

RESEARCH ARTICLE

Mercury in the human thyroid gland: Potential implications for thyroid cancer, autoimmune thyroiditis, and hypothyroidism

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Abstract

Objective

Mercury and other toxic metals have been suggested to be involved in thyroid disorders, but the distribution and prevalence of mercury in the human thyroid gland is not known. We therefore used two elemental bio-imaging techniques to look at the distribution of mercury and other toxic metals in the thyroid glands of people over a wide range of ages.

Materials and methods

Formalin-fixed paraffin-embedded thyroid tissue blocks were obtained from 115 people aged 1–104 years old, with varied clinicopathological conditions, who had thyroid samples removed during forensic/coronial autopsies. Seven-micron sections from these tissue blocks were used to detect intracellular inorganic mercury using autometallography. The presence of mercury was confirmed using laser ablation-inductively coupled plasma-mass spectrometry which can detect multiple elements.

Results

Mercury was found on autometallography in the thyroid follicular cells of 4% of people aged 1–29 years, 9% aged 30–59 years, and 38% aged 60–104 years. Laser ablation-inductively coupled plasma-mass spectrometry confirmed the presence of mercury in samples staining with autometallography, and detected cadmium, lead, iron, nickel and silver in selected samples.

Conclusions

The proportion of people with mercury in their thyroid follicular cells increases with age, until it is present in over one-third of people aged 60 years and over. Other toxic metals in thyroid cells could enhance mercury toxicity. Mercury can trigger genotoxicity, autoimmune reactions, and oxidative damage, which raises the possibility that mercury could play a role in the pathogenesis of thyroid cancers, autoimmune thyroiditis, and hypothyroidism.

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Introduction

Environmental factors are estimated to contribute more than 40% to the risk of thyroid cancers [1] and about 25% to the risk of autoimmune thyroiditis [2]. The nature of these risk factors remains largely unknown [3], but one topic of interest has been the possibility that heavy metals may play a role in thyroid disorders since the thyroid gland appears to be a target for environmental toxic metals [4, 5]. Evidence for a role for toxic metals comes from epidemiological and experimental studies showing the effects of toxic metals on thyroid function, with most studies focusing on mercury, cadmium, and lead [4, 6–8]. However, conflicting results, underpowered epidemiological studies, problems in establishing environmental sources of heavy metal exposure, and difficulties in translating the findings of animal toxicant studies to human toxicant exposure, have hampered research in this field. Animal studies indicate that the thyroid gland of the rat, dog and monkey are predisposed to take up heavy metals such as mercury [5, 9, 10], but animal studies cannot replicate the human experience of being exposed long-term to continuous or repeated low levels of toxic metals that bioaccumulate in tissues. They also do not account for potential human genetic susceptibilities to heavy metal toxicity [11, 12]. Finding environmental influences on thyroid disorders remains an important topic, particularly since many of these disorders are reported to be increasing in incidence [1, 13–17], raising suspicion that environmental pollutants may contribute to this increase [3].

Few studies of toxic metals in the human thyroid are available [5, 18, 19]. Levels of mercury in autopsy-sampled thyroid, measured by atomic absorption, were five times higher in people with more than 11 occlusal mercury-containing amalgam fillings than people with fewer than four such fillings [18]. Similarly, levels of mercury in autopsy-sampled thyroids, measured by inductively coupled plasma-mass spectrometry, correlated with the numbers of dental amalgam surfaces present [19]. In tissue samples removed at surgery from euthyroid subjects, levels of elements such as mercury and cadmium, measured with inductively coupled plasma-mass spectrometry, were higher in the thyroid than in adjacent muscle and fat samples [5]. In these studies, thyroid tissue had to be digested before analysis so elements could not be located within specific cells. This is relevant to the thyroid, since its follicular architecture means the overall cellularity of the thyroid is low, so elements present at low levels, or at high levels in only a few cells, are difficult to detect.

To gain a clearer picture of the role of toxic metals in thyroid disorders, we designed a project with two differences to previous studies. First, we used two elemental bio-imaging techniques that allowed us to study the cellular distribution of mercury and other toxic metals in sections of human thyroid glands. Second, we analysed autopsy-derived thyroid samples from people with a spectrum of clinicopathological conditions, and from a wide range of ages, from which we could estimate the prevalence of mercury-containing thyroid glands and the effect of aging on the mercury content of the thyroid.

Materials and methods

Ethics

This study (X14-029) was approved by the Human Research Committee, Sydney Local Health District (Royal Prince Alfred Hospital Zone). This institutional review board waived the need for written informed consent from relatives of individuals studied since this was a de-identified retrospective study of archived paraffin-embedded tissue. Data were fully anonymised on the research database after initial access to Department of Forensic Medicine records.

Sample collection

Paraffin-embedded thyroid tissue blocks were obtained from The New South Wales Department of Forensic Medicine tissue archive. These had been taken as part of standard tissue sampling from the autopsies of 115 people (68 male, 47 female) with a mean age of 54 years, median age of 47 years, age range 1–104 years, and SD 27 years (Table 1). Major medical conditions were: none known (N = 50), neurodegenerative disease (N = 33), psychosis (N = 27),

Table 1. Age, gender, and follicular cell autometallography of samples.

