The relationship between iodine intake and serum thyroglobulin in the general population.

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Short running title: Thyroglobulin and iodine intake.

Summary

The relationship is shown between a concentration of urinary iodine and serum thyroglobulin in population studies carried out on a general population that was randomly selected from the registry of the General Health Insurance Company (individuals aged 6-98 years, 1751 males, 2420 females). The individuals were divided into subgroups with a urinary iodine concentration of <50, 50-99, 100-199, 200-299 and ≥300 μg/l. The mean and median of thyroglobulin were calculated in these subgroups.

Tg concentrations were dependent on gender (males < females), age (thyroglobulin increased with age) and statistically significant negative relationship was observed between thyroglobulin and urinary iodine in individuals with urinary iodine < 300 μg/l and the age under 65 years. Upper nonparametric tolerance limits of thyroglobulin in relation to iodine intake were calculated in subgroup of normal individuals (n=1858, thyroglobulin, urinary iodine, thyrotropin and free thyroxine were within the normal reference range). Upper limits were dependent on gender and age. The total value of upper limits is 44 μg/l; for individuals aged 6-17 years it is 39.1; 18-65 years=51.4 and 66-98 years=60.6 μg/l. In general, thyroglobulin serum concentrations higher than 40 μg/l should be an indicator for determining urinary iodine.

Keywords: Thyroglobulin, Iodine, Population studies, Tolerance limits, Normal individuals

Introduction

About 31% (1900.9 million) of the world's population is estimated to have insufficient iodine intake, with the most affected World Health Organization regions being South-East Asia and Europe (WHO, UNICEF, ICCIDD 2007). The Czech Republic belongs historically to the area of iodine deficiency; however, since the year 2000 the iodine nutrition has not been a serious public health problem (Zamrazil *et al.* 2004). The recommended daily intake of iodine should be 90 μg for preschool children (0 to 59 months), 120 μg for schoolchildren (6 to 12 years), 150 μg for adolescents (above 12 years) and adults and 250 μg for pregnant and lactating women (WHO, UNICEF, ICCIDD 2007). Several indicators are used to assess the iodine status of a population: thyroid volume, urinary iodine (UI) and the blood constituents, thyrotropin (TSH), and thyroglobulin (Tg) (WHO 2004). A major indicator corresponding to iodine nutrition and reflecting recent changes in iodine intake in period of days is concentration of iodine in urine. According to World Health Organization (WHO), United Nations Children's Fund (UNICEF) and International Council for Control of Iodine Deficiency Disorders (ICCIDD), urinary iodine below 20 μg/l denotes severe iodine deficiency, between 20-49 moderate, between 50-99 mild iodine deficiency, UI between 100-199 is adequate iodine intake, UI between 200-299 more than adequate, and UI more than 300 μg/l is excessive iodine intake (WHO, UNICEF, ICCIDD 2007, WHO, UNICEF, ICCIDD 2001). UI concentrations of pregnant women under 150 μg/l indicate an insufficient iodine intake, between 150-249 is adequate, between 250-499 is above the required level and over 500 μg/l is excessive iodine intake (WHO, UNICEF, ICCIDD 2007). Median urinary iodine concentration of 100 μg/l is adequate iodine intake for lactating women and children below 2 years of age (WHO, UNICEF, ICCIDD 2007).

Thyroid volumes reflect a population's history of iodine nutrition, but not its present iodine status (Zimmermann 2004). Thyroid volume may not return to its normal size for

months or years after an iodine deficiency correction (Delange *et al.* 2001), thus it is not a good indicator of iodine deficiency disorders after the introduction of iodized table salt. Iodine deficiency lowers circulating T4 and raises the serum TSH, so that iodine-deficient populations generally have higher serum TSH concentrations than do iodine-sufficient groups, but the difference is not great and much overlap occurs between individual TSH values. Therefore, the blood TSH concentration in school-age children and adults is not recommended for routine use as an indicator of iodine intake (WHO, UNICEF, ICCIDD 2001).

