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Thyroid Cancer, Volcanic Areas and Heavy Metals

PhD Thesis

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1. Introduction

1.1 Changes in Thyroid Cancer Epidemiology

Thyroid cancer is the most frequent endocrine cancer (1.0%–1.5% of all new cases in the US), and its incidence, which was stable until the 1980s, has constantly increased in the last decades [1,2]. It is now the fourth most frequent cancer in women [3], whereas it ranked 14th in the early 1990s.

Increased incidence of thyroid cancer has occurred worldwide, as documented by the annual percent change in all countries where this parameter has been calculated, with very few exceptions [4,5].

The reasons underlying this increase are unknown. Many experts believe that the increase is mostly apparent, due to the diagnosis of a large reservoir of small papillary thyroid tumors that have no significant clinical relevance in terms of patient health and survival and that were not detected in the past but have been identified in the last decades because of the increasing diffusion of sensitive imaging procedures such as ultrasound scans [6]. Although there is a general consensus that a higher detection rate has contributed to the increasing thyroid cancer incidence, much evidence indicates that this cannot be the only explanation. In fact, large thyroid cancers have also increased [7], and these tumors are very unlikely to have gone undetected in the past. Moreover, thyroid cancer-related mortality, which should have decreased because of early detection and better treatment, is stable or increasing [8]. Finally, a temporal trend has been observed with changes in the thyroid cancer molecular profile with an increasing prevalence of BRAF and RAS mutations [2,9,10]. All these evidence support the possibility that also a true increase in thyroid cancer incidence is occurring.

The causes of the recent changes in both quantitative and qualitative thyroid cancer characteristics are most likely environmental, as suggested by the sharp increase in incidence during the last decades. Most malignancies of other tissues and organs have not increased within the same period, indicating that the potential carcinogenic factors involved must be in some way thyroid specific. Therefore, general factors that are known to favor cancer, such as the *obesity epidemic*, are unlikely to play a major role favoring specifically thyroid cancer.

Different and more plausible environmental risk factors have been suggested. Since the thyroid is very radiosensitive, especially at a young age, and *radiation exposure* has doubled in the last 25 years in most industrialized countries (mainly because of medical diagnostic procedures), radiation is the most commonly referenced cause [11,12]. For instance, frequent

dental X-rays in children/adolescents may have favored higher radiation-related carcinogenicity in the thyroid.

Another possible risk factor specifically favoring thyroid cancer is the progressive increase in *iodine intake* due to prophylaxis programs diffused worldwide in recent decades to prevent iodine-deficient diseases and goiter. Iodine enrichment may favor chronic lymphocytic thyroiditis [13], which may in turn promote thyroid cancer by increasing thyroid-stimulating hormone (TSH) levels and inducing proinflammatory cytokine production and oxidative stress in the gland [14–16].

However, as possible risk factors also the large number of *potential carcinogens* associated with the westernized postindustrial lifestyle should be considered. In recent decades, the population has been highly and progressively exposed to *compounds and chemicals* that may interfere with biological functions, including hormone homeostasis (endocrine disruptor chemicals) [17]. Many compounds used in agro-industrial activities (fertilizers, pesticides, repellents, and preservatives) may directly cause cancer or indirectly produce conditions that favor malignant transformation. For instance, the increased ingestion of nitrates, which are a frequent contaminant of drinking water in areas of intense agricultural industry and are present at high levels in processed meat, has been associated with an increased risk of thyroid cancer [18,19]. Other potential thyroid-specific carcinogens can originate from other industrial activities such as the organic compounds polybrominated diphenyl ethers and bisphenols [20–22]. However, many other *environmental pollutants* (solvents, plastic, heavy metals, diet preservatives, etc.) may also be responsible for the increased thyroid cancer incidence. For most of these compounds, no standards for safe values are available, and the effect on the thyroid of chronic, everyday exposure to these pollutants is difficult to evaluate.

In any case, all evidence indicates that the causes of the true component of the worldwide increase in thyroid cancer are recent, environmental, and multiple [16]. The generalized low-level heavy metal pollution of our environment meets all these requirements.

1.2 Volcanic Environment and Thyroid Cancer

A specific natural example of the relationship between thyroid cancer and the environment is the increased incidence of this cancer observed in residents of volcanic areas.

This association was first reported 40 years ago [23] and was then confirmed by observations made in islands harboring active volcanoes, such as Hawaii [24,25] and Iceland [26,27], in the late 1980s. An elevated thyroid cancer incidence has since been reported in numerous volcanic areas in the Pacific Ocean, such as Vanuatu [28], French Polynesia [29], and New Caledonia [30], leading to the hypothesis that some components of the volcanic environment could be involved in the pathogenesis of thyroid cancer [31].

Many possible causative factors have been proposed, including the possibility that *genetic* characteristics of the small population resident in isolated volcanic islands might be responsible. However, the observation that residents of Hawaii exhibit much higher rates of thyroid cancer relative to individuals with the same ethnic background living in other geographic areas suggest that environmental rather than genetic influences play a major role [24].

Among volcanic *environmental factors*, geothermal causes such as high-temperature water containing hydrogen sulfide and radon [32] have been hypothesized to play a role. More specifically, the increased natural radioactivity present in the volcanic areas and mainly due to ^{222}Radon emission has been hypothesized to be involved, but recent studies found no association between Radon levels and thyroid cancer [33,34].

In the early 2000s, an epidemiological study on thyroid cancer was carried out in Sicily, a large Mediterranean island with over five million inhabitants and hosting a continuously active volcano (Mt. Etna, the highest volcano in Europe). Mt. Etna is located in the northeastern area of Sicily, in the province of Catania that has a population of over 1,000,000 inhabitants. For the first time, therefore, the study compared two large populations (volcanic and non-volcanic residents) with the same ethnic background, similar sex and age distributions, similar lifestyles, and similar access to medical assistance.

Thyroid cancer incidence was more than doubled in residents of the volcanic area compared to the remaining population of Sicily [35]. In both areas, there was no significant difference in the F/M ratio of thyroid cancer patients but in the volcanic area an increased incidence was observed in pediatric age [36]. Environmental factors such as iodine intake and industrial pollution did not differ in the two areas. A relevant finding is that only the papillary histotype of thyroid cancer was increased in the volcanic area, reflecting the similar specific increase observed in the worldwide increase of thyroid cancer incidence (**Table 1**).

Area	Thyroid Cancer Incidence (ASRw)		PTC/FTC Ratio
	F	M	
Volcanic Area (Catania province)	31.7	6.4	25.9
Control Area (Sicily without Catania)	14.1	3.0	9.8

Table 1. Thyroid cancer incidence in Sicily: age-standardized rates for the world population (ASRw) in the volcanic and the control areas and the PTC/FTC histotypes ratio. PTC= Papillary Thyroid Cancer; FTC= Follicolar Thyroid Cancer. Represented data are derived from ref. [35]

1.3 Heavy metals in the Mt. Etna Volcanic Area and Resident Biocontamination

It is well documented that in volcanic areas non-anthropogenic pollution with many trace elements is present [37,38] as a consequence of the volcanic activity with the emission of gases, ashes and lava. More specifically, an increased level of heavy metals such as B (Boron), Fe, Mn (Manganese), and V (Vanadium) was reported in the groundwater of the Mt. Etna volcanic aquifer [35]. Mt. Etna harbors a large aquifer that provides water to over 700,000 residents and is used for irrigation in most of Catania Province. Therefore, volcanic aquifer-originated water is an important vehicle for population biocontamination, both directly and indirectly via locally grown vegetal and animal food. Therefore, a careful comparative study of heavy metal environmental pollution in the Mt. Etna area and of biocontamination of residents was carried out [39].

To investigate *environmental pollution,* metal concentrations were measured in water and lichens. Lichens are composite organisms that bioaccumulate elements present in the atmosphere and are therefore used for the biomonitoring of atmospheric pollution [40,41].

To investigate *human biocontamination*, urine specimens were collected from two matched groups of individuals living in the volcanic and control areas. In fact, under conditions of chronic exposure, urine is considered a reliable indicator of the chemicals absorbed by a subject through contact, inhalation, and ingestion.

By measuring 27 trace elements and heavy metals in the environment (water and lichens) and human biological samples (urine), considerable volcano-derived heavy metal pollution and consequent biocontamination were found. In the volcanic area, where the thyroid cancer incidence is doubled relative to the Sicilian non-volcanic areas, many metals were significantly increased in both water and lichens, documenting metal pollution in the environment. The

differences relative to the control areas were more marked in water, in which the concentrations of metals such as As, B, Cd, Hg, Mn, Mo (Molybdenum), Pd (Palladium), Se, U (Uranium), V, and W (Tungsten) were increased by three- to 50-fold, although their average concentrations never exceeded the reference values indicated by the World Health Organization [42].

When these elements were measured in the urine specimens of residents of the volcanic area, 18 metals were found at a significantly increased level compared to values measured in the urine samples collected in the control areas (**Figure 1**).

For most metals, the average increase in the urine of residents of the volcanic area is small relative to the average value in the urines of residents of the control area: a twofold or more than twofold increase was present only for cadmium (Cd), mercury (Hg), manganese (Mn), Pd, thallium (Tl), uranium (U), vanadium (V), and W. Moreover, in most cases, the individual values were only moderately increased and for all metals examined the average value in the urines of residents of the volcanic area was still within the normal range [39]. The human biocontamination with heavy metals was confirmed by the increased metal concentration observed in other studies in the scalp hair of children living in the Mt. Etna volcanic area [43].

These studies, therefore, documented relevant heavy metal pollution of the volcanic environment with consequent human biocontamination in the same area where thyroid cancer incidence was doubled.

The well-established carcinogenic effect of some heavy metals and the observation that individuals living in volcanically active areas exhibit DNA damage more frequently than subjects living in non-volcanic areas [44] may support the hypothesis of a cause–effect relationship between increased heavy metal biocontamination and increased thyroid cancer incidence.

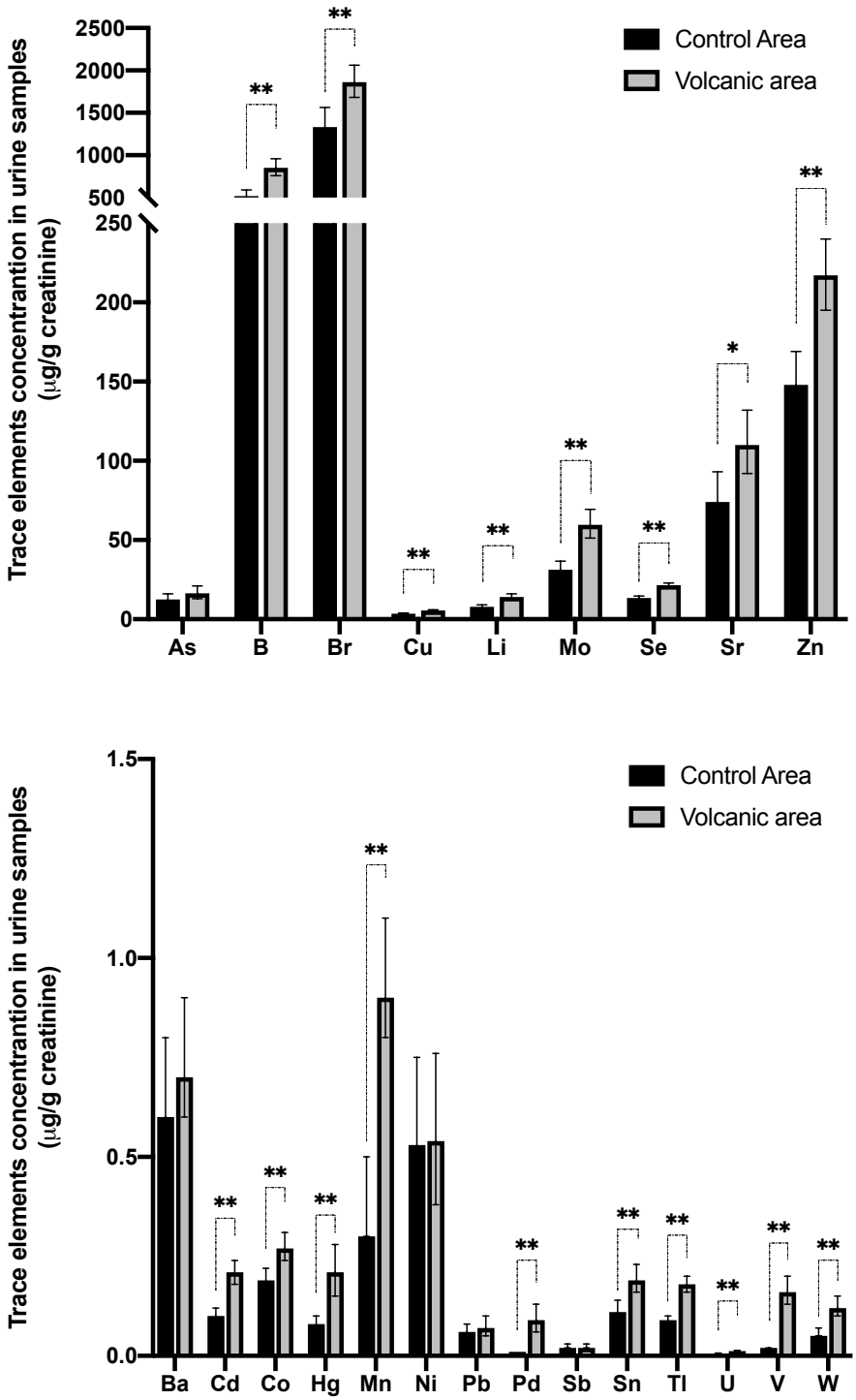

Figure 1. Trace elements concentration in the urine of residents of the Mt. Etna volcanic (grey) and the control areas (black) in Sicily. For all elements except As, Ba, Ni, Pb, and Sb, the values were significantly higher (* $p < 0.05$, ** $p < 0.01$) in the urine of volcanic area residents. Data from [39].