ID	Age	Gender	AMG	ID	Age	Gender	AMG	ID	Age	Gender	AMG
T1	1	Female	0	T40	39	Male	++	T79	70	Male	+
T2	2	Male	0	T41	39	Male	0	T80	71	Female	0
T3	4	Male	0	T42	39	Male	0	T81	72	Female	++
T4	9	Male	0	T43	40	Female	0	T82	72	Female	0
T5	16	Male	0	T44	40	Female	0	T83	74	Male	+
T6	18	Male	0	T45	41	Male	0	T84	74	Female	++
T7	18	Female	0	T46	41	Female	0	T85	75	Male	0
T8	18	Female	0	T47	41	Male	0	T86	76	Female	++
T9	20	Male	0	T48	42	Male	0	T87	77	Female	+
T10	20	Male	0	T49	43	Male	0	T88	77	Female	0
T11	20	Female	0	T50	43	Male	0	T89	77	Male	0
T12	20	Male	0	T51	43	Male	0	T90	80	Male	0
T13	21	Female	0	T52	44	Male	0	T91	81	Female	++
T14	22	Female	0	T53	44	Female	0	T92	83	Male	++
T15	23	Male	0	T54	45	Male	++	T93	86	Male	+
T16	24	Male	0	T55	45	Male	0	T94	86	Female	0
T17	25	Female	0	T56	45	Male	0	T95	87	Female	0
T18	26	Female	0	T57	46	Female	0	T96	89	Female	+
T19	26	Male	0	T58	47	Male	0	T97	95	Female	0
T20	28	Male	0	T59	47	Male	+	T98	95	Female	0
T21	29	Female	0	T60	48	Female	0	T99	95	Male	0
T22	29	Male	0	T61	49	Female	+	T100	95	Female	++
T23	29	Male	++	T62	49	Male	0	T101	95	Female	+
T24	30	Male	0	T63	49	Male	0	T102	96	Female	0
T25	30	Male	0	T64	49	Male	0	T103	96	Male	+
T26	30	Male	0	T65	53	Male	0	T104	96	Male	0
T27	33	Male	0	T66	54	Male	0	T105	96	Female	0
T28	34	Male	0	T67	55	Male	0	T106	96	Female	0
T29	35	Male	0	T68	58	Male	0	T107	97	Female	0
T30	35	Female	0	T69	59	Female	0	T108	97	Female	0
T31	35	Female	0	T70	59	Male	0	T109	97	Female	0
T32	36	Male	0	T71	61	Male	0	T110	98	Male	0
T33	36	Female	0	T72	61	Male	0	T111	98	Male	+
T34	37	Male	0	T73	61	Female	++	T112	99	Male	0
T35	37	Male	0	T74	62	Male	++	T113	100	Male	0
T36	37	Female	0	T75	66	Male	0	T114	104	Female	0
T37	38	Female	0	T76	67	Male	+	T115	104	Female	0
T38	38	Male	0	T77	69	Male	0				
T39	38	Female	0	T78	70	Male	0				

Age: years, AMG: autometallography staining of follicular cells, ID: sample identity number, 0: no AMG-positive follicular cells, +: AMG-positive follicular cells but fewer than five follicles with 50% or more AMG-positive cells, ++: at least five follicles with 50% or more AMG-positive cells.

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epilepsy (N = 2), and one each of anorexia nervosa, cancer, and Down syndrome. Causes of death were: suicide (N = 26), trauma (N = 17), drowning (N = 16), cardiovascular (N = 14), drug overdose (N = 14), infection (N = 8), undetermined (N = 6), choking (N = 5), cerebrovascular (N = 3), cancer (N = 2), and one each of hypothermia, respiratory insufficiency, sudden unexpected death from epilepsy, and undernutrition.

Autometallography

Paraffin blocks were sectioned at 7 μm with a Feather S35 stainless steel disposable microtome blade and deparaffinised. Sections were stained for inorganic mercury using silver nitrate autometallography, which represents the presence of mercury as black silver grains surrounding the mercury [20]. Autometallography is a sensitive amplification technique that can detect as few as 10 mercury sulphide/selenide molecules in a cell [21]. Briefly, sections were placed in physical developer containing 50% gum arabic, citrate buffer, hydroquinone and silver nitrate at 26°C for 80 min in the dark then washed in 5% sodium thiosulphate to remove unbound silver. Sections were counterstained with mercury-free hematoxylin and viewed with bright-field microscopy. Each staining run included a control section of mouse spinal cord where motor neuron cell bodies contained mercury following an intraperitoneal injection of mercuric chloride, with archived paraffin blocks used from a previous experiment approved by the University of Sydney Animal Ethics Committee [22]. Sections were stained with hematoxylin only to act as a control for the autometallography-stained sections, to ensure any black grains seen were from the autometallography and not from the melanin-like pigment that can occasionally be seen in follicular cells [23]. Autometallography staining of the thyroid was categorised as either: 0: no AMG-positive follicular cells, +: AMG-positive follicular cells but fewer than five follicles with 50% or more AMG-positive cells, or ++: at least five follicles with 50% or more AMG-positive cells.

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS)

To confirm which metal autometallography was demonstrating (since autometallography can also detect inorganic silver and bismuth) and to look for the presence of other toxic metals, 7 μm paraffin sections of selected thyroid samples were deparaffinised and subjected to LA-ICP-MS for mercury, silver, bismuth, aluminium, gold, cadmium, chromium, iron, nickel and lead, as well as for phosphorus (contained in cell nuclei) to assess cellular density. Analyses were carried out on a New Wave Research NWR-193 laser or a Teledyne Cetac LSX-213 G2+ laser hyphenated to an Agilent Technologies 7700x ICP-MS, with argon used as the carrier gas. LA-ICP-MS conditions were optimised on NIST 612 Trace Element in Glass CRM and the sample was ablated with a 50 μm spot size and a scan speed of 100 $\mu\text{m}/\text{s}$ at a frequency of 20 Hz. The data were collated into a single image file using in-house developed software and visualised using FIJI.

