

# Determinants of TSH change in a community-based cohort

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## THE THREE ORIGINAL PAPERS ARE:

1. Bjergved L, Jørgensen T, Perrild H, Carlé A, Cerqueira C, Krejbjerg A, Laurberg P, Ovesen L, Bülow Pedersen I, Banke RL, Knudsen N. Predictors of change in serum TSH after iodine fortification: an 11-year follow-up to the DanThyr study. *J Clin Endocrinol Metab.* 2012 Nov; 97(11):4022-9.
2. Bjergved L, Jørgensen T, Perrild H, Laurberg P, Krejbjerg A, Ovesen L, Rasmussen LB, Knudsen N. Thyroid function and body weight: a community-based longitudinal study. *PLoS One.* 2014 Apr 11;9(4):e93515.
3. Bjergved L, Carlé A, Jørgensen T, Perrild H, Laurberg P, Krejbjerg A, Ovesen L, Bülow Pedersen I, Rasmussen LB, Knudsen N. Parity and 11-year serum TSH and thyroid auto-antibody change: A longitudinal population-based study. Under review.

## INTRODUCTION AND OBJECTIVE

Thyroid disorders are common, with a lifetime risk of 12% of the population [1] and with occurrence being primarily determined by the availability of dietary iodine [2,3]. Evidence shows that developmental brain damage caused by iodine deficiency and goitre can be eradicated by programmes aiming to increase iodine intake in iodine-deficient populations [4,5]. International recommendations on iodine intake, and many national iodine fortification programmes are based on this knowledge. Iodized salt is considered the most effective way of iodine supplementation [5].

As an initial voluntary programme starting in June 1998 to add iodine to all salt products manufactured in Denmark proved to be insufficient, a mandatory iodine fortification of household salt and salt used in commercial production of bread in Denmark was implemented on July 1, 2000 [6]. The programme was based on

the results of several Danish studies [7–10] showing an iodine intake in the population below the levels recommended by WHO, UNICEF, and ICCIDD [4,11]. Furthermore, a high prevalence of goitre and hyperthyroidism caused by autonomous nodules in older age groups [12], and indications of an insufficient iodine intake in pregnant women was found [13]. A programme monitoring the Danish iodine fortification was initiated before the fortification was implemented, The Danish investigation of iodine intake and thyroid disease (DanThyr) [6]. Geographical variations in iodine intake across Denmark, explained by different concentrations in groundwater [14], made it possible to compare the western and eastern regions with different iodine exposure levels. The Danish Institute for Food and Veterinary Research independently monitors the iodine content in food [6].

The impact of the Danish iodine fortification programme on the thyroid function has been investigated previously [15–21], finding marked differences in the pattern of thyroid disease types with increased iodine intake. An increased incidence rate of overt hypothyroidism was observed after fortification of salt, most pronounced in young and middle aged individuals with previous moderate iodine deficiency [19]. The incidence rate of overt hyperthyroidism also increased [15,17], primarily in young adults [15,17], and with clear indications of a transient increase [17]. Limited data exist regarding the impact of iodine fortification on the individual development in thyroid function. Furthermore, the association between serum thyrotropin (TSH) and body mass index (BMI) [22–27] as well as reproductive factors [24,28–32] are well described in cross-sectional observational studies, two of which used the baseline data of this study [26,28]. However, as yet only a few longitudinal studies of the associations have been carried out on these topics [23,27,33–35]. Hence there is a need for longitudinal data to assist in clarifying these relationships ensuring temporality of the studied associations.

Serum TSH is a sensitive indicator of changes in thyroid function [36]. Hence the development in thyroid function was measured as the observed change in serum TSH. To enable detection of very subtle changes in thyroid function we used TSH as a continuous variable.

The overall aim of this PhD project was to analyse the effect of a nationwide iodine fortification programme on the individual development in thyroid function and to identify concurrent determinants for these possible changes. More specifically the following questions were addressed:

How was the individual development in thyroid function affected by the Danish nationwide iodine fortification programme and which factors determined a possible change?

Do we find any association between thyroid function and weight in longitudinal data?

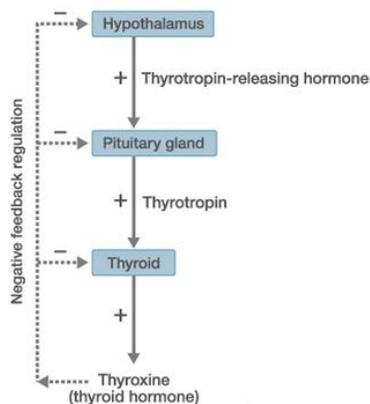
How are parity, abortions, oestrogen use, age at menarche, menopausal status and change in serum TSH as well as thyroid peroxidase autoantibody status related in longitudinal data?

## BACKGROUND

### PHYSIOLOGY OF THE THYROID GLAND

Iodine is a mineral obtained from the diet, dietary supplements, and food additives. It is essential for the synthesis of thyroid hormones [37]. Thyroid hormones, thyroxine (T4) and triiodothyronine (T3) are synthesised by the thyroid follicle cells, and play a key role in regulation of gene transcription and metabolism throughout the body [37].

TSH is a glycoprotein hormone secreted by thyrotroph cells of the anterior pituitary gland [38]. It acts on receptors on the membrane of the follicle cells and controls all aspects of thyroid hormone synthesis, e.g. release of thyroid hormones, the uptake of iodide by follicle cells through the synthesis of the transport proteins, the synthesis and secretion of thyroglobulin, the generation of hydrogen peroxide, endocytosis and proteolysis of thyroglobulin, the blood flow through the gland and transcription of the thyroperoxidase and thyroglobulin genes [37]. Thyroid hormones have a role in the feedback regulation of TSH secretion [38] (Figure 1).



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Figure 1 The hypothalamic-pituitary-thyroid axis

### DETERMINANTS OF THYROID DISORDERS

#### Genes and environment

The etiology of most thyroid disorders is believed to be multifactorial; a combined effect of multiple susceptibility genes and environmental triggers needed to be present for the clinical phenotype to develop. This applies to the most common phenotypes with autoimmune origin, such as Graves' Disease (GD) [39,40], Hashimoto's Thyroiditis (HT) [40], and subclinical autoimmune thyroid disease (AITD) [41], and to those with a non-autoimmune origin such as simple goitre [42,43] and thyroid nodularity [44].