2. Research aims and objectives

Many epidemiological observations indicate that the incidence of thyroid cancer is increased in volcanic areas. Indeed, in the volcanic area of Sicily, where thyroid cancer incidence is greatly increased, residents are biocontaminated with volcano-originated heavy metals, but the observed biocontamination is low-level, multi-elemental and chronic. For most of these metals, no data are available regarding their effect on the thyroid gland.

The aim of this thesis is to better understand the nature of the relationship between this type of heavy metal biocontamination and thyroid cancer and to investigate the potential mechanisms involved.

According to these considerations I carried out two independent experimental approaches using human thyroid tissue

First, I carried out an *ex vivo* study in order to evaluate whether the thyroid has a tissue-specific capacity to differentially accumulate some metals, explaining why thyroid cancer seems to be a specific consequence of metal pollution. To this aim, I examined the concentration of metals in normal thyroid tissue by measuring the content of iodide and 25 additional trace elements in the human thyroid and compared the values to those measured in the muscle and adipose tissue of the same euthyroid individuals. To reinforce the observed data, I repeated the same protocol with rat tissues.

Second, I explored *in vitro* the effect of five heavy metals (Cu, Hg, Pd, W and Zn) at very low concentrations (nanomolar, the biocontamination level in residents of the volcanic area in Sicily) on the biology of both undifferentiated (thyrospheres) and differentiated human thyroid cells. In this study, each metal was examined as a single component as well as their mixture to obtain information on the multi-elemental effect which better reflects the condition observed in the volcanic area.

3. Concentration of Metals and Trace Elements in the Normal Human and Rat Thyroid: Comparison with Muscle and Adipose Tissue and Volcanic Versus Control Areas

3.1 Background

Metals are chemical elements that are not biologically synthesized but are present in the environment and may be acquired by living cells. Some metals (e.g., zinc, copper, iron, and selenium) are essential for life and are considered necessary micronutrients that must be available in a defined range to allow normal physiological processes of living cells. Other metals are called nonessential and may even be toxic in small amounts (e.g., arsenic, cadmium, and mercury).

Excessive exposure to the metals in this latter group may interfere and damage normal biological processes. In endocrinology, metals and trace elements have been investigated mainly as chemical agents that may interfere with hormone production and function (endocrine-disrupting chemicals or endocrine disruptors) [45]. Much less studied is their potential role in endocrine cell proliferation, differentiation, apoptosis, and mutagenesis.

Metal homeostasis and concentration in tissues are dependent on metal uptake, compartmentalization, retention, and clearance. Appropriate metal availability in the environment and normal absorption and distribution to different tissues may not be sufficient to assure correct cell function when congenital or acquired abnormalities of mechanisms involved in metal cellular metabolism are present. These mechanisms have been well studied for the thyroid because of its peculiar activity regarding iodide uptake, retention, and compartmentalization and thyroid hormone synthesis and secretion. Diseases derived from an abnormality in these different steps of iodide uptake and metabolism have been widely studied.

Because less relevant to thyroid physiopathology and clinical consequences, the capacity of the thyroid to uptake and metabolize other chemicals and metals is scarcely studied, even if some of them (i.e., selenium) are essential for the thyroid function.

Reference concentration values of metals in the thyroid are a prerequisite to plan biological and molecular studies on the role of these elements in thyroid physiology and pathology, but our present knowledge of the normal concentration range of these elements and metals in the thyroid is poor and is sometimes affected by preanalytical and analytical

problems [46]. Moreover, for many metals, the thyroid tissue concentration has never been investigated.

As already mentioned, recent studies in Sicily have confirmed that thyroid cancer incidence is greatly increased in volcanic areas and have documented for the first time that this increase is associated with a relevant, nonanthropogenic, multi-elemental environmental pollution concerning many metals and metalloids, with the consequent contamination of residents. The carcinogenic effect of the volcanic pollution appears to be specific for the thyroid. These observations have raised the possibility of a relationship between one or more of the increased metals and thyroid cancer, via a mechanism related to the thyroid capacity to accumulate some of the environmentally increased metals.

For these reasons in the present study, I investigated the concentration of a series of metals in normal thyroid tissue by measuring the content of iodide and 25 additional trace elements and metals in the thyroid, as well as in the muscle and adipose tissues of the same euthyroid individuals. The aim of this study was to evaluate whether the thyroid has a tissue-specific capacity to differentially accumulate some metals or trace elements. To reinforce the experimental significance, I also measured these elements in the same tissues (thyroid, muscle and fat) of normal rats to evaluate possible species differences in an experimental animal commonly used for thyroid research. Finally, I evaluated the possible differences in element concentration in the thyroid tissue of individuals exposed to volcanic environmental contamination relative to control subjects living in adjacent nonvolcanic areas.

To carry out these studies I obtained the multi-disciplinary collaboration of the Surgical Oncology service of the Garibaldi-Nesima in Catania (for human tissues collection), of the CNR, National Research Council, Institute od Cell Biology and Neurobiology in Rome (for animal studies) and of the Laboratory of Experimental and Clinical Toxicology at the IRCCS Maugeri in Pavia (for sensitive and accurate trace element measurements).

3.2 Methods

3.2.1 Human Subjects and Tissues

Seventy-seven adult subjects with normal thyroid function (assessed by thyroid hormone and TSH serum levels in the reference range and negative anti-thyroid antibodies) undergoing thyroidectomy at the Surgical Oncology Service of the Garibaldi-Nesima Medical Center (a tertiary referral center for thyroid diseases) because of the presence of a discrete

(maximum diameter ≤ 2 cm) single thyroid nodule. Signed informed consent to donate small tissue fragments of thyroid, muscle and adipose tissue (each<1 g) was given by all patients: Tissues were collected at surgery and subsequently used in the study. On pathological examination, 42 nodules (55.5%) were benign and 35 were malignant (differentiated papillary thyroid cancer in all cases).

Donated tissues for each patient included macroscopically normal thyroid tissue (at least 1 cm distant from the nodule) and sternothyroid muscle tissue and neck subcutaneous adipose tissue. Average patient age was 48.7 ± 16.7 years and there were 54 (70.1%) women and 23 men. Their residence at least during the least 10 years was either in the Mount Etna volcanic area (province of Catania, 43 cases) or in adjacent non-volcanic areas in Sicily (34 cases). All patients were and had always been euthyroid as evaluated by their medical records, clinical examination and biochemical measurements of TSH, thyroid hormones, and negative antibodies prior to surgery. At enrolment time, serum TSH values ranged from 0.42 to 4.50 mIU/L (reference range 0.35 - 4.94), free triiodothyronine 3.25 to 5.21 pmol/L (reference range 2.63 – 5.70), and free thyroxine 10.30 to 17.85 pmol/L (reference range 9.0 - 19.05).

The following exclusion criteria were adopted: previous clinical or laboratory evidence of abnormal thyroid function and/or treatment for thyroid diseases, history of previous head/neck irradiation, obesity (BMI>30 kg/m2), smoking, taking medications that can interfere with thyroid function including iodide and nutritional supplements, chronic disease with specific attention to gastrointestinal, liver and kidney diseases.

All tissue specimens were collected at surgery using a titanium scalpel, divided in two fragments and placed in conical tubes containing saline. One portion was used for morphological examination. The other was weighed and frozen in liquid nitrogen within one hour from excision and stored deep frozen until the experimental measurement.

This study was approved by the Local Institutional Ethics Committee (n.12/2015/CECT2).

3.2.2 Experimental animals and sample collection

All animal experiments were carried out at the CNR, National Research Council, Institute of Cell Biology and Neurobiology in Rome and approved by the ethical committee of the Catholic University of Rome, Italy (n. Q42), in accordance with institutional guidelines, which are in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circular

No. 8, G.U., July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996) on the ethical use of animals.

Eight female Wistar rats (9 weeks old, 200-230 g) were kept under standard housing conditions (temperature 21°-23°C, relative humidity 45-65%, and 12h:12h light/dark cycle) fed with a standard pellet diet for rodents (Mucedola 4RF2) and tap water *ad libitum*. All animals were housed in plastic cages containing two animals/cage.

For specimen collection, rats were sacrificed by CO_2 inhalation. The thyroid gland, the hindlimb muscle and abdominal visceral fat were surgically removed with a sterile scalpel and immediately snap-frozen in liquid nitrogen for subsequent metal measurements.

3.2.3 Measurements of trace elements

Multi-elemental quantitative determination was performed in wet tissue specimens in the Laboratory of Experimental and Clinical Toxicology at the IRCCS Maugeri in Pavia. In most previous studies with metal measurement in the thyroid, element values were normalized based on the weight of dry tissue specimens. Therefore, to better compare our data with previous studies, we converted our measured concentrations from wet to dry weight under the assumption that the water content in the thyroid tissue is 74.2% ± 3.4 [47].

The following 26 elements were measured: Silver (Ag), Arsenic (As), Boron (B), Barium (Ba), Bromine (Br), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Mercury (Hg), Iodide (I), Lithium (Li), Manganese (Mn), Molybdenum (Mo), Nickel (Ni), Lead (Pb), Palladium (Pd), Antimony (Sb), Selenium (Se), Tin (Sn), Strontium (Sr), Thallium (Tl), Vanadium (V), Tungsten (W), Zinc (Zn) and Zirconium (Zr).

Measurements were performed using an Inductively Coupled Plasma Mass Spectrometry (DRC-ICP-MS) equipment (ELAN 6100 DRCII ICP-MS PerkinElmer SCIEX Instruments, Canada) with a dynamic reaction cell (DCR) and a quadrupole mass filter, a cyclonic spray chamber with a concentric nebulizer, and an AS90 plus auto-sampler (PerkinElmer). DRCICP-MS is a very sensitive technique that is suitable for the determination of trace and ultra-trace elements. This multi-elemental technique measures different elements at the same time based on their mass-to-charge ratio (m/z). The dynamic reaction cell (DRC) allows reduced chemical interference [48] and allows the acquisition of background values < 1 cps and detection limits on the order of ng/L.

The mass spectrometer also allows to work on complex matrices (biological and environmental samples) in a linear way in a wide dynamic range to analyse the various analytes at different concentrations with comparable precision and accuracy.

The following procedure was used to assure measurement accuracy:

a) Before the analysis, tissue samples underwent a pre-treatment in microwave to reduce interferences caused by the matrix. Tissue samples (ap proximately 100 mg) were added to the digestion vessels with 4 mL HNO_3 (65% m/v) and 0.5 mL H_2O_2 (30% m/v). After complete digestion and cooling, samples were filtered and transferred to 50 ml graduated polypropylene tubes and diluted to volume with deionized water.

b) The dynamic reaction cell was vented and pressurized with reactive gases (CH_4, NH_3) under computer control and provided online chemical modifications of the ion beam were provided to eliminate interferences.
The specificity of interference rejection was obtained through the selection of the reaction gas and the operating conditions. Ag, B, Ba, Br, Cd, Co, Cu, Hg, I, Li, Mn, Mo, Pb, Pd, Sb, Sn, Sr, Tl, W, Zn and Zr were determined in “standard mode” while As, Cr, Ni, Se and V were determined in “enhanced mode” (CH_4, NH_3).

c) To optimize the ICP-MS signal, a standard solution containing 1 µg/L of three elements that covered the entire mass range (^{24}Mg, ^{115}In, ^{238}U) was used. Calibration was performed by an “external calibration” method. Multielement standard solutions (calibration from 0.1 to 20 µg/L) were prepared from ICP-MS Multi-elemental standard solutions 10 mg/L (Standard 3, 4, MS3, MS1 CPI International, Amsterdam, NL), from 1000 mg/L I and Br standard solutions (CPAchem Ltd) and from Standard solution 1 µg/L ELAN 6100 DRC SETUP/STAB/MASSCAL SOLUTION (Perkin Elmer Life and Analytical Sciences, Shelton, USA). Standards were diluted with high purity water containing the same amount of acids as the samples. The stability of the calibration curve was assessed by generating linear calibration curves of all multi-elemental standard solutions. To evaluate the day-to-day stability of the calibration curves, spiked aqueous solutions were analysed on five consecutive days.