Statistical analyses

Prism v8.4 software was used for chi-square analyses with Fisher's exact test to compare categorical variables, chi-square analysis for trend to look for age-effects in groups, and t-tests to compare continuous variables. Significance was assessed at the 0.05 level.

Results

Autometallography

Mercury-staining black grains were seen in the cytoplasm of follicular cells in 22 of the 115 (19%) samples, 15 with category + and 7 with category ++ autometallography (the latter all

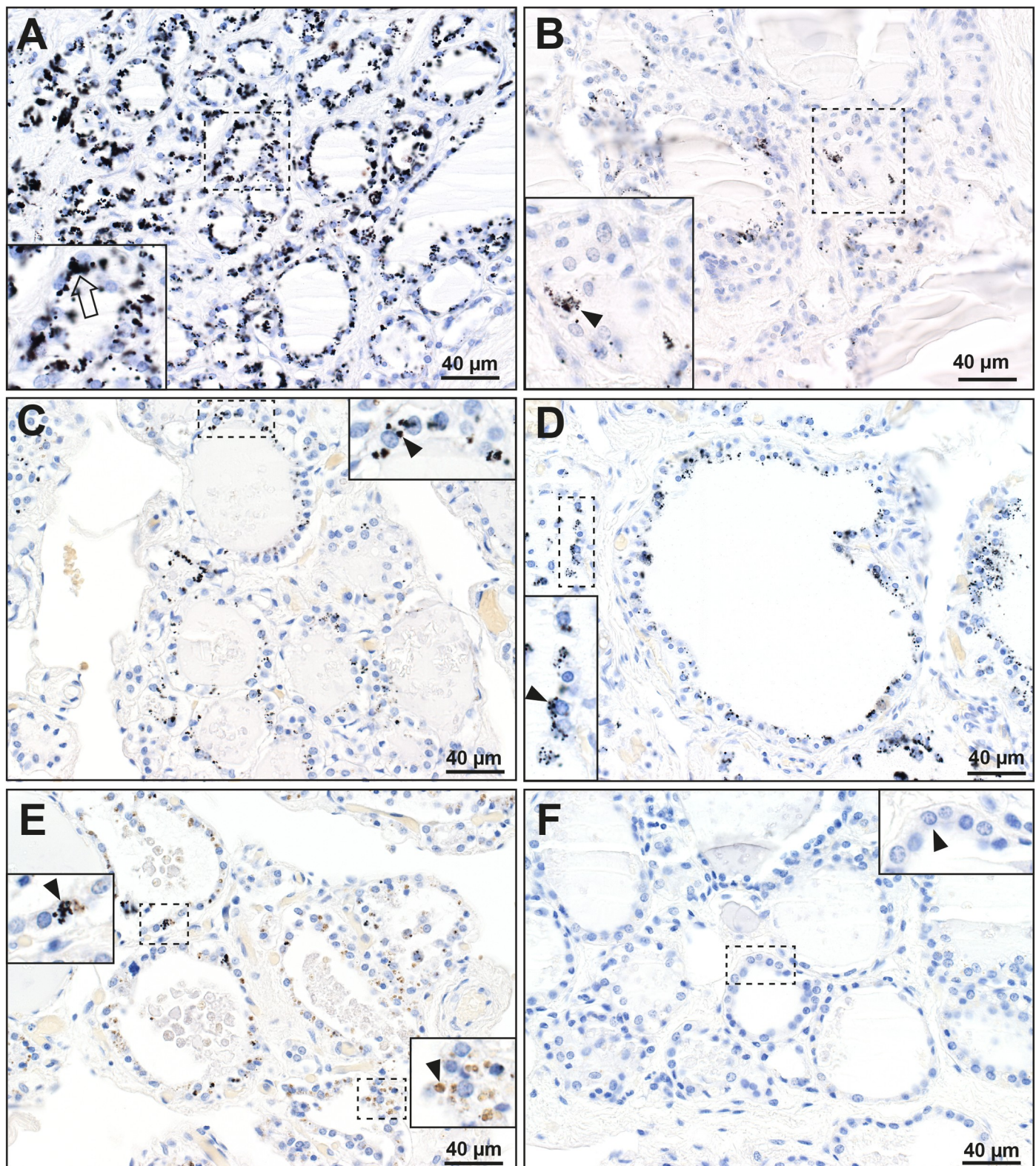


Fig 1. Mercury staining in the thyroid gland. Insets are enlarged views from the dashed-line rectangles. (A) Dense mercury grains (eg, arrow in inset) are present in most cells of these thyroid follicles (T81). (B) Another region of the thyroid in sample A shows mercury grains in only a few scattered follicular cells (eg, arrowhead), showing the variability of mercury uptake in different regions of the thyroid (T81). (C) Black mercury grains (eg, arrow) are seen in fewer than 50% of the follicular cells in this thyroid (T87). (D) Multiple small mercury grains (eg, arrowhead) are present in most cells of this follicle (T23). (E) In some follicles, a mixture of cells containing mercury (arrowhead, left) and lipofuscin (eg, arrowhead, right) were seen (T103). (F) No black mercury grains are seen in the cells (eg, arrowhead) of these follicles (T97). Autometallography/hematoxylin. T: sample identity number (see Table 1).

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aged over 70 years) (Fig 1, Table 1). The density of mercury staining in individual follicular cells varied within and between samples. The proportion of follicular cells containing mercury also varied within samples, as well as between samples.