The familial clustering seen in e.g. GD is suggestive of a genetic contribution in disease development [45], whereas a high spouse correlation for GD indicates the importance of environment [45]. Estimated liabilities to disease development attributable to genetic factors of approximately 79% in GD [46], 67% in thyroid nodularity [44], 71% in thyroid size estimated by ultrasonography [47] in twin studies, suggest that the genetic contribution is substantial. A detailed description of the genes so far identified is not included in this thesis. The scientific background for a role of the three environmental factors studied in this thesis on development of thyroid disorders (or TSH changes) and other factors that may also influence serum TSH levels are briefly reviewed below.

#### Iodine and thyroid disorders

The daily dose of iodine intake recommended by WHO, UNICEF, ICCIDD is 150 µg in adults who are not lactating or pregnant, but intakes up to a threshold of 1,100 µg per day are tolerable in healthy adults [4,48]. Median urinary iodine excretion (UIE) concentrations in a population are widely used as a biomarker of the iodine intake [4], due to a substantial day-to-day variation in iodine intake [49]. UIE levels < 20 µg/liter are considered severe iodine deficiency, between 20-49 µg/liter moderate iodine deficiency, and 50-99 µg/liter mild iodine deficiency, whereas 100-199 µg/liter is considered adequate iodine nutrition [4].

In the presence of iodine deficiency the normal thyroid gland is able to conserve the limited iodine resource and improve its utilization by autoregulatory intrathyroidal mechanisms [3,37]. Another compensatory mechanism is induction of hyperactivity of the thyroid gland through hypothalamic/pituitary feedback to keep thyroid hormone production within the normal range. Follicular proliferation and mutations causing multifocal autonomous thyroid function might be a consequence of prolonged mild to moderate iodine deficiency [50]. The prevalence of thyroid enlargement and nodularity is high in populations with mild to moderate iodine deficiency especially in the elderly [3,51], in some cases resulting in hyperthyroidism. If iodine deficiency is severe goitre, overt hypothyroidism and developmental brain damage (or less severe intellectual impairment) are dominating disorders, as compensation of the thyroid gland fails [2].

As regard occurrence of iodine excess, animal studies have shown that organic binding of iodide (I<sup>-</sup>) within the thyroid is blocked when plasma iodide levels increase to a critical level, the so-called acute Wolff-Chaikoff effect [52]. This effect is in rats replaced by normalization in thyroid hormone synthesis within a few days, called the "escape" phenomenon [53]. A higher prevalence of hypothyroidism is seen in populations with higher iodine intake compared with low-iodine-intake populations in observational studies [3,54].

In response to increased iodine intake, such as under an iodine fortification programme, the thyroid gland of susceptible individuals might have an increased risk of failing to compensate. Patients with autoimmune thyroid disease, or early stages of thyroid autoimmunity, might be susceptible to developing iodine-induced hypothyroidism in increased exposures [3,48,53-55]. A failure in acute Wolff-Chaikoff effect have been suggested, but the mechanisms in humans remains unclear [48]. Iodine-induced hyperthyroidism as a response to an increased iodine intake is well-documented [48,56], presumably caused by an increase in substrate for thyroid hormone synthesis [56]. Moreover, an earlier development of Graves' Disease with increased iodine intake has been suggested [15,51].

Both low and high iodine intake associate with an increased disease risk, leaving the optimal interval of iodine intake as a relatively narrow window [50]. Monitoring of iodine fortification programmes is therefore essential.

#### **Body weight and thyroid disorders / thyroid function changes**

Thyroid hormones play an important role in body energy metabolism, particularly in regulating total energy expenditure, adaptive thermogenesis and muscle metabolism [57–59]. An enhanced energy expenditure, protein breakdown and lipolysis [60,61] and typical weight loss is seen in overt hyperthyroidism, and the reverse in hypothyroidism [62]. Adipose tissue is involved in the regulation of food intake and energy expenditure [59]. Various studies suggest that thyroid hormones and bodyweight are connected. Higher serum TSH levels are consistently seen in obese [63] and morbidly obese adults [64] and in obese children [65] compared with normal weight individuals. Studies of weight reduction by bariatric surgery [66,67] and gastric banding [68] have found a significant postoperative decrease in TSH in the two studies of bariatric surgery whereas the study of gastric banding found unchanged levels. Likewise, a significant decrease in TSH was also found after weight loss induced by 6 months of medical treatment for obesity, however, only in individuals with a weight loss > 10% body weight [63]. It has been suggested that the adipocyte derived hormone leptin affects thyroid hormone status, particularly through the stimulation of hypothalamic thyrotropin-releasing hormone gene expression [69]. Interestingly, a recent prospective study of a 2-month rapid weight loss programme in obese children found no association between change in TSH and leptin change, but a positive association with change in free T3 and free T4 [70]. The key questions is whether body weight and levels of TSH and thyroid hormones are causally connected.

#### **Reproductive measures and thyroid disorders**

Autoimmune thyroid diseases have an often 5-10 times higher prevalence in women than in men [41]. A role of immunological and/or hormonal differences between men and women is often suggested as an explanation for this female predominance. Women with GD tend to undergo clinical remission during pregnancy only to relapse in the postpartum period [71]. Following pregnancy 5.4% of all women develop postpartum thyroiditis [72]. These pregnancy-induced alterations could be explained by the immunosuppressive shift from a helper T-cell 1 dominance towards a T-helper-2-cell dominance, a change in trophoblast molecule expression or immunoregulatory effects of increased progesterone and estrogen levels [71]. Also, an immunomodulatory role of fetal microchimerism, i.e. presence of a small number of cells in the mother originating from the fetus might caused by transplacental passage during pregnancy, in development of thyroid autoimmunity has been suggested [73]. Furthermore, a prolonged exposure to endogen estrogenous metabolite 2-methoxyestradiol led to apoptosis and increased TPO-Ab release in rats [74], suggestive of a role sex hormones in the etiology of thyroid autoimmunity. If this were the case, the marked changes in the rates of hormone secretion induced by OCP, HRT, menarche, pregnancy, abortions, and menopause might also have an impact on development of thyroid autoimmunity.

#### **OTHER FACTORS AFFECTING TSH LEVELS**

Thyroid hormones and serum TSH levels exhibit diurnal-, day-to-day- [75,76], and a seasonal variations [77]. Acute or chronic

illness can affect thyroid hormone levels in clinically euthyroid patients causing low concentrations of thyroid hormones, called non-thyroidal illness [78]. Furthermore, a wide range of pharmacological agents are known to have an effect on thyroid hormone synthesis, secretion, binding and metabolism [79,80].

