

New Reference Intervals for Thyrotropin and Thyroid Hormones Based on National Academy of Clinical Biochemistry Criteria and Regular Ultrasonography of the Thyroid

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Background: The aim of our present study was to establish new reference intervals for thyrotropin (TSH) and thyroid hormones based on National Academy of Clinical Biochemistry (NACB) criteria and regular thyroid ultrasonography. We also assessed the effect of potentially confounding factors to modulate the limits of these intervals.

Methods: We investigated 870 apparently healthy persons and excluded, step by step, those with a family history of thyroid disease, pathologic thyroid ultrasonography results, and increased anti-thyroid peroxidase or anti-thyroglobulin antibodies. Accordingly, only 453 of the 870 persons in the entire group were finally included as reference collective. We measured serum concentrations of TSH, total and free thyroxine (T_4 and FT_4), and total and free triiodothyronine (T_3 and FT_3) of the whole and the reference collective on the ELECSYS system assays (Roche Diagnostics) and calculated the 2.5th and 97.5th percentiles for comparison.

Results: The calculated lower limit for TSH differed significantly between the reference intervals for healthy persons with an assessed normal thyroid gland vs the nonselected group of healthy blood donors. Age was the only independent factor and was significantly inversely associated with TSH ($P < 0.0001$). Use of oral contracep-

tives was a significant predictor for variation in T_4 concentrations ($P < 0.001$). Age and oral contraceptives were independently associated with T_3 variations ($P < 0.05$). For FT_4 vs FT_3 variation, gender and (inversely) age ($P < 0.01$) were independent modulating factors.

Conclusions: The selection of healthy persons according to NACB criteria combined with sonographic confirmation of a normal thyroid gland provide a valid basis for the reference interval for TSH. Factors indicating a preclinical disease state, such as family history, pathologic ultrasonography result, or increased anti-thyroid peroxidase and anti-thyroglobulin antibodies, can be associated with normal hormone concentrations. Additionally, patient age and gender as well as use of contraceptives should be considered in diagnostic evaluation of thyroid diseases.

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The diagnostic specificity and sensitivity of laboratory tests is mainly affected by the validity of reference intervals. Several years ago the IFCC and the National Committee for Clinical Laboratory Standards (now the CLSI) recommended that each laboratory define its own reference intervals (1–3). However, the selection and clinical characterization of suitable populations for the establishment of reference values is time-consuming, and the mandatory measurement of high numbers of samples is expensive. Moreover, reference intervals are dependent on analytical quality parameters as sensitivity, specificity, precision, and accuracy of the applied assay system. Published reference interval data are very limited; therefore, clinical laboratories frequently use only insufficiently approved reference intervals given by the manufacturers of commercially available assays.

Reference intervals of the thyroid hormones thyro-

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tropin (TSH),⁴ total and free triiodothyronine (T₃ and FT₃), and total thyroxine and free (T₄ and FT₄) are typically established by measuring the serum concentrations in so-called "apparently thyroid-healthy subjects" without further characterization of the thyroid and clinical chemical or demographic data (4–10). However, considering the abundance and variety of the many factors that may affect thyroid function, standardized recommendations need to be established to ensure the validity of reference intervals on the basis of a thyroid-healthy population. According to the results from the National Health and Nutrition Examination Survey (NHANES) studies on regular thyroid function (11), the National Academy of Clinical Biochemistry (NACB) proposed that for the establishment of new TSH reference intervals, only euthyroid healthy volunteers be included, who should be free from detectable autoantibodies against thyroid peroxidase (TPOAbs) or thyroglobulin (TgAbs) and any personal or family history of thyroid dysfunction (12). In addition, no visible or palpable goiter and no medication except estrogens are allowed. Moreover, it was proposed that for the establishment of reference intervals of thyroid antibodies, only male goiter-free nonsmokers <30 years of age, without any family or personal history of thyroid or nonthyroid autoimmune disease and with serum TSH concentrations between 0.5 and 2 mIU/L should be included. However, it is unclear whether these very complex selection criteria are necessary to establish an improved reference interval for TSH. On the other hand, it has not yet been elucidated whether thyroid ultrasonography to exclude nonpalpable thyroid enlargement and thyroid nodules should be mandatory for reference studies of thyroid hormones.