No significant variations were observed, and the analytes were therefore considered to be stable over this time.

d) The *precision* of the method was determined in terms of repeatability (intra-assay precision) and is expressed as relative standard deviations (RSD) calculated from ten replicate measurements on six samples at different concentrations. The CV% was 3 - 4% at concentration levels between 0.01 µg/L and 1 - 2% at 10 µg/L.

   The *trueness* was evaluated by analyzing ten replicates of five different samples spiked with three different concentrations (0.01, 10, 20 µg/L) of all studied analytes.

   The *recoveries*, expressed as the percentage recovery mean ± standard deviation (n=5), were between 98.2 and 100.6% (± 1.0 – 1.8%).

   The *detection limits* (LOD, defined as three times the standard deviation of five repeated readings of blank sample prepared on five consecutive days) for all trace elements were in the range 0.0001 - 0.016 µg/L.

3.2.4 Statistical analysis

Chemical concentrations are expressed as median and range or either arithmetic mean (± standard deviation) or geometric mean with 95% confidence interval. The geometric mean (GM) was calculated to estimate the central tendency by taking the log of each concentration and then computing the mean and its 95% confidence interval (CI) of the log-transformed values. Values below the limit of detection (LOD) were also included by assigning a value equal to the LOD divided by the square root of 2 [49]. If the number of measurements below the LOD was greater than 40 % of specimens examined, the GM was not calculated, and the element was classified as ‘‘nondetectable’’ in that tissue. To verify the difference in chemical concentrations between the different tissue specimens, linear regression analysis was used by including the log-transformed values of each chemical in the model. Age and sex of tissue donors were also included in the linear regression model to control the differences for these matching variables. P values lower than 0.05 were considered statistically significant for a two-tailed test. All statistical analyses were performed using the STATA 13.1 statistical package (StataCorp LP, College Station, Texas, USA).

3.3 Results

3.3.1 Element concentration in the human thyroid compared to muscle and adipose tissue

Comparing the thyroid tissue concentrations of the studied elements to those measured in the sternothyroid muscle and the subcutaneous neck fat tissue of the same individual, we observed a significant difference in the tissue accumulation of different chemicals.

In addition to iodide, seven other elements were significantly more concentrated in the thyroid tissue than in muscle and adipose tissue ($p < 0.01$ for all) (**Table 2**); among them were the halogen Br, as well as Se, an indispensable component of selenoenzymes involved in thyroid hormone metabolism, and Mn and Sn, whose role in thyroid biology is not known. The bromine concentration was 2–3-fold higher than iodide in muscle and adipose tissue, in contrast to the thyroid tissue where iodide was present at an over 50-fold higher concentration relative to bromine. However, Br is significantly more concentrated in the thyroid than in the other tissues. The specific ability of the thyroid to concentrate Br- is probably related to its chemical similarity with iodide: the two halides are transported inside thyroid follicular cells through the NIS (sodium–iodide symporter), specifically expressed at a high level in those cells [50].

Of note, three recognized carcinogens (As, Cd and Hg) were significantly more concentrated in the thyroid relative to the other tissues studied. Finally, the [Zn]: [Cu] ratio was 12.6 in the thyroid versus 5.9 in adipose tissue and 18.2 in the muscle. The relatively similar ratios suggest that the human thyroid has no specific mechanism for the uptake and accumulation of these two metals that both have a relevant role in cell enzyme function.

	Thyroid tissue		Muscle tissue		Adipose tissue	
	GM (95% CI)	No. Of cases <LOD	GM (95% CI)	No. Of cases <LOD	GM (95% CI)	No. Of cases <LOD
A-Trace Elements significantly more prevalent in the thyroid tissue						
I	433.7 (329.1-571.5)	0	0.37 (0.21-0.68)**	4	1.05 (0.61-1.8)**	4
As	0.07 (0.05-0.09)	7	ND**	51	ND**	40
Br	8.2 (7.6-8.9)	0	1.29 (0.95-1.76)**	0	2.30 (1.83-3.12)**	0
Cd	0.14 (0.11-0.17)	0	0.02 (0.02-0.03)**	6	ND**	40
Hg	0.04 (0.03-0.05)	7	ND**	44	ND**	58
Mn	0.22 (0.19-0.25)	0	0.08 (0.06-0.10)**	2	0.09 (0.07-0.11)**	2
Se	0.28 (0.24-0.33)	0	0.07 (0.06-0.08)**	0	0.03 (0.03-0.04)**	0
Sn	0.02 (0.02-0.03)	0	0.01 (0.01-0.02)**	27	0.01 (0.01-0.02)**	26
B-Trace Elements significantly less prevalent in the thyroid tissue						
B	ND	53	0.47 (0.36-0.62)**	2	0.53 (0.39-0.73)**	3
Cr	0.30 (0.20-0.40)	0	0.55 (0.45-0.67)**	1	0.37 (0.29-0.46)**	0
Ni	0.18 (0.11-0.24)	0	0.40 (0.33-0.47)**	0	0.29 (0.22-0.36)**	0
C-Trace Elements at similar concentration in the examined tissue						
Ba	0.17 (0.14-0.20)	0	0.14 (0.11-0.17)	0	0.16 (0.13-0.20)	0
Sr	0.18 (0.15-0.21)	0	0.15 (0.11-0.19)	0	0.11 (0.03-0.19)	0
W	0.03 (0.02-0.03)	20	0.02 (0.02-0.03)	29	0.03 (0.02-0.04)	16
Zr	0.03 (0.03-0.04)	0	0.03 (0.03-0.04)	0	0.04 (0.03-0.05)	0
D-Other conditions						
Ag	0.03 (0.01-0.05)	22	ND**	63	0.05 (0.01-0.2)	26
Cu	0.95 (0.90-1.10)	0	1.07 (0.95-1.20)	0	0.56 (0.49-0.65)**	0
Mo	0.02 (0.02-0.02)	2	0.02 (0.02-0.02)	3	0.01 (0.01-0.02)	5
Pb	0.02 (0.02-0.02)	19	0.03 (0.02-0.03)*	12	0.03 (0.02-0.03)	10
Zn	11.97 (10.49-13.67)	0	19.5 (16.8-22.5)**	0	3.3 (2.76-3.94)**	0

Table. 2 Concentration of Trace Elements and Metals (μg/g of Wet Tissue) in Human Thyroid, Muscle, and Adipose Tissue of the Same Individuals. ND: nondetectable, because values are <LOD (LODs) in over 40% specimens examined; GM: geometric mean. *$p < 0.05$; **$p < 0.01$ relative to thyroid tissue.

3.3.2 Element concentration in the rat thyroid

Given the relevance of rat as the most widely used experimental animal in thyroid studies, species differences between man and rats for metal concentrations in tissues were evaluated. Eleven metals were measured in the thyroid, muscle, and adipose tissue of eight normal rats (**Table 3**). As in the human thyroid, after iodide, the highest concentrated element in the rat thyroid was Zn, followed by Br and Cu, all of which were in the µg/g tissue range and at a concentration similar to that measured in the human thyroid. In contrast, Mn was markedly more concentrated in the rat thyroid compared to the human thyroid (rat/man ratio approximately 5:1).

Of the remaining elements Se, As, Mo and Hg values were in the range of ng/g of tissue (250 to 10) as observed in human thyroid while Ba, Cd and W were neither detectable (lower than detection limits) in the rat thyroid nor in the muscle and adipose tissue of the examined animals. When comparing the rat thyroid concentration to that of muscle and adipose tissue, Ag, Br, Hg, Mn and Se were all more concentrated in the thyroid, reflecting a similar behavior observed in humans. In contrast to human specimens, Zn, Cu and Mo were more concentrated in the rat thyroid relative to the other tissues examined. As in humans, however, the [Zn]: [Cu] ratio was remarkably similar in the tissues evaluated: 7.9 for the thyroid and 7.0 in both muscle and adipose tissues. Moreover, as in man, both the carcinogenic metals As and Hg were found at a higher concentration in the thyroid, while no comparative evaluation was possible for Cd since this metal was present at a lower than measurable concentration in all rat tissues examined.

Element	Thyroid	Muscle	Adipose	Human Thyroid
I	245.7 (129.0-506.1)	0.18 (0.12-0.26)*	0.58 (0.22-0.81)*	**433.7 (329.1-571.5)**
Zn	14.14 (13.47-14.81)	7.62 (6.39-8.84)*	5.54 (3.34-7.75)*	**11.97 (10.49-13.67)**
Br	8.95 (7.95-9.94)	6.41 (5.14-7.68)*	5.80 (3.35-8.25)*	**8.2 (7.6-8.9)**
Cu	1.79 (1.53-2.04)	1.09 (0.91-1.27)*	0.79 (0.56-1.02)*	**0.95 (0.90-1.10)**
Se	0.25 (0.19-0.32)	0.10 (0.09-0.12)*	0.07 (0.03-0.10)*	**0.28 (0.24-0.33)**
Mn	1.28 (0.79-1.79)	0.11 (0.09-0.12)*	0.18 (0.10-0.27)*	**0.22 (0.19-0.25)**
Ba	ND	ND	0.01 (0.01-0.03)	**0.17 (0.14-0.20)**
Cd	ND	ND	ND	**0.14 (0.11-0.17)**
As	0.12 (0.08-0.15)	0.05 (0.03-0.06)*	0.08 (0.04-0.12)	**0.07 (0.05-0.09)**
Hg	0.01 (0.003-0.44)	ND	ND	**0.04 (0.03-0.05)**
W	ND	ND	ND	**0.03 (0.02-0.03)**
Mo	0.07 (0.05-0.08)	0.02 (0.02-0.03)*	0.08 (0.05-0.10)	**0.02 (0.02-0.02)**

Table 3. Trace element concentration (μg/g of Wet Tissue) in Rat Tissues. Eight female Wistar rats were used. Data are presented as GM and 95% CI (between brackets). Values for human thyroid are indicated in the fourth column for comparison. *$p < 0.01$ relative to the thyroid tissue. ND: nondetectable.

3.3.3 Element concentrations in the thyroid of residents in the volcanic vs non volcanic areas

As previously mentioned in the Sicilian volcanic area of Mount Etna, where thyroid cancer incidence is double relative to adjacent non-volcanic areas, a significant environmental pollution is present. Many metals were at significantly higher concentrations in the urines of residents of the volcanic area relative to values in the urine of residents of adjacent non-volcanic areas. I, therefore, investigated whether this difference in biocontamination was present also in tissues.

I comparatively examined the concentration of these elements in the thyroid of residents of the two areas, 43 from the volcanic area (mean age 45±18 y, F=33) and 34 from the control area (mean age 52.2 ±14.6, F=21). For many elements (As, Ba, Br, Cr, Cu, Hg, Mn, Ni, Se, Zr, Zn) concentrations in the thyroid were slightly higher in individuals living in the volcanic area compared to residents of control areas. However, a large overlap of individual values was present, and differences were not significant (**Table 4**). In addition, in the muscle

and adipose tissues of residents of the volcanic area, the concentrations of most examined elements examined were also slightly higher, suggesting that the increase was the general consequence of the increased exposure for all tissues and that no specific accumulation occurs in the thyroid. Only As and Hg were increased in the thyroid but not in the muscle and adipose tissues of residents of the volcanic area. Also, for these carcinogenic metals, however, differences were small and nonsignificant relative to values found in the thyroids of the control group.

Element	Volcanic Area (no. of cases 43)	Control Area (no. of cases 34)
Ag	0.02 (0.01-0.05)	0.03 (0.01-0.06)
As	0.07 (0.05-0.1)	0.06 (0.04-0.1)
Ba	0.18 (0.15-0.23)	0.16 (0.12-0.21)
Br	8.83 (7.4-9.7)	7.44 (6.7-8.25)
Cd	0.13 (0.09-0.19)	0.14 (0.1-0.2)
Cr	0.33 (0.19-0.48)	0.27 (0.13-0.40)
Cu	1.03 (0.9-1.2)	0.87 (0.8-1.0)
Hg	0.04 (0.03-0.07)	0.03 (0.02-0.05)
Mn	0.24 (0.2-0.3)	0.19 (0.15-0.23)
Mo	0.02 (0.01-0.02)	0.02 (0.02-0.03)
Ni	0.18 (0.09-0.27)	0.17 (0.09-0.25)
Pb	0.02 (0.01-0.03)	0.02 (0.02-0.03)
Se	0.29 (0.23-0.36)	0.28 (0.22-0.36)
Sn	0.02 (0.02-0.03)	0.02 (0.01-0.03)
Sr	0.16 (0.13-0.20)	0.20 (0.16-0.25)
W	0.02 (0.02-0.03)	0.03 (0.02-0.04)
Zr	0.04 (0.03-0.04)	0.03 (0.02-0.04)
Zn	12.2 (10.0-14.8)	11.7 (9.8-14)

Table 4. Trace elements concentration (μg/g of Wet Tissue) in the thyroid of residents of the Mt. Etna volcanic and the control areas in Sicily.