## **METHODS**

### **STUDY POPULATION**

The same study population was used in the analysis in all three papers. The DanThyr C1 cohort is a population-based study of Danish adults aiming at monitoring presence and development of thyroid disease under the introduction of mandatory iodine fortification of salt, July 1 2000, examined before (1997-98) and after (2008-10) fortification. The cohort comprised a population of 40,233 persons within defined age groups, born in Denmark, living in two regions of Denmark, Aalborg and Copenhagen, with different baseline iodine intake, using the national Civil Registration System. In this system, all inhabitants of Denmark can be identified by a unique 10-digit number. A region, age- and sex-stratified random sample of 9,677 individuals was drawn from the original study population of whom 9,274 were eligible for invitation to a health examination at Aalborg Hospital, Aalborg or Bispebjerg Hospital, Copenhagen. Of those invited to participate during 1997-98, 4,649 (50.1%) did so [43]. At the follow-up examination 72 of the 4,649 baseline participants had emigrated and 403 had died, leaving 4,174 to be invited of whom 2,465 participated (59.1%) (Figure 1, Paper I) [81]. Mean follow-up time was 11.2 years (range, 10.1-12.8 years). Mean exposure time to mandatory iodized salt was 8.6 years (range, 7.7-9.6 years). Women of four age groups (18-65 years) and men of one age group (60-65 years) were chosen due to the female predominance of thyroid diseases and the increased prevalence of thyroid disease with increasing age. All study participants of the DanThyr C1 cohort answered a standardized questionnaire and went through an interview, physical examination, and laboratory test (non-fasting levels of free T4, free T3, TSH, TPO-Ab and Tg-Ab, thyroglobulin, spot urine iodine excretion and, for a small subsample, 24 hour urine iodine excretion).

### **ETHICS STATEMENT**

Participants gave their written informed consent, and the studies were approved by the North Denmark Region Committee on Health Research Ethics (Nos. 2-16-4-0001-97, VN 96/208mch, and N-VN-19960208mch), and the Danish Data Protection Agency. The recommendations of the Declaration of Helsinki were followed.

### **EXPOSURES**

#### **Iodine fortification**

Everyone is exposed to dietary iodine to some extent. In studying such exposures the population should be classified according to their exposure status [82], in this case their level of iodine intake. Urinary iodine excretion is the best indicator of iodine intake in a population [4]. An individual measure of a person's iodine intake would be preferable, but due to considerable intra-individual variation in urinary iodine excretion the use of single value results for individuals is not feasible [83–86]. The design of this study made it possible to compare two regions with similar populations

but with different spot urine iodine excretion levels, using region as a proxy for iodine exposure. Spot urine iodine concentrations were determined at both examinations using the Ce/As method after digestion by alkaline ashing, as previously described [87,88]. The analytical sensitivity of the assay was 2 µg/L. The iodine laboratory was certified by the U.S. Center for Disease Control and Prevention EQUIP program.

### Body mass index /weight change

Weight and height were measured when participants were wearing light clothes and no shoes. BMI was calculated as weight (kg) divided by square of height (m<sup>2</sup>). As height was assumed to be constant in the follow-up period we used weight change instead of change in BMI in the prospective analysis. The difference between the follow-up and the baseline measurements, Δweight, was assumed to be approximately normally distributed.

### Gynaecological exposures

Data on gynaecological history were self-reported. The same questionnaire was filled out at baseline and follow-up, with the exception of a few questions that were omitted. We examined the following exposures: parity, abortions, miscarriages, age at menarche, menopausal status, use of oral contraceptives, hormone replacement therapy.

## OUTCOMES

### Serum TSH change

Serum TSH change was used as outcome measure in all three papers. TSH measurements relied on a single measurement at each examination. Blood samples were collected between 8.00 and 17.30, stored at -20 °C, and analysed in random order with respect to region, age and season of the year. Concentrations were measured with LUMItest assay (BRAHMS, Berlin, Germany) at baseline and with the Roche Modular E system by electrochemical luminescence using ELECSYS (Roche Diagnostics, Mannheim, Germany) at follow-up. An internal validation study confirmed that the results were comparable throughout the study period as previously described [89]. The normal reference interval for serum TSH used in this study, 0.4-3.6 mU/liter, was defined by the 2.5th and 97.5th percentiles of TSH in participants of the DanThyr C1 cohort baseline examination, without known thyroid disease, TPO-Ab levels under 60 kU/L (Dytest, RIA, BRAHMS), without thyroid enlargement and thyroid nodules determined by ultrasonography [90].

### TPO autoantibodies

TPO autoantibody status was used as outcome in Paper III. The presence of thyroid autoantibodies indicate an ongoing autoimmune process in the thyroid gland. A subsequent change in serum TSH concentration would therefore be expected. However, most of those with a positive thyroid autoantibody test are euthyroid as the degree of injury in the thyroid gland is not sufficient to cause hypothyroidism [72]. Thus, we expected thyroid antibody measurements to be a more sensitive indicator of early stage autoimmune thyroid disease than serum TSH. TPO-Ab measurements were analysed by radioimmunoassay (RIA) technique by DYNtest anti-TPO method (BRAHMS Diagnostica, Berlin, Germany) at baseline, as described earlier [91] and KRYPTOR anti-TPOn (BRAHMS Diagnostica, Berlin, Germany), an automated antibody assays were used at follow-up [92]. An internal validation study confirmed that the results were comparable throughout the study

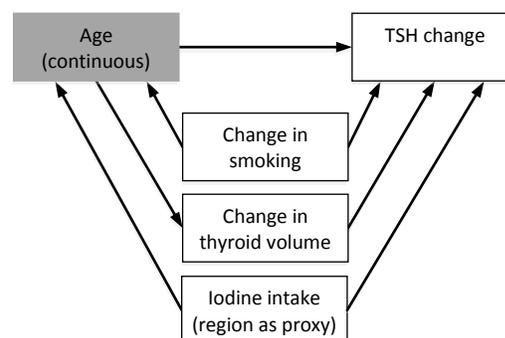
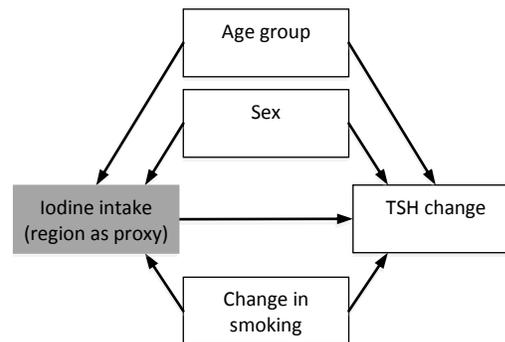
period [93]. Positive TPO-Ab status was defined as 30 kU/liter or higher according to the functional sensitivity given by the manufacturer and the limits used in previous DanThyr studies [91]. Change in TPO-Ab status was classified as 'increased during follow-up' in individuals with a TPO-Ab level below assay cut-off (30 kU/liter) at baseline and a positive TPO-Ab level (≥30 kU/liter) at follow-up (n=195), and 'not increased' when TPO-Ab level was unchanged during follow-up (n=1,480) or switched from positive to negative (n=35).