The aim of our present study was to establish new reference intervals for TSH based on both the NACB criteria and regular thyroid ultrasonography. In addition, we studied whether these criteria could be also helpful for the establishment of reference intervals for T₃, T₄, FT₃, and FT₄. Finally, we assessed the effect of potentially confounding factors such as gender, age, body mass index, and the use of oral contraceptives for their capacity to modulate the limits of these intervals.

Participants and Methods

PARTICIPANTS

For this study, we included 870 healthy blood donors [experienced and first-time donors, which met the general principles of donor selection and guidelines for deferral according to the German Guidelines (13)] without a personal history of a thyroid disease. All donors filled out a questionnaire requesting information concerning a fam-

ily history of thyroid diseases, anthropometric data, smoking habits, and medications. A total of 445 male and 425 female donors (age range, 18–68 years) were randomly included (Table 1). Thyroid ultrasonography was performed with an EUB-405 (Hitachi) with a 7.5 MHz transducer. The thyroid volume was calculated as length × width × depth × 0.479 for each lobe (14). Goiter was defined as a thyroid volume exceeding 18 mL in women and 25 mL in men (15). Solid nodules were identified as differing from the healthy thyroid tissue in pattern and ultrasonic echo intensity. The nodules were classified as isoechoic if their texture closely resembled that of healthy thyroid tissue, hyperechoic if more echogenic, and hypoechoic if less echogenic.

Venous blood was collected before the blood donation procedure started. The time of blood collection was between 0800 in the morning (mean, 1025) and 1800 in the evening (mean, 1445). After blood was centrifuged at 3000g for 15 min, the serum was aliquoted and stored frozen at –25 °C until analysis 2–3 months after blood sampling. All blood donors gave written consent for the participation in this study.

METHODS

Serum concentrations of TSH, FT₄, FT₃, T₄, and T₃ were measured by assays on the ELECSYSTM system (Roche Diagnostics). The immunoreactivity of serum TPOAbs and TgAbs was measured on the same platform.

Intra- and interassay CVs (n = 16) were <5.1% for TSH (concentrations, 2.04 and 10.7 mIU/L) and the thyroid hormones (4.39 and 21.3 pmol/L for FT₃, 10.0 and 37.0 pmol/L for FT₄, 2.83 and 5.69 nmol/L for T₃, and 108 and 187 nmol/L for T₄; n = 21–26) and <12.3% for TPOAb activity (37 and 102 units/L) and <4.6% for TgAb antibody activity (81 and 187 units/L).

STATISTICS

The data for TSH and the thyroid hormones did not adhere to a gaussian distribution; descriptive statistics are therefore reported as median and empirical percentiles. The reference intervals of the examined hormones are reported as 2.5th–97.5th empirical percentiles. Confounding factors for thyroid reference intervals were identified by univariate analysis of correlation according to Spearman and by stepwise forward multiple regression analysis. Gender dependency of thyroid hormone concentrations was confirmed by the Mann–Whitney *U*-test. *P* values ≤0.05 were defined as statistically significant. All calculations were performed with Statistica 6.0 software (Statsoft).

Results

CHARACTERIZATION OF THE INVESTIGATED COLLECTIVE OF HEALTHY PARTICIPANTS

Of the 870 healthy persons included into the whole study group, 34 were 18–19.9 years of age, 382 were 20.0–29.9 years of age, 174 were 30.0–39.9 years of age, 172 were 40.0–49.9 years of age, 70 were 50.0–59.9 years of age, and 37

⁴ Nonstandard abbreviations: TSH, thyrotropin; T₃, total triiodothyronine; FT₃, free triiodothyronine; T₄, total thyroxine; FT₄, free thyroxine; NHANES, National Health and Nutrition Examination Survey; NACB, National Academy of Clinical Biochemistry; TPOAb, anti-thyroid peroxidase antibody; TgAb, anti-thyroglobulin antibody; and TBG, thyroxine-binding globulin.