Proportion of people with mercury in their thyroid glands

The proportion of people with mercury in their thyroid follicular cells was 4% in the 1–29 years age range, 9% in the 30–59 years age range, and 38% in the 60–104 years age range (trend $p < 0.0001$) (Fig 2). The mean age of people with thyroid follicular cell mercury was

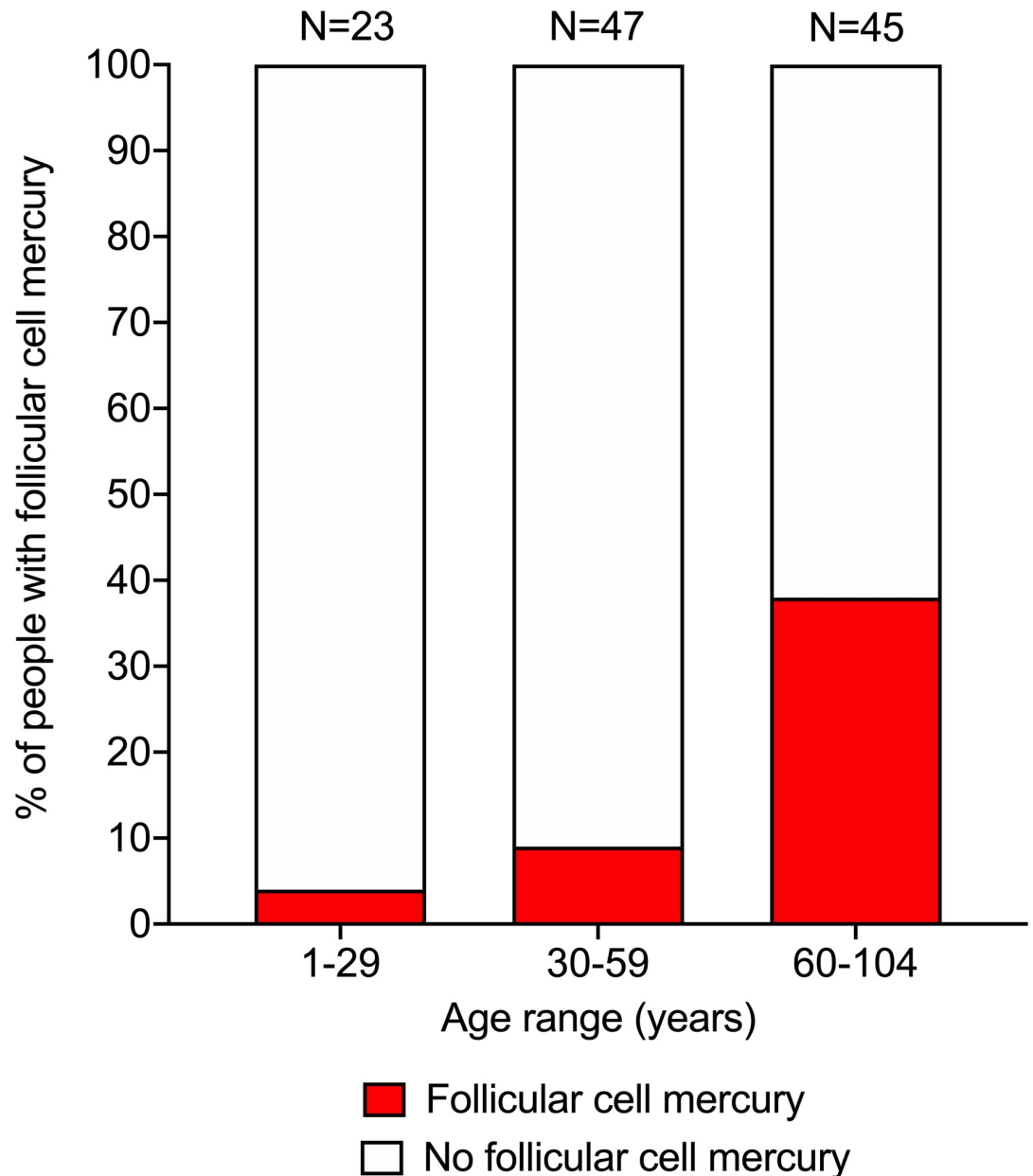


Fig 2. Proportion of people with follicular cells containing mercury. Mercury was seen on autometallography in the thyroid follicular cells of 4% of people aged between 1–29 years old, 9% of people aged between 30–59 years old, and 38% of people aged between 60–104 years old. Numbers above bars = numbers in age groups.