## OTHER COVARIATES

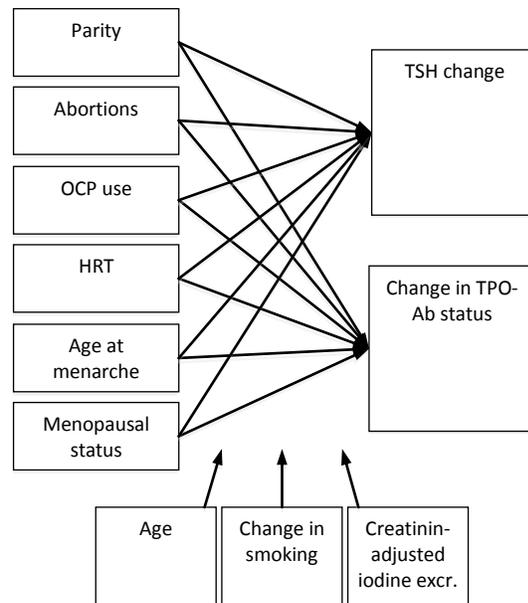
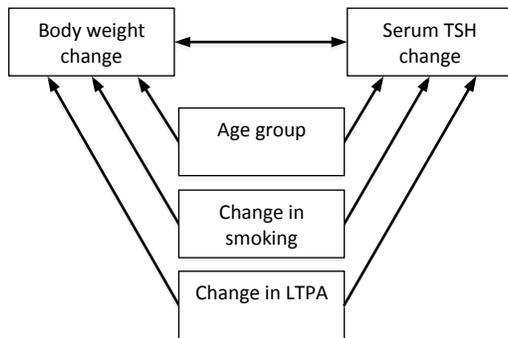
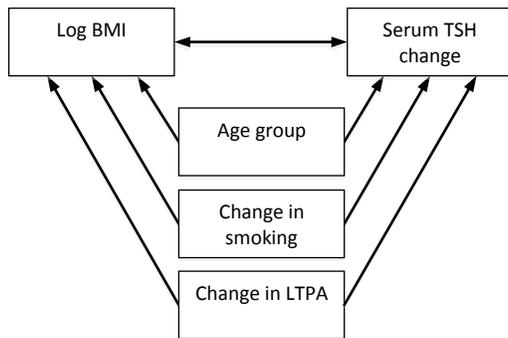
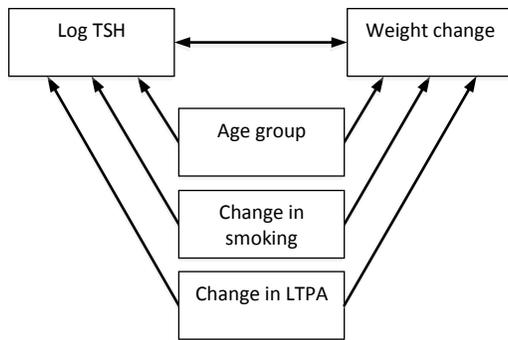
Age and change in smoking status were considered possible confounders in all analyses described in this thesis (diagrams in Figure 2 and Paper I, II, III). Sex stratified analyses were used in Paper II. Only women were included in the prediction model of TSH change and in the analysis of age effect (Paper I) due to the distribution of sex and age in the cohort, and in Paper III due to the nature of the exposures. To reduce a possible bias of the impact of thyroid volume on TSH levels with increasing age, we adjusted for change in thyroid volume, used as a proxy for multinodular goitre, in the analysis of the age effect on TSH changes (Appendix I, Paper I). Leisure time physical activity was included as a possible confounder in all analyses of the association between weight/BMI and TSH (Paper II). In Paper III we further adjusted for creatinine-adjusted urinary iodine excretion thereby attempting to reduce a possible bias of the increased iodine intake following the iodization (Paper III).

Figure 2 Using a diagram to identify variables that need to be adjusted for in estimating exposure effects

A) Analysis of the adjusted TSH change estimates and the age effect on TSH change (Paper I).



B) Analysis of the association between BMI/weight and TSH. LTPA leisure time physical activity (Paper II).



C) Analysis of the association between reproductive factors and TSH or TPO-Ab changes. OCP oral contraceptive pills, HRT hormone replacement therapy, TSH thyrotropin, TPO thyroid peroxidase antibodies (Paper III).

#### STATISTICAL ANALYSIS

Data were analysed using SAS version 9.2 in Paper I and II and version 9.3 (SAS Institute Inc., Cary, NC USA) in Paper III. Two-tailed  $P < 0.05$  was considered statistically significant. Repeated measurements in an individual are not independent of each other. Thus, the applied statistical technique in longitudinal studies must take this within-subject correlation into account [94]. To obtain adjusted TSH change estimates for each period in Paper I a multiple linear regression model with generalized estimating equations (GEE) and exchangeable correlation matrix was used. Also, in analysing age effect and sex-related differences in TSH change we used the GEE model. The assumption of linearity of age was tested by including age<sup>2</sup> in the age-effect model (Paper I). Linear regression models were used to determine the association between exposures (the prediction model in Paper I, Paper II, Paper III) and TSH change during follow-up. Logistic regression models were used in the analyses of the association between gynaecological exposures and change in TPO-Ab status (dichotomous) and estimates presented as odds ratios with 95% CI (Paper III). Sex stratified analyses were used in Paper II as described above. In the linear models (Papers I, II and III) adjustments were made for the baseline value of the outcome to adjust for regression to the mean and to allow changes in exposure variables to be interpreted as effects on outcome change from baseline to follow-up [95]. The Bonferroni method was used to adjust for multiple comparisons (Table 3, Paper III). The validity of the linear models was tested by checking normality of residuals, homogeneity of residuals, and linearity of the relationship between response variable and predictors. Logistic regression models were tested using the Hosmer-Lemeshow test.

#### KEY RESULTS

##### IODINE FORTIFICATION AS EXPOSURE (PAPER I)

Urinary iodine excretion levels are shown in Table 1 and 2 [96]. Before iodine fortification spot urinary iodine excretion was 68 µg/liter in Copenhagen and 53 µg/liter in Aalborg increasing to 84 µg/liter and 83 µg/liter, respectively, at follow-up.