Table 1. Comparison of anthropometric and other relevant data between the whole group of healthy blood donors and the constraint group selected according to the NACB recommendations and with sonographically assessed normal thyroid gland.^a

Variable	All healthy blood donors (n = 870)		Healthy donors with normal thyroid gland (n = 453)	
	Females (n = 425)	Males (n = 445)	Females (n = 174)	Males (n = 279)
Age, years	32.7 (12.0)	34.8 (12.4) ^b	30.6 (11.3)	32.4 (11.7)
Height, cm	168 (6)	180 (7) ^c	167 (6)	180 (7) ^c
Weight, kg	65.7 (10.3)	79.2 (11.1) ^c	63.7 (9.1)	77.5 (10.4) ^c
Body mass index, kg/m ²	23.4 (3.7)	24.4 (3.0) ^c	22.9 (3.6)	23.9 (2.8) ^c
Thyroid volume, mL	14.7 (6.6)	19.9 (8.7) ^c	12.0 (2.8)	16.2 (3.8) ^c
Daily number of cigarettes (for smokers)	1.9 (4.2)	3.3 (6.4) ^c	1.6 (3.6)	3.4 (6.3) ^d
Oral contraceptives (yes), n (%)	234 (55.4%)		108 (62.1%)	
Systolic blood pressure, mmHg	123 (15)	133 (15) ^c	122 (15)	131 (15) ^c
Diastolic blood pressure, mmHg	78 (10)	80 (12) ^c	77 (9)	78 (11)
Heart rate, beats/min	77 (11)	74 (12) ^c	77 (11)	74 (12) ^d

^a All values are the mean (SD) except for use of oral contraceptives.

^{b-d} Significantly different between males and females: ^b $P < 0.05$; ^c $P < 0.001$; ^d $P < 0.01$.

were older than 60 years. Gender was comparably distributed within all age groups (data not shown). Thyroid volume was significantly higher in males than in females (Table 1). Other physiologic variables such as blood pressure and heart frequency showed values and gender-dependent differences within the expected intervals for healthy persons.

Approximately one-fifth of the donors (16.2% males and 26.1% females) reported a family history of thyroid diseases. Goiter and/or hyperechoic or hypoechoic areas were newly detected in 26.8% of the investigated females and 23.8% of the investigated males. Of the 870 donors recruited, 220 showed irregularities of the thyroid or had a positive family history for thyroid diseases; thus, 650 (74.7%) fulfilled the NACB criteria for the absence of family history and showed a sonographically normal thyroid.

REFERENCE INTERVALS FOR TPOABs AND TgABs

Nonsmoking males <30 years of age with no goiter as assessed by ultrasonography, without any personal or family history of thyroid or nonthyroid autoimmune disease, and with TSH concentrations between 0.5 and 2 mIU/L were included for the calculation of new reference intervals of thyroid antibody assays. Only 69 of 440 male donors fulfilled the inclusion criteria of the NACB. We reached the recommended number of 130 by extending the age interval to 50 years. The subsequent reference intervals (97.5th percentile) for TPOABs and for TgABs were <37.1 IU/mL and <98.1 IU/mL, respectively. Of all persons examined, 9.6% had increased TPOAb immunoreactivity and 10.3% had TgAb immunoreactivity; 16.0% of these showed immunoreactivity for both antibodies.

REFERENCE INTERVALS FOR TSH AND THYROID HORMONES

In the next step we excluded all data for persons with increased antibody activity from the group with no family history and a sonographically assessed normal thyroid

gland. The remaining 453 data sets (52.1%; constraint group of 279 males and 174 females) were used for establishing reference intervals for the thyroid hormones (Table 1). There was no significant difference in age between the female and male groups, and there was also no difference in age distribution between the whole group and the constraint group. The distributions of data for TSH and thyroid hormones in the constraint group were neither normally nor log-normally distributed according to the Kolmogorov–Smirnov index ($P < 0.10$). When we compared the reference data from healthy persons with an assessed normal thyroid gland with the data from the whole group of healthy blood donors, we found a definite difference only in the calculated lower limit for TSH (2.5th percentile; Table 2 and Fig. 1). This difference was more than twice as high as the mean interassay CV of the analytical method used. Additionally, when we compared previous reference intervals from Elecsys studies with “apparently healthy subjects” without a known personal and family history of thyroid diseases (15), we found marked differences ($\pm 5\%$ deviation from the limits of previous studies) for TSH (0.27–4.20 mIU/L; $n = 516$) and FT₄ (12–22 pmol/L; $n = 801$), and for the upper limits for T₃ (1.3–3.1 nmol/L; $n = 606$) and T₄ (66–181 nmol/L; $n = 2526$). At the time of our study, no data had been published about reference intervals for FT₃ obtained with the recently developed assay, which was also used in this study.

