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PROGETTO DI RICERCA:

THYROID CARCINOMA AND ENVIROMENT: A MULTICENTRIC STUDY ON A POSSIBLE EZIOPATHOGENIC ROLE OF ENDOCRINE DISRUPTOR CHEMICALS AND OVERWEIGHT.

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1. INTRODUCTION

1.1 Nodular Thyroid Pathology: Differentiated Thyroid Carcinoma

Nodular thyroid pathology is a quite common clinical problem. Epidemiological studies have shown the prevalence of palpable thyroid nodules that are approximately 5% in women and 1% in men living in iodine-sufficient parts of the world. In iodine-deficient areas this percentage can exceed 50%. On the other hand, by using ultrasound techniques (US), the prevalence of these lesions in the general population may rise to 19-68% depending on the considered case studies, with a higher frequency in women and the elderly [1]. Non palpable nodules detected on US or other anatomic imaging studies are called ‘‘incidentalomas”.

Role of clinicians is to exclude thyroid cancer, which occurs in 10% of cases, depending on different factors [2]. For this reason, it is important to take a complete patient history, to make a physical examination focusing on the thyroid gland and adjacent cervical lymph nodes by ultrasonography and to perform a fine needle aspiration (FNA) when necessary, according to risk stratification score systems [1-5].

Historical factors predicting malignancy include a history of childhood head and neck irradiation or exposure to ionizing radiation, family history of thyroid carcinoma, or thyroid cancer syndrome (e.g., Cowden’s syndrome, Familial Polyposis [FAP], Carney complex, Multiple Endocrine Neoplasia [MEN 2], Werner syndrome), rapid nodule growth with hoarseness, dyspnoea, or dysphagia [3].

Differentiated thyroid carcinoma (DTC) is the most frequent endocrine cancer.

This histological class includes papillary thyroid carcinoma or PTC with its variants (classic, tall cell, hobnail, NIFTP, etc..) and follicular thyroid carcinoma or FTC with its subtypes (minimally or widely invasive).

As claimed by Italian Consensus [4], treatment, usually, provides surgery resolution, more or less conservative according to patient's clinical conditions and preference, tumour size and localization, presence of comorbidity and evidence of lymphatic metastasis.

It is recommended lobectomy for microcarcinomas with no clinical or US evidence of lymphadenopathy. It is recommended total thyroidectomy for lesions over 4 cm or less extensive but with lymphatic recurrences or if is likely an iodine treatment. Lesions with main diameter from1 to 4 cm may be treated with lobectomy if other conditions already listed do not exist. Prophylactic central compartment neck dissection is not routinely recommended, it is just indicated when there is clinical, US or intraoperative evidence of lymphatic involvement in central or lateral neck stations.

Radio iodine treatment, then, should be planned on the basis of three factors: TMN staging [6], ATA risk classification [1] and thyroglobulin (Tg) value during L-thyroxine (L-T4) therapy or after TSH stimulation (TSHs). In fact, Iodine treatment is strongly recommended for high-risk tumour, whereas may be considered for intermediate class. Moreover, if Tg values under L-T4 treatment or after TSHs is respectively undetectable or less than 5 ng/ml, recurrence probability is very low and iodine treatment is avoidable.

Anyway, if Iodine treatment must be done, an activity of 30-50 mCi should be considered for low and intermediate risk tumour, while more than 100 mCi should be administered for patients at high risk [1,4].

The incidence of DTC appears to be steadily increasing [4], environment and life-style may pay a role in thyroid carcinogenesis [7-25]. This trend involves not only micro-carcinomas but lesions of all sizes and the evidence that a plateau has not been achieved, despite long-term use of sensitive diagnostic procedures, supports an etiologic role for environmental contamination and life-style. This hypothesis was recently empowered by the observation that increased incidence mainly involves DTC with BRAF and RAS mutations [26], which seems to occur more frequently in chemically polluted areas [27]. Despite the improved knowledge of molecular genetics of DTC [26,28], aetiology of genetic damage is still unclear. Furthermore, genetic alterations, such as RAS mutations and RET rearrangements, have been detected in benign nodules, being considered as markers of pre-cancerous lesions [29].