3.4 Comments

a) Quality of our experimental data

Using an advanced spectrochemical technique, we measured the concentration of 26 trace elements in the human thyroid gland, paying special attention not only to the quality of the analytical technique but also to preanalytical factors such as patient euthyroidism, normal tissue selection, sample handling and processing in a highly specialized laboratory and postanalytical calculations.

I carefully analyzed the measurements in previous studies and found several weaknesses, limitations, and pitfalls. For instance, in some studies measurements were carried out in the thyroid of deceased individuals [47,50–52] with no information on post mortem period duration or on possible interfering factors such as previous thyroid disease, disorders in other organs, or intake of medications. In contrast, the strict inclusion criteria that we used are one of the strengths of this study: we investigated thyroid tissue collected during planned surgery for a single non-functioning thyroid nodule in patients with confirmed past and present euthyroid status. The normal morphology of the excised thyroid tissue was further controlled at pathology, minimizing the possible variability due to altered tissue architecture. The normal thyroid, in fact, has a large colloid component (approximately 20% of wet weight) and this component may be increased, reduced or absent in different thyroid conditions such as iodide deficiency, hyperthyroidism, adenomas and cancer. Since the relative concentration of metals in cells vs. colloid has never been measured, the abnormal cellular/colloid ratio may influence the results. For this reason, the comparison of the chemical element concentration per gram of tissue in the normal and pathological thyroid may be of limited significance.

As far as the analytical procedure is concerned, the DRC-ICP-MS method is currently the most widely used method for the detection of trace elements and metals. In fact, it ensures excellent detection limits that, for most elements, are one or two orders of magnitude below the µg/L concentration. Moreover, it allows the possibility of multi-element analysis of isotope mixtures with extremely accurate results.

Another strength of our study is data calculation. Most previous studies presented data as the arithmetic mean of measured values. Metal concentrations in tissues, however, follow an asymmetric rather than normal (Gaussian) distribution and nonparametric models such as the median with range values or the geometric mean with 95% C.I. are preferable.

Different calculations provide different values with the arithmetic mean levels often significantly higher (increased from 20-50% up to 2-3-fold) than values obtained with non-parametric analyses (**Table 5**). These differences are due to the influence of occasional exceptionally high values on the arithmetic mean and variation. Since data from most previous studies were presented as the mean±SD, when comparing data from different series, we also present our data in this form in **Table 6**.

Element	GM (95%CI)	M ± SD	Median (range)	GM calculated in dry weight
I	433.7 (329.1-571.5)	757.5 ± 764.4	548.6 (25.3-3969.2)	1682.8
Zn	11.97 (10.49-13.67)	13.91 ± 7.31	12.41 (2.05-37.52)	46.4
Br	8.2 (7.6-8.9)	8.67 ± 2.9	8.37 (2.40-16.71)	31.8
Cu	0.95 (0.90-1.10)	1.06 ± 0.51	1.04 (0.32-3.40)	3.69
Cr	0.30 (0.20-0.40)	0.48 ± 0.45	0.30 (0.02-1.68)	1.12
Se	0.28 (0.24-0.33)	0.35 ± 0.23	0.28 (0.02-0.98)	1.08
Mn	0.22 (0.19-0.25)	0.26 ± 0.15	0.23 (0.06-0.70)	0.85
Ni	0.18 (0.11-0.24)	0.30 ± 0.28	0.21 (0.01-0.98)	0.70
Sr	0.18 (0.15-0.21)	0.22 ± 0.13	0.20 (0.04-0.81)	0.70
Ba	0.17 (0.14-0.20)	0.20 ± 0.19	0.14 (0.01-0.95)	0.66
Cd	0.14 (0.11-0.17)	0.21 ± 0.21	0.14 (0.01-1.31)	0.54
As	0.07 (0.05-0.09)	0.10 ± 0.07	0.08 (ND-0.28)	0.27
Hg	0.04 (0.03-0.05)	0.09 ± 0.14	0.03 (ND-0.8)	0.16
Zr	0.03 (0.03-0.04)	0.04 ± 0.02	0.02 (ND-0.14)	0.12
Ag	0.03 (0.01-0.05)	0.09 ± 0.16	0.02 (ND-0.8)	0.12
W	0.03 (0.02-0.03)	0.07 ± 0.22	0.03 (ND-1.96)	0.12
Sn	0.02 (0.02-0.03)	0.03 ± 0.22	0.03 (0.01-0.1)	0.08
Pb	0.02 (0.02-0.02)	0.04 ± 0.04	0.02 (ND-0.29)	0.08
Mo	0.02 (0.02-0.02)	0.02 ± 0.02	0.02 (ND-0.20)	0.08

Table 5. Trace elements concentration (μg/g of Wet Tissue) in human thyroid tissue.

Country and Reference	**As**	**Br**	**Cd**	**Cu**	**Hg**	**Mn**	**Pb**	**Se**	**Zn**
Japan [47]	0.87± 0.9	18.1± 5.1	16.1± 22.5			1.34± 0.68		3.69± 2.33	129± 67
India [53]	125.1± 12.0			54.9± 5.5	98.5± 10.0	17.3± 1.8	17.2± 1.7		149.8± 15
Turkey° [54]				2.7± 0.8					58.2± 23.3
Kuwait [55]				1.2± 2.7		0.31± 1.0		0.49± 1.6	32.0± 72.4
China [50]	0.053 (0.009-0.08)	1.28 (0.7-1.7)	0.59 (0.1-5.4)	2.05 (0.7-3.1)	0.006 (0.001-1.1)	0.49 (0.13-0.7)	0.067 (0.04-0.09)	0.7 (0.4-1.2)	28.8 (14.4-31)
Poland [51]			ND	5.24± 0.51		0.68± 0.10			101± 10.9
Russia [52]		13.9± 12		4.23± 1.52					112± 44
Italy Present study	*0.39± 0.27*	*33.6± 11.3*	*0.81± 0.81*	*4.11± 1.98*	*0.35± 0.54*	*1.0± 0.58*	*0.15± 0.15*	*1.36± 0.89*	*54.0± 28.4*

Table 6. Comparison of Trace elements concentration (μg/dry Human Thyroid Tissue) with published data. ° Measured in tissue wet weight and converted to dry weight values. All data are presented as arithmetic mean±SD except data from reference [50].

b) Analysis and comparison with data reported in other studies

Considering these pre-analytical, analytical, and post-analytical differences, and considering the different ethnicities, diet and environment of the subjects studied, the difference in observed values between the present and previous data can be easily explained **Table 6**. The concentration values obtained in studies performed in the last twenty years are in most cases in the same range found in this study (**Table 6**) except for the study of Reddy et al. [53], which was performed in only 4 normal thyroid specimens and in a very different ethnic and environmental context. In that study, a surprisingly low iodide/zinc ratio has been reported (1.54 vs. 36.2 in our series), and the concentration values of most elements are one or two orders of magnitude higher than in other studies (**Table 6**), suggesting severe overestimation because of possible problems for tissue selection, tissue specimen processing, including those related to the freeze-dry procedure of the samples [54], or the analytical procedure [56].

c) Analysis of metal concentration in the thyroid vs. muscle and fat

Considering all the trace elements measured in our study and excluding iodide, nearly 90% of all trace elements in human and rat thyroid is represented by Zn and Br.

Bromine has no recognized specific physiological function in any organ and is minimally incorporated into organic compounds. Its elevated concentration in the thyroid was significantly higher than in muscle and fat in humans (**Table 2**) and was also higher in the rat thyroid (**Table 3**), likely the consequence of its chemical similarity and functional competition with iodide (both halogens) [57]. In our series, the observed [I]: [Br] ratio was 52.9 in humans and 27.4 in rats.

In contrast to Br, Zn is an essential metal that is involved in many aspects of cell biology as a structural element and as a regulatory factor. The free intracellular (Zn+2) level is very low since most Zn is associated with proteins, predominantly metallothioneins and metallo-enzymes [56,58]. Zn, together with Cu, has an important role as a cofactor for the superoxide dismutase (SOD), a key enzyme that protects cells from superoxide toxicity, a very relevant function in the biology of the thyroid. In the human thyroid the [Zn]: [Cu] ratio is greater than 10 in all studies except the one by Reddy et al. [53]. In the rat, the two metals were more abundant in the thyroid relative to muscle and fat (**Table 3**), but this was not the case in humans, in which both metals were present at a higher level in muscle (**Table 2**). The difference between human and rat may be due to species-specific differences in physiological mechanisms but also to the different environmental conditions of our experimental models, including the limited mobility and artificial standardized diet given to the rat, as well as the different localization of excised muscle and fat in measured specimens from humans and rats. Zn and Cu also serve as signaling factors for the regulation of cell proliferation, differentiation and death [56,59,60] and have been suggested to play a role also in thyroid cancer etiology and progression [61–63]. However, until now, the chemical speciation, compartmentalization and role in thyroid physiopathology of these two essential metals remains unknown and requires future research.

The thyroid concentration of Se is remarkably similar in humans and rats; in both species, the values in the thyroid are higher than in muscle and fat. Selenoenzymes (type I, II and III deiodinases, as well as glutathione peroxidase and thioredoxin reductase) are known to play an essential role in thyroid hormone metabolism and redox processes. Whether the

increased concentration of Se in the thyroid is due to facilitated uptake, or increased retention due to incorporation into proteins, or both, is not known.

Another metal with a concentration in the human thyroid that is more than twice as high relative to muscle and fat is Mn. This metal is potentially toxic at high levels and is possibly carcinogenic [64], but at the same time, it is an essential constituent of many enzymes including the Mn-SOD, a superoxide dismutase that is a major antioxidant for neutralizing the toxic effect of reactive oxygen species. In the rat thyroid, Mn was found at a much higher levels that in the human thyroid, and at approximately ten times greater levels than in muscle and fat. Since Mn concentrations in muscle and fat tissues are similar to those found in humans, this observation suggests that, for unknown reasons, the rat thyroid accumulates Mn more than the human thyroid.

Another interesting observation is the significantly higher concentration of three important carcinogens in the thyroid relative to the other tissues studied. The levels of As, Cd and Hg in the human thyroid were significantly higher ($p < 0.01$) than those in muscle and fat (**Table 2**). This same increase was observed for As and Hg (but not for Cd) in the rat thyroid (**Table 3**). How the relative abundance of these toxic metals can influence the very frequent occurrence of benign and malignant nodules in the thyroid is not known. In middle and advanced age human individuals, thyroid nodules occur in 50% or more cases [65,66], and the thyroid mutation rate is calculated to be 8–10-fold higher than in other organs [67]. Mutations may accumulate because follicular cells are constantly exposed to the toxic effect of free radicals produced by the continuous generation of hydrogen peroxide necessary for the oxidation of iodide (I^-) to derivatives like hypoiodite, hypoiodous acid, and iodinium [68]. This unfavorable microenvironment promotes “spontaneous” mutagenesis [67] and the increased presence and activity of carcinogenic metals may favor cell transformation leading to the formation of benign and malignant nodules.

d) Metal concentration in tissues of residents of the volcanic vs. non-volcanic areas

Many elements and metals are present at a higher concentration in the thyroid of residents of the Mount Etna volcanic area, where thyroid cancer incidence is doubled than in the thyroid of residents of adjacent non-volcanic areas. The differences between the two groups are not statistically significant, and therefore, no conclusion can be drawn until more cases are studied. It is noteworthy, however, that in residents of the volcanic area, the small increase of most elements observed in the thyroid also occurs in muscle and fat, which

suggests generalized biocontamination from a polluted environment. But this is not the case for As and Hg, two carcinogens that are increased (+16.6% and +25%, respectively) in the thyroid but not in the two other examined tissues. As already mentioned, in our limited series, the increases are small and not statistically significant, but a deleterious biological effect of the very low dose increase is possible, as a consequence of a hormetic dose-response that follows a biphasic pattern [69]. This hormetic effect has already been reported for chemicals such as As [70], Cd [71,72], Hg [72,73], W [74], and Bisphenol A [75].

e) Conclusion

In conclusion, with these studies I provide novel information on the concentration of numerous elements in the human thyroid relative to other tissues of the same individual and in similar tissues in the rat.