Thyroglobulin (Tg) may be a promising functional biomarker of both iodine deficiency and excess (Zimmermann *et al.* 2013). Thyroglobulin originates only in the thyroid gland where the synthesis of thyroid hormones is involved. It is the major iodoglycoprotein of the thyroid gland (molecular weight of soluble dimer about 660 kDa, 0.1-2.0% iodine; 8-10% total carbohydrate with galactose, mannose, fucose, N-acetyl glucosamine and sialic acid residues (Venkatesh and Deshpande 1999), which consists of two identical subunits (homodimer), and belongs to the type B carboxylesterase/lipase family (Park and Arvan 2004). The serum Tg concentrations primarily reflect three factors: a) the mass of differentiated thyroid tissue present; b) any physical damage to or inflammation of the thyroid gland; and c) the magnitude of thyrotropin receptor stimulation (Spencer *et al.* 1996). The thyroid hyperplasia and goitre characteristic of iodine deficiency increases serum Tg levels, and, in this setting, the concentration of serum Tg reflects iodine nutrition over a period of months or years (WHO, UNICEF, ICCIDD 2007).

WHO proposed in 1994 that a median Tg concentration of less than 10 μg/l in a population indicated iodine sufficiency (WHO, UNICEF, ICCIDD 1994). The value of thyroglobulin as an indicator of global IDD status has not yet to be fully explored (WHO 2004), but the results from population studies show that thyroglobulin seems to be a valuable

indicator of thyroid status in respect to its sensitivity to recent changes in iodine nutrition (Zimmermann 2004, Benmiloud *et al.* 1994, Knudsen *et al.* 2001, European Commission, Scientific Committee on Food 2002). The main problem is the specification of Tg cut-off value corresponding to various levels of iodine intake. The use of Tg for monitoring iodine status is limited by a large interassay variability and a lack of reference data for Tg in healthy, iodine-sufficient individuals (Zimmermann *et al.* 2006). In iodine-deficient areas the serum Tg concentration is elevated due to TSH hyperstimulation or thyroid hyperplasia (Zimmermann *et al.* 2006). Serum thyroglobulin and urinary iodine concentration are the most appropriate indicators of iodine status and thyroid function under conditions of increasing iodine supply (van den Briel *et al.* 2001), because thyroid volume, thyroid nodularity or iodine excretion had close associations to serum Tg (Knudsen *et al.* 2001). Significant inverse correlations were found for relationships between measures of urinary iodide excretion and serum thyroglobulin (Thomson *et al.* 2001, Simsek *et al.* 2003, Skeaff *et al.* 2012, Raverot *et al.* 2012). For example, Buchinger, *et al.* (1997) have shown that the mean serum thyroglobulin of 2311 untreated euthyroid patients decreased progressively as the urinary iodine concentration rose, but that this change did not reach statistical significance. In a cross-sectional study in primary schools in 12 countries with children 6 to 12 years of age (n=2512) it was found that, over a range of iodine intakes from severely deficient to excessive, Tg concentrations showed a clear U-shaped curve (Zimmermann *et al.* 2013). According to the authors Zimmermann MB et al. (2013, 2006), median values of Tg in dried whole blood spots between 13 and 40 μg/l indicate iodine sufficiency in the population of 5 to 14 year-old children. The question is if this reference range can be used for other groups in the population.

Serum Tg is currently measured by immunoassay, and Tg belongs among the difficult serum assays in current routine diagnostic due to the inhomogeneity of the large Tg molecule,