**Table 1 Urinary iodine excretion levels from spot urine samples in the DanThyr C1 cohort**

	C1 Cohort	
	Baseline, 1997-98 Median (IQR)	Follow-up, 2008-10 Median (IQR)
Iodine excretion (µg/liter)		
Copenhagen, n=1,224	68 (39-112)	84 (48-137)
Aalborg, n=1,212	53 (30-93)	83 (46-129)

IQR interquartile range

Including persons taking iodine supplements

**Table 2 Urinary iodine excretion levels from spot urine samples in the DanThyr C2 cohort**

	C2 Cohort	
	2004-2005 Median (IQR)	
Iodine excretion (µg/liter)		
Copenhagen, n=1,785	108	
Aalborg (n=1,785)	93	

IQR interquartile range

Including persons taking iodine supplements

TSH increased significantly during the 11-year follow-up, from 1.27 mU/liter (95% CI, 1.23, 1.30) to 1.38 mU/liter (CI, 1.34, 1.43),  $P < 0.01$ . The increase was most marked in persons living in Copenhagen with the highest iodine intake, from 1.30 mU/liter (CI, 1.25, 1.35) to 1.49 mU/liter (CI, 1.43, 1.55),  $P < 0.01$ , whereas for persons living in Aalborg the increase was not statistically significant, from 1.24 mU/liter (CI, 1.19, 1.29) to 1.28 mU/liter (CI, 1.23, 1.34). The results were essentially unchanged after adjustment for sex, age, and change in smoking status. Age (continuous) was not associated with a significant TSH change after adjustment for region, change in smoking, and change in thyroid volume ( $P=0.93$ ).

In the prediction model, living in Aalborg, with the lowest iodine intake levels, determined a less steep serum TSH increase compared to living in Copenhagen, with a higher iodine intake (Table 3). Other determinants of TSH increase seemed to be presence of TPO-Ab at baseline and absence of goitre and multiple nodules at the baseline examination. Familial disposition for thyroid disease, a hypoechogenic thyroid ultrasound pattern at baseline examination, and changed smoking status during follow-up were not significantly associated with the serum TSH changes.

**Table 3 Predictors of TSH change at 11-yr follow-up in women (n=1,458)**

	Estimate	P
Age		0.03
Women, 18-22 yr	-0.029	
Women, 25-30 yr	-0.013	
Women, 40-45 yr	0.028	
Women, 60-65 yr (reference)	0	
Region (proxy for iodine intake)		< 0.01
Aalborg	-0.056	
Copenhagen (reference)	0	
Change in smoking during follow-up <sup>a</sup>		0.18
Current smoker (baseline and follow-up)	-0.041	
Former smoker (baseline and follow-up)	-0.020	
Stopped smoking during follow-up	-0.005	
Never smoker (reference)	0	

TPO-Ab ≥30 KU/liter at baseline		< 0.01
Yes	0.117	
No (reference)	0	
Hypoechogenicity by ultrasonography at baseline		0.83
Yes	0.006	
No (reference)	0	
Thyroid enlargement (volume >18/25 ml by ultrasonography)		< 0.01
Yes	-0.148	
No (reference)	0	
Multiple nodules at baseline		< 0.01
Yes	-0.104	
No (including solitary nodules) (reference)	0	
Familial disposition of thyroid disease		0.93
Yes	0.002	
No (reference)	0	

The DanThyr C1 cohort (Denmark) was examined at baseline (1997-1998) and reexamined 11yr later (2008-2010). Subjects treated for thyroid disease (n=228), subjects with no TSH measurements (n=36), and men (n=403) were excluded. Multiple linear regression model included serum TSH at follow-up as outcome variable and TSH at baseline, age, region, change in smoking status, TPO-Ab status, ultrasound hypoechogenicity, thyroid enlargement, multiple nodules, and familial disposition as predictor variables. The estimate defines the number of units of change in log (TSH) (y) in the specific class of the predictor (x) compared with the reference group. The reference groups are indicated.

<sup>a</sup> Persons who started smoking during follow-up (n=26) and occasional smokers (n=266) were excluded from analysis. Bjergved L et al., *J Clin Endocrinol Metab.* 2012 Nov;97(11):4022-9.

Persons with a low-normal TSH at baseline were more likely to have a below-normal TSH at follow-up (4.8%) compared to those with a TSH in the upper-normal tertile (0.2%). Likewise, individuals with a high-normal TSH at baseline had a higher probability of a TSH over the normal reference range at follow-up (10.5%) compared to those in the low-normal tertile (0.5%) (Paper I, Table 3).

#### BODY MASS INDEX AND WEIGHT CHANGE AS EXPOSURES (PAPER II)

No association was found between baseline log TSH and weight change during 11-year follow-up in either sex in a multiple linear regression model adjusted for age, change in smoking and change in leisure time physical activity ( $P = 0.17$ ; men,  $P = 0.06$ ). Baseline log BMI was not associated with change in serum TSH during follow-up when similarly adjusted (women,  $P = 0.21$ ; men,  $P = 0.85$ ). In the adjusted model, serum TSH change and weight change during follow-up were significantly associated in both sexes (Table 4). Similar results were found when excluding those with a serum TSH level outside the normal reference range at baseline.

**Table 4 Multiple linear regression of the prospective association between 11-year change in body weight and change in serum TSH in women and men**

		Number	Weight change per one-mU/L TSH increase	
			Model <sup>a</sup> kg (95 % CI)	Model <sup>b</sup> kg (95 % CI)
Individuals without thyroid disease	Women	1,703	0.2 (0.05–0.4)	0.3 (0.1–0.4)
	Men	399	0.8 (0.9–1.4)	0.8 (0.1–1.4)
			P=0.01	P=0.005

Individuals without thyroid disease and euthyroid at baseline	Women	1,577	P=0.01 0.6 (0.4–0.9)	P=0.02 0.6 (0.4, 0.9)
	Men	367	P<0.001 0.8 (0.1–1.4)	P<0.01 0.7 (0.02–1.3)
			P=0.02	P=0.04

CI confidence interval, TSH thyrotropin

<sup>a</sup> Unadjusted model

<sup>b</sup> Model adjusted for age, change in smoking and change in leisure time physical activity.