The reference data provided by our study involve many trace elements that play an important role as determinants of thyroid function, such as the antioxidant defense system (metalloenzymes), hormone production and metabolism, and thyroid cell growth, differentiation and death. Some of the studied elements, when in excess, may also have toxic effects via the metal-mediated formation of free radicals, DNA damage and enhanced lipid peroxidation [76], possibly influencing some genetic alterations predisposing to thyroid cancer [77–79]. It must be stressed, however, that the biological meaning of the data reported in this study, as well as in previous studies, is hampered by the consideration that the values observed represent the average of heterogeneous levels present in the different structural and functional compartments of the gland. Follicular cells and colloid, C cells, stromal components and vessels might have a markedly different concentration of the studied chemical, as has been observed for iodide.

Thus, further interdisciplinary endocrine research is required to connect variations in the overall tissue concentration of metals with the chemical, biochemical and molecular aspects of thyroid physiology and pathology.

4. Thyroid Stem Cells but Not Differentiated Thyrocytes Are Sensitive to Slightly Increased Concentrations of Heavy Metal

4.1 Background

In the Sicilian volcanic area, a significant non-anthropogenic heavy metal pollution is present and causes the bio-contamination of residents, suggesting the possibility of a cause-effect relationship with the increased incidence of thyroid cancer. However, in the urine of volcanic area residents, only boron, molybdenum, palladium and tungsten are at higher levels than the 95th percentile of Italian standard values [39], posing the question of whether chronic exposure to a small increase in environment metals, often within the "normal" limits for each single metal, can have a detrimental effect on thyroid cell biology.

As already mentioned, detrimental effect of metals at slightly increased concentrations is possible, as indicated by the biological effects of many metals (A) at very low concentrations, activating a biphasic, non-linear hormetic response in exposed cells [69]. Moreover, (B) a combined effect of different metals that are only slightly increased but that are acting synergistically is also possible. Finally, (C) another possibility is that cells at a lower level of differentiation, such as embryonic cells, may be more sensitive to the detrimental effects of metals even only slightly increased [80].

I planned to evaluate these different possibilities through an *in vitro* study aimed at investigating whether heavy metals that are slightly increased in the volcanic environment may differently affect immature vs. mature thyroid cells and whether a metal mixture can be more detrimental than each single component acting alone.

To this end I decided to investigate five metals that are increased in the volcanic area and to test their effects in human mature thyrocytes and undifferentiated thyrocytes in the form of thyrospheres (aggregates of thyroid stem cells and precursors of thyrocytes at different level of differentiation). Depending on the culture medium used, thyrospheres are able to either differentiate in mature thyrocytes or produce additional thyrospheres by a process named self-renewal.

4.2 Materials and Methods

4.2.1 Investigated Heavy Metals

The following metals were investigated: zinc and copper because of their relevance in cell biology; mercury and tungsten because of their potential carcinogenic capacity and

palladium because no information at all is available in the literature regarding its effects on the thyroid. All of them are significantly increased in the urines of residents of the volcanic area [39].

All these five metals were studied in the chemical form of salt compounds: copper (Cu) used as $CuSO_4$, zinc (Zn) as $ZnCl_2$, mercury (Hg) as $HgCl_2$, palladium (Pd) as $PdCl_2$ and tungsten (W) as Na_2WO_4. All these salts were obtained from Sigma-Aldrich (St. Louis, MO, USA). $CuSO_4$ and Na_2WO_4 were dissolved in deionized water, $ZnCl_2$ and $PdCl_2$ were dissolved in deionized water containing 10% 1N HCl and $HgCl_2$ was dissolved in absolute ethanol.

Stock solutions were prepared for all these salts at 10 mM concentrations and stored at 4°C until used, when they were further diluted to the indicated concentrations in RPMI medium containing 0.1% BSA.

4.2.2 Preparation of Human Thyroid Cell Cultures

Normal human thyroid tissue specimens were obtained from euthyroid female patients aged 30–65 years who had undergone surgery (Oncology Surgery unit of the Garibaldi-Nesima Medical Center in Catania) for a solitary thyroid nodule which was always shown to be benign at pathological examination. Written informed consent was obtained in all cases and the study was approved by the local Ethics Committee (n.12/2015/CECT2).

The isolation and culture of human thyroid cells were performed as follows:

a) *fresh thyroid tissue handling:* after meticulous removal of fibrous tissue, the just excised fresh thyroid tissue was first minced with sterile scissors and then digested with collagenase IV (1 mg/ml, Sigma-Aldrich) for 2 h at 37°C. The resulting cell suspension containing intact and fragmented thyroid follicles was centrifuged (400 *g* for 10 min), and the pellet was suspended in RPMI 1640 culture medium (Sigma) supplemented with 2 mM glutamine (Sigma), 2.5% heat-inactivated fetal bovine serum (FBS, from Invitrogen), B-27 (1:100, Thermo), insulin-transferrin-sodium selenite liquid medium supplement (ITS, 1:200, Thermo), and epidermal growth factor (EGF, 1 ng/ml; Sigma), before being incubated at 37°C in a 5% CO_2 atmosphere. After 12–24 h, viable thyroid cells attached to the flasks and supernatants containing unattached cells were transferred into fresh flasks, and the cells were cultured at 100%

confluence. Residual fibroblasts, when present, were depleted using magnetic anti-fibroblast beads (Miltenyi Biotec) according to the manufacturer's instructions.

Under these conditions, follicular cell monolayers are formed after 1–2 days. The culture medium was then renewed every 2–3 days and all experiments were performed with cells at passages 3–7.

b) differentiated thyrocytes in primary culture were grown in the above described RPMI medium supplemented with 1 mU/ml bovine thyroid-stimulating hormone (TSH) (Sigma) and deprived of EGF.

c)stem/progenitor thyroid cells were obtained by the trypsinization of primary culture of thyroid cells and seeding cells at a density of 1–5 × 10^4 cells/ml. These were cultured in ultralow attachment plastic flasks (Eppendorf) in the standard stem cell medium, RPMI 1640 supplemented with 2% B-27 (Thermo) enriched with EGF (20 ng/ml, from Sigma). Under these conditions, stem/progenitor thyroid cells form free-floating thyrospheres, aggregates of thyroid stem cells and precursors of thyrocytes at different levels of differentiation. Human thyrospheres generated with these procedures were characterized for phenotypic and genetic markers [81].

4.2.3 Cell Proliferation Measurement

Cell proliferation was evaluated by 5-bromo-2'deoxyuridine (BrdU) incorporation. Thyrocytes and precursor/stem cells disaggregated from thyrospheres were seeded at a cell density of 10,000–15,000 cells/well in a 96-well microtiter plate in phenol red-free RPMI medium supplemented with 2% FBS. After adhesion and overnight starvation in RPMI medium with 0.1% BSA, heavy metals were added to the culture medium at the indicated concentrations. After 48 hours of exposure to metals, cells were labeled with BrdU (DELFIA cell proliferation kit, PerkinElmer) for an additional 24 hours and BrdU incorporation was evaluated according to the manufacturer's instructions.

To further evaluate the metal effect on thyroid stem/progenitor cell growth, we also measured thyrosphere numbers and volume after exposure to metal. Thyrospheres were first dissociated mechanically and enzymatically into single cells and then seeded at a cell density of 3000 cells/well in a 96-well microtiter plate in phenol red-free RPMI medium supplemented with 0.1% B-27, before being exposed for 8–10 days to metals at the concentration causing

the greatest effect at BrdU incorporation. The secondary thyrosphere number was counted in each well (96-well plate, 100 µL) and the average of four wells was averaged for each condition. Phase-contrast images of morphological changes of secondary thyrosphere formation were captured by an Olympus optical microscope supported by a DP20-5E digital camera. Thyrosphere size was calculated by measuring sphere areas using Image J software (NIH, Bethesda, MD, USA) [82].

To ascertain whether changes in thyrospheres' size were due to proliferation rather than endoreplication, thyrospheres were dissociated into single cells by trypsin-EDTA treatment for 10 min with gentle pipetting and the number of viable cells counted.

4.2.4 Immunoblot Analyses

To measure the extracellular signal-regulated protein kinase (ERK1/2), thyrospheres were lysed and subjected to Western blot analysis, as previously described [83]. The following antibodies against total and phosphorylated ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA): anti-ERK1/2, anti-P-ERK1/2 (T202/Y204) and anti-Vinculin. Bands were detected digitally using the Odyssey Fe imaging system (Li-COR Bioscience, Lincoln, NE, USA) and the blots were then quantified using the Li-COR Image Studio software version 5.2.5.

The metal concentration used for evaluating ERK1/2 activation at 5-, 15- and 30-min time points was selected based on the peak effect of each metal on BrdU incorporation.

ERK phosphorylation was also measured in cells exposed to metals in the presence of PD98059 (20 µM) that was kept in the culture medium throughout the entire period of exposure to metals.

4.2.5 Cell biology analyses in early studies with Tungsten

In the early studies carried out with cell exposure to tungsten I investigated additional biological effects. Briefly:

- <u>DNA damage/repair</u> was investigated evaluating the expression of DNA damage/repair proteins H2AX in immature thyroid cells exposed to Na_2WO_4 30 nM for 90 min by immunofluorescence.

- Cell transformation was examined by three different experimental procedures:

  (a) anchorage-independent growth in soft agar by measuring colony formation: cells were suspended in 0.35% agar and seeded into 12-well plate pre-coated with 0.5% agar, incubated for 3 weeks with or without Na_2WO_4 30 nM and the number and size of colonies analyzed with Image J software.

  (b) clonogenic assay: cells were seeded into a 12-well plate at a density of 500 cells and incubate for 3 weeks. Colonies were stained with 0.1% crystal violet and counted.

  (c) invasiveness was evaluated studying the ability of cells to migrate: cells were seeded in a 12-well plate precoated with poly-L-lysine and, after cells formed a confluent monolayer, a scratch was made manually by scraping the cells with a pipette tip of 200 ml. The scratched area was photographed at intervals of 0, 24, and 48 h using an inverted microscope.

4.2.6 Statistical Analysis

All data were expressed as mean ± SEM. Statistical analyses were performed using the GraphPad Prism 5.0 Software. All differences between mean values were evaluated by the Student's *t*-test. A two-sided $P < 0.05$ was accepted as significant.

4.3 Results

4.3.1 *In vitro* effects of Tungsten in immature and mature thyroid cells

I choose to first carry out some in vitro studies with Tungsten (W) because this is the most increased heavy metal in drinking water of Mount Etna volcanic area where it exceeds the normal range in the urine of 27% inhabitants.

Tungsten use in the industrialized world has steadily expanded in the recent decades from 32,200 tons in 1998 to 88,100 tons in 2016 (https://www.usgs.gov/centers/nmic/tungsten-statistics-and-information). Cement carbides (hard metals, superalloys) and military applications are the most important tungsten usages (International Tungsten Industry Association; http://www.itia.info/tungsten-primary-uses.html) and, unavoidably, the increased use has spawned greater contamination in the natural and human environment [84]. Therefore, tungsten must be considered an emerging toxicant, acting either alone or by augmenting the effect of other toxicants and cell stressors

[85,86]. Additionally, tungsten has been reported to have also potential carcinogenic effects both *in vitro* [87,88] and *in vivo* [74].

Since in the Mount Etna volcanic area residents' exposure to increased tungsten is a chronic, life-long condition starting in prenatal life because the fetus and the newborn are exposed by transplacental and breast-feeding routes [89,90], I hypothesized that early exposure to increased tungsten might predispose individuals to thyroid cancer risk later in life. Stem/progenitor cells, in fact, may be more susceptible to the detrimental effect of increased toxicant and transmit the damage to their differentiated progeny, as previously observed in other human and animal models [91–95]. To test this hypothesis, I planned experimental procedures examining in vitro the effects of (a) chronic exposure with progressively increasing tungsten doses and (b) comparing immature vs. mature thyroid follicular cells as well as thyrocytes derived from stem/precursor cells exposed to tungsten.

In cultured human thyrospheres chronic (15 days) exposure to low doses of W (applied as sodium tungstate dihydrate) caused a series of biological effects. First, very low concentrations of W stimulated thyrosphere proliferation, as indicated by 5-bromo-2-deoxyuridine (BrdU) incorporation, increased DNA levels, and morphological changes observed under phase-contrast microscopy (**Figure 2**) [96]. These effects were observed at very low W concentrations (nM) within the same range measured in the urine of the residents of the Mt. Etna volcanic area (where thyroid cancer incidence is markedly increased) and disappeared at higher (µM) concentrations.

In parallel experiments, no effect of W was observed in differentiated human thyrocytes in primary culture (**Figure 2A and 2B**).