1.2 Endocrine Disruptor Chemicals

Endocrine disruptor chemicals (EDCs) are exogenous substances, persistent in the environment and widely absorbed by humans, altering the function of the endocrine system [25].

Although there may be hundreds or more environmental chemicals with EDC activity, several classes are most commonly studied .

In this work we consider bisphenols and phthalates as principal EDCs.

Bisphenol A (BPA) was first synthesized in 1891 and was discovered to be estrogenic in 1936 [30]. More BPA is produced annually than any other chemical, with 15 billion pounds produced in 2013 [31]. It is used in a very wide array of manufacturing, food packaging, toys, and other applications, and BPA resins are

found in the lining of many canned foods and beverages such that virtually everyone is exposed continuously [32]. In food contact materials, BPA may leach into food or water under high heat, physical manipulation, or repetitive use. Due to its ubiquitous nature and continuous exposure, 93% of Americans have a measurable amount of BPA in their urine [33,34]. It is also detected in breast milk of some women [35]. BPA is so prevalent in our daily environment that elimination of BPA contamination during carefully controlled quantitative procedures has proven difficult [36,37]. BPA is rapidly metabolized to non-bioactive forms and has a short half-life of approximately 4–5 hours in adult humans, with lower metabolic rates in the fetus and infants [38,39]. Measurements of bioactive or free BPA in human serum is controversial at present, with some documenting nanograms per milliliter quantities in samples using contamination-free conditions [39,41], whereas others report that ordinary exposures result in picograms per milliliter levels or lower [42]. Although relevant internal exposure remains a critical issue that is still unresolved, it is noteworthy that industrial exposures, vulnerable populations, and individual variations in metabolism and susceptibility must be taken into consideration [43]. Currently, the US Environmental Protection Agency (EPA) safety level of BPA is set at 50 μg/kg/d, whereas the European Food Safety Authority's temporary tolerable daily intake was recently lowered to 4 μg/kg/d. Several studies in the present report will document BPA effects in mammalian systems at or below these current safety levels.

Phthalates. Phthalates and phthalate esters are a large group of compounds used as liquid plasticizers found in a wide range of products including plastics, coatings, cosmetics, and medical tubing. These compounds were first introduced as additives in the production of plastic in the 1920s and resulted in the rapid

widespread use of polyvinyl chloride plastic in the 1930s and later. Because they are not chemically bound to the plastic, phthalates can leach into the environment. Moreover, a variety of consumer products use various phthalates, including personal care products, medical tubing, vinyl flooring materials, and toys. They are detectable in human urine, serum, and milk samples too [44-46], and the estimated daily exposure to one major phthalate, di(2-ethylhexyl)phthalate (DEHP), ranges from 3–30 μg/kg/d [47].

So, chronic lifelong exposure to EDCs may predispose individuals to pathologies.

Thyroid is a target of EDCs, which may affect both metabolism and transport of thyroid hormones, and also their transcriptional activity [49-51].

BPA is one of the most produced and well-studied EDCs, which has been shown to interfere with thyroid hormone signaling and action via various mechanisms, including inhibition of the sodium/iodide symporter (NIS) and altering the expression of thyroid function related genes [49]. Some recent epidemiological studies indicate that BPA exposure may either enhance or decrease serum T4 levels in humans. These observations are in line with in vitro data showing that BPA is a weak ligand for thyroid receptors (TRs), therefore acting as an indirect antagonist, and may also interfere with thyroid hormone action by a nongenomic mechanism [50].

Likewise, animal experiments have shown that exposure to DEHP and its metabolites reduces the expression of NIS, decreases the level of transthyretin, one of the main thyroid hormone-binding proteins, and increases the levels of deiodinase 1 and UDP glucuronosyltransferase (UGT) in the liver, which metabolizes thyroid hormones. These observations suggest that DEHP can affect

the thyroid hormone levels through effects on thyroid hormone synthesis, transport, and metabolism [51].

A higher prevalence of autoimmune thyroid diseases (AITD) was observed in people living in polluted areas near to petrochemical plants, and in petrochemical workers, but also in area contaminated with organochlorine pesticides, or with polychlorinated biphenyls, or near aluminum foundries [52].

