

DIRECT ESTIMATION OF REFERENCE INTERVALS FOR THYROID PARAMETERS IN THE REPUBLIC OF SRPSKA

DIREKTNA PROCENA REFERENTNIH INTERVALA ZA PARAMETRE ŠTITNE ŽLEZDE U REPUBLICI SRPSKOJ

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Summary

Background: The aim of this study was to determine the reference values for thyrotropin (TSH), thyroid hormones (total and free thyroxine, T4 and fT4; total and free triiodothyronine, T3 and fT3), thyroglobulin (Tg) and thyroid antibodies (thyroid peroxidase, TPOAb and thyroglobulin antibody, TgAb) in the population of the Republic of Srpska.

Methods: A total of 250 euthyroid subjects were enrolled in this study. A direct method for choosing reference subjects was used to establish reference intervals. The hormones and thyroid antibodies were measured by an electrochemiluminescence immunoassay method (ECLIA, Roche Diagnostics, Mannheim, Germany). We calculated the reference intervals by MedCalc, version 12.1.4.0 (MedCalc software, Belgium) as recommended by the IFCC (CLSI C28-A3).

Results: Using guidelines recommended by the National Academy of Clinical Biochemistry (NACB) and based on standard statistical approaches, the reference intervals derived for TSH, fT4, T4, fT3, T3 were 0.75–5.32 mIU/L, 12.29–20.03 pmol/L, 73.49–126.30 nmol/L, 4.11–6.32 pmol/L, 1.15–2.32 nmol/L and for Tg, TPOAb, TgAb were 3.63–26.00 µg/L, <18.02 mIU/L, < 98.00 mIU/L, respectively. We found a significant difference ($p < 0.05$) in TSH and fT3 values between different age groups as well as in T4, fT4 and fT3 values between gender groups.

Kratak sadržaj

Uvod: Cilj ovog rada bio je da se utvrde referentne vrednosti tireotropnog hormona (TSH), tireoidnih hormona (ukupni tiroksin, T4 i slobodni tiroksin, fT4; ukupni trijodtironin, T3 i slobodni trijodtironin, fT3), tireoglobulina (Tg) i antitela štitne žlezde (antitela na tireoperoksidazu, TPO-At i tireoglobulinska antitela, Tg-At) kod stanovništva Republike Srpske.

Metode: Ukupno 250 eutireoidnih ispitanika bilo je uključeno u ovo istraživanje. Korišćen je direktan način izbora referentnih osoba za utvrđivanje referentnog intervala. Hormoni i antitela štitne žlezde određivani su u serumu elektrohemioluminiscentnom metodom (ECLIA, Roche Diagnostics, Mannheim, Germany). Referentne granice su određene korištenjem programa MedCalc, verzija 12.1.4.0 (MedCalc software, Belgium) po preporuci IFCC (CLSI C28-A3).

Rezultati: Koristeći smernice Nacionalne akademije za kliničku biohemiju (NACB) i na temelju standardnih statističkih pristupa utvrđeni referentni intervali za TSH, fT4, T4, fT3, T3 bili su 0,75–5,32 mIU/L, 12,29–20,03 pmol/L, 73,49–126,30 nmol/L, 4,11–6,32 pmol/L, 1,15–2,32 nmol/L kao i za Tg, TPOAt, TgAt 3,63–26,00 µg/L, <18,02 mIU/L, < 98,00 mIU/L. Takođe, postojala je i značajna razlika između različitih starosnih grupa za TSH i fT3 vrednosti, kao i razlika za T4, fT4 i fT3 vrednosti između žena i muškaraca.

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Conclusions: The established reference values for the population of the Republic of Srpska were significantly different from the values recommended by the manufacturer of reagents (Roche Diagnostics). Our results showed that a laboratory needs to establish its own reference values in order to set up a proper diagnosis, as well as to treat patients successfully.

Keywords: reference values, TSH, thyroid hormones

Introduction

The improvement of sensitivity and specificity of biochemical thyroid tests has had a strong influence on the clinical strategy for detecting and treating thyroid diseases in the last forty years. TSH is a first-line test for the assessment of thyroid function, mainly due to suspicion of subclinical hypothyroidism, if it is determined by the third generation methods with a functional sensitivity of 0.01 mIU/L (1).