where various isoforms of Tg exist with differences both in the primary structure and iodine or carbohydrate content. These factors determine the three-dimensional conformation of the molecule, and thus they can reduce the epitopes important for immunoanalytical interactions. In addition, the presence of circulating autoantibodies against Tg may substantially interfere in determining serum Tg, and these autoantibodies are an important limitation concerning both the precision and accuracy of immunoanalytically measured serum Tg, especially if immunometric techniques (false-decreased results, (Giovanella and Ceriani 2011)) are used. Competitive radioimmunoassay seems to be more resistant to circulating autoantibodies against Tg (Spencer *et al.* 2005). Nevertheless, the reduced availability of tracer (thyroglobulin labelled with radioiodine) due to binding to autoantibody may lead to false-positive interference in competitive radioimmunoassays. More than adequate iodine intake (Teng *et al.* 2011) or iodization program (Bülow Pedersen *et al.* 2011) was associated with an increase in the prevalence of autoantibodies against Tg. Heterophilic antibodies also interfere in Tg assays and generally result in false elevations of Tg (Clark and Franklyn 2012, Preissner *et al.* 2003), but treatment using heterophile-blocking agents prevented the potential impact of heterophilic antibodies on Tg analysis (Giovanella *et al.* 2009). A collaborative effort, sponsored by the Community Bureau of Reference of the Commission of the European Communities, produced a Tg standard CRM-457 (Feldt-Rasmussen *et al.* 1996a, 1996b). The applications of this serum Tg reference material can improve the interassay variability (Zimmermann *et al.* 2006), but in respect to the complicated three-dimensional structure of Tg and the various Tg isoforms, the standardization of antibodies used to determine circulating Tg can be a more important step for obtaining the comparable results across many commercially available Tg assays (Bilek *et al.* 2009). The application of CRM-457 reduced the interassay coefficient of variations from 47 % to 37 % (Spencer *et al.* 2005) which means that significant differences among various kits remain.

In the 1960s the first hemagglutination techniques were developed to measure Tg in serum (Torrigiani *et al.* 1969). More convenient RIA techniques were introduced in the 1970s (Van Herle *et al.* 1973). Immunoradiometric assays used since the 1980s (Mariotti *et al.* 1982) improve the functional sensitivity of Tg from 3-5 ng/ml (Schlumberger and Baudin 1998) to less than 1 ng/ml (Smallridge *et al.* 2007), and the correlation with previous RIAs was excellent in sera not possessing an interference in the assay (Schlumberger and Baudin 1998). Novel serum Tg assays that use mass spectrometry may avoid the issue of autoantibody interference and other problems with currently available immunoassays for Tg (Hoofnagle and Roth 2013, Kushnir *et al.* 2013). In the work we presented our results concerning the relationship between the concentration of urinary iodine and serum Tg in a randomly selected general healthy population.

Materials and methods

The concentration of urinary iodine was determined using the alkaline ashing of urine specimens preceding the Sandell-Kolthoff reaction with brucine as a colorimetric marker. Details about the spectrophotometric method were published formerly (Bilek *et al.* 2005).

Serum Tg was determined in 1997-1999 by the 2-step enzymoimmunometric (ELISA, code Enzymun-Test Tg, Boehringer-Mannheim, Germany) sandwich assay (1932 individuals aged 5-96 (30+/-21) years, 819 males, 1113 females) using monoclonal mouse antibodies and streptavidin technology. The standards have been calibrated against the BCR standard CRM 457 (reference range 0-85 μg/l). From 2000 to 2006 serum Tg was determined with the 1-step electrochemiluminometric (ECLIA, code 1820834 Elecsys Tg Immunoassay, Roche Diagnostics, Mannheim, Germany) sandwich assay (2239 individuals aged 6-98 (29+/-22) years, 932 males, 1307 females) using monoclonal mouse antibodies and streptavidin technology. The standards have been also calibrated against BCR standard CRM 457

(reference range 0-85 μg/l). A linear regression of Elecsys Tg (Y) with Enzymun-Test Tg (X) using 98 clinical samples gave the following correlations according to the Roche information in the package insert: $Y = -3.20 + 1.02X$, correlation coefficient $r=0.995$.