FACTORS MODULATING REFERENCE INTERVALS FOR THYROID HORMONES

Gender, age, weight, height, body mass index, smoking, time of blood collection, and the use of oral contraceptives potentially modulate thyroid hormone reference intervals. We therefore performed a stepwise forward multiple

Table 2. Reference intervals for thyroid hormones established from the whole group of healthy blood donors and the constraint group selected according to the NACB recommendations and with sonographically assessed normal thyroid gland.

Hormone	Whole group (n = 870)			Constraint group (n = 453)		
	Median	Minimum–Maximum	2.5th–97.5th percentiles	Median	Minimum–Maximum	2.5th–97.5th percentiles
TSH, mIU/L	1.31	0.05–14.5	0.30–3.63	1.36	0.12–5.29	0.40–3.77 ^b
T ₄ , nmol/L	101	52.1–209	71.5–158	98.3	61.8–173	70.5–157
T ₃ , nmol/L	1.77	0.89–4.00	1.23–2.80	1.77	0.89–3.96	1.27–2.79
FT ₄ , pmol/L	16.2	8.24–28.6	12.7–20.8	16.2	9.30–24.7	12.8–20.4
FT ₃ , pmol/L	5.10	2.52–9.96	3.89–6.66	5.13	2.52–9.96	4.02–6.79

^a Data are reported as empirical percentiles.

^b The lower limit of the TSH reference interval in the constraint group was >5% different from the comparable limit of the whole group.

regression analysis to identify the independent predictive value of these variables on the variance of hormone concentrations in the constraint group: Age was the only independent factor that was significantly inversely associated with TSH ($r^2 = 6.0\%$; $P < 0.0001$). TSH concentrations of persons <40 years of age ($n = 338$; median, 1.46 mIU/L; 2.5th–97.5th percentiles, 0.52–3.50 mIU/L) were significantly higher than concentrations in the corresponding group above 40 years of age ($n = 113$; median, 1.14 mIU/L; 2.5th–97.5th percentiles, 0.30–3.94 mIU/L).

The use of oral contraceptives was a significant predictor for the variations in T₄ ($r^2 = 36.3\%$; $P < 0.001$) and T₃ concentrations ($r^2 = 22.9\%$; $P < 0.001$). Additionally, age was weakly associated with T₃ variations ($r^2 = 0.7\%$; $P < 0.05$), but was of negligible statistical relevance. For the FT₄ vs FT₃ variation, gender (7.3% and 9.1%, respectively; $P < 0.001$) and (inversely) age (1.9% and 1.9%; $P < 0.01$) were independent modulating factors. The median concentrations and reference intervals for the thyroid hormones and their dependence on gender and the use of oral contraceptive are shown in Table 3.

Discussion

To the best of our knowledge this is the first reference interval study for TSH that applies the stringent NACB criteria and ultrasonographic assessment of a normal thyroid gland for the selection of the reference collective. We also extended these criteria to the evaluation of reference intervals for thyroid hormones. Apparently healthy persons with a suggestive subclinical thyroid disease were excluded to avoid any bias on the reference intervals. Of the 870 healthy blood donors [who all met the German Guidelines for blood donor selection criteria (13)], 47.9% did not meet all criteria for normal thyroid function or morphology. The high portion of excluded persons may reflect a considerable incidence of thyroid diseases in the subclinical state, even in a preselected long-term supervised collective of blood donors. Previous studies for the evaluation of reference intervals did not consider the high incidence of subclinical thyroid diseases in a generally healthy population (4–10, 16). Interestingly, persons meeting all of our selection criteria showed a good congruence in the reference intervals for T₃, T₄, FT₃, and FT₄ with our unselected total group of