To use this test, the following conditions must be fulfilled as follows:

1. intact hypothalamic pituitary function
2. stable thyroid status.

Since the clinical signs and symptoms are often unclear and non-specific, in the case of increase or deficiency of thyroid hormones (total and free thyroxine, T4 and fT4; total and free triiodothyronine, T3 and fT3) laboratory determinations are necessary to confirm hyperthyroidism or hypothyroidism.

Thyroperoxidase (TPO) is a key enzyme in the formation of thyroid hormones and a major autoantigen in autoimmune thyroid diseases (2). Thyroid peroxidase antibodies (TPOAb) are very important for the diagnosis of autoimmune diseases of the thyroid gland, particularly Hashimoto's disease. They are often present in euthyroid subjects (prevalence of 12–26%) (3) and they are important for the evaluation of future clinical status of euthyroid persons (4). The measuring of TPOAb has much greater influence than measuring of TgAb in euthyroid persons, which can be used to identify people at increased risk of hypothyroidism.

A reference interval is a range of reference values obtained in healthy individuals of the same or similar characteristics. A direct or an indirect method are most commonly used for the preparation of a reference interval. Using a direct method, reference persons are selected from a reference population to precisely define the criteria. By using an indirect method, instead of reference persons, values with the required characteristics are selected from an existing database. The direct method of selecting reference persons is in line with the Clinical and Laboratory Standards Institute/International Federation of Clinical Chemistry and Laboratory Medicine (CLSI/IFCC) recommendation (5) and it can be applied in all situations, but its

Zaključak: Dobijene referentne vrednosti za populaciju Republike Srpske znatno su se razlikovale od vrednosti preporučenih od strane proizvođača reagensa (Roche Diagnostics). Rezultati su ukazali da laboratorije moraju da izrade sopstvene referentne vrednosti sa ciljem postavljanja prave dijagnoze, kao i uspešnog lečenja pacijenata.

Ključne reči: referentne vrednosti, TSH, hormoni štitne žlezde

lack is that the selection of a reference group is followed by major problems and costs.

For example, the reference interval for TSH should be determined in order to represent the limits that are a 95% confidence interval of the log-transformed values obtained by determining TSH in at least 120 rigorously selected normal euthyroid volunteers, in whom no thyroid autoantibodies (TPOAb and TgAb measured by sensitive immunoassays) were detected, with no personal or family history of thyroid dysfunction, with no visible palpable goiter and with no medication (except estrogen) (6). The sample for the determination of reference values should be taken under conditions as similar as possible to the conditions that exist in clinical practice. It is necessary to conduct a complete standardization of the preanalytical conditions under which samples are taken in order to reduce the random error. The basic rule which must be complied with in the standardization of these conditions is to control only those conditions that significantly affect the intra- and inter-individual variation, as well as those that are easy to control in the clinical setting.

The preanalytical factors which affect the reference values can be divided into biological (preparation, or the patient's condition prior to sampling and time of sampling) and methodology (method of sample collection, transport and handling of the sample prior to treatment and processing of the sample itself) (6). The most of preanalytical factors have no impact on the determination of TSH in the serum, as well as the hormones of the thyroid gland. Physiological variables, individual variables such as genetic abnormalities of thyroid binding proteins or variables present in heavy non-thyroid diseases can affect determination. Also, iatrogenic factors such as thyroid and non-thyroid drugs (glucocorticoids or beta blockers), the presence of autoantibodies to the thyroid hormones or heterophilic antibodies (human anti-mouse antibodies, HAMA) can affect the diagnostic accuracy (6). Variables such as gender, age, phase of the menstrual cycle, smoking, exercise, taking a sample of fasting or track during phlebotomy have minimum impact on the reference intervals of thyroid tests without patients (3). According to the fact that the differences in these physiological variables are less than the differences of the method, they do not have importance in the clinical practice. The aim of this study was to determine the reference values for thyrotropin (TSH), thyroid hormones (total and free thyro-

xine, T4 and fT4; total and free triiodothyronine, T3 and fT3), thyroglobulin (Tg) and thyroid antibodies (thyroid peroxidase, TPOAb and thyroglobulin antibody, TgAb) in the population of the Republic of Srpska, because there are recommendations that users should establish reference intervals for their own population depending on the methods and reagents used.