### REPRODUCTIVE FACTORS AS EXPOSURES (PAPER III)

No association was found between parity, abortions, OCP use, HRT use, age at menarche, menopausal status and 11-year serum TSH change in a multiple linear regression model adjusted for age, change in smoking, and creatinin-adjusted iodine excretion (Paper III, Table 2). Use of HRT and its duration were both associated with a significantly decreased risk (odds ratio) of positive TPO antibody status during follow-up. These associations were not statistically significant when applying the Bonferroni adjusted significance level. Parity, abortions, OCP use, age at menarche, and menopausal status did not seem significantly to influence the risk of change in TPO-Ab status during follow-up (Paper III, Table 3).

## DISCUSSION

### DISCUSSION OF RESULTS

Urinary iodine excretion levels increased significantly as expected during iodine fortification. Interestingly, another post-iodization DanThyr study in 2004-2005 [20] found a higher spot urinary iodine excretion concentration of the population than we did. This could suggest a decreased iodine intake in the population in the recent years despite an unchanged iodization programme.

A significant difference in TSH change was found in similar populations with different iodine exposure levels although the latter differences were small. Thus, individual development in thyroid function seems, at least partly, to be affected by the 8.6 years of exposure to mandatory iodized salt. As the normal thyroid gland is able to compensate by enhancing or limiting the use of available iodine [52,53], the thyroid function in most people in the studied cohort is probably unaffected. Nevertheless, multinodular goitre at baseline was a determinant of a less pronounced TSH increase which might be explained by iodine-induced hyperthyroidism in individuals with autonomous nodules [15,17,97,98]. Positive TPO-Ab status at baseline was strongly associated with a subsequent TSH increase which could be due to an iodine-induced abnormal inhibition of the thyroid function in individuals with thyroid autoimmunity [53–55]. Thus, it seems likely that susceptible persons in the cohort were subject to well-known adverse effects of an iodine fortification programme. A quantification of this possible impact would rely on a randomized trial which was not possible in our setting.

To the best of our knowledge there has been only one previous longitudinal study of an adult population examined before and after introduction of mandatory iodized salt, and,

in line with our results, it found a significant increase in TSH [99]. That study, by Golkowski et al. [99], however, has serious methodological limitations: information on the exposure time to iodized salts, and possible consequences of the shift in TSH assay were not available, no adjustment was made for possible confounders, and the allowed induction time of iodine-induced hyperthyroidism might be too short.

Using serum TSH as outcome measure makes it difficult to measure the benefits of the iodine-fortification programme in Paper I. First, to quantify the number of prevented cases of multinodular goitre or the prevention of deterioration of existing goitres after the fortification programme, reducing the risk of development of hyperthyroidism, would require a control group. Second, thyroid volume determined by thyroid ultrasonography might be a more specific indicator of iodine deficiency than serum TSH, which is also one of the measures recommended in adults by WHO [4,53,55].

Age could be a key variable in explaining the significant serum TSH increase during follow-up in our cohort (Paper I). A cross-sectional observational study in a population considered iodine sufficient has found a positive association between serum TSH and age in men and women independent of thyroid autoantibody status [100]. In iodine-deficient populations an inverse association between age and TSH was found in cross-sectional data [21,101], the former in baseline data of this study. Nevertheless, age is just a marker of time and an apparent effect of age might be confounded by factors as yet unknown [82]. The observed lack of association between age and TSH change (Paper I) after adjustment for iodine intake (region), change in smoking and change in thyroid volume (a proxy for multinodular goitre) imply that age was unlikely to have had a major effect on the observed TSH increase. The main age effect cannot be distinguished from the period and cohort effect in a single longitudinal cohort study [94], which challenges the interpretation of longitudinal data.

Our results regarding a low-normal TSH level as a determinant of future decreased serum TSH (Paper I), conform with those from a previous longitudinal study [54,102]. Also, our finding of a high-normal TSH values as determinant of a TSH over the normal reference range is in line with the results of Guan et al. [102]. Thus, development of thyroid disease seems to be a gradual process starting with small changes within the normal TSH range. The definition of the normal reference ranges upper limit remains contentious [103–105]. The National Academy of Clinical Biochemistry found that 95% of people without evidence of thyroid disease or thyroid autoantibodies had TSH concentrations below 2.5 mU/liter [36], followed by suggestions of lowering the upper-normal limit [103,105]. On the other hand, lowering the upper limit might lead to unnecessary or even harmful treatment of subclinical hypothyroidism [104,106] and the evidence for levothyroxine treatment of subclinical hypothyroidism is still scarce [107,108]. Our results of this gradual change in thyroid function towards disease (plausibly followed by a gradually increased risk of complications), could support the recently stated suggestions of considering thyroid hormone levels as “risk factors” for disease, rather than to regard a particular TSH level to be abnormal [103,109]. There is, however, a need for studies identifying TSH levels associated with symptoms and complications, and to determine which patient groups benefit from treatment.

BMI or body weight seems to be a positive correlate of TSH within the normal reference range in most [22–27], but not all [110,111] cross-sectional observational studies. The observed lack of association between baseline BMI and TSH change in our study (Paper II) is inconsistent with a recent study of Svare et al. [34], identifying baseline BMI as a negatively associated determinant of TSH change. On the contrary, baseline TSH might be a consistent non-determinant of weight change [23,34] which agrees with our results. Our results of a positive, significant association between change in weight and TSH change is in line with several previous longitudinal studies [23,27,33,34], though restricted to women in one study [33] and to non-smokers in another [27]. It is, however not possible to differentiate cause and effect in this association, and it does not indicate which of the two might be the causal factor or necessarily reflect a causal relationship. Data are still lacking on the phenotypic consequences of variation in free T<sub>4</sub> and free T<sub>3</sub>.

A lack of association between parity and TPO-Ab status has been found in several [28,29,32], but not all [112], cross-sectional population-based studies. Our finding of parity to be a non-determinant of increased TPO-Ab status has, to the best of our knowledge, not been reported in longitudinal data before. Somewhat supportive of our results is a study by Vanderpump et al. [113] who found during their 20-year follow-up that parity at first survey was not associated with an increased risk of development of hypothyroidism. In contrast to our result, a registry-based study [114] found an increased risk of a hospital contact with the diagnosis of Hashimoto's thyroiditis in mothers compared with nulliparous women, and a study [115] of 17,298 pregnant women screened during their first 20 weeks of gestation found a significant association between parity and TPO-Ab level > 500 IU/mL, but not for lower cut-off levels. Recently, a case-control study by Carlé et al. [116] found that women aged 55 and diagnosed with autoimmune overt hypothyroidism (defined as elevated TSH and low free T<sub>4</sub> and diagnosis by examination by their GP or in hospital) reported previous pregnancy, childbirths and induced abortions more often than their controls. Interestingly, an increasing risk (odds ratio) of autoimmune hypothyroidism was seen with increasing number of childbirths and induced abortions [116].

