Practical Laboratory Medicine 26 (2021) e00250

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Evaluation of a new automated assay for high-sensitivity thyroglobulin measurement and comparison with two established high-sensitivity thyroglobulin assays☆

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ARTICLE INFO

Keywords:
Thyroglobulin
Tg
Highly sensitive assay
Differentiated thyroid carcinoma
Tumor marker
Follow-up
Recurrence
Reference interval

ABSTRACT

Objective: Thyroglobulin (Tg) is an important tumor marker for therapy control and follow-up of differentiated thyroid carcinoma (DTC). Over the past decade, assays for highly sensitive Tg measurement have become increasingly established. We evaluated a newly developed high-sensitive Tg assay running on an automated platform (LIAISON® Tg II Gen assay, DiaSorin), with a limit of quantification of 0.10 ng/ml.

Design and Methods: Tg values of 166 sera from subjects without thyroid diseases and of more than 500 sera of well-defined DTC patients were determined with the new LIAISON® Tg II Gen assay and compared with two established assays (Elecsys® Tg II/Roche, and Medizym® Tg REM/Medipan).

Results: Tg reference values from healthy subjects were up to 37.93 ng/ml (women) resp. 24.59 ng/ml (men) with the LIAISON® Tg II Gen assay. Tg values showed good correlations in healthy subjects and patients with active tumorous disease. In contrast, Tg values in the very low range from cured thyroidectomized patients were poorly comparable between the three assays, while clinical differences between the cohorts were correctly reflected by all assays.

Conclusions: With the new LIAISON® Tg II Gen assay, another automated assay standardized against the first International Reference Preparation CRM-457 for highly sensitive measurement of Tg values is available.

1. Introduction

Thyroglobulin (Tg) is a tumor marker for differentiated thyroid carcinoma (DTC) originating from thyroid follicular cells. As a tumor marker, Tg is only specific after thyroidectomy with the specificity increasing with the radicality of thyroid ablation since Tg is

☆ Dedicated to our esteemed colleague Ina Binse, who had devoted herself to thyroid research in our clinic and died of serious illness in 2019 at the age of 41.

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[1] This publication contains substantial data from the doctoral thesis of Irina Mehnert.

https://doi.org/10.1016/j.plabm.2021.e00250
Received 14 June 2021; Received in revised form 25 July 2021; Accepted 26 July 2021
Available online 27 July 2021

Abbreviations

Tg	thyroglobulin
DTC	differentiated thyroid carcinoma
IMA	immunometric assay
IRMA	immunoradiometric assay
IEMA	immunoenzymometric assay
ICMA	immunochemoluminometric assay
hsTg	highly sensitive thyroglobulin
TSH	thyrotropin
LoQ	limit of quantification
TgAb	anti-Tg autoantibodies
fT3	free triiodothyronine
fT4	free tetraiodothyronine
TPO-Ab	anti thyroid peroxidase autoantibodies
LoB	limit of blank
CLSI	Clinical and Laboratory Standards Institute
LoD	limit of detection
ECLIA	electrochemiluminescence immunoassay
CLIA	chemiluminescence immunoassay
SD	standard deviation
CI	confidence interval
NACB	National Association of Clinical Biochemistry
ATA	American Thyroid Association
fAS	functional assay sensitivity
HAMA	human anti-mouse antibodies
HAAA	human anti-animal antibodies
ATA	American Thyroid Association

also released from normal or benign altered thyroid tissue. The high importance of Tg for therapy monitoring and follow-up of DTC patients is expressed in all guidelines and consensus papers of professional associations [1–4].