Materials and Methods

Subjects

A total of 250 participants were enrolled in this study. The subjects were voluntarily included in the study at the University Clinical Centre of the Republic of Srpska, Banja Luka and Health Centre Laktaši (Banja Luka, Bosnia and Herzegovina). The study was approved by the National Ethics Committee. Written consent was obtained from the participating subjects.

The study included all subjects in which on the basis of thorough history and physical examination, as well as insight into existing medical records, a sick condition could not be determined that would affect change in the results of laboratory tests for which the reference values were determined. Subjects on medication for thyroid diseases and subjects with a positive history of thyroid diseases were excluded from this study. Also, people who take medications that can affect the hypothalamus-pituitary-thyroid gland (amiodarone, glucocorticoids) were excluded from the study. The final decision about a reference person is given with the help of laboratory findings (according to the NACB recommendation).

For the measurement of thyroid parameters in serum, blood samples were taken in the morning between 7:00 and 9:00 a.m. after 12–14 h of fasting.

Methods

The serum thyroid hormones and antibodies concentration was measured by an electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics, Mannheim, Germany). The reference range for the TSH assay was from 0.27 to 4.2 mIU/L, and the functional sensitivity provided by the manufacturer was 0.014 mIU/L. The expected values for euthyroid subjects were: 66–181 nmol/L of T4; 12–22 pmol/L of fT4; 1.3–3.1 nmol/L of T3; 3.1–6.8 pmol/L of fT3; 3.5–77 µg/L of Tg; borderline value of 34 IU/mL of TPOAb and threshold value of 115 IU/mL of TgAb. Lower limit of TgAb measurement is 10.00 IU/mL. Values below the lower detection limit are reported as <10.00 IU/mL. Reliability of the measurement results was regularly checked through the assessment of appropriate controls and application of internal and external quality control principles.

Statistics

All calculations were performed using MedCalc v. 12.1.4.0 (MedCalc software, Belgium). The Kolmogorov–Smirnov test was used to test normality. Outliers were identified and omitted using the Tukey method incorporated into the MedCalc software (7, 8). TSH, fT4 and fT3 values were normally distributed variables. After logarithmic transformation of the non-normally distributed variables, T4, T3, Tg and TPOAb also achieved normality. For TgAb, normality was not achieved even after the logarithmic transformation. For the parameters with normal distribution (with or without log transformation) we calculated both the central 95% of the distribution and the 90% confidence limits on both ends. Also, we calculated the values of the 2.5th and 97.5th percentiles, with 90% confidence intervals for lower and upper limits, as recommended by the IFCC (CLSI C28-A3). We analyzed different groups according to gender by Student *t* test and age groups by ANOVA test.

Results

Women were the majority in the group of subjects for reference values (68%). The median age of subjects was 39; the youngest person was 19, and the oldest person was 70. The reference intervals are shown in *Table I*. We found a significant difference of TSH values ($p < 0.05$) in the third, fourth and fifth decade of life and significant difference of fT3 values in the third and fifth decade of life (*Table II*). A statistically significant difference ($p < 0.05$) was found between males and females for fT4, T3 and fT3 (*Table III*). Distribution values of the thyroid parameters are shown in *Figure 1* and *Figure 2*.

Discussion

The reference values obtained in our research on the population of the Republic of Srpska vary significantly from the values recommended by the manufacturer of reagents that we use in our laboratory (9). The reference intervals (RI) for TSH obtained on different populations and different analyzers show significant differences in the lower and upper limits of RI ranging from 0.17 to 0.6 and also from 3.63 to 5.95 mIU/L (10–14). Our results are not in accordance with these and the results of many other studies, where the upper TSH values were 3.6 mIU/L (15, 16), 3.35 mIU/L (17), 3.77 mIU/L (18), 3.37 mIU/L (19), and 3.7 mIU/L (20). Although these studies used ultrasound to rule out non-obvious or potential abnormalities of the thyroid gland, this should not be the reason for non-compliance with our results, because there are no recommendations for necessary ultrasound examination of the thyroid gland by the NACB (6). Our results are not similar to the results of studies in which ultrasound was not used for the assessment of patients' health status (21, 22). The

Table I Reference values of the thyroid gland parameters.