As regard our finding of an inverse association between ever using HRT and the time on HRT treatment and the risk of an increased TPO-Ab status during 11-year follow-up could be a false positive result after multiple testing. A larger, adequately powered longitudinal study could perhaps add something to the discussion. It would be interesting to measure absolute protective effect against thyroid autoimmunity and to see whether the magnitude of a possible reduced risk of thyroid autoimmunity is comparable to that associated with delaying menopause.

Our choice of a relatively low TPO-Ab cut-off level (30 kU/liter) as the definition in the outcome measure in Paper III could be an important explanation for the dissimilarities in the results outlined above. The prevalence rate of TPO antibodies ( $\geq 30$  kU/liter) is high, 13.1% in baseline data of this study [91], but their clinical importance in development of autoimmune thyroid disease should be considered. Using too low a TPO antibody cut-off level would make our antibody measure a less sensitive indicator of thyroid autoimmunity which could lead to misclassification tending to bias our estimates toward the null hypothesis. The chosen measures of thyroid autoimmunity in the three studies finding a

positive association with parity, hospital contacts with Hashimoto's thyroiditis [114], TPO-Ab levels >500 IU/mL [115], and a combined measure of a positive TPO-Ab status and hypoechogenic pattern by thyroid ultrasonography [112], could indicate that this might be the case. If it is the case that the studied exposure has an effect only on "real" disease development, the fact that we excluded all those undergoing treatment for thyroid disease at baseline and follow-up would reduce our chances of finding an effect of the exposures. Another explanation for the negative results in Paper III, besides the possibility that the studied gynaecological exposure (Paper III) does not influence our outcome, could be, as suggested by Thomas Brix et al. [41], that the predictive value of the chosen exposures is minor against the background of substantial environmental and genetic diversity. This presumably characterizes the complex phenotypes of thyroid diseases and many other chronic multifactorial diseases.

## METHODOLOGICAL CONSIDERATIONS

### *Study design*

The main strengths of this study are the longitudinal design ensuring temporality in the studied associations, that cause precedes effect in time; the long-term follow-up; the objective measurements of BMI, thyroid hormones, and thyroid autoantibodies; the detailed gynaecological history; and the detailed information on covariates. Temporality is a crucial causal criteria [82]. Nevertheless, it can be difficult to provide the proper time sequence for cause and effect, and other important criteria should be considered in causal inference e.g. biological plausibility and consistency with previous research. Thus, the longitudinal design does not solve the problem of causality [94]. The main superiority of the longitudinal over the cross-sectional study design is that the individual development of a specific outcome variable over time can be studied and its relation to development in other variables can be described [94]. Limitations of the design are that it is time-consuming, expensive, can be statistically challenging, and, as seen in the present study, the inevitable drop-outs during follow-up can reduce the possibility of studying rare outcomes.

### *Internal validity*

#### Selection bias

Factors that influence study participation could bias our results due to the relatively low participation rate. Willingness to participate could be influenced by a family history of thyroid disease or nonspecific symptoms of thyroid disease which might increase motivation to participate due to worries about disease risk [82]. Compared to participants at follow-up, nonparticipating individuals eligible for invitation to follow-up examination were more often regular smokers, had a higher frequency of bad or very bad self-rated health, a lower thyroid volume, and a lower frequency of positive autoantibody titre (Paper I, Table 1). The latter two measures could indicate that participants of the study were more prone than nonparticipants to suffer from thyroid disease, in line with the findings of Völzke et al [117] for early responders. No difference was, however, found regarding the levels of TSH, free T<sub>4</sub>, free T<sub>3</sub>, family disposition for thyroid disease, or frequency of thyroid disease at baseline, between the two groups in our study. Thus, selection due to thyroid disease seems less likely. A higher participation in the healthiest people was found in several studies [82], which is in line with our findings regarding regular smoking

and self-rated health status. In contrast, no significant difference in BMI, hypertension, or high risk alcohol consumption was found between participants and non-participants in our study.

#### Information bias

The use of region as a proxy for iodine exposure (Paper I) may have led to misclassification. Some participants moved to a region with different exposure status in the follow-up period. This nondifferential misclassification would, however, tend to bias our estimates toward the null hypothesis. Also, the observed change in spot urinary iodine excretion in our cohort [20,96] (Table 1 and 2) could even out the regional differences in iodine exposure and question the validity of region as a proxy for different iodine exposure levels. Nevertheless, this would also bias our estimates towards the null hypothesis. An individual measure of a person's iodine intake would have been preferred, as described in the methods section, but this was not feasible. In addition, a misclassification of information obtained by questionnaires regarding some exposures could be a methodological concern e.g. 'family history of thyroid disease' could be subject to misclassification. A low validity of self-reported history of thyroid disease was described by Brix et al. [118], and the validity of family members thyroid disease is probably even lower.

#### Confounding

A confounding factor is an unaccounted-for factor associated with both exposure and outcome, resulting in a confusion of effects of the studies association. The effect of the exposure is mixed together with the effect of another variable biasing the results [82]. Residual confounding from unmeasured variables such as non-thyroidal illness (NTI), defined by low thyroid hormone concentrations in clinically euthyroid patients following any acute or chronically illness [78], cannot be ruled out (Paper II). Also, pharmacological agents affecting thyroid function and body weight could be an unmeasured confounding factor (Paper II). Nevertheless, our study population is relatively healthy and the impact of NTI and pharmacological treatment on our estimates is probably small.

Moreover, a possible confounding effect of period (time of measurement) and/or cohort effects (group of subjects born in the same year) should be considered when analysing longitudinal data[94]. We consider the impact in this study to be small.

#### **External validity**

The distribution of sex in the studied cohort and the low participation rate might limit the validity of generalization from the results. Despite the absence of statistical representativeness of the Danish population, the biological representativeness based on scientific knowledge in our study should be considered, as stated by Rothman [82]. We believe that the biological mechanisms thought to be responsible for the observed changes in TSH in our study could, to a high extent, be applied to a broad population.

#### **CONCLUSIONS AND PERSPECTIVES FOR FURTHER RESEARCH**

Observational studies serve a wide range of purposes from refutation or confirmation of previous findings to the exploration of new ones [119].