Human subjects

Basal concentration of urinary iodine and serum Tg were determined in the general healthy population of 4171 randomly selected individuals from the registry of the General Health Insurance Company aged 5-98 (30+/-22) years (1751 males, 2420 females, **total population**). This population study was conducted in 1997-2006 in the Czech Republic. All individuals included in the study were in addition to biological sampling also examined by an endocrinologist. In 87.2 % of total population described as **normal population** on the basis of laboratory diagnostics and medical examination (89.7 % of males, 85.5 % of females) was found simultaneously measured serum TSH and FT4 in reference ranges. Subclinical hypothyroidism was observed in 5.0 %, hypothyroxinemia in 4.4 %, clinical hypothyroidism in 1.6 %, subclinical hyperthyroidism in 0.8 %, clinical hyperthyroidism in 0.4 %, hyperthyroxinemia in 0.4 %, central hyperthyroidism in 0.1 %, central hypothyroidism in 0.1 % of total population and 0.1 % of subjects were missing some of TSH or FT4 determination. The investigation was approved by the local ethical committee.

Statistical methods

All statistical calculations (means, standard deviations, medians, frequency distributions, descriptive tables, Mann-Whitney U or Wilcoxon rank-sum test for difference in medians, tolerance intervals) were performed using the statistical computer program NCSS 2004 (Number Cruncher Statistical Systems, Kayville, Utah, USA).

Results

The individuals were divided into subgroups according to their level of iodine intake, i.e. to the subgroup with urinary iodine concentration < 50 (moderate iodine deficiency), 50-99 (mild iodine deficiency), 100-199 (adequate iodine intake), 200-299 (more than adequate iodine intake) and ≥300 (excessive iodine intake) μg I/l of urine. In these subgroups the mean and median of Tg were calculated. The distribution by age and gender of the total population is shown in Figure 1. Tg values were not distributed normally, therefore nonparametric tests were used for statistical analysis. The mean±SD (sample standard deviation) and median values of Tg were 27.9±54.8, 18.0 μg/l for total population (n=4171) and 27.4±53.2, 18.3 μg/l for normal population (n=3639). We observed the statistically significant differences ($p<0.0000$) of Tg concentrations between males and females both in total population (males: mean 21.4±19.3, median 16.8; females: mean 32.6±69.7, median 19.4) and normal population (males: mean 21.5±19.5, median 16.9; females: mean 31.8±68.1, median 19.8) according to Mann-Whitney U or Wilcoxon rank-sum test for difference in medians. The increased concentrations of Tg in females existed in all subgroups of subjects with given levels of urinary iodine (ie. moderate, mild iodine deficiency, adequate, more than adequate and excessive iodine intake). The dependence of Tg values on age and gender in normal population, where both Tg and UI are in reference ranges, is shown in Table 1.

Figure 2 shows that both the mean and median of Tg decreased in relation to the increase in urinary iodine. However, a slight increase in both mean and median values of Tg is shown in the subgroup with excessive iodine intake. A partial U shape is shown on the resulting curve. Because the distribution of Tg values was not normal in particular subgroups, it was used the Mann-Whitney U or Wilcoxon Rank-Sum Test for Difference in Medians for evaluation of statistical significant differences of Tg among particular subgroups based on the concentration of urinary iodine. The differences of Tg concentrations were statistically

significant both in the total and normal population between subgroups of moderate and mild iodine deficiency (total population p=0.0047, normal population p=0.0199), between mild iodine deficiency and adequate iodine intake (total population p=0.0019, normal population p=0.0010), adequate and more than adequate iodine intake (total population p=0.0039, normal population p=0.0103), but no significant difference was found between more than adequate and excessive iodine intake (total population p=0.2015, normal population p=0.2087). It is clear from Figure 2 that substantially higher mean Tg values were found in the subgroup of total population with moderate iodine deficiency (mean Tg=42.7 μg/l), compared to the same subgroup of the normal population (mean Tg=35.6 μg/l). The mean values in other subgroups are nearly the same.