Parameter	n	Reference interval					
		Parametric method			Non-parametric method		
		-1.96 SD Lower limit of 95% distribution	\bar{x}	+1.96 SD Upper limit of 95% distribution	0.025 2.5 Percentile	0.50 Median	0.975 97.5 Percentile
TSH (mIU/L)	224	0.75	1.99	5.32	0.65	1.96	5.39
T4 (nmol/L)	228	73.49	96.34	126.30	73.01	96.22	127.70
fT4 (pmol/L)	227	12.29	16.16	20.03	12.41	16.06	20.18
T3 (nmol/L)	229	1.15	1.63	2.32	1.17	1.62	2.40
fT3 (pmol/L)	226	4.11	5.22	6.32	4.09	5.25	6.33
Tg (μ g/L)	120	3.63	9.72	26.00	3.48	9.51	27.58
TPOAb (mIU/L)	120	5.17	9.65	18.02	5.00	9.65	18.81
TgAb (mIU/L)	120	-	-	-	<10	10.00	98.00

\bar{x} , mean value; SD, standard deviation; n, number of subjects; TSH, thyrotropin; T4, total thyroxine, fT4 free thyroxine; T3, total triiodothyronine; fT3, free triiodothyronine; Tg, thyroglobulin; TPOAb, thyroid peroxidase; TgA, thyroglobulin antibody.

Table II Thyroid parameters in different age groups.

Parameter	Groups	n	Mean values	Different ($p < 0.05$) from group
TSH (mIU/L)*	1 (age ≤ 30 years)	46	2.36 (2.03/2.73)	2,3
	2 (age 31–40 years)	84	1.79 (1.62/1.98)	1
	3 (age 41–50 years)	40	1.77 (1.49/2.10)	1
	4 (age 51–60 years)	36	2.15 (1.83/2.50)	–
	5 (age > 60 years)	18	2.39 (1.84/3.10)	–
T4 (nmol/L)*	1 (age ≤ 30 years)	46	94.57 (91.22/98.03)	–
	2 (age 31–40 years)	85	96.12 (93.55/98.76)	–
	3 (age 41–50 years)	41	95.15 (89.97/100.62)	–
	4 (age 51–60 years)	36	97.97 (94.21/101.89)	–
	5 (age > 60 years)	19	103.28 (96.55/110.49)	–
fT4 (pmol/L)	1 (age ≤ 30 years)	48	16.60 \pm 1.85	–
	2 (age 31–40 years)	84	16.29 \pm 1.94	–
	3 (age 41–50 years)	40	15.89 \pm 2.24	–
	4 (age 51–60 years)	36	15.50 \pm 1.82	–
	5 (age > 60 years)	19	16.29 \pm 1.93	–
T3 (nmol/L)*	1 (age ≤ 30 years)	47	1.65 (1.57/1.74)	–
	2 (age 31–40 years)	86	1.64 (1.58/1.70)	–
	3 (age 41–50 years)	41	1.55 (1.45/1.67)	–
	4 (age 51–60 years)	36	1.65 (1.57/1.73)	–
	5 (age > 60 years)	19	1.64 (1.52/1.78)	–
fT3 (pmol/L)	1 (age ≤ 30 years)	46	5.45 \pm 0.55	3
	2 (age 31–40 years)	85	5.22 \pm 0.52	–
	3 (age 41–50 years)	41	5.05 \pm 0.74	1
	4 (age 51–60 years)	36	5.25 \pm 0.43	–
	5 (age > 60 years)	19	5.05 \pm 0.57	–

The values are presented as arithmetic mean \pm standard deviation and geometric mean (95% CI) for variables marked with *. ANOVA test was used. Abbreviations: n, number of subjects; p, level of significance.

Table III Thyroid parameters in different gender groups.

Parameter	Male		Female		p
	n	Mean values	n	Mean values	
TSH (mIU/L)*	73	2.09 (1.87/2.34)	151	1.95 (1.80/2.11)	0.322
T4 (nmol/L)*	72	95.08 (91.95/98.32)	155	97.16 (95.13/99.24)	0.266
fT4 (pmol/L)*	73	16.48 (16.00/16.97)	154	15.83 (15.54/16.14)	0.023
T3 (nmol/L)*	74	1.72 (1.66/1.79)	155	1.59 (1.54/1.63)	<0.001
fT3 (pmol/L)	72	5.46±0.56	155	5.12±0.55	<0.001

The values are presented as arithmetic mean ± standard deviation and geometric mean (95% CI) for variables marked with *. Student's t test was used for comparison. Abbreviations: n, number of subjects; p, level of significance.