The results of this PhD project are important for several reasons:

First, this study is the first to describe thyroid function changes after long term (8.6 years) exposure to mandatory iodine fortification of salt and important determinants of the

observed TSH change (Paper I). Urinary iodine excretion levels increased significantly as expected during iodine fortification. A significant increase in serum TSH was found, and the difference found between regions with different iodine intakes could indicate that iodine explains, at least partly, the TSH increase. The presence of baseline TPO-Ab determined a more pronounced TSH increase than its absence, and that of thyroid multinodularity determined a less pronounced TSH increase. This may indicate that susceptible individuals were subject to well-known adverse effects [15,17,53–55,97,98]. It was not possible in this study design to measure the benefits of the iodine fortification programme, such as prevented cases of multinodular goitres nor the prevention of aggravation of existing goitres, nor to quantify the number of individuals affected by adverse effects.

Second, TSH values in the upper and lower normal range at baseline were identified as determinants of future (11-year) serum TSH abnormalities (Paper I), suggestive of a gradual development of thyroid disorders. Also, a significant weight increase for every one unit TSH increase was found, but without baseline BMI being a determinant of 11-years TSH change and without baseline log TSH being a determinant of future weight change (Paper II). These results are an important contribution to the discussion about the role of thyroid hormone levels within the normal range, set points and the association with body weight. Whether body weight and serum TSH are causally connected remains to be proven. A more precise outline of the shape of their relationship with a large number of subjects repeatedly studied could be informative.

Third, our study of the etiology of female reproductive factors in development of thyroid autoimmunity using longitudinal data is a valuable supplement to the literature which as yet has almost exclusively been based on cross-sectional data (Paper III).

The observed significantly lower urinary iodine excretion levels in 2008-10 than in a Danish cohort in 2004-05 [20], could indicate a decrease in iodine intakes in the recent years. Our study thereby is an example of the importance of persistent monitoring of fortification programmes if the goal is sustainable iodine sufficiency. Another challenge faced is the sodium-reducing interventions requiring coordination with salt iodization programmes to sustain adequate iodine intake levels as salt intake declines [120]. A future investigation of transnational cohorts with some countries undergoing iodine fortification and others not, might be informative.

It is important to understand whether, and to what extent, modifiable factors affect development of thyroid dysfunction, and to understand the role of thyroid hormones within the normal range for optimal treatment. A stronger focus on determinant research using adequately powered longitudinal studies, rather than repetition of cross-sectional associations, could improve the understanding of risk factors of development of thyroid dysfunction. It is, however, important to realize that the longitudinal study neither solve the problem of causality nor is the optimal design to measure the impact of an intervention, e.g. iodization of salt, due to the lack of control group, but can be informative in the study of determinants. Furthermore, the identification of environmental factors with a relatively small effect on the development of thyroid dysfunction compared to the importance of the background risk of the diverse spectrum of genes and environmental factors remains a challenge [41].

## ABBREVIATIONS

AITD autoimmune thyroid disease  
BMI body mass index  
CI confidence interval  
DanThyr Investigation on Iodine Intake and Thyroid Disease  
GD Graves' Disease  
GP general practitioner  
HT Hashimoto's Thyroiditis  
HPT axis Hypothalamic-pituitary-thyroid axis  
HRT hormone replacement therapy  
ICCIDD International Council for the Control of Iodine Deficiency Disorders  
LTPA leisure time physical activity  
NTI non-thyroidal illness  
OR odds ratio  
OCP oral contraceptive pills  
T<sub>3</sub> triiodothyronine  
T<sub>4</sub> thyroxine  
TSH thyrotropin  
Tg thyroglobulin  
TPO-Ab thyroid peroxidase autoantibody  
UIE urinary iodine excretion  
UNICEF United Nations Children's Fund  
WHO World Health Organisation

## SUMMARY

**Introduction:** Thyroid disorders are common with occurrence primarily determined by the availability of dietary iodine. Iodine fortification programmes are internationally recommended to ensure sufficient iodine intake in populations. An understanding of the role of thyroid hormone levels within the normal range, set points and etiological factors related to thyroid disease development is important for optimal prevention and treatment. Limited data, however, exist regarding the impact of iodine fortification on thyroid function development. Additionally, the relation between body weight and thyrotropin (TSH) within the normal range and the role of female reproductive factors in the etiology of thyroid autoimmunity is debated.

**Objective:** The aim of this PhD project was to analyse the effect of a nationwide iodine fortification programme on individual development in thyroid function and to identify concurrent determinants for the possible changes. Furthermore, we aimed to investigate the association between weight and serum TSH change as well as the association between female reproductive factors and change in TSH and thyroid peroxidase antibody (TPO-Ab) status.

**Methods:** A longitudinal population-based study of the DanThyr C1 cohort examined before (1997-1998) and after (2008-2010) the introduction of mandatory iodine fortification of salt on July 1 2000. A total of 2,465 individuals participated in the follow-up examination. The main outcome measure was change in serum TSH. Change in TPO-Ab status was additionally used in Paper III.

**Results:** Urinary iodine excretion levels increased significantly during follow-up. Serum TSH also increased significantly, most pronounced in the region with the highest iodine intake, whereas the increase was not significant in the low-iodine-intake region. The presence of TPO-Ab at baseline and absence of goitre and multiple nodules were identified as determinants of TSH increase. Moreover, a low-normal TSH at baseline was a determinant of future decreased serum TSH, while likewise a high-normal baseline TSH values determined a TSH above normal reference range at follow-up. A positive association between 11-year serum TSH change and weight change was found, but without baseline body

mass index being a determinant of future weight change and without baseline TSH being a determinant of future weight change. An inverse association between the time on HRT treatment and the risk of increased TPO-Ab status during follow-up was found, but the association was not significant when applying the Bonferroni adjusted significance level and not associated with TSH change. Parity, OCP use, abortions, age at menarche and menopausal status were associated neither with TSH change nor with increased TPO-Ab status during follow-up.

**Conclusion:** TSH increased significantly, and the difference between regions with different iodine intakes could indicate that iodine, at least partly, explains the TSH increase. The identified determinants of TSH change may indicate that susceptible individuals were subject to well-known adverse effects of iodine fortification. The predictive value of TSH on future TSH levels suggests a gradual development of thyroid disease. Whether body weight and TSH are causally connected remains to be proven. These results are an important contribution to the discussion of the role of thyroid hormones level within the normal range, set points and the association with body weight. A minor role, if any, is suggested for the studied female reproductive factors in development of thyroid autoimmunity. The longitudinal study neither solves the problem of causality nor is of the optimal design to measure the impact of iodization of salt, but can be informative in the study of determinants.

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