The influence of age on the mean and median values of Tg in subgroups of total or normal population according to their iodine intake is shown in Figure 3. The differences of Tg concentrations were statistically significant ($p<0.0000$) both in the total and normal population (Table 1) among subgroups of individuals aged 6-17, 18-65 and 66-98 years. The trend of Tg decrease with the increase of urinary iodine is evident in subjects aged 6–65 years. The situation in individuals over 65 years of age is quite different. There both the mean and median Tg values increase with the increased iodine intake. The mean Tg values in the subgroup of the elderly with excessive iodine intake were more than 110 μg/l, and the median values of Tg were above 38 μg/l both in the normal and total population. The difference of mean or median values of Tg exists between the total and normal population in the subgroup of moderate iodine deficiency. The difference is higher in individuals aged 6-17 years (total population: mean Tg=37.7 μg/l, median Tg=20.2 μg/l; normal population: mean Tg=26.8 μg/l, median Tg=17.2 μg/l), in comparison with individuals aged 18–65 years (total population: mean Tg=46.1 μg/l, median Tg=34.7 μg/l; normal population: mean Tg=41.0 μg/l, median Tg=36.1 μg/l).

Upper tolerance limits of Tg in relationship to iodine intake are shown in Table 1. Limits were calculated from individuals of the normal population, where both the concentration of Tg and urinary iodine were in normal reference range (Tg=0–85 μg/l, UI=100–200 μg/l). If the serum Tg is higher than the tolerance limits, one of the possible factors is insufficient iodine intake and the evaluation of urinary iodine in these individuals can be recommended. It is evident from Table 1 that the tolerance limits in terms of upper one-sided 95 % nonparametric tolerance bound of Tg are influenced by gender and age. Limits are higher in women than in men and they increase with the age. The total value of tolerance limits is 44 μg/l, for individuals aged 6–17 years the value is 39.1, 18–65 years=51.4 and 66-98 years=60.6 μg/l.

Discussion

Difficulties in the immunoanalytical determinations of Tg are mainly the prevalence of anti-Tg autoantibodies and their influence to underestimation (immunometric assays) or overestimation (competititve immunoassays) of Tg and the large interassays variability. This has made it difficult to establish normal reference ranges and/or cutoffs to distinguish the severity of iodine deficiency (Zimmermann 2009). An analysis of data from the 10 intervention studies showed that Tg does appear to be a useful marker of iodine status in children and adolescents, but there was little evidence of its usefulness in other groups (infants, adults, postmenopausal women, elderly) and it does not appear to be useful during pregnancy and lactation (Ristic-Medic *et al.* 2009).

Based on our results, we tried to find answers to some of the issues of Tg as a marker of iodine supply in the population. We found statistically significant differences of Tg concentrations between men and women. The mean values of Tg were about 30% higher in women than men. Our results are in agreement with the fact that serum Tg is elevated in

subjects with deficient iodine intake. As can be seen in Figures 2 and 3, the increase of urinary iodine is accompanied by a decrease of serum thyroglobulin concentrations. In our examined subjects aged 6–98 years the relationship between a urinary iodine and concentration of Tg was statistically significant in all subgroups based on iodine intake except for differences between the subgroups of more than adequate and excessive iodine intake. Figures 2 and 3 show that the dependence corresponds to the partial U-shaped curve. Similar results were found for example by Zimmermann *et al.* (2013, 2006). However, as shown in Figure 3, the results are dependent on the age and they are not valid for the population over 65 years of age. In elderly people there was shown only an increase of mean or median values of Tg with an increase of UI across all subgroups based on iodine intake and in more than adequate to excessive iodine intake the mean and median values were 112 (mean) or 39 (median) μg/l. It is important that, while in the subgroups of mild deficiency to excessive iodine intake in the total and normal population the mean and median values of Tg are almost the same, they differ in the subgroup corresponding to a moderate iodine deficiency. From Figure 3 it is clear comparing the normal and total population that an increase of Tg was higher in children and in people over 65 years, while in adults aged 18-65 years, the difference was not so significant. In other words, it is possible to say that children and elderly people with any thyroid disorders are more sensitive to iodine deficiency than adults.