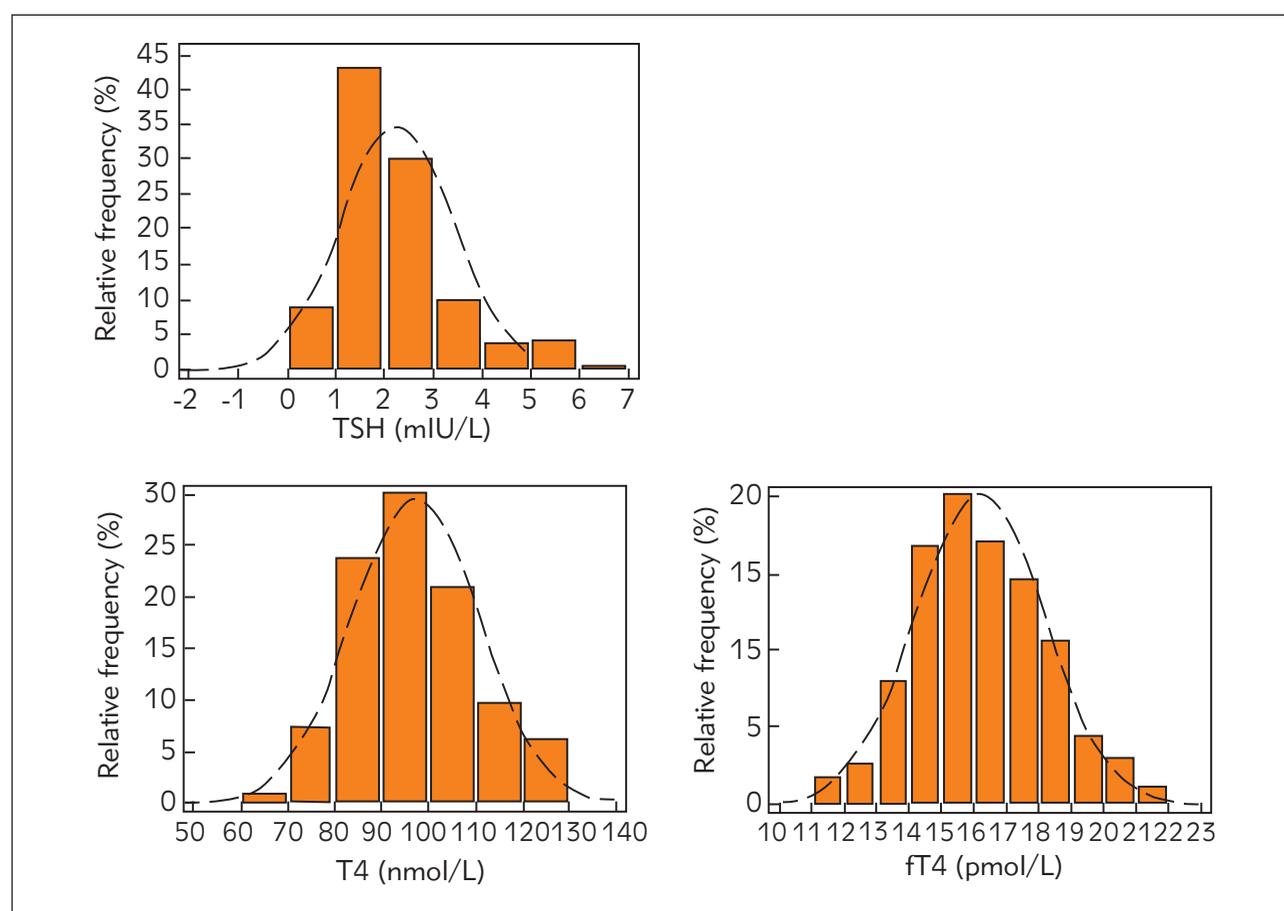


Figure 1 Distribution of TSH, T4 and fT4 values

reference values of thyroid hormones are dependent on the population, methodology and apparatus on which the analysis is performed, as our study confirmed (23–26).