To date, exposure to environmental pollutants has been associated to impairment of thyroid-hormone profile, but data about carcinogenic activity in human thyroid are scarce and unclear [53-55].

1.3 Obesity and Thyroid Cancer

As we said, DTC is the most common endocrine malignancy into the world and its incidence has increased dramatically in recent years. In parallel, the prevalence of overweight and obesity has also increased, suggesting a possible link between these two diseases. Indeed, obese patients present chronic inflammation, altered cytokine levels, insulin resistance, oxidative stress, and hormonal changes that are factors involved in carcinogenesis [7,56].

According to the World Health Organization (WHO), body mass index or BMI (kg/m2) is used as a measure of body fat. For optimal health, a BMI between 18.5 and 24.9 kg/m2 is recommended. Individuals with a BMI in the range of 25.0–29.9 kg/m2 are overweight, whereas obesity occurs in subjects with a BMI greater than 30 kg/m2 .

Moreover, several other parameters can be used to assess the clinical relevance of obesity, including visceral or subcutaneous adipose tissue, waist-to-hip ratio, waist circumference (WC).

About WC, different normal range for sex exist; for Caucasian population we consider normal a Wc <80cm in women and <94cm in men.

Indeed, BMI is the most commonly used but sometimes can be inaccurate because it is unable to distinguish adipose tissue from lean mass.

It has been estimated that a five-point increase in BMI and a 0.1-point increase in waist-to-hip ratio raise the risk of DTC by 30% and 14%, respectively [57].

Furthermore, it would seem that obesity was also associated with an increased risk of anaplastic thyroid cancer, suggesting a possible role of obesity in the progression and dedifferentiation of PTC [57].

Adipose tissue (AT) is considered an endocrine organ due to its ability to release various active molecules like adiponectin (APN), leptin, resistin, and many immune system cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and complement factor D or adipsin [58]. Dysregulation of the immune system in AT of obese individuals leads to chronic low grade inflammation, which is linked to cancer development, as published in a lot of recent studies [57-59]. As evidence, serum levels of leptin and apnea were found higher in patients with DTC [58,59].

2. OBJECTIVES

This is a multicenter analytical cross-sectional study evaluating chemical exposure, lifestyle and thyroid cancer diagnosis in patients affected by thyroid nodules submitted to FNA. The aim of the current study is to evaluate if EDCs exposure, and/or lifestyle are associated with the incidence of DTC.

3. PATIENTS AND METHODS

3.1 Patients

From May 2019 to February 2021 we enrolled and subjected to FNA, and any molecular test, consecutive patients affected by thyroid nodules in euthyroidism with ultrasonographic features of risk for thyroid cancers according to ATA guidelines [1], admitted to Endocrinology Clinics of Experimental Medicine Department of Sapienza University of Rome and Medicine Department of Federico II University of Naples.

Inclusion criteria: a) age ≥18 years, b) thyroid cytology consistent with thyroid tumor or benign nodule (TIR2-TIR5 SIAPEC categories [60]), c) clinical management, including surgery (when performed), entirely performed in one of the involved centers, d) euthyroidism.

Exclusion criteria: a) inconclusive cytology (TIR1 SIAPEC category [60]), b) clinical and/or cytological and/or histological features consistent with medullary thyroid cancer, c) Thyroiditis.

The study received the approval of the Ethic Committee of the coordination Institute (Sapienza University of Rome) (Prot. 0945/2022, Rif 6870). Informed consent was obtained from each enrolled patient.

3.2 Methods

Cytological samples were evaluated at the Units of Cytopathology of Federico II and Sapienza University and classified according to SIAPEC systems [60]. All patients with TIR3B, TIR4, and TIR5 cytological categories were addressed to surgery (±radioiodine treatment according to guidelines) with pathological examination and definition of clinicopathological features. Patients with TIR3A cytological category underwent further FNA within 6 months and then were followed up or addressed to surgery according to the last FNA. Patients with TIR2 cytological category were followed-up or operated according to physician recommendation [2] and their preference. Molecular evaluation was performed in thyroid nodules undergone to surgery.

