shorter length of hospital stay, a reduction in the risk of bilateral laryngeal paralysis, and a reduced risk of intra and post-operative bleeding (2,3). In the last decade, stimulated by the current advances in endoscopic and robotic surgery, new approaches for thyroidectomy procedures were investigated to substitute the classic cervical incision, and are currently a theme for several scientific debates.

Previously described techniques using endoscopic and small incisions through the chest wall were proven not to be efficient and were soon taken out of practice. The same happened with the robotic techniques using the axillary route. It is worth mentioning that most of the robotic apparatus was designed for abdominal and thoracic cavity surgeries. To date, no specific instrument in the market was designed specifically for Head and Neck Surgery.

However, new technique proposals for thyroidectomy using remote access via cervicofacial routes have recently emerged. The most used pathways for access are the retro-auricular route (an incision is made at the hairline and access granted through a subcutaneous tunnel) and the transoral routes (TOETVA – where the surgical instruments are adapted from the ones used on abdominal endoscopic surgery – laparoscopies, and TOETRA – using the same robotic instruments mentioned above) (4-6).

In Brazil, about ten percent of the head and neck surgeons (out of about a thousand) are enthusiastic about the transoral technique, advocating for it to be included as a standard of care treatment in the surgical treatment of the thyroid gland (7).

In this letter, a group composed of Brazilian Head and Neck Surgery specialists is bringing their opinion in including the technique as a standard of care procedure. We will bring some points and concerns that are should be highlighted and the doubts about having these procedures (TOETVA or TOETRA) considered routine standard of care.

Publications advocating TOETVA (or TOETRA) present clear data of feasibility and remarkable aesthetic results. Some publications demonstrate similar (incipient) oncological results when compared to the classic procedure (7-10).

However, the literature fails to report some data that our group believes is of utmost importance when admitting the procedure as a possible standard of care technique. The literature lacks data on time spent on the operation, the need for conversion to the classic route, presence of bleeding, rate of infection, complications due to the route used for thyroid resection such as injury to mental nerves, and aesthetic complications such as tissue fibrosis due to the extensive dissection and inadvertent opening of the soft tissues on the anterior cervical region. Furthermore, we believe that the introduction will play an important impact on surgical costs and that there are ethical issues in terms of equity (public system and private system).

DISCUSSION

Limitations

It is important to highlight that one of the main limitations of endoscopic thyroidectomy is that the operative field is visualized in 2 dimensions. Furthermore, it uses rigid instruments (which mainly produce linear movements, lacking the "filtering" characteristics from the hands of surgeons (3). In this context, we should also consider the difficulties of dissecting vital structures or tissues close to those noble structures in a confined space as the endoscopic thyroidectomy narrows the visualization of the operative field. For example, there are no literature reports on the preservation of the external branches of the superior laryngeal nerve, a crucial technical aspect when operating patients that are voice professionals.

Learning curve and surgery duration

The literature reports that a surgeon must perform around 15 TOETVA cases as a learning curve (7). In addition, during the training, operative time can be two or three times longer when compared to the classic thyroidectomy and remains long even post-learning curve. Could this curve be ethically justified in daily practices? Wouldn't it be more appropriate to place the procedure under a research protocol? It is important to emphasize that high morbidity and unusual complications after conversion to a classic thyroidectomy have been reported (9). Therefore, when would it be appropriate to convert the procedure into classic access – acting time after a complication has been identified?

Bleeding

Almost no major bleeding has been reported in the published case series of TOETVA and TOETRA, neither intra nor postoperative. It is noteworthy that every thyroid surgeon has already experienced the serious adverse event of having to reopen, as an emergency, the conventional cervicotomy to evacuate a hematoma and alleviate the patient's respiratory failure. A cervical hematoma is a serious and worrisome complication. Although it is a common complication of thyroidectomy (expected in 1% of thyroidectomies), there are no reports of this occurrence in the published series of TOETVA and TOETRA. How would those cases be managed since there is no cervical incision to be opened? Wouldn't a cervical incision with local anesthesia add more morbidity to an event that is already serious?

Infections

There are few reported cases of infections in classic thyroidectomies. The same remains true in the published series of TOETVAS and TOETRA. However, although infection is an occasional risk in classical surgery, in TOETVA it becomes an assumed risk once the thyroid is accessed through the oral cavity, which is considered a contaminated or potentially contaminated territory (7-10).

New complications

Entering the cervical area through unusual "portals" places risk to several anatomical structures (such as lips, mental nerves, mimic muscles, thyrohyoid membrane, and others) that are not manipulated during a conventional thyroid incision. Corroborating this statement, the literature reports complications such as labial paresthesia, due to mental nerve injury. In addition, other complications never described in conventional thyroidectomies, have been reported such as changes in smell and taste caused by using antiseptics in the areas accessed and by surgical positioning.

A new type of complication should also be highlighted: The quality of the surgical specimen. Although small incisions allow instruments to be introduced and used for dissection, they do not permit, in a considerable number of cases, the removal of the entire surgical specimen without imposing damage to the tissue. Tissue quality is of utmost importance for the histopathological assessment of lesions that are crucial for future decisions on adjuvant therapies.

It is also important to mention that TOETVAS and TOETRA imposes a risk of CO2 embolism, once it is used for tissue insufflation to maintain the operative cavity distended and allow for the visualization of anatomical structures. This new complication is not mentioned in the literature, although the theoretical risk of it happening should be more frequent than what is currently seen in abdominal and thoracic surgeries, as the neck does not have a real anatomical cavity. To insufflate gas into the cervical region, a territory of many important vessels, greater pressure is needed to create the necessary space for the operation. In addition, the literature reports a few cases of subcutaneous emphysema in patients submitted to endoscopic thyroidectomy through lateral incisions, a technique that has now been abandoned. In those cases, insufflation caused not only emphysema of the neck but also of the face and chest, with indisputable aesthetic impairments, in addition to severe pain.