Also, the high prevalence of subclinical hypothyroidism (27) interferes with the proper selection of healthy people in order to create reference values. A limiting factor could be that the sampling time is not standardized, as pointed out in the study of Mirjanic-Azaric et al. (28). Further, it is known from the Middle

Ages that the Balkan region is strumogenic, and recent researches in the Republic of Srpska (29) indicated a lack of iodine in the diet, which could certainly affect some reference values for TSH in the Republic of Srpska. Nevertheless, it is interesting that our results do not correspond to the RI population of the Republic of Serbia (0.75–5.32 vs. 0.42–3.67 mIU/L), although they used an indirect method of determining the reference values in their study (30). It is important to indicate that the study of Hollowell

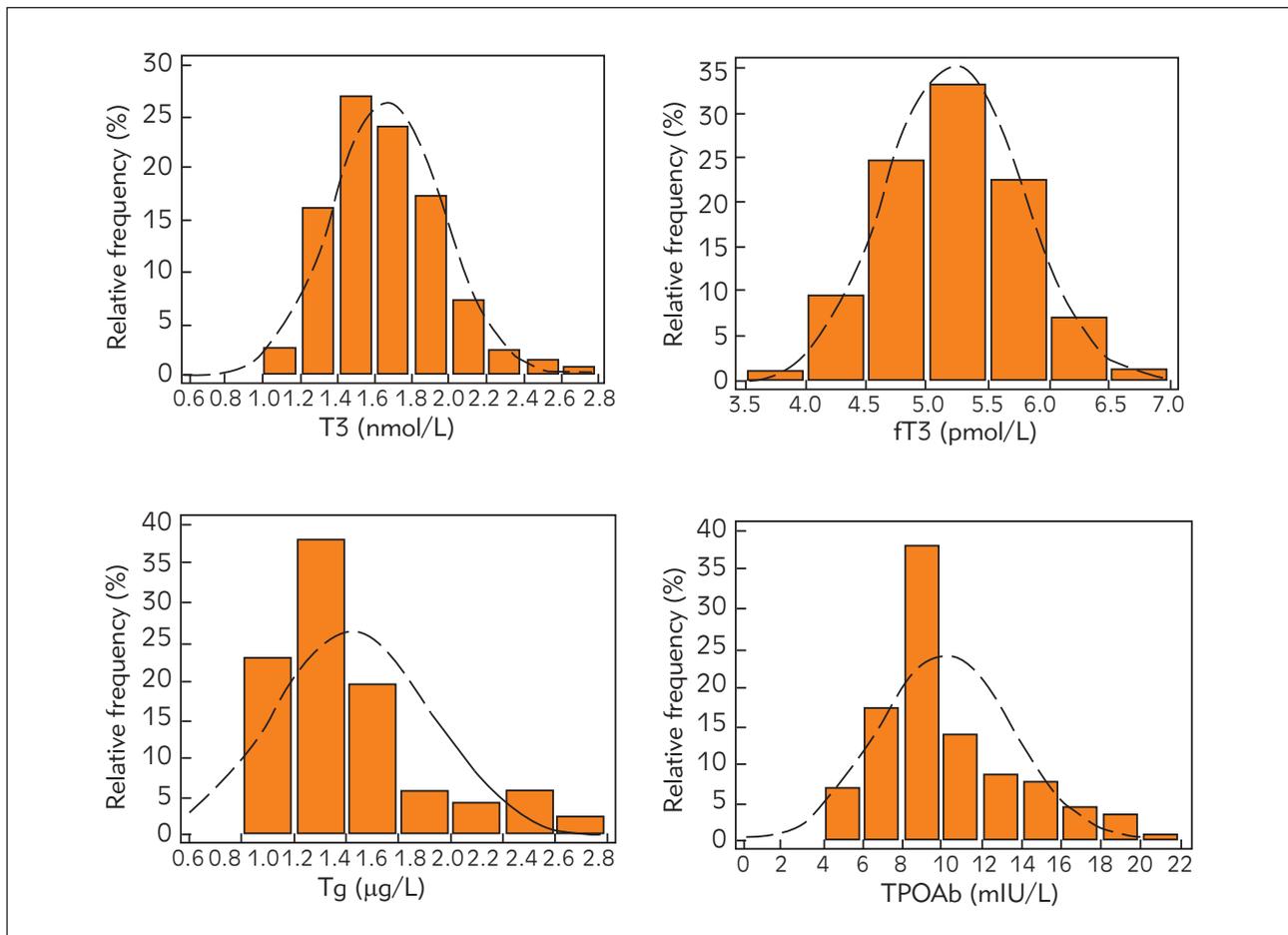


Figure 2 Distribution of T3, fT3, Tg and TPOAb values.