apparently healthy persons. These unexpected findings may be explained by the lower comorbidity in blood donors as a population basis for our whole group compared with persons included from the general population, as were used in studies such as NHANES III (11). However, we found a small but significant increase in the lower limit of the reference interval for TSH, from 0.3 to 0.4 mIU/L, suggesting that thyroid volume and structure have an impact on TSH reference data. This hypothesis is supported by results of a multiple regression analysis in the whole group, including age, gender, the use of oral contraceptives, and thyroid volume as independent and TSH or thyroid hormones as dependent variables (data not shown). Thyroid volume was a relatively strong independent predictor only for the TSH variation ($r^2 = 9.5\%$; $P < 0.001$), whereas the predictive value was definitely lower ($r^2 < 2.2\%$) for the thyroid hormones. Therefore, a healthy thyroid gland appears to be an important inclusion parameter for establishing reference intervals, at least for TSH. Moreover, the potential association between thyroid volume/structure and thyroid hormones may be of particular relevance if collectives with a higher comorbidity than in blood donors are included to establish reference intervals. This hypothesis is supported by findings of a recently published population-based reference interval study from Völzke et al. (17). They demonstrated marked differences between TSH concentrations of a “disease free” and a “reference” group based on regular ultrasonography (17). In addition, reduced thyroid echogenicity demonstrated by ultrasonography has been shown to be a strong predictor of autoimmune thyroid disease (18).

In the case of TSH, which represents the most important tool for the diagnostic exclusion of thyroid diseases, the new lower limit, which was calculated on the basis of the constraint group, could be helpful to avoid misidentification of patients with (sub)clinical peripheral hyperthyroidism or central hypothyroidism. However, it must be pointed out that on the basis of these new reference values, TSH concentrations were normal in 77% of our apparently healthy donors with a pathologic finding in the thyroid ultrasonography.

With regard to the calculated upper limit of our TSH reference interval, the use of increased TSH as indicator of preclinical hypothyroidism was affected only slightly by the

Table 3. Medians and reference intervals for thyroid hormones, established in 453 healthy blood donors with sonographically assessed normal thyroid gland and selected as recommended by the NACB, showing their dependence on sex and use of oral contraceptive by females.

	TSH, mIU/L	FT ₄ , pmol/L	FT ₃ , pmol/L	T ₄ , nmol/L	T ₃ , nmol/L
Males (n = 279)					
Median	1.35	17.0	5.25	93.4	1.72
2.5th–97.5th percentiles	0.36–3.44	13.0–21.4	4.09–6.80	68.4–125	1.28–2.33
Females (n = 174)					
Median	1.42	15.5	4.77	113	1.94
2.5th–97.5th percentiles	0.44–3.94	12.2–20.0 ^a	3.84–6.70 ^a	71.4–166 ^a	1.23–2.97 ^a
Females not using OC ^b (n = 66)					
Median	1.29	15.4	4.76	90.8	1.62
2.5th–97.5th percentiles	0.42–4.25	11.7–20.1	3.66–6.03	68.0–138	1.08–2.90
Females using OC (n = 108)					
Median	1.56	15.6	4.78	124	2.12
2.5th–97.5th percentiles	0.44–3.50	12.3–20.1	4.07–7.08	81.8–168 ^a	1.35–3.35 ^a

^a Statistically significantly different ($P < 0.001$) by the Mann–Whitney U -test.