In the 1970s, a competitive radioimmunoassay with polyclonal antibodies, which enabled a valid measurement of Tg values in serum was developed [5] and this assay architecture was the basis for the first commercially offered Tg assays. Immunometric assays (IMA) working with monoclonal antibodies or combinations of mono- and polyclonal antibodies became predominantly established for Tg measurement from the late 1980s onwards. For signal detection both isotopic (immunoradiometric assay, IRMA) and non-isotopic modalities - such as immunoenzymometric assays (IEMA) or immunochemoluminometric assays (ICMA) - are used. The functional sensitivities of these IMAs are in the order of 0.5–1 ng/ml; they are offered in conjunction with automated platforms as well as manual assays, and are still widely used. The recommendations of most international guidelines are based on these functional assay sensitivities.

Around the turn of the millennium, manual IEMA with a functional sensitivity of up to 0.03 ng/ml were available for the first time [6–8]. Starting in 2004, the first automated highly sensitive Tg assay was introduced (Access®, Beckman Coulter), followed around 2013 by highly sensitive Tg assays run on further automated platforms (Elecsys® Tg II, Roche; BRAHMS Kryptor hTg sensitive, Thermo Fisher). These assays, also referred to as “second generation assays” to distinguish them from the preceding assay generation [9] had a functional sensitivity of about 0.1 ng/ml.

While the usefulness of such high-sensitivity Tg (hsTg) measurements was discussed controversially in the beginning, this new standard gained increasing acceptance over time and is also implicitly referenced in current guidelines: For example, in DTC patients after thyroidectomy in most cases hsTg levels <0.1 ng/ml (under ongoing thyroid hormone medication with resulting low TSH levels) eliminate the need for Tg measurement under maximal thyrotropin (TSH) stimulation (either endogenously by temporary discontinuation of thyroid hormone replacement medication, or exogenously by recombinant human TSH), which was previously recommended to demonstrate absence of tumor [3,10,11].

The current consensus paper on the use of hsTg assays [11] emphasizes that the limit of quantification (LoQ) must be determined by the manufacturer according to standardized criteria and communicated in a comprehensible manner. When dealing with Tg values, it is also important to know and consider influencing parameters. Apart from stimulation of the TSH receptor, which usually leads to a significantly increased Tg release, the most important interfering factors include anti-Tg autoantibodies (TgAb), which can lead to false-low or even false-negative Tg values in IMA - with the risk of underestimating or missing active tumor growth.

The aim of our study was to evaluate a newly developed hsTg assay running on the LIAISON® XL automated platform (LIAISON® Tg II Gen assay, DiaSorin). We compared this with two well-established hsTg assays (Elecsys® Tg II running on the Cobas automated platform/Roche, and Medizym® Tg Rem, a manual two-step sandwich IEMA/Medipan).

2. Material and methods

2.1. Patients/serum samples

Male and non-pregnant female patients ≥18 years of age were included in the study. Patients who were unable to understand the study information sheet due to limited language proficiency were not included. Sera collected were frozen at −20 °C until use, unless laboratory determinations were performed promptly after blood collection. Histologically diagnosed papillary, follicular, oncocytic (Hürthle cell), or poorly differentiated thyroid carcinomas are referred to as thyroid follicular cell-derived differentiated thyroid carcinomas (DTC) in the following.

Cohort A: For establishing Tg reference ranges in subjects without thyroid abnormalities, 99 women and 67 men were consecutively recruited. All these patients visited the doctor's practice for nuclear medicine in Duisburg for clarification of a possible thyroid disease, which, however, could be excluded if the following criteria were met:

- Normal-sized thyroid gland (thyroid volume ≤ 18 ml in women and ≤25 ml in men).
- No thyroid nodules or other circumscribed structural abnormalities in ultrasonography
- Euthyroid metabolic status (values for TSH, free triiodothyronine (fT3) and free tetraiodothyronine (fT4) within the reference range).
- No evidence of an inflammatory or autoimmune thyroid disease (inconspicuous anti-thyroid peroxidase autoantibodies (TPO-Ab) value, thyroid parenchyma with isoechogenic homogeneous pattern in ultrasonography).

In addition, there was no evidence of acute extrathyroidal diseases in these subjects, and no thyroid-specific drugs were taken.