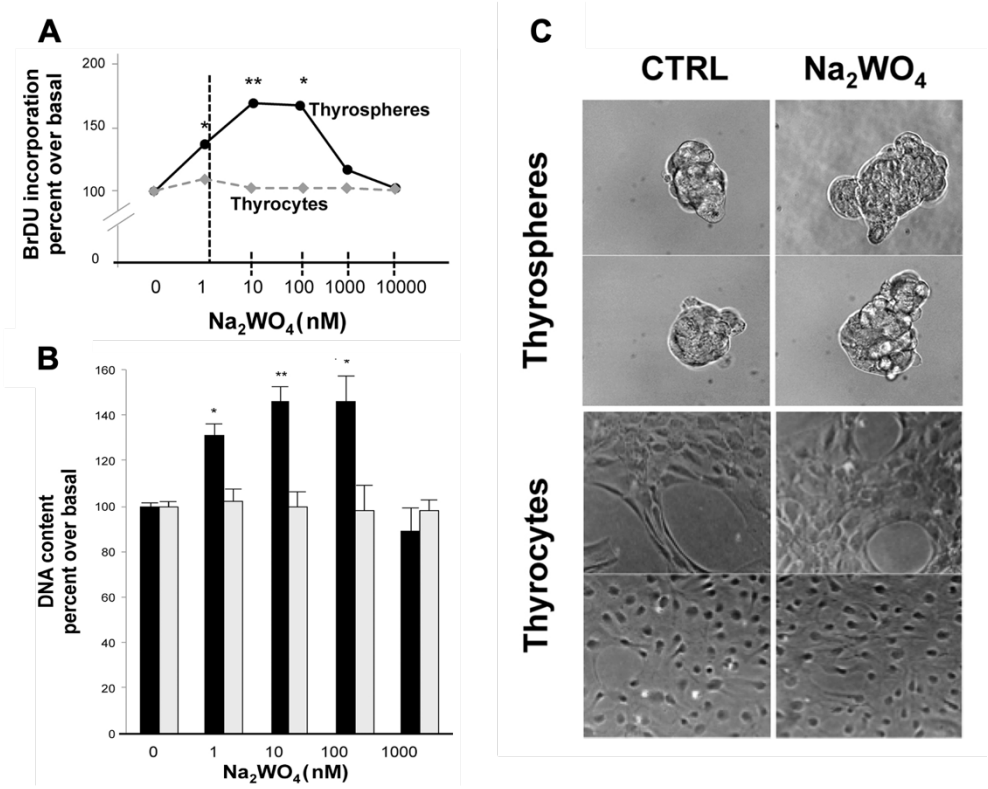

Figure 2. Chronic exposure to Na_2WO_4 affects thyrosphere growth, but not differentiated thyrocyte. (A)BrdU incorporation in thyrospheres and thyrocytes after exposure to increasing concentrations of Na_2WO_4 (*n* = 6 separate experiments). The vertical dotted line indicates the average W concentration in urines of residents of the volcanic area in Sicily (0.2 µg/L of W). (B) DNA content measured in thyrospheres and thyrocytes after chronic exposure to increasing concentrations of Na_2WO_4 (*n* = 4 separate experiments). (C) Representative phase-contrast microscopy images of morphological changes in thyrospheres and thyrocytes incubated with or without tungstate (30 nM, 14 days, *n* = 4 independent experiments). For all data: mean values are indicated with S.E.M.; paired samples are compared by two-tailed Student *t*-test (NS, non-significant; **P* < 0.05, ***P* < 0.01).

In the same model and at the same low concentrations, W reduced thyroid stem cell differentiation as indicated by the expression of thyroid-specific and stemness genes. Moreover, mature thyrocytes derived from thyrospheres chronically exposed to tungstate presented some characteristics typical of transformed cells: they formed more and larger colonies in soft agar and in the clonogenic assay, and they showed a greater migration capacity in the scratch wound-healing assay [**103**].Moreover, also DNA repair protein activity was affected [96] (**Figure 3**).

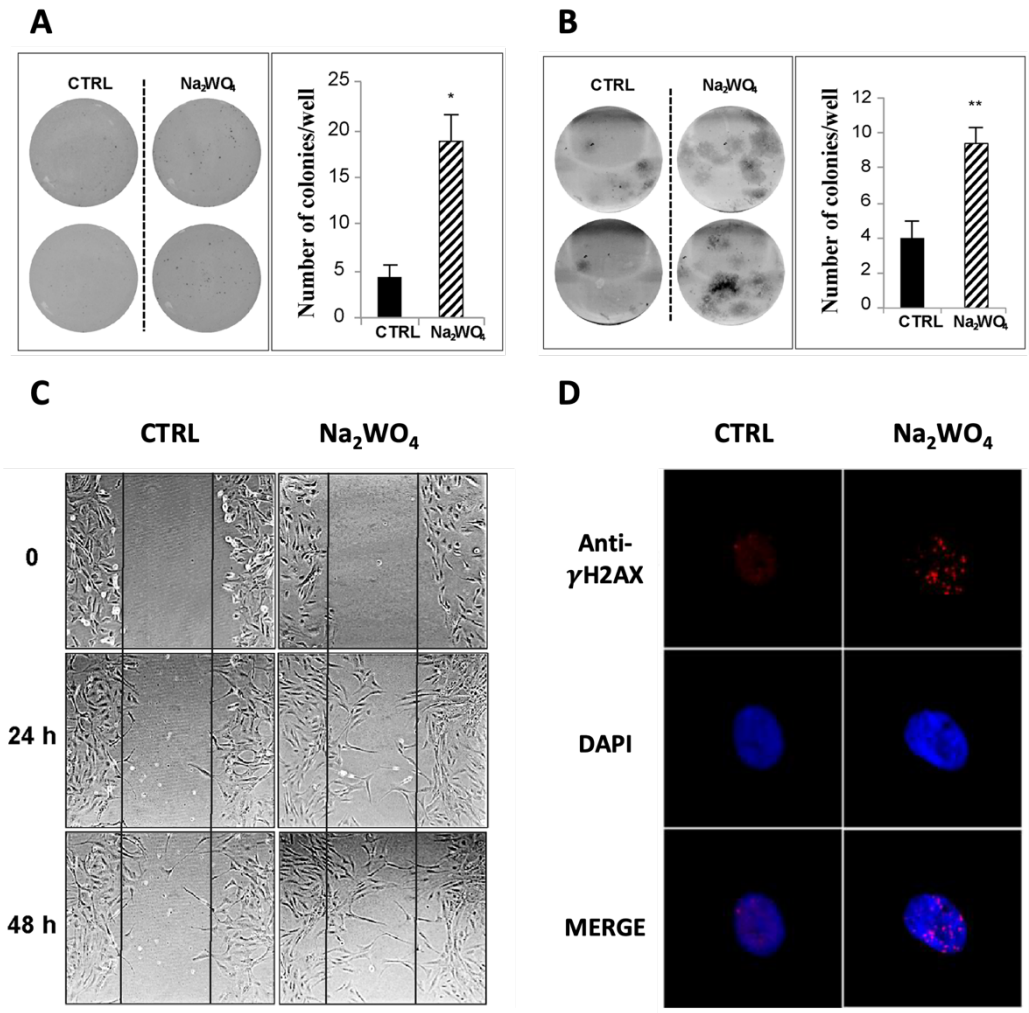

Figure 3. *In vitro* transformation assays in thyrocytes differentiated from thyrospheres either chronically exposed or not exposed (controls) to Na_2WO_4. Both the adherence-independent growth in soft agar (A) and the clonogenic potential in plate formation assay (B) show that thyrocytes differentiated from thyrospheres chronically exposed to Na_2WO_4 (30 nM) form more and larger colonies than thyrocytes differentiated from control, not Na_2WO_4-exposed thyrospheres. Representative images and the average values (number of colonies/well, mean ± S.E.M.) of three separate experiments are shown (*$P < 0.05$; **$P < 0.01$ vs control thyrocytes). (C) Microphotographs of the scratch wound-healing assay show that thyrocytes from W-exposed thyrospheres partially healed the scratch progressively at 24 and 48 h indicating an increased migration activity compared to controls ($n = 3$). (D) Exposure to a low dose of tungsten affect DNA repair proteins in human thyrospheres. The exposure of human thyrospheres to a low dose of W (Na_2WO_4, 30 nM for 90 min) increases the expression of the DNA repair protein γH2AX. γH2AX (red) was detected using a γH2AX antibody followed by an Alexa Fluor-594-conjugated secondary antibody. Nuclei were visualized with DAPI (4',6-diamidino-2-phenylindole) (blu).

These data indicate that chronic exposure to very low concentrations of W, while being harmless to mature thyrocytes, has relevant effects on undifferentiated or partially

differentiated thyroid cells. Biphasic hormetic responses to metals have already been described for other types of undifferentiated cells, such as lung embryo fibroblasts and human embryonic kidney cells [80,96–99]. The major novelty of our observations with Tungsten in stem/precursor thyroid cells is that abnormalities of progenitors exposed to tungsten may produce a population of mature thyrocytes with biological characteristics compatible with a preneoplastic state.

4.3.2 Metal Effect on Thyroid Cell Proliferation

Based on the experimental data obtained with tungsten, I planned parallel experiments in both thyrospheres and differentiated thyrocytes with all the five selected metals and their mixture.

Metals were investigated in a wide range of concentrations spanning 1,000-fold the lowest dose tested and always included the average metal concentration previously documented in the urine of residents of the volcanic area (µg/g creatinine assuming 1g of creatinine equivalent to 1 L of urine): Cu= 5.5, Zn= 217.0, W= 0.12, Hg= 0.21, Pd= 0.09 [39].

In thyrospheres, BrdU incorporation was significantly increased after exposure to each metal examined, with peak values being significantly higher than basal values: Cu +36.7% ± 6.9, $p<0.001$; Zn +58.8% ± 10.1, $p<0.001$; W +59.5% ± 13.3, $p<0.01$; Hg +36.7% ± 10.1, $p<0.01$; and Pd +36.2% ± 7.2, $p<0.001$. Values then declined in all cases when the metal concentration was further increased (**Figure 4**). In parallel experiments, thyrospheres were exposed to a mixture of the five examined metals, each at a concentration causing the greatest BrdU incorporation (Pd at 0.01 nM, Hg at 0.1 nM, Cu and W at 10 nM and Zn at 100 nM).

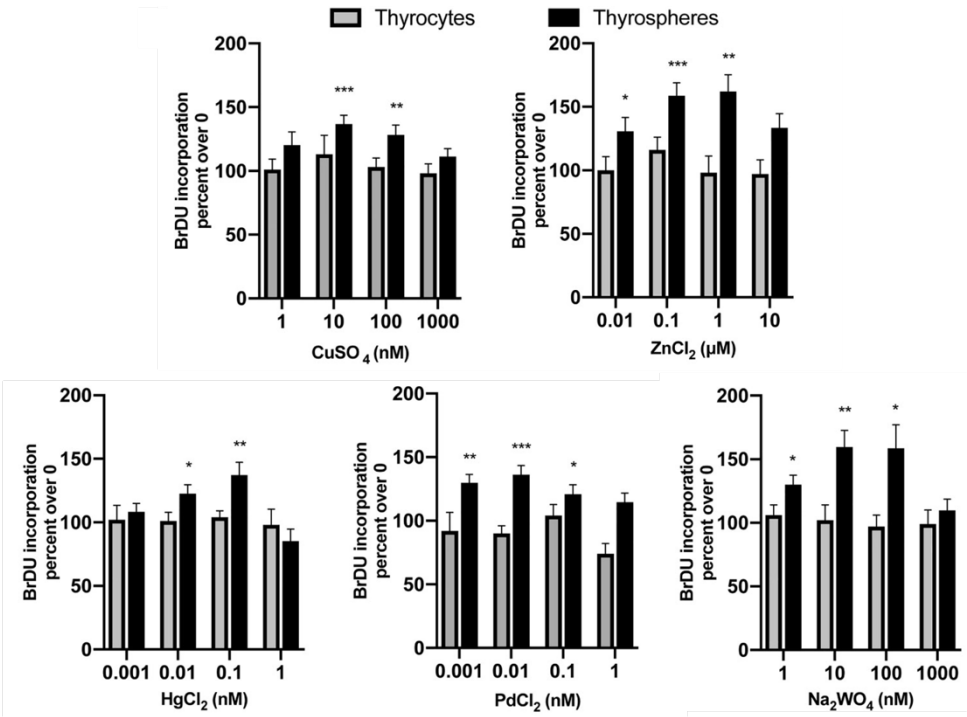

Figure 4. Chronic exposure of human thyrospheres (aggregates of stem/precursor thyroid cells) to heavy metals at the indicated salt concentrations significantly increased BrdU incorporation in all cases. Basal values in untreated cells were always considered equal 100 and values of BrdU incorporation after cell exposure to metals were expressed as percent changes over basal. The dose-response curves followed in all cases a biphasic pattern, declining after the peak value when metal concentrations were further increased. Data shown for each metal indicate the average values ± SEM of four separate experiments except for W (ten separate experiments). In differentiated thyrocytes none of the five heavy metals studied or their mixture had any effect on BrdU incorporation. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0.