The risk of DTC and thyroid-specific genetic damage related to EDCs were stratified for thyroid cytology category as well as for factors demonstrated/suspected to be involved in thyroid tumorigenesis or affecting the grade of environmental contamination: age, gender, BMI, WC, education level, living area (time since living there), presence of industrial areas, waste dumping sites (legal or illegal), current and previous occupation, history of parental illnesses, smoker status, alcohol consumption, type and frequency of foods consumed during a standard week. These data were obtained through self-reported questionnaires.

Serum samples used for EDCs evaluation were obtained at the time of enrolment and stored at -20° C. Assessment of BPs and phthalates presence and

concentration within the serum were performed at the Pharmacy Unit, Federico II University. Molecular analyses were performed on cytology specimens at the Cytopathology Unit, Federico II University. Thyroid function tests and calcitonin outcome were exhibited by patients at the beginning of screening [61].

3.3 Assessment of chemical exposure

3.3.1 EDCs

Blood samples for EDCs evaluation were obtained at the time of enrolment, in the same day of the FNA execution. Five mL were collected in Vacu-test® tubes from the antecubital vein and centrifuged at 3000 rpm for 20 min, subsequently transferred in an Eppendorf, and stored at -20°C until the analysis. Sample preparation was carried out as follows: 300 mL of serum was added to 150 mL of perchloric acid in 25% w/v aqueous solution (to precipitate the proteins), 150 mL of distilled water, 585 mL of ethylacetate/n-hexane 50/50 v/v, 100 mg NaCl, and 15 mL of a 10 mg/mL solution of biphenyl in ethylacetate as internal standard (IS). The supernatant was used for the detection of endocrine disruptors. Assessment of BPs and phthalates (BPA, BPF, BPE, BPB, BPS, BPAF, DCB, TCS, BADGE, DEHP, MEHP, TCB, 4_CP, 2_CP) serum concentration was performed at the Pharmacy Unit, Federico II University of Naples. EDCs screening included: BPA and some of its structural analogues and bis(2-ethylhexyl) phthalate (DEHP) and its main metabolite, mono-ethylhexyl phthalate (MEHP). The analytical procedures for the detection of the reported analytes in human serum (essential for assessing the total internal exposure) were validated by the Pharmacy Unit, Federico II University of Naples. Determination was performed using HPLC coupled with fluorescence detection (FD) and/or mass spectrometry (MS).

Total BPs were determined after hydrolysis by ß-glucuronidase enzyme, extraction by solid phase extraction (SPE), followed by LC/FD/MS analysis; DEHP and MEHP, extracted by SPE were analysed by LC/Ultraviolet Detection (UV)/MS analysis. The analyses were performed at least three times and the results are the averages of three determinations.

3.3.2 Molecular Test

The molecular analysis was performed on cytology specimens at the Cytopathology Unit, Federico II University of Naples.

During each FNA procedure, fresh cells were prospectively collected and stored

in eppendorf with DNAsi free solution at -20°C, while waiting for the cytological report, to obtain nucleic acid with optimal quality and quantity. Molecular analysis involved indeterminate and malignant thyroid nodules.

An aliquot of the aspirated material was suspended into a vial of nuclease-free water (Ambion, Invitrogen, Thermofisher, Waltham, MA, USA). The vial was stored at -20°C until the final cytological diagnosis was available. The DNA and RNA were simultaneously extracted using the AllPrep DNA/RNA kit (Qiagen). The nucleic acid extracted from the vial was analyzed using a real-time PCR (RT-PCR)-based procedure on a Quant Studio 5 platform (Applied BioSystem, Thermofisher) using the Entrogen Thyroid Cancer Mutation Analysis Panel kit (EntroGen Inc,Woodland Hills, CA, USA) that detects BRAFV600E, KRAS codons 12 and 13, NRAS codon 61, HRAS codon 12, 13 and 61 point mutations, and the RET/PTC1, RET/PTC1, RET/PTC3 and PAX8/PPARg fusions. The assay was performed in two runs: one run to detect point mutations in the BRAF and RAS genes on DNA, and the second to detect the fusion genes on RNA. Fusion

detection reactions were performed with a one-step procedure that combines cDNA synthesis and RT-PCR. The resulting RT-PCR amplification curves were visualized on QuantStudioDesign&Analysis software v 1.2 (Thermofisher)**.**