Cohort B: To investigate the correlation of measured Tg values between the LIAISON® Tg II Gen assay and the already established Elecsys® Tg II over the entire functional assay range, 126 TgAb-negative sera from 29 different DTC patients with the widest possible range of Tg values. All patients were on ongoing thyroid hormone medication and had subnormal TSH values. Tumor status was variable in these patients.

Cohort C: To investigate the influence of TgAb on measured Tg values, 55 serum samples from 17 different DTC patients (status post thyroidectomy and radioiodine therapy; 1–7 serum samples per patient) with measurable TgAb levels (23–1970 U/ml) were analyzed. These patients were on ongoing thyroid hormone medication, too. Tg levels in these patients were predominantly below the detection limit or in the low measurable range. The current tumor status was also variable in this group; 10 of these patients (38 samples) had structural tumorous disease despite of non-measurable or inadequately low Tg levels, while in 7 patients (17 samples) no structural tumorous disease could localized.

Cohort D: This cohort comprises serum samples from total thyroidectomized low- and intermediate-risk DTC patients obtained after completion of initial therapy, in whom the long-term course was monitored over a median period of 7 years and there was no evidence of tumor persistence or recurrence (TgAb negative and/or undisturbed low-dose recovery test). This cohort was used to investigate the range of highly sensitive measured Tg values with the different assays in cured DTC patients, depending on influencing parameters such as TSH stimulus and adjuvant radioiodine therapy. The following subgroups were studied:

Subgroup D1: 104 different DTC patients (status post total thyroidectomy and adjuvant radioiodine therapy) with two serum samples each. One sample (D1b) was obtained at the time of diagnostic radioiodine whole body diagnosis (usually about 1 year after last radioiodine therapy) under maximal TSH stimulation (TSH >30 mU/ml). The other sample (D1a) was obtained between last radioiodine therapy and radioiodine whole body diagnosis under TSH-suppressive thyroid hormone medication.

Subgroup D2: 46 serum samples from 23 different DTC patients (status post total thyroidectomy and adjuvant radioiodine therapy) under ongoing thyroid hormone medication (2 samples per patient) that were selected according to the criterion that they were all in the measurable range above the LoQ of the Medizym® Tg Rem assay (see below).

Subgroup D3: 110 serum samples from 27 different DTC patients (status post total thyroidectomy but WITHOUT adjuvant radioiodine therapy) under ongoing thyroid hormone medication (2–5 serum samples each per patient).

Cohort E: 20 DTC patients with 54 serum samples (2–5 samples per patient), which were obtained over a longer time period (median 15 months), in whom tumor recurrence was finally confirmed. The samples from these patients are used to control whether serial hsTg measurements correctly reflect the tumor progression by continuously increasing values even in the low measuring range.

2.2. Assays used

The Medizym® Tg Rem assay (Medipan, Blankenfelde-Mahlow, Germany) is a manual two-step sandwich IEMA with 2 monoclonal antibodies directed against different epitopes of the Tg molecule. The capture antibody is fixed to the surface of a microtiter plate, the free 2nd antibody is conjugated with horseradish peroxidase. Overnight incubation of the sample with the antibodies is followed by enzymatic color reaction with 3,3′,5,5′-teramethylbenzidine and measurement of optical density at 450 nm. The functional assay standard range is up to 3 ng/ml. Details on this assay have been published elsewhere [6,12]. This assay is the standard assay for routine follow-up of DTC patients unless they have Tg values above the functional assay standard range in our clinic of nuclear medicine since 2005. The limit of blank (LoB) according to CLSI EP-17A2 [13] was calculated after repeated measurement of blank values and diluted control samples with low Tg concentration. LoB in our lab is 0.007 ng/ml while the limit of detection (LoD) is 0.026 ng/ml. LoQ was calculated according to CLSI EP-17A2 by repeated measurement of serially diluted control samples and reflects a relative squared mean error of measurement of 20%. LoQ of the Tg Rem assay is 0.09 ng/ml which we also used in the current study (values below the LoQ

were reported as <0.09 ng/ml).