The mean or median values of Tg was found nearly 2 times higher in our study than was published for the normal population with sufficient iodine intake by WHO (WHO, UNICEF, ICCIDD 1994), Spencer and Wang. (1995) or Vejbjerg *et al.* (2009), which states that a median serum Tg in the population less than 10 μg/l is a marker of iodine sufficiency using the immunoluminometric assay (Lumitest, Brahms, Germany). The problem with cut-off value of Tg in relationship with iodine nutrition is that for example serum Tg normal reference values in a group of healthy subjects (209 males, 229 non-pregnant females, age

34.7±13.1 years) were determined in the range of 1.40-29.2 ng/ml (males) or 1.50-38.5 ng/ml (females) using Beckman Coulter UniCel DxI 800 immunoanalyzer (Giovanella *et al.* 2012). However, the influence of iodine intake in this group of subjects is not known and, on the other hand, median values of Tg between 13 and 40 μg/l determine the iodine sufficiency in the population (Zimmermann *et al.* 2013). The tolerance limits of Tg, whose excessive values should be considered, along with iodine deficiency, as one of the factors that increase Tg, are listed in Table 1. The limits were calculated from the values of normal population, where both the UI and Tg were inside the reference range. Our results suggest that the limits in the term of upper one-sided 95 % nonparametric bound of Tg are dependent on the gender and age. They are higher in women than men and they increase with age. The total tolerance limit of Tg was 44.2 μg/l for the whole normal population with values of UI and Tg inside the reference ranges. These limits were 39.1 μg/l for children aged 6–17 years, 51.4 μg/l for adults aged 18–65 years and 60.6 μg/l for people aged 66–98 years. There is agreement between our results and the results published by the authors Zimmermann et al. (Zimmermann *et al.* 2013) for school children aged 6-12 years (n=2512) where median values of Tg determined in standardized dried blood spots between 13 and 40 μg/l are indicative of iodine sufficiency.

In conclusion, Tg seems to be a useful marker of iodine deficiency in a population, where thyroid diseases are not too frequent. Tg values are dependent on gender and age, and our results also show that, under conditions of iodine deficiency, the dietary iodine factor that increases the concentration of Tg in the circulation is inadequate, and thus directly points to the fact that thyroid disorders are amplified by iodine deficiency. Children and the elderly are more affected in this respect by insufficient iodine supply.

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Gender	Age (years)	Number of subjects	Mean Tg (μg/L)	Median Tg (μg/L)	Upper tolerance limit of Tg (μg/L)
Both	6 – 98	1858	21.4	17.5	44.2
Female	6 – 98	1038	22.6	18.3	48.4
Male	6 – 92	820	19.9	16.6	40.0
Both	**6 – 17**	**970**	**19.5**	**16.3**	**39.1**
	18 – 65	**798**	**23.2**	**19.1**	**51.4**
	66 – 98	**90**	**25.7**	**19.8**	**60.6**
Female	6 – 17	471	20.1	16.9	40.6
	18 – 65	504	24.3	19.8	53.8
	66 – 98	63	27.7	22.7	73.4
Male	6 – 17	499	18.9	15.9	38.6
	18 – 65	294	21.5	17.7	49.2
	66 – 92	27	21.1	18.7	60.3

Table 1. Upper tolerance limits of Tg.

Upper one-sided 95 % nonparametric tolerance bound of Tg based on the gender and age in the normal group of subjects where the concentration of Tg was within the reference range 0-85 μg/L and the values of urinary iodine correspond to the adequate iodine intake with urinary iodine 100-200 μg/L.