3.4 Study groups definition

Study population was divided into two subgroups: group A, patients with benign thyroid nodules or with low cytological risk to have thyroid cancer (TIR2 or TIR3A wilde thype); and group B, patients with high cytological risk to have thyroid cancer (Indeterminate nodules with high cancer risk, TIR4, and TIR5**).**

3.5 Statistical analysis

Statistical analysis was performed using Pearson's Chi-square and Fisher's test of frequency differences and t-test for paired and unpaired data for comparison of means. Univariate and multivariate logistic regression analysis (Odd ratio and 95% confidence interval 95%CI) were also performed to investigate the association between the individual factors analysed and thyroid cytology. The results were considered statistically significant for p values <0.05. Statistical analysis was performed using IBM-SPSS version 25 software (IBM Corporation, New York, United States of America).

4. RESULTS

In the study were enrolled 201 consecutive patients, among them 11 were excluded due to inconclusive cytology. Finally, the population included 190 subjects, 138 female and 52 males, with age 52.89±13.94y (mean±SD).

51.3% of patients were overweight/obese (BMI 25-30 kg/m2 in 69 subjects; BMI> 30 kg/m2 in 28 subjects).

Among EDCs analysed, we found BPAF exposure in 24.7% (47 pt) and DEHP exposure in 85.3% (162 pt) of subjects of overall population, with a median serum concentration of 7.23 ng/mL (range 0.02-21.66) and 29.54 ng/mL (range 0.81-1027.95), respectively.

According to cytological categories, 15 of 24 patients who underwent thyroidectomy had DTC. Three subjects of group B refused surgery, two choose for active surveillance and one for radiofrequency ablation.

The prevalence of obese subjects was higher in group B than in group A (33.3% vs 11.7%, $p<0.007$); so also BMI mean±SD (27.6±5.3 vs 25.1±4.7 kg/m2; $p<0.013$). The prevalence of visceral obesity, evaluated as WC above the reference range for sex, was higher in group B than in group A (61.5% vs 35%, $p<0.016$), so as WC mean±SD (94.8±16.5 vs 86.8±13.0cm; $p=0.06$).

The prevalence of BPAF exposure was higher in group A than in group B (27,6% vs 7,4%; p=0,04). Analogue data was found for DEHP contamination (86,5% vs 77,7%; p= 1,0 respectively in group A and B), although with a non significant statistically difference.

At the T-test for independent samples, DEHP concentrations in the two groups were not significantly different (27.5±88.3 in group A vs 13.9±34.4 in group B, p=0.4). At ROC analysis, it was not possible to identify a serum concentration of DEHP capable of significantly differentiating patients with low risk cytology from those at risk of cancer.

At univariate analysis, male gender (OR 2.46, 95% CI 1.06-5.69, p=0.035), BMI (OR 3.1, 95% CI 1.26-7.86, p=0.014) and WC (OR 2.9, 95% CI 1.25-6.91, p=0.013) were significantly associated with tumor risk cytology.

The results of the multivariate analysis showed a statistically significant association of tumor risk citology with gender (OR 2.7, 95% CI 1.13-6.47, p=0.025) and WC (OR 2.9, 95% CI 1.24-7.03, p=0.014), but not with BMI (OR 2.2, 95% CI 0.82-5.92, p=0.12).

A subgroup analysis was performed in patients undergone to surgery (24 thyroidectomies: 8 goiters, 16 DTC): no statistically significant differences were observed for BMI, WC, DEHP concentration and BPA concentration.

No somatic mutations were discovered in the latter samples.

5. DISCUSSION

Our work has examined a heterogeneous population of patients residing in the italian regions of Lazio and Campania.

The incidence of thyroid carcinoma, confirmed on operating pieces, in the overall population was in line with the data present in the literature (8.4% excluding the 3 missing data) [1,2,4].