For the measurement of Tg values that are above the functional measuring range of the Tg Rem assay, we used in our clinic the SELco® Tg assay (Medipan, Blankenfelde-Mahlow, Germany), a manual IRMA with two monoclonal antibodies. The functional measuring range is 0.3–250 ng/ml Tg. More details on this assay were previously published by our study group [7]. In the context of this study this assay was only used for the preselection of the serum samples for cohort B. Since the SELco® Tg assay was not calibrated 1:1 against the 1st International Reference Preparation (see below) and is no longer available, it was not used for the correlation calculations between the measured Tg values.

The Elecsys® Tg II (Roche, Basel, Switzerland) is an electrochemiluminescence immunoassay (ECLIA) for the Cobas automated system which uses biotinylated monoclonal Tg-specific antibodies and monoclonal Tg-specific antibodies labeled with a ruthenium complex that form a sandwich complex with Tg molecules in the sample. The complex then becomes bound to the streptavidin-coated microparticles that are magnetically captured onto the surface of the electrode. Application of a voltage to the electrode induces chemiluminescent emission, which is measured by a photomultiplier. The LoB of this assay is 0.02 ng/ml, LoD was reported to be 0.04 ng/ml and LoQ is 0.10 ng/ml (values below the LoQ were reported as <0.10 ng/ml). Measuring range is up to 500 ng/ml.

The LIAISON® Tg II Gen assay was run on a LIAISON® XL analyzer according to the instructions of the manufacturer (DiaSorin, Saluggia, Italy). The LIAISON® XL analyzer is a fully automated chemiluminescence analyzer that adopts a "flash" chemiluminescence technology (CLIA) with paramagnetic microparticle solid phase. The measuring range of the LIAISON® Tg II Gen assay is 0.1–500 ng/ml. Data collected on the system in use at the time of our measurement revealed that LoB was 0.025 ng/ml, LoD was reported to be 0.057 ng/ml, while LoQ was 0.10 ng/ml. Values below the LoQ were reported as <0.10 ng/ml.

The Medizym® Tg Rem assay, the LIAISON® Tg II Gen assay and the Elecsys® Tg II assay were standardized 1:1 against the 1st International Reference Preparation CRM-457 (now described as BCR® 457).

TgAb determinations were performed on the Immulite 2000XPi Immunoassay system (Siemens Healthineers, Eschborn, Germany) according to the instructions of the manufacturer. The anti Tg assay is a solid phase, enzyme-labeled, chemiluminescent sequential immunometric assay. Reportable range of values is 20–3000 IU/ml. According to the relevant recommendations [14,15], TgAb determinations performed to authenticate measured Tg values were not based on the reference range of thyroid healthy individuals, but each measurable TgAb value was considered a potential TgAb interference.

2.3. Statistics

The data were collected using MS Excel v.16. Statistical analysis was performed using software IBM SPSS v.27 and the Abacus 2.0 software package (Abacus Validation Systems, Jena, Germany).

Tg-values were represented by ranges and reference intervals and the assays were compared by Passing-Bablock Regression and Bland-Altman-Plots. Group comparison of clinical characteristics were carried out by Mann-Whitney-U-Test. Correlations between Tg-Values and age or clinical characteristics were computed by pearson's correlation.

The significance level was set by 0.05.

2.4. Interchangeability (method comparisons)

Method comparisons were performed between the LIAISON® Tg II Gen assay and the Elecsys® Tg II assay, and (for Tg values up to 3 ng/ml) the Medizym® Tg Rem assay, respectively. Method comparisons were calculated according to the CLSI EP09-A3 guideline [16] using the Abacus 2.0 software package (Abacus Validation Systems, Jena, Germany).