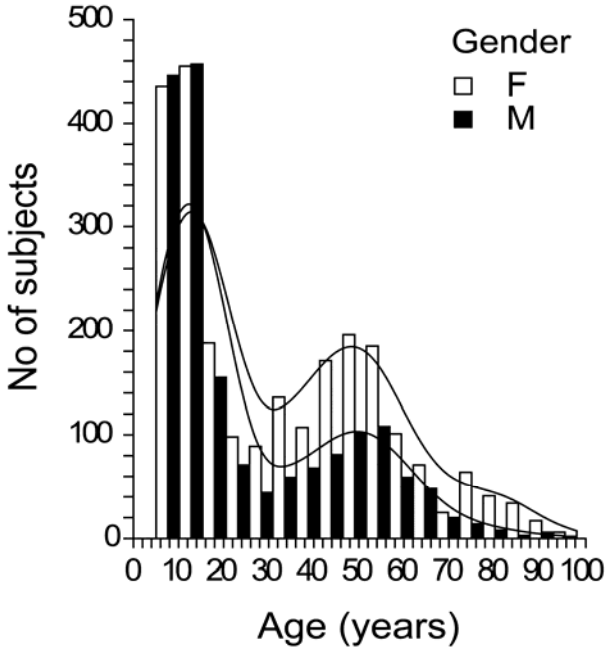

Fig. 1. Histogram of age by gender (F, female; M, male) in total populations. Results of population studies concerning the situation of iodine intake conducted in the Czech Republic. 4 171 randomly selected individuals (1 751 males, 2 420 females) participated in this research.