Esthetical complications

It sounds strange to discuss esthetics once TOETVA and TOETRA are techniques that propose superior esthetic results for the patients. However, it should be mentioned that there are reports of severe skin lesions happening in the surgical instrument's pathway on aesthetically significant areas of the face and neck. Punctures and burns can present significant and unacceptable esthetical complications. For example, if the damage is inflicted on the lower lip, either sensory or motor, wouldn't it be more visible and esthetically unpleasant than the presence of a scar from a classic neck incision? Undoubtedly, this requires consideration. Moreover, fibrosis-related skin and deep tissue retraction is an esthetical complication that has not been described in the literature, probably due to the longer time needed to occur post-operatively. Tissue fibrosis can also present with functional impairments such as dysphagia and movement discomfort. Besides this, the presence of undulations and irregularities in the skin of the cervical region, in addition to the sensation of "neck tightness" and "burning", can also be caused by a larger dissection area needed for surgical access.

Financial and social burdens

It is important to assess the financial and social costs of routinely considering TOETVA and TOETRA on the standard of care practices. Although not measured in the literature, there is a clear increase in real costs. Exact quantification of the increase in costs is difficult, especially when considering the subjectivity of the data. Nevertheless, qualifying is not. The procedure requires investments in surgical training, it has a longer execution time, requires a larger amount of medications to be given to the patient (for example, antibiotics are given for at least 7 days (4)), and require the use of expensive tools such as endoscopic materials. Those materials are considerably more expensive even when permanent tools are used instead of disposable ones.

The social burden of routinely offering TOETVA and TOETRA as the standard of care treatment for thyroid surgical pathologies cannot be ignored, especially when considering the reality of our country. In the Public Health System (SUS), several patients are waiting in large queues for an opportunity to be surgically treated. It does not seem appropriate to routinely offer a procedure that takes at least twice as

long to be completed, as it will increase the time those patients will be waiting for treatment.

Besides this, routinely performing TOETVA and mainly TOETRA, would highly impact both the public and private health care systems due to the significant cost increase of the procedure. It would certainly impose an undesirable revision in the actuarial calculations of thyroidectomy costs. This would be particularly undesired in the difficult times in which we live now.

Finally, we would like to highlight that the literature also states the TOETVA and TOETRA techniques cannot be considered for all patients. The exclusion criteria include thyroid gland with high volume, tumor staging, presence of previous treatments, and patient anthropometrics (7-10). The defenders of the procedure consider TOETVA and TOETRA to be minimally invasive. However, transoral access should not be considered minimally invasive as they require larger tissue dissection, and access through potentially contaminated areas. Although it does not present with a visible neck incision, in our opinion, those procedures are even more invasive than the classic thyroidectomy.

CONCLUSION

In our opinion, there is only one gain from the TOETVA and TOETRA approach which is the aesthetical appearance of the neck. We also believe that this benefit should be considered limited due to the risk of complications motioned above. Moreover, it is important to emphasize that classic thyroidectomy incisions are almost always unnoticeable and have little impact on quality of life (11,12). We strongly believe that further discussions on the risk versus benefits of those procedures should be clearly and honestly described in professional forums. We also vehemently repudiate the use of media vehicles, outside the scope of ethical and scientific discussions, for the dissemination of techniques that, in our point of view, still lack a solid foundation for routine adoption. In our opinion, as many questions remain to be answered, those procedures should stand as an exception and should be further studied before entering the routine standard of care.

In conclusion, TOETVA and TOETRA are procedures that add several unprecedented complications to thyroidectomies, take longer to perform (approximately two to three times when compared to conventional thyroidectomy), and adds costs. There is a large learning curve and it is inadequate to be carried out outside of an educational institution, as it requires experienced mentorship. The aesthetical gain of this procedure makes little sense given the excellent results obtained with the traditional procedures. The technique, mistakenly entitled "minimally invasive", lacks the advantages widely found in video-laparoscopic surgeries. TOETVA and TOETRA are not offered as a surgical option at the main cancer treatment centers in the USA (Memorial SK Cancer Center and MD Anderson) nor in the guidelines of the American Society of Endocrine Surgeons (ATA). We strongly believe that those procedures should be considered experimental until further data is available to ensure patient safety.

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- Pedigrees should be drawn according to published standards (See Bennett et al. J Genet Counsel (2008) 17:424-433 - DOI 10.1007/s10897-008-9169-9).

Nomenclatures

- For genes, use genetic notation and symbols approved by the HUGO Gene Nomenclature Committee (HGNC) – (http://www.genenames.org/).
- For mutation nomenclature, please use the nomenclature guidelines suggested by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/)
- Provide information and a discussion of departures from Hardy-Weinberg equilibrium (HWE). The calculation of HWE may help uncover genotyping errors and impact on downstream analytical methods that assume HWE.
- Provide raw genotype frequencies in addition to allele frequencies. It is also desirable to provide haplotype frequencies.
- Whenever possible, drugs should be given their approved generic name. Where a proprietary (brand) name is used, it should begin with a capital letter.
- Acronyms should be used sparingly and fully explained when first used.

Papers must be written in clear, concise English.

Avoid jargon and neologisms. The journal is not prepared to undertake major correction of language, which is the responsibility of the author. Where English is not the first language of the authors, the paper must be checked by a native English speaker.

For non-native English speakers and international authors who would like assistance with their writing before submission, we suggest Voxmed Medical Communications, American Journal Experts or PaperCheck.

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