et al. (3), as one of the rare studies which has shown consistency with our research in terms of the upper limit of the reference range for TSH, refers to the results obtained for the white race in the United States of America with reagents of the same manufacturer: 0.75–5.32 vs. 0.48–5.73 mIU/L. Also, laboratory guidelines are published today which show that more than 95% of normal people have TSH below 2.5 mIU/L (31) which cannot be said for our group of respondents (*Figure 1*). The establishing of more precise and more accurate RI for TSH has important implications both for retrieval as well as for the treatment of diseases of the thyroid gland.

Our study revealed very high similarity to the reference value obtained with the value of fT4 listed by the manufacturer *i.e.* 12–20 pmol/L vs. 12–22 pmol/L (9), while for the other reference values of hormones T4, T3 and fT3 this was not the case. Some other studies (32–34) showed large discrepancies with our obtained values, which might be the result of all those facts previously listed for TSH. The best coherence of our reference values for T4 and T3 with the reference values of hormones in the population of the Republic of Serbia (30) was achieved for the follow-

ing T4: 73–126 vs. 66.0–136.10 nmol/L and T3: 1.15–2.32 vs. 1.10–2.39 nmol/L. For fT4 and fT3, there is a huge difference: 12.29–20.03 vs. 10.20–18.40 pmol/L and 4.11–6.33 vs. 3.17–5.59 pmol/L. Tg reference interval was set for the development of reference values for Tg according to the guidance, except that we included in the group persons up to 50 years. Inter-individual variations are particularly high for the serum concentration of Tg, because there are differences in the weight of the thyroid gland in different individuals, the status of TSH, and there are also conditions associated with thyroid injury (thyroiditis) – all of these conditions are affected by the serum concentration of Tg (31). Although there is statistically significant difference in the values of TSH and fT3 between different age groups (*Table II*), we cannot say that our study is consistent with the results of other studies (35–37) in which the levels of TSH and free T3 gradually declined in older age. When we analyzed the TSH and T4 levels in the male and female population, we found no statistically significant difference between these two groups, but for the fT4, T3 and fT3 values we found significant difference (*Table III*). On the basis of currently available data, we cannot fully explain this difference. Nevertheless, the magnitude of significant

differences was small, suggesting that gender-specific reference intervals are not necessary. Since Tg is dependent on the intake of iodine, it is necessary to create its own reference range. In countries with iodine deficiency, the mean value of Tg of the population and the upper Tg reference limit could be increased in relation to the degree of iodine deficiency.

Yet, it is a big surprise for us that there is so large a difference in the upper limit of the reference value for thyroglobulin in our population in relation to the reference values recommended by the manufacturer of reagents, *ie.* 4–26 vs. 5–77 $\mu\text{g/L}$. Also, an unexpected distribution of Tg values can be seen in Figure 2. Therefore, we recommend new research to confirm the obtained results, but this time with a larger number of subjects and the use of indirect method which is much cheaper and also recommended by the NACB.

The reference interval for TPOAb and TgAb was determined by the recommendations of the NACB in order to minimize the inclusion of people with a predisposition to autoimmune thyroid disease, except the fact that we included subjects up to age of 50 in order to achieve the recommended number of respondents. Our results for TPOAb are significantly lower than

the values recommended by the manufacturer of reagents that we use.

In one study (38), the upper limit of our reference interval for TPOAb and TgAb almost coincides with the values of their subjects, *i.e.* 18 vs. 19 IU/L and 98 vs. 99 IU/L. Finally, the determination of precise and accurate RI for thyroid hormones will enable the conditions for the establishment of proper diagnosis and better treatment of the thyroid gland than using RI made by the manufacturer.

Conclusion

The establishing reference values for the population of the Republic of Srpska were significantly differed from the recommended values by the manufacturer of reagents (Roche Diagnostics). The results showed that laboratories have to establish their own reference values in order to set up a proper diagnosis, as well as to treat patients successfully.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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