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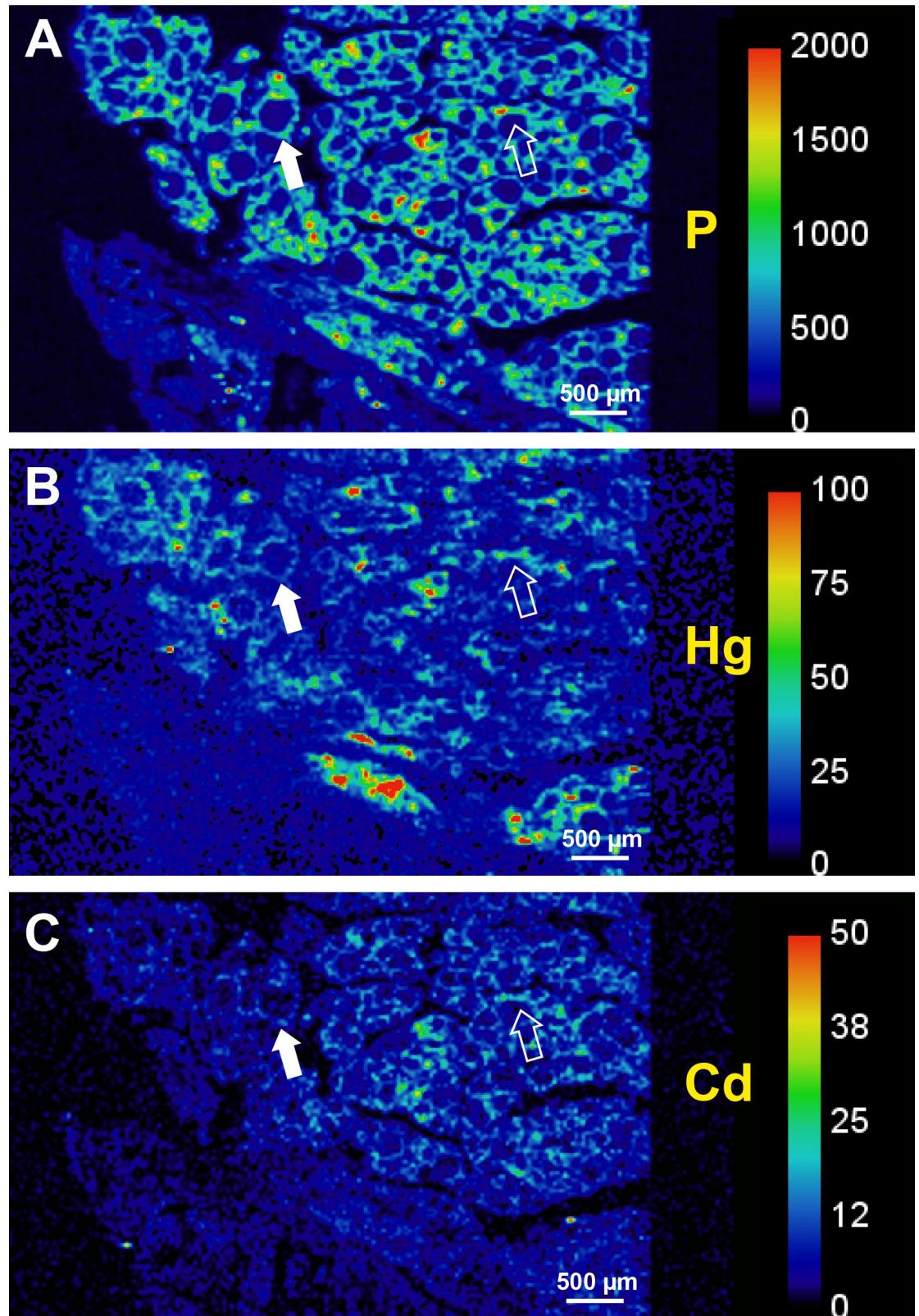


Fig 3. Localisation of mercury and cadmium with LA-ICP-MS. This sample (T40) showed autometallography staining of most thyroid follicles. (A) Phosphorus imaging of cell nuclei demonstrates the follicular architecture of the thyroid. The filled arrow

shows an example of one complete follicle. The open arrow shows cells at one edge of a follicle. (B) Mercury is present in most follicular cells. (C) Cadmium is present in scattered follicular cells. Scale = counts per second (proportional to abundance). T: sample identity number (see Table 1).

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higher (mean age 71 years, SD 20 years, range 29 to 98 years) than those without mercury (mean age 50 years, SD 28 years, range 1–104 years) ($p = 0.001$). The proportion of thyroid samples containing mercury did not differ between males (12 out of 68, 18%) and females (10 out of 37, 21%) ($p = 0.64$), despite females having a higher mean age (61 years, SD 30 years) than males (49 years, SD 25 years) ($p = 0.028$). There were insufficient numbers in subgroups of pre-mortem medical conditions, or of causes of death, to undertake robust statistical analysis of thyroid mercury in these subgroups.

LA-ICP-MS

LA-ICP-MS phosphorus images demonstrate cell nuclei and outlined the follicular architecture of the thyroid (Fig 3). Metals such as mercury and cadmium could therefore be localised to follicular cells using LA-ICP-MS (Fig 3). LA-ICP-MS images showed follicular cell mercury in all the three autometallography-positive thyroid samples (Fig 4, Table 2), but no LA-ICP-MS mercury was seen in three samples that did not stain with autometallography (Fig 5, Table 2). Apart from mercury, four other potentially toxic metals were detected in the six LA-ICP-MS samples (Figs 4 and 5, Table 2): follicular cadmium was detected in all six samples, iron in five samples, lead in four samples, and nickel in two samples. Some background silver was seen in two samples. Chromium, aluminium, bismuth and gold were not detected in any samples.

Discussion

A key finding of this study is that mercury is commonly present in human adult thyroid follicular cells, raising the possibility that mercury could contribute to several thyroid disorders (S1 Fig). Other toxic metals such as cadmium and lead are also found in the human thyroid, suggesting synergistic interactions between toxic metals could enhance mercury toxicity in thyroid cells [24].

We were unable to ascertain why usually only some follicular cells contained mercury, but this variability in cellular mercury appears to be common in human tissues such as the brain [25], pituitary [26], pancreas [27] and breast [28]. Of note, follicular cells within a single follicle can be flattened on one side and cuboidal or columnar on the other, indicating the presence of functional polarity [23]. Theoretically, either increased mercury uptake or decreased elimination in subsets of follicular cells could underlie this variability. Future studies of autometallography combined with immunohistochemistry for a range of transporters of mercury both into and out of cells [29, 30] would be needed to see if variability in these cellular transporters underlies the patchy presence of mercury in follicular cells.