The growth effect of this metal mixture on thyrospheres was +91.0% ± 14.8, which was significantly higher (p<0.001) than control thyrospheres and also significantly higher than the effect observed with each single component of the mixture acting alone at the same concentration (p<0.05 for Cu, W and Zn, p<0.01 for Hg and Pd, see **Figure 5**).

No effect of the examined metals was observed on the proliferation of differentiated thyrocytes at any concentration tested and the metal mixture had no effect.

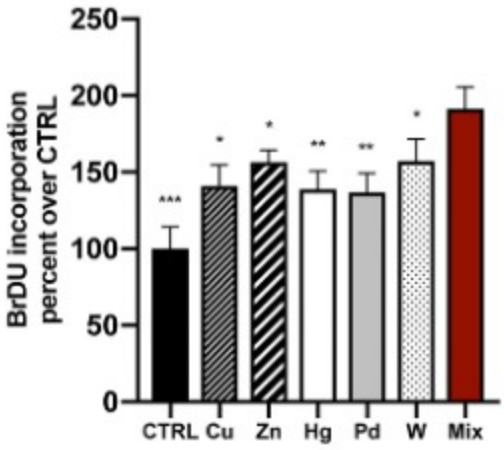

Figure 5. The mixture of the five metals studied (Mix), each at the concentration causing the maximum BrdU incorporation, promoted BrdU incorporation significantly more than control thyrospheres (CTRL) and also significantly more than each metal acting alone (average values ± SEM of four separate experiments). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. Mix.

This growth effect caused by metals in thyrospheres was also evaluated using an independent method. The number and size of thyrospheres was measured 8 days after exposure to each metal at the concentration causing the maximum effect on BrdU incorporation and after exposure to the metal mixture. All metals caused an increase in the thyrosphere size and altered thyrospheres' morphology (**Figure 6A**). Average size values indicated that the increase (μM^2) was significant for all metals ($p<0.05$ for all metals except zinc $p<0.01$; **Figure 6B**). The metal-induced growth effect observed with this procedure roughly reflected the effect observed with the BrdU incorporation method, again with the greatest effect caused by thyrosphere exposure to the metal mixture. In contrast, chronic exposure to metals did not increase the number of thyrospheres. Only the metal mixture had such an effect, significantly increasing the number of thyrospheres ($p<0.01$) relative to both control thyrospheres and thyrospheres exposed to each individual metal (**Figure 6A**).

Counting viable cells of dissociated thyrospheres indicated that proliferation rather than endoreplication was involved in the effect of metals on thyrospheres (data not shown).

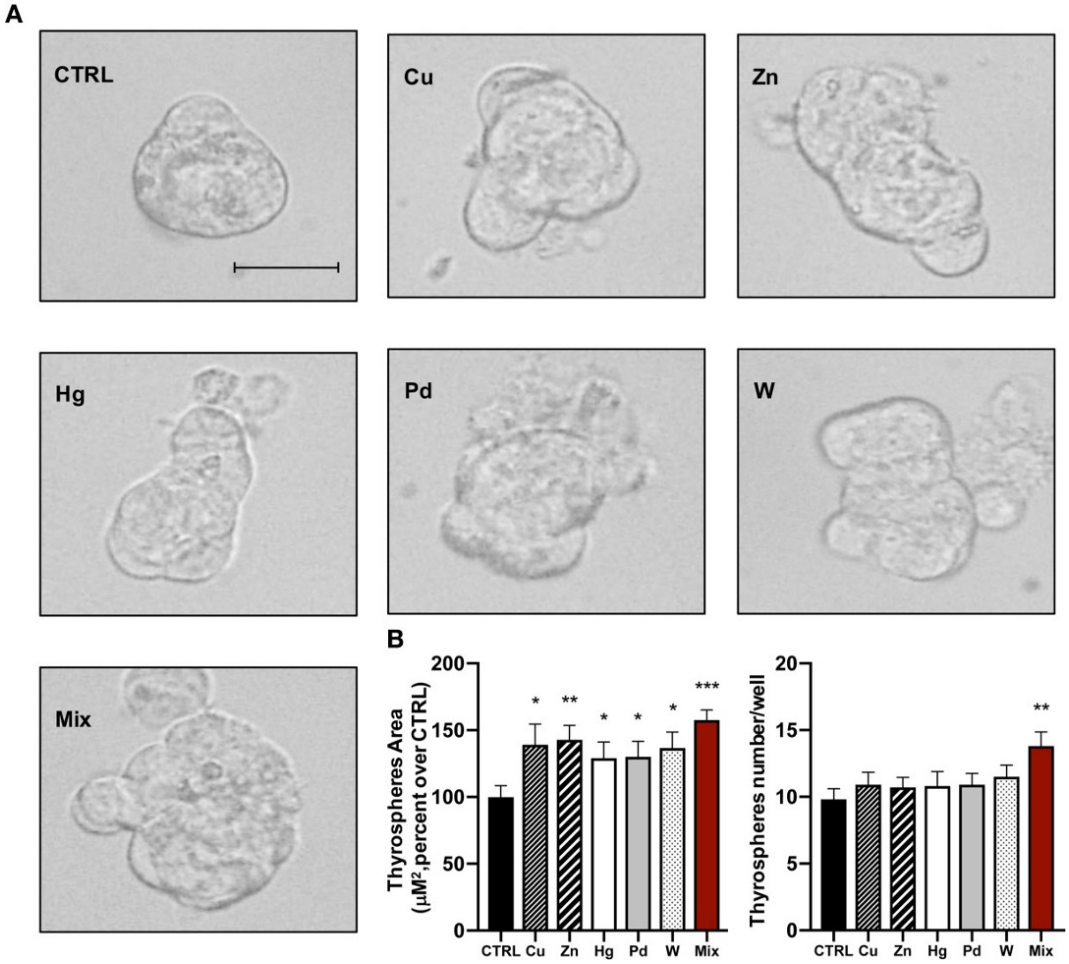

Figure 6. (A) Representative phase–contrast microscopy images of thyrospheres grown in standard medium (CTRL) or in medium added with each of the five heavy metals tested (at the salt concentration causing the maximum BrdU incorporation) or their mixture for 8 days (Scale bar: 30 µM). (B) Histograms indicate the mean value ± SEM of three separate experiments for measuring the size (μM^2) and the number of thyrospheres after exposure to each metal or their mixture. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CTRL.

4.3.3 Metal Effect on ERK1/2 Phosphorylation

To investigate the mechanism involved in the metal-induced growth in human thyrospheres, we measured the extracellular signal-regulated protein kinase phosphorylation (ERK1/2), a pathway that is already reported to be activated by cell exposure to metals and that it is well known to be involved in cell proliferation [73,96,98,100,101].

Each single metal rapidly and significantly ($p<0.01$) stimulated ERK1/2 phosphorylation with an increase that was over 100% for all metals except Hg. The peak value occurred at 5 min and then values decreased, returning to basal levels after 30 min. Hg had a more prolonged effect, but ERK1/2 phosphorylation returned to basal values at 30 min also with this metal (**Figure 7A**).

Again, thyrosphere exposure to the metal mixture caused ERK1/2 phosphorylation that was significantly greater than that observed with each single metal alone (**Figure 7B**).

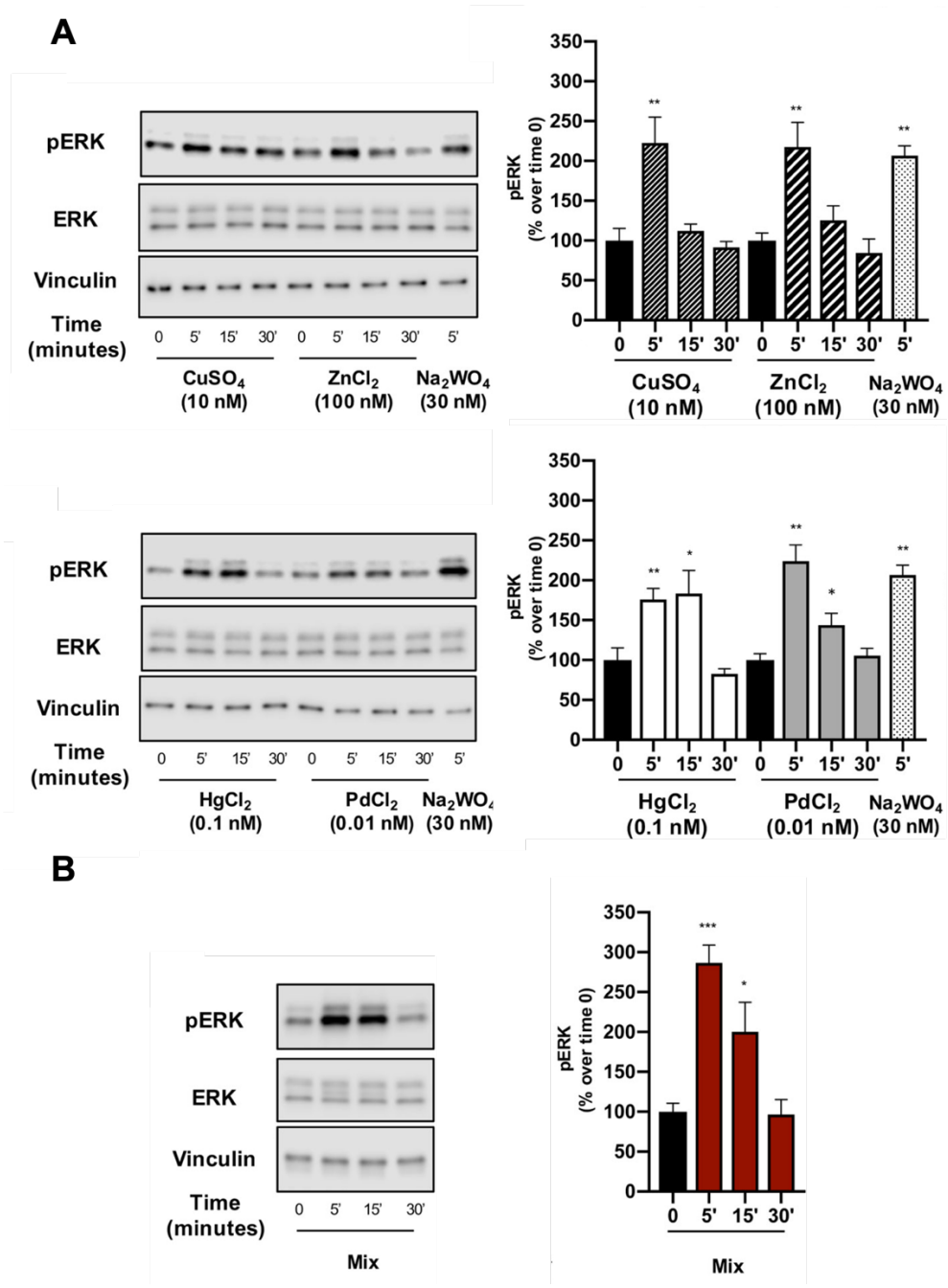

Figure 7. The effect of every single metal alone (A) and their mixture (B) on ERK1/2 phosphorylation in thyrospheres. Thyrospheres were treated for 30 min with the indicated metals at the concentrations causing maximum BrdU incorporation, lysed at the indicated times and analyzed for ERK1/2 phosphorylation by Western blot. Representative immunoblots from six separate experiments. Histograms represent the mean value ± SEM of densitometric values normalized to vinculin and expressed as percent of time 0. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To confirm that the ERK1/2 signaling pathway plays a major role in thyrosphere growth after exposure to metals, we measured BrdU incorporation after thyrospheres' incubation with metals in the presence of the ERK1/2 inhibitor PD98059. In this condition, thyrospheres proliferation was significantly reduced for all metals, with a different effect for different metals: the ERK1/2 inhibition was greater for Cu, Zn and for the metal mixture ($p<0.001$), was less significant for Hg ($p<0.01$) and smaller for Pd and W ($p<0.05$) (**Figure 8**).

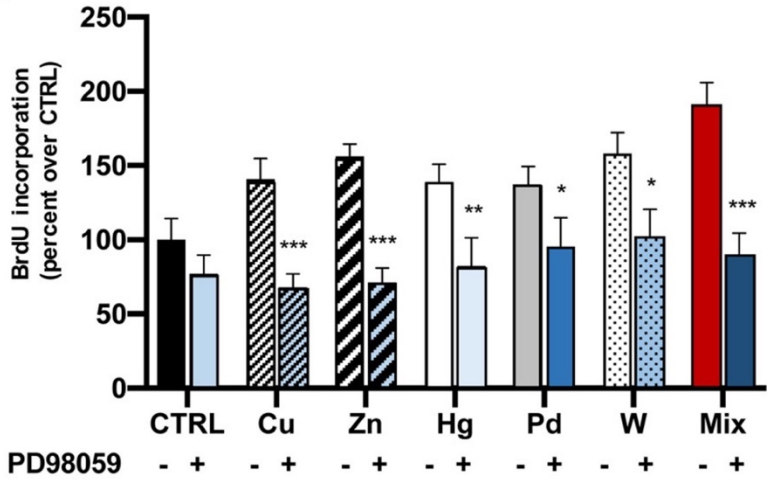

Figure 8. Effect of the ERK1/2 inhibitor PD98059 on metal-induced BrdU incorporation. Histograms represent the mean value ± SEM of three separate experiments. For each metal and their mixture values are expressed as percent of CTRL (untreated thyrospheres). After thyrospheres' exposure to metals for 3 days in the absence or the presence of PD98059 (20 µM) BrdU incorporation was significantly iblunted in all cases by the inhibition of the ERK1/2 pathway. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ vs. CTRL.