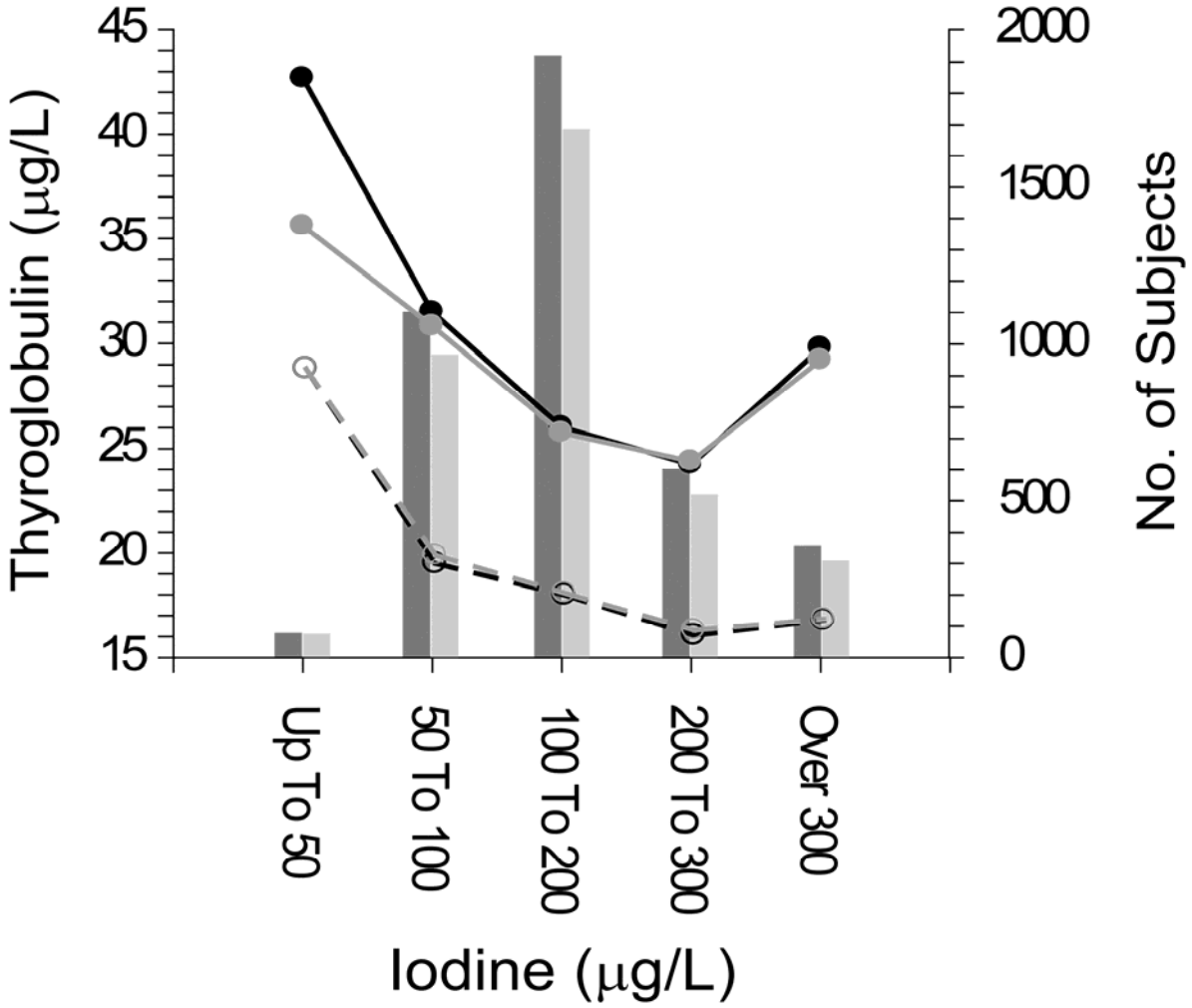

Fig. 2. The relation of mean (solid lines) and median (dashed lines) concentrations of serum thyroglobulin (Tg) on urinary iodine in total (black lines) and normal (shadow lines) populations. Number of subjects is shown as bars. Subjects were divided into subgroups according to their iodine intake and the mean or median of serum thyroglobulin was calculated in these subgroups.

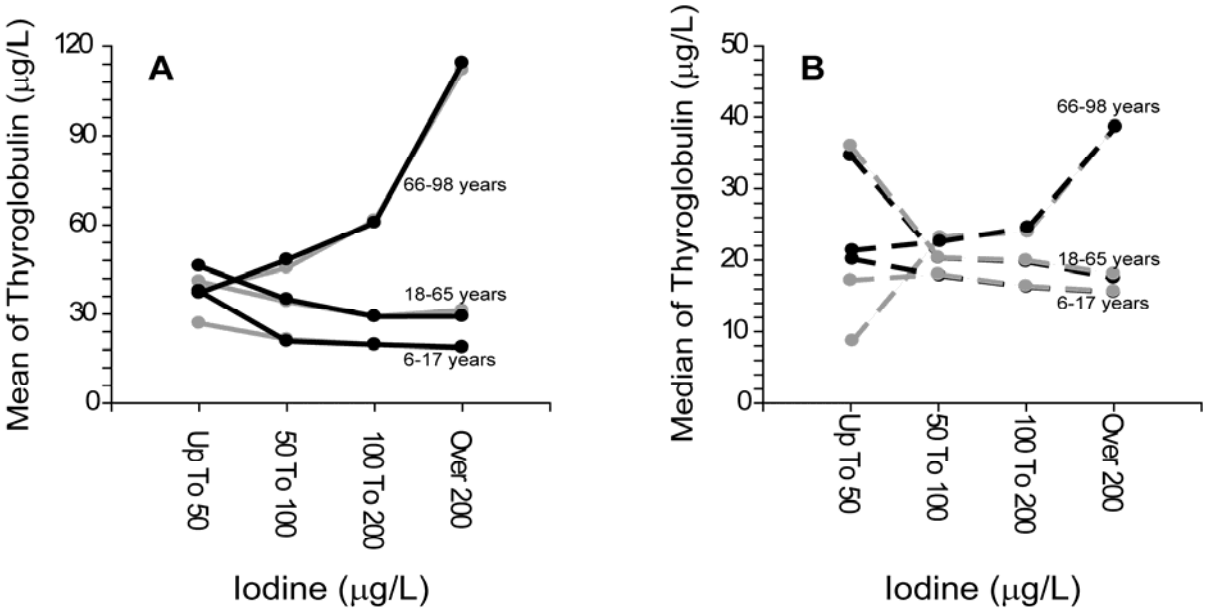

Fig. 3. The influence of age on the relation between mean (part A, solid lines) and median (part B, dashed lines) concentrations of serum thyroglobulin and urinary iodine in total (black lines) and normal (shaded lines) populations in subgroups according to their iodine intake. Number of examined subjects was 1 961/1 738 (total/normal population) for 6-17 years of age, 1 832/1 609 for 18-65 years of age and 256/193 for 66-99 years of age.