Toxic heavy metals such as mercury could trigger a variety of thyroid disorders since mercury can initiate pathways leading to genetic mutations [31, 32], autoimmune reactions [33], and oxygen free radical production [34, 35], mechanisms suspected to underlie the pathogenesis of thyroid cancers [36], autoimmune thyroiditis [37], and hypothyroidism [38]. Mercury has genotoxic properties that could promote the formation of cancer-causing mutations [31, 32]. Mouse thyroid stem cells contain the mercury transporter ABCG2/BCRP, which suggests thyroid stem cells (though not follicular cells that lack ABCG2/BCRP) have adapted to mercury uptake and make efforts to rid themselves of this metal [39, 40]. Mercury is a known promoter of autoimmune reactions [33, 41], so when the amount of mercury in thyroid follicular cells reaches a critical level in people with a genetic predisposition to autoimmunity, autoimmune

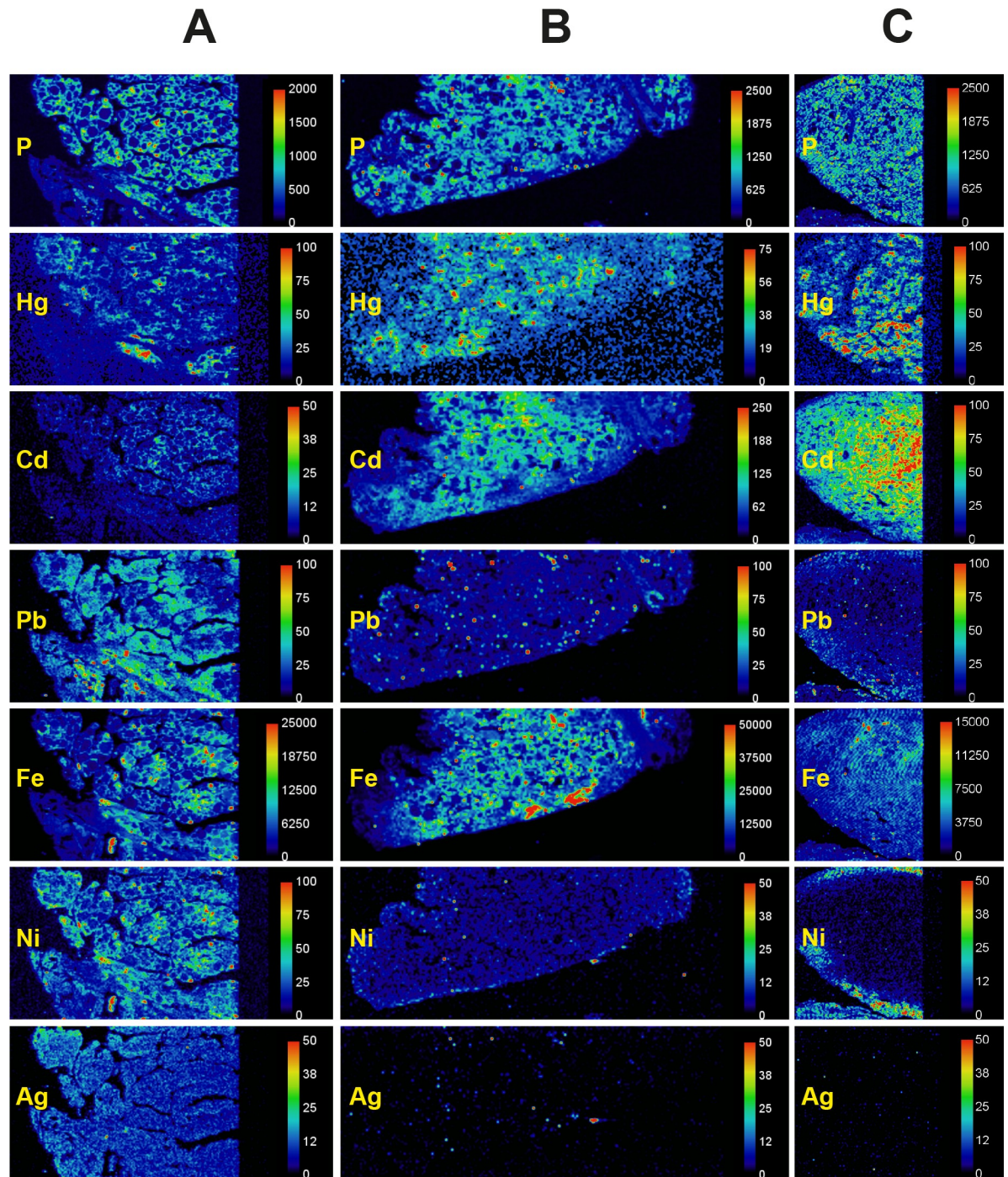


Fig 4. LA-ICP-MS of AMG-positive thyroid samples. Phosphorus images show the cellularity of the samples. (A) Follicular cells contain mercury, cadmium, lead, iron, and nickel (T40). (B) Follicular cells contain mercury, cadmium and iron (T86). (C) Follicular cells contain mercury, cadmium and iron (T91). Small discrete red dots, eg, in the lead image in B, are from surface contamination. An artefactual nickel edge effect is seen in C. Scale = counts per second (proportional to abundance). T: sample identity number (see Table 1).

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thyroiditis could result [42, 43]. Some cases of hypothyroidism are thought to arise from sub-clinical autoimmune thyroiditis [38], and the generation of oxygen free radicals by mercury [35] is a further mechanism by which this metal could contribute to thyroid underactivity [44].

Table 2. Potentially toxic metals found by LA-ICP-MS in six human thyroid glands.