^b OC, oral contraceptives.

strict inclusion criteria of the NACB plus thyroid ultrasonography. If we used the 97.5th percentile as proposed by the NACB, we found a comparable upper TSH limit of 3.77 mIU/L for the constraint and 3.63 mIU/L for the whole group. On the one hand, these concentrations are considerably lower than the currently used upper reference limit of ~4.2 mIU/L proposed by Roche Diagnostics (19) and the 97.5th percentile of 4.1 mIU/L from the NHANES III study (11). On the other hand, our values are markedly higher than 2.12 mIU/L, which was the upper limit in the above-mentioned study of Völzke et al. (17). Moreover, these values are also higher than the cutoff of 2.5 mIU/L that has been proposed recently by the NACB for distinguishing between euthyroidism and preclinical hypothyroidism (12). These discrepancies may be attributable to differences in number, age distribution, ethnicity, and iodine supply of the investigated groups as well as to the different inclusion criteria of the respective studies. Most importantly, we performed an ultrasonographic thyroid assessment of our volunteers to exclude persons with subclinical thyroid diseases and still negative TPOAb antibodies, which could be responsible for a skewed shift to the “right” from the gaussian distribution of TSH data (12). However, our TSH concentration data from the constraint group also demonstrate a skewed shift to the right from the gaussian distribution, even if ultrasound is used; therefore, our ultrasound procedure and/or the assay used for measuring anti-TPO immunoreactivity could be too insensitive for detecting occult thyroid dysfunction. Furthermore, assay-dependent differences in the analytical results may also have a major impact on the absolute values of TSH reference limits, even if the assay calibrators are calibrated against the same reference preparation, MRC 88/558. For example, when we used the Centaur method (Bayer Diagnostics) to measure serum TSH, we calculated an upper limit of 2.92 mIU/L (97th percentile) in our constraint group (data not shown). This value is much closer to the cutoff of 2.5 mIU/L recommended by the NACB than is the 3.77 mIU/L calcu-

lated from the data we obtained with the Roche system. However, it must be emphasized that possible consequences of a TSH concentration between 2.5 and 4.0 mIU/L for therapeutic interventions to prevent clinical manifestation of hypothyroidism are still being discussed (20–22).

Our calculated new reference intervals show some limitations. For example, previous studies have suggested that the ratio of within- to between-individual variation is low for TSH but also the thyroid hormones (23, 24). Thus, population-based reference intervals for these hormones are relatively insensitive to aberrations from normality in the individual. This may cause uncertainty in the diagnosis of overt and, in particular, subclinical thyroid disease. In addition, to establish reference intervals for thyroid autoantibodies, we could not fulfill the suggested NACB age restriction to 30 years because the number of eligible males was too low for a powerful statistical evaluation. However, the extension of the age criterion to 50 years for this group did not show any age dependency of autoantibody concentrations (data not shown). Finally, the age association of all thyroid hormones studied in the constraint group was limited to persons <60 years of age. The question of whether the upper reference limit for TSH should be lower for persons older than 60 years remained open in our study. This limitation also applies to the age dependency of reference intervals for FT₃ and FT₄. However, the variance that is predicted with age for the concentrations of both hormones was negligible.

Our results provide additional information about confounding factors for reference values in laboratory tests used for thyroid diagnostics. With the exception of the TSH results, all other thyroid hormones were strongly dependent on gender or the use of oral contraceptives in the multiple regression analysis. This dependency is attributable to the well-known impact of sex hormones and oral estrogens on thyroid function (11, 25, 26). The estrogen-dependent increases in total T₃ or T₄ may be adjusted for the concentration of thyroxine-binding globulin (TBG)

by its direct determination or by the measurement of T-uptake values. However, in our study, the significant effect of gender on FT₃ concentrations was surprising and points to a potential interference of binding proteins on automated analog methods, such as those used by the Elecsys system. Moreover, reference intervals for total T₃ and T₄ that take into account the use of estrogens in females may give improved insight into thyroid hormone activity and potentially make unnecessary measurements of TBG in patients without any TBG-influencing diseases.

In general, the sequential selection of healthy persons according to the NACB criteria combined with ultrasonographic confirmation of a healthy thyroid gland provides a valid new basis for determining reference intervals for TSH. For the clinical use of serum values in thyroid diagnostics, it must be considered that factors pointing to a preclinical disease state, such as a family history, pathologic ultrasonography finding, or presence of TPOAb and TgAb antibodies, can be associated with normal hormone concentrations. Finally, patient age (for TSH, FT₃, and FT₄) and gender (for FT₃ and FT₄) as well as the use of contraceptives (for T₃ and T₄) ought to be considered as selection criteria for further reference interval studies in the diagnostic evaluation of thyroid diseases.

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