Inhibition of the ERK1/2 pathway also affected the thyroid stem cell self-renewal, reducing the number of secondary thyrospheres. No significant change was observed in thyrospheres' size or morphology. The decrease in thyrosphere number was greater in control (not metal-exposed) thyrospheres ($p<0.001$ in comparison with thyrospheres grown in the absence of the inhibitor). The PD98059 effect on secondary thyrosphere number was present to a lesser extent for all metals (range -23 to -40%) and was significantly lower than observed in control thyrospheres (**Figure 9**).

It should be noted that in thyrospheres exposed to Hg or to the metal mixture, the number was reduced relative to thyrospheres cultivated in the absence of PD98059, but the difference was not statistically significant (**Figure 9**).

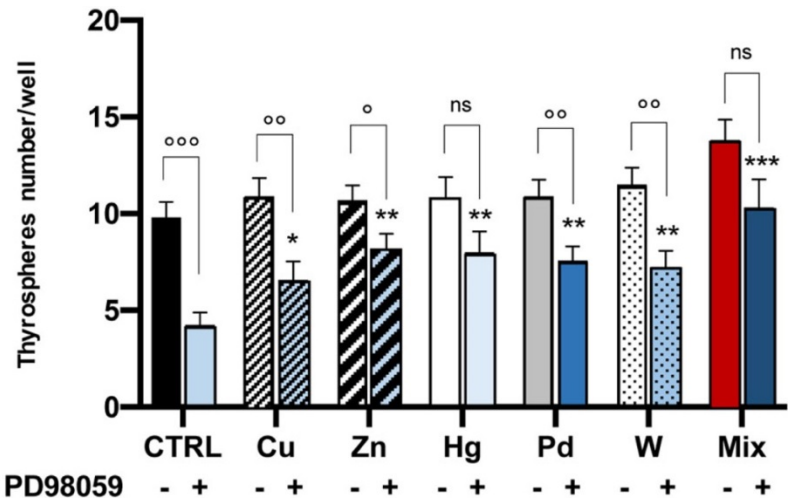

Figure 9. Effect of the ERK1/2 inhibitor PD98059 on metal-induced secondary thyrospheres' formation. Inhibition of the ERK1/2 pathway reduces secondary thyrospheres' formation. Cells from dissociated thyrospheres were plated at a density of 3,000 cells in nonadherent 96-wel plates and exposed to metals in the absence or the presence of PD98059 (20 µM) for 8 days. Spheres were counted in 4 wells for each condition. The results are expressed as mean value ± SEM of three separate experiments. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ comparing thyrospheres' number in the presence of PD98059 to the number measured under the same condition in control thyrospheres. ns, non significant; °$p < 0.05$; °°$p < 0.01$ and °°°$p < 0.001$ comparing thyrospheres' number in the absence or the presence of PD98059.

4.4 Comments

a) Heavy metals, at a very low dose, differently affect thyroid cell proliferation depending on the differentiation level

Using both differentiated and undifferentiated human thyroid cells in primary culture, the present study demonstrates that five heavy metals (Cu, Hg, Pd, W and Zn) at a very low concentration promote proliferation in thyroid stem/precursor cells, but not in differentiated thyrocytes.

Exposure to the studied metals increased BrdU incorporation in thyrospheres with a maximum growth increase ranging from +35% to +59% for the different metals. This peak effect was reached at metal concentrations in the nanomolar range, similar to those measured in the urine of residents from the volcanic area in Sicily, where the thyroid cancer incidence is doubled in comparison to adjacent non-volcanic areas. For all metals, the effect on BrdU incorporation followed a biphasic pattern, decreasing after the peak value when the metal concentration was further increased. This bimodal dose-response of a biological effect is

typical of hormesis, a well-recognized phenomenon occurring at very low concentrations of the stimulating agent and observed both *in vitro* and *in vivo* [69,102]. Chemically-induced hormesis occurs for many compounds and trace elements, including heavy metals [71], and can be cell-specific [103], life-stage specific [104] and also depend on the duration of exposure [105].

We now observe that a hormesis-driven growth response is observed in immature but not in well-differentiated human thyroid cells after chronic exposure to nanomolar concentrations of Cu, Hg, Pd, W and Zn. The different response is certainly based on the different genetic and biological characteristics of cells at different stages of differentiation. In translational terms, this observation highlights the increased sensitivity of immature thyroid cells to environmental heavy metals. This information might be of special relevance during fetal life but also for stem/immature cells present in the adult thyroid. Immature thyroid cells, in fact, may suffer cytotoxic damage from metal concentrations that are not unhealthy for well-differentiated thyrocytes. If progenitors exposed to such a low dose of metals produce a progeny of mature thyroid cells prone to transformation this process could favor an increased incidence of thyroid cancer in the population [93–96]. Our studies with tungsten, in fact, indicate that thyroid progenitor cells' exposure to low dose of this metal influence the characteristics of their progeny (mature thyrocytes). This effect is reminiscent of the transgenerational transfer of the hormetic effects of metals reported in plants [106,107] and animals [108]. These findings may have important implications for the estimation of hazard assessments for carcinogenesis and cancer risk in later life. During the prebirth period and neonatal life, when tissues (including the thyroid) exhibit a high prevalence of stem/precursor cells, the "safe" concentration of Tungsten in the environment is not defined and should be investigated.

Our studies also provide morphological evidence indicating that this growth effect concerns the size rather than the number of thyrospheres (except for the metal mixture) suggesting that exposure to metals primarily affects the proliferating capacity of thyroid precursor cells rather than the self-renewal of thyroid stem cells. Moreover, since thyrospheres exposed to slightly increased metals show an abnormal shape, the orderly cell aggregation in spheres is also affected. The mechanisms and consequences of this phenomenon require additional studies.

b) The potentializing effect of the metal mixture

An important observation is that the mixture of the five metals, each at a fixed concentration determined by its maximal effect on stimulating proliferation, has a significantly greater effect than each single metal used alone at the same concentration used in the mixture. The more potent effect of the mixture can be the final result of different mechanisms concerning the synergistic and antagonistic relationship between different metals that can interfere in the uptake, accumulation and interaction of other metals with cellular biological mechanisms [109]. As reported in the introduction, the environmental condition present in the Mt. Etna volcanic area is characterized by multiple low-level increase of different metals in both water and atmosphere and the consequent multiple biocontamination of the residents.

The model we have studied in vitro concerns a simplified and limited chemical mixture: actually each component of the mixture, at a different concentration than used in our study, could differently interact with the other components determining different thresholds for their biological effects [102,110]. For this reason, our data on the metal mixture are of limited general significance. They are sufficient, however, to highlight the complexity of defining the effect of a multiple and variable environmental pollution on the cell biology, including the transformation and carcinogenic potential. As a consequence, it will be difficult for government agencies to assess the public health risk when dealing with the exposure to multiple chemicals, even when they are increased at a low-level [111].

c) Role of the ERK signaling pathway in metal-induced proliferation

Our studies also demonstrate that metal-induced proliferation occurs via activation of the ERK1/2 pathway and that the mixture of the five metals has a significantly greater effect than each single metal acting alone.

The ERK1/2 signaling pathway is a major effector of metal-induced proliferation. This is an already reported mechanism, but we observe it at nanomolar metal concentrations, much lower than usually tested [73,98,100]. Quantitatively, the effect of metals on the ERK1/2 activation was similar for all compounds examined, but this does not necessarily imply similar activation mechanisms. The relative potency of each metal may be the final result of different biological effects, possibly combined, and including the possible generation of reactive oxygen

species, the inhibition of phosphatases and also different metal-specific mechanisms, such as the activation of zinc-sensing receptors for the Zn effect [101].

A major role of the ERK1/2 signaling pathway in the metal-induced growth of thyrospheres is confirmed by the significant decrease in BrdU incorporation when metal stimulation occurred in the presence of the specific ERK1/2 inhibitor PD98059 (**Figure 8**). The inhibition of ERK1/2 signaling significantly reduced the number of secondary thyrospheres, an effect that was mostly evident in control thyrospheres (not exposed to metals), suggesting that this pathway is relevant for immature thyrocyte self-renewal and proliferation. The metal-induced growth of thyrospheres, however, was only partially inhibited by the presence of PD98059 (**Figure 9**), suggesting that the ERK1/2 pathway is indeed involved, but that other unexplored mechanisms may be activated. In addition to ERK, in fact, other mitogen activated protein kinases (MAPK), such as c-Jun NH_2-terminal kinase (JNK) and p38 MAPK, may be activated by extracellular signals and regulate cell functions like growth, differentiation and apoptosis. All of these pathways could respond to changes in the cellular environment, like the increase of heavy metals [112], and initiate the downstream induction of transcription factors such as the Nuclear Factor-kappa B (NF-kb) which, in turn, may mediate a variety of cell processes [113]. In this complex network, most data indicate that ERK is generally activated by mitogenic stimuli (as in our study), while both JNK and p38 are more involved in the regulation of apoptosis [114,115]. This evidence, however, is not univocal, as it has always been observed with metal concentrations in the micromolar range (2-3 orders of magnitude greater than in our study), and may be metal and cell-type specific [112].

5. General Conclusions

The *ex vivo* and *in vitro* studies that I carried out in recent years provide indirect but consistent evidence that the multi-metal pollution that characterizes the Mt. Etna volcanic area may contribute to the increased incidence of thyroid cancer in residents of that area in comparison with individuals living in adjacent non-volcanic areas.

A series of novel information were produced by these studies.

First, these studies provide the reference data of metal concentrations in the normal thyroid tissue compared to other tissues in both humans and rats. These data concern many trace elements that play an important role as determinants of thyroid function, such as the antioxidant defense system (metalloenzymes), hormone production and metabolism, and thyroid cell growth, differentiation, and death. Some of the studied elements, when in excess, may also have toxic effects via the metal-mediated formation of free radicals, DNA damage and enhanced lipid peroxidation [76], possibly influencing some genetic alterations predisposing to thyroid cancer [77–79]. A limitation, however, regards the biological meaning of the data measured in this study as well as in all previous studies. It must be considered that thyroid tissue specimens were investigated, and the values measured, therefore, represent the average of heterogeneous metal levels present in the different structural and functional compartments of the gland. Follicular cells and colloid, C cells, stromal components and vessels might have a markedly different concentration of the studied chemical, as has been already observed for iodide.

Second, in both humans and rats many metals accumulate in the thyroid more than in other tissues (muscle and fat) of the same individual. Among the metals more concentrated in the thyroid are As, Cd and Hg, well recognized carcinogens. Moreover, some heavy metals are found at a higher concentration in the thyroid of residents of the volcanic area relative to subjects living in adjacent non-volcanic areas. These differences, however, are not statistically significant, probably the consequence of a limited number of cases studied.

Third, investigated heavy metals can stimulate the growth of thyroid stem/precursor cells at environmentally relevant concentrations that have no effect on mature thyrocytes. Stem/precursor thyroid cell stimulation occurs at very low metal concentrations and follows a biphasic patter characteristic of hormesis. Immature thyroid cell increased sensitivity, therefore, should be considered when establishing the safe metal values in the environment.

Forth, the mixture of the five heavy metals studied has an increased effect relative to each single component of the mixture. This synergistic effect may occur through a variety of mechanisms that probably include the relative concentration of each component, the time of exposure, the biological and molecular activities elicited by each metal and the direct and indirect interference of the involved metals. These complex interactions of the many metals increased in the volcanic environment may determine the final biological response in terms of function, proliferation, differentiation, and carcinogenesis.

Fifth, the molecular mechanism involved in metal effects on stem/precursor thyroid cell proliferation requires the activation of the intracellular ERK signaling pathway, as confirmed also by the reduced effect when a specific inhibitor of ERK is present. This mechanism, however, is not exclusive since ERK inhibition does not completely blunts metal effect.

In conclusion, our studies are compatible, but do not demonstrate, that chronic exposure to the mixture of slightly increased metals may affect undifferentiated thyroid cells, making them (and possibly their progeny) more susceptible to future damaging factors.

Thus, further interdisciplinary research is required to connect the increased thyroid cancer incidence in the volcanic areas with the metal-induced biological, biochemical, and molecular changes of thyroid physiology reported in the present studies.

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