ID	Site	Hg	Cd	Pb	Fe	Ni	Ag	Cr	Al	Bi	Au
T40	Follicular	+	+	+	+	+	-	-	-	-	-
	Background	-	-	-	-	-	+	-	-	-	-
T86	Follicular	+	+	-	+	-	-	-	-	-	-
	Background	-	-	-	-	-	-	-	-	-	-
T91	Follicular	+	+	-	-	-	-	-	-	-	-
	Background	-	+	-	+	-	-	-	-	-	-
T97	Follicular	-	+	-	+	-	-	-	-	-	-
	Background	-	+	-	+	-	-	-	-	-	-
T45	Follicular	-	+	+	+	-	-	-	-	-	-
	Background	-	+	-	-	-	-	-	-	-	-
T70	Follicular	-	+	+	+	-	-	-	-	-	-
	Background	-	-	-	-	-	+	-	-	-	-

Follicular: follicular epithelial cells, ID: sample identification number, —not detected, + detected.

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Evidence for a link between thyroid follicular cell mercury and oxidative stress could be sought by combining autometallography with a histochemical marker of nucleic acid oxidative damage [45]. A link between autoimmune thyroiditis and thyroid cancer has been noted by several researchers, with the proposal that the cancer may arise from the chronic inflammation of the thyroiditis [46, 47]; our finding of mercury in follicular cells suggests that mercury toxicity underlying both disorders could be another reason these two disorders often co-exist.

Increases over time in the incidence of thyroid cancer, autoimmune thyroiditis, and hypothyroidism have been reported [1, 13–17]. The amount of mercury in the global atmosphere has increased since 1950 [48], mostly due to the burning of coal [49]. Atmospheric mercury enters water and then seafood, especially in predatory fish where tissue mercury levels are rising [50]. Fish consumption is the commonest cause of human mercury exposure [51], and our finding that mercury is often present in thyroid cells suggests this metal is a candidate to explain the increasing incidence of a variety of thyroid disorders.

Of the non-mercury metals found in the thyroid, cadmium was most common, being present in the follicular cells of all LA-ICP-MS samples. Cadmium is a known genotoxin that has been implicated in thyroid cancer [52] and has endocrine-disrupting activities that could interfere with thyroid function [8]. Cigarette smoke is a major source of cadmium exposure in humans, but smoking appears to reduce rather than increase the risk of thyroid cancer [1], with contradictory evidence concerning its role as a risk factor for autoimmune thyroiditis [3, 42, 53] and hypothyroidism [3, 7, 53]. Although from our LA-ICP-MS images it appears likely that mercury and cadmium are co-located in some follicular cells, we cannot be sure this is the case since the spot size for LA-ICP-MS is 50 μm , which is larger than a typical cuboidal follicular cell (about 10 μm , see Fig 1F). Other metals detected in the thyroid on LA-ICP-MS were lead, iron, nickel and silver. Studies of the association of human thyroid hormones and lead exposure have given inconsistent results [7] and a meta-analysis has not provided evidence that occupational lead exposure affects thyroid function [54]. However, lead may recruit antibodies that attack the thyroid [55], and could play a part in autoimmune thyroiditis [56] and colloid goitre [57]. Iron is an essential metal but in large quantities, such as found in hemochromatosis, it may cause hypothyroidism with antithyroid antibodies [58]. A systemic allergy to nickel could result in an autoimmune reaction in people who have nickel in their thyroid cells [59]. Silver nanoparticles can induce oxidative stress in cells [60]. Finally, all these metals could interact with mercury to enhance thyroid cellular toxicity [24].