The distribution by gender of consecutive patients enrolled, confirmed the higher prevalence of nodular disease in the female gender [1].

On the other hand, uni- and multivariate analyses showed a statistically significant prevalence of male gender in the group B, confirming the postulate that nodules are "less frequent but more suspicious in males".

The anthropometric evaluation in the overall population did not allow particular speculations on the role of fat in general nodular pathology (A + B), while the study of prevalences in the 2 sub-populations showed a statistically significant difference with a predominance of obese / overweight in the group of subjects with high risk cytology. This different distribution, at a preliminary examination by univariate analysis, was significant considering both parameters that attest to weight gain: BMI and WC. Instead, at a confirmatory multivariate analysis only the different distribution of WC retained significance. However, the data is in line with what is emerging from numerous scientific evidences [57-59].

In fact, it is known that WC is a parameter that best represents android obesity and visceral fat, unlike BMI which fails to discriminate lean and fat mass and examines body weight in its entirety [57]. Moreover, it is known from the

literature that visceral fat represents in all respects an endocrine organ, capable of producing cytokine and creating inflammation preparatory to cardiovascular and tumor pathological processes[56-59,62]. In particular, some recent works showed higher values of insulin and leptin present in patients with thyroid cancer[59, 63]. Insulin is able to stimulate cell growth through the link with its receptor (IR), which is overexpressed in different cancer cells. Hyperinsulinemia, so, may stimulate oncogenesis through different cell signalling pathways, enhancing growth factor dependent cell proliferation, and exerting a mitogenic effect [63]. In the context of thyroid cancer, leptin seems to play a key role in promoting DTC cell proliferation and angiogenesis instead, promoting aggressive histopathological features of thyroid cancer [59].

With regard to EDCs, emerged data may seem to be at odds with the literature [53-55,64], but on the contrary they appear as a source of important food for thought.

BPAF exposure and its mean concentration, in fact, were higher in group A; while there were no statistically significant differences in relation to exposure or concentration of DEHP in the two sub-populations. This data, at first reading, could suggest a null effect (DHEP) or even protective effect of BPAF.

As shown before, however, the two subpopulations see a heterogeneous distribution of visceral fat, predominant in group B, which tends to be less exposed to BPAF. A recent study by Marotta et al. [64] showed a higher risk of thyroid cancer in an overweight group exposed to Bisphenol A (BPA) coming from Campania region, suggesting an interaction between BPA exposure and adipose tissue excess in promoting thyroid carcinogenesis. Indeed, detectable serum BPA levels were related to significantly higher risk (5.3 fold) of DTC

only in overweight/obese subjects. It is conceivable, therefore, that the absence of excess visceral fat in group A acted as a protective factor against BPAF.

In addition, the patients enrolled in our study (A + B) were exposed at average concentrations of EDCs significantly lower than that ones showed in the two studies previously conducted on the Campania population [55,64]. This fact may depend on the heterogeneous origin of the samples of our work (Lazio and Campania), allowing us to speculate on the possibility of not having exceeded a presumed average cut-off of high risk in population B.

It should also be noted that some EDCs have extremely short half-lives (BPAF 4-5 hours), and a single sample may not be fully representative of a daily exposure. Additional dedicated tools, so, will be needed for a more accurate exposure study.

Further elements concerning the "life-style" (smoking, alcohol, food, education) collected from the self-administered questionnaires were not presented, as they were all distributed homogeneously in the two groups, and therefore without statistically significant differences. Finally, the subanalysis performed on patients undergoing surgery showed no significant correlations between DTC incidence and exposure to ECDs, BMI, WC. This result is probably linked to the smallness of the sample.

In fact, the limitations of the study are as follows: the cross-sectional design and the short half-life of EDCs which do not allow a causal relationship to be established precisely; the small sample size that hinders interesting sub-analyses.

6. CONCLUSIONS

Our study reports a significant association between visceral fat and risk of differentiated thyroid carcinoma in patients with nodular goiter.

No statistically significant data were emerged about exposure to EDCs and incidence of Thyroid Cancer. Further study will be necessary to evaluate this association.

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8. FIGURES