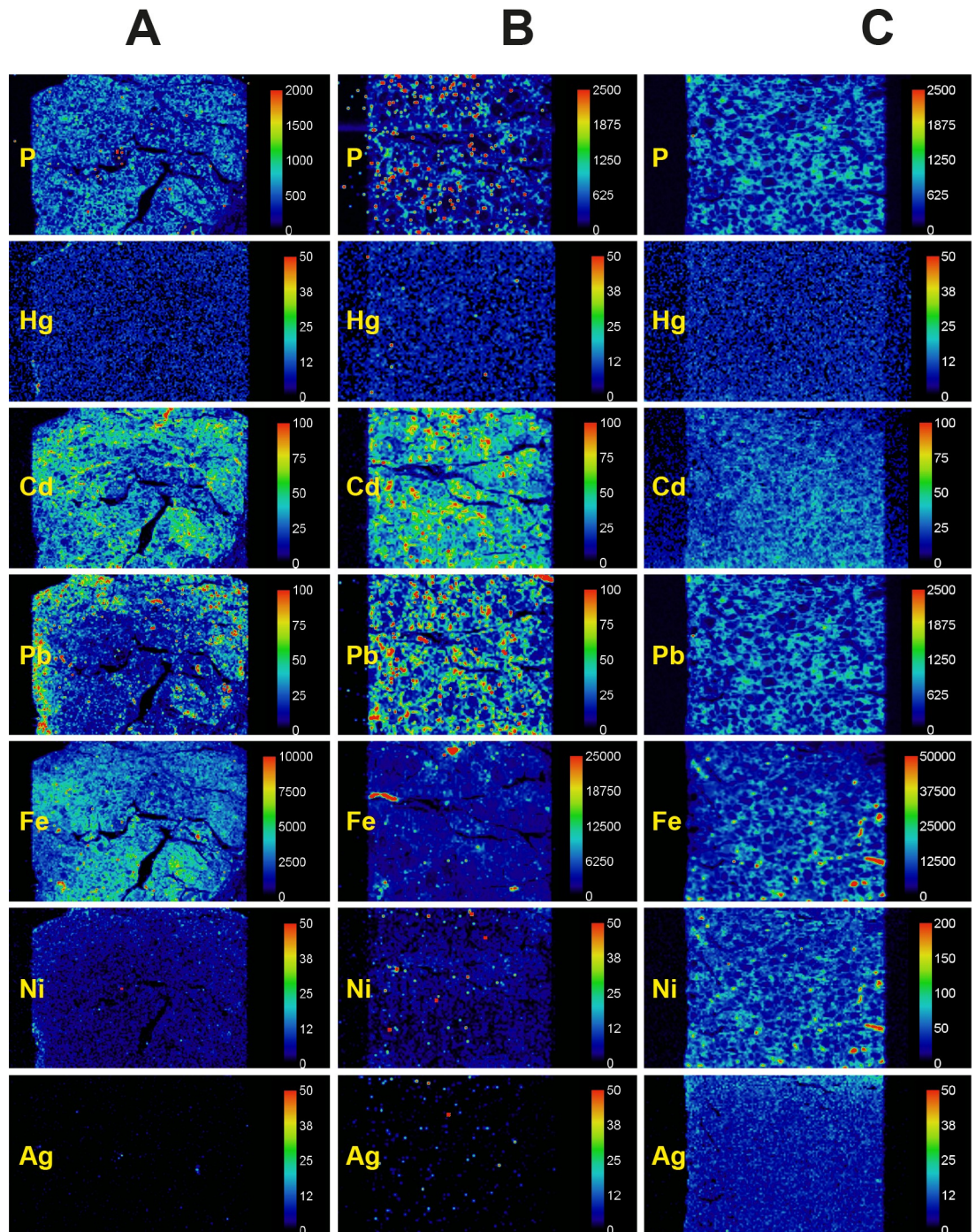


Fig 5. LA-ICP-MS of AMG-negative thyroid samples. Phosphorus images show the cellularity of the samples. (A) Cadmium, lead and iron are present in follicular cells (T97). (B) Cadmium, lead and iron (sporadically) are present in follicular cells (T45). (C) Follicular cells contain cadmium, lead, iron and nickel (T70). Scale = counts per second (proportional to abundance). T: sample identity number (see Table 1).

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Human exposure to environmental toxic metals is common, so it is likely that other risk factors are needed to interact with metal toxicants before thyroid cells are damaged. Several metabolic processes act to protect cells from metal toxicants, and genetic variants affecting

proteins in these pathways have been identified that could increase susceptibility to metals such as mercury [11]. Selenium detoxifies cellular mercury, and low selenium levels have been implicated in thyroid autoimmunity, cancer, and colloid goitre [56, 57, 61, 62]. However, we have no evidence from our study that follicular intracellular selenium levels were low, since LA-ICP-MS is not a sensitive method of detecting selenium and no histological methods are available to detect this element.

This study has several limitations. (1) Our forensic/coronial autopsy series provided thyroid samples from people over a wide range of ages and from varied clinic pathological conditions. However, no autopsy series can precisely replicate prevalence data from a living human population. (2) We did not have enough clinical information to know if any of the people in our study had clinical thyroid disease. Future prospective elemental studies of thyroid tissue from people with clinical data, thyroid function studies and thyroid autoantibodies would be needed to correlate individual thyroid disorders with metal content of the thyroid gland. (3) Prospective *in vivo* biochemical and genomic studies will be needed to examine the roles of selenium deficiency and genetic susceptibilities as susceptibility factors for metal toxicity in the thyroid. (4) Nobody in our study population had thyroid cancer. A future study looking at the heavy metal content of structurally normal thyroid tissue adjacent to thyroid cancers, as has been undertaken for breast cancer and mercury [28], will be needed to determine whether genotoxic metals are more commonly found in thyroid tissue from people with, compared to those without, thyroid cancer. (5) We were unable to determine why mercury enters thyroid follicular cells selectively. The presence of sulfhydryl groups and metallothionein in thyroid cells, which bind metals such as mercury and cadmium, could be one factor in the accumulation and persistence of these metals in thyroid cells. Future studies on toxic metals in the thyroid and their relationship to the thyroid content of sulfhydryl groups and to metallothionein expression could provide more information on this issue. (6) Autometallography can detect only inorganic mercury, but since this appears to be the proximate toxic form of mercury in cells [63] it is the most important form to identify.

In conclusion, mercury is found commonly in follicular cells of the human thyroid, the proportion of people having mercury in their thyroid follicular cells increases with aging, and other toxic metals such as cadmium are found often in the thyroid. Many toxic metals have damaging actions which may contribute to the pathogenesis of thyroid cancer, autoimmune thyroiditis, and hypothyroidism. Most effects of toxic metals on the human thyroid remain hypothetical (S1 Fig), so future prospective experiments correlating the presence of toxic metals in the thyroid with specific thyroid disorders will be needed to shed further light on the role toxic metals such as mercury play in thyroid diseases.

Supporting information

S1 Fig. Hypothetical pathway indicating how mercury and other toxic metals could increase the risk of thyroid disorders. Human exposure to mercury from (1) consumption of marine or freshwater fish, crustaceans and molluscs, (2) occupations, or (3) dental amalgam fillings results in inorganic or methylmercury being deposited in thyroid follicular cells. Methylmercury is slowly converted into inorganic mercury in cells. The toxicity of mercury could be enhanced by genetically susceptibilities, selenium deficiency, or the presence of other toxic metals. After bioaccumulation, critical intracellular level of mercury could produce genetic mutations triggering cancer, autoimmune reactions causing thyroiditis and hypothyroidism, and oxidative damage contributing further to hypothyroidism.

(TIF)

Author Contributions

Conceptualization: Roger Pamphlett.

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Project administration: Roger Pamphlett.

Resources: Roger Pamphlett, Philip A. Doble.

Software: Philip A. Doble, David P. Bishop.

Visualization: Roger Pamphlett, David P. Bishop.

Writing – original draft: Roger Pamphlett.

Writing – review & editing: Philip A. Doble, David P. Bishop.

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