2.5. Reference intervals

Reference intervals for women and men for the LIAISON® Tg II Gen assay and the Elecsys® Tg II assay were calculated according to the CLSI C28-A3c guideline [17] using the Abacus 2.0 software package (Abacus Validation Systems, Jena, Germany). The robust method according to Horn & Pesce [18] was used. This method was developed for small non-normally distributed samples ($n < 120$) or samples with many outliers, where neither the parametric nor the non-parametric method can be used. Robust indicators of location and distribution are used instead of the mean and standard deviation. The 90% confidence intervals for the limits are calculated using a bootstrap procedure.

The study was granted a positive vote by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (16-7084-BO).

3. Results

3.1. Tg reference values of healthy subjects without thyroid abnormalities

In cohort A (healthy individuals without thyroid abnormalities), Tg values of the 99 women were measured in the range of 0.40–63.50 ng/ml (median 11.70 ng/ml) with the LIAISON® Tg II Gen assay and in the range of 0.17–75.80 ng/ml (median 12.50 ng/ml) with the Elecsys® Tg II assay. Tg values of the 67 men were in the range of 1.20–46.90 ng/ml (LIAISON® Tg II Gen; median 10.20 ng/ml) and 2.45–50.60 ng/ml (Elecsys® Tg II; median 11.30 ng/ml). However, gender differences did not reach the level of statistical significance (Mann Whitney *U* test $p > 0.05$ for both assays).

Reference intervals of healthy subjects (cohort A) were calculated up to 37.93 ng/ml (90% confidence interval (CI) for the upper value: 36.44–39.67 ng/ml) for women and up to 24.59 ng/ml (23.07–26.10 ng/ml) for men with the LIAISON® Tg II Gen assay. Reference values for the Elecsys® Tg II assay were calculated as up to 35.60 ng/ml (90% CI for the upper value: 33.76–36.78 ng/ml) for women and up to 27.35 ng/ml (25.48–29.33 ng/ml) ng/for men with the Elecsys® Tg II assay.

Tg values of healthy individuals showed no correlation with age (p values of Pearson's correlation coefficients were 0.950 for the LIAISON® Tg II Gen assay and 0.948 for the Roche Elecsys® Tg II assay).

3.2. Method comparison

3.2.1. Samples of healthy subjects (cohort A)

Comparison of the LIAISON® Tg II Gen and the Elecsys® Tg II results obtained from samples of healthy persons by Passing-Bablock regression yielded a slope of 0.930 (95% CI: 0.887 to 0.980), an intercept of −0.413 (95% CI: 0.94 to −0.03) and Kendall's tau of 0.861. Bland Altman analysis revealed a medium inaccuracy of −10.8% (95% CI: 13.9 to −7.7%; Fig. 1).

3.2.2. Samples of DTC patients with active tumorous disease

In cohort B (126 TgAb-negative sera with the widest possible range of Tg values from 29 different DTC with variable tumor status), results were in the range of 0.18 ng/ml to 488.4 ng/ml in the LIAISON® Tg II Gen assay while in the Elecsys® Tg II assay the range was <0.1–422 ng/ml with one sample below the LoQ of 0.1 ng/ml. Correlation of Tg values measured with the LIAISON® Tg II Gen assay with the already established Elecsys® Tg II assay yielded a Passing-Bablock regression with a slope of 1.065 (95% CI:1.030–1.088), an intercept of −0.176 ng/ml (95% CI: 0.46-0.30) and Kendall's tau of 0.964. Bland Altman analysis showed a medium inaccuracy of 4.3% (95% CI:2.1 to 6.5; Fig. 2).

In cohort E (54 serum samples with Tg values in the lower range from 20 different DTC patients with tumor recurrences at an early stage), results were in the range of 0.11 ng/ml to 3.00 ng/ml in the LIAISON® Tg II Gen assay, while in the Elecsys® Tg II assay the range was <0.1 ng/ml to 2.90 ng/ml with 2 samples below the LoQ of 0.10 ng/ml. Additionally, we compared results of both assays with results of the Medizym® Tg Rem assay, our routinely used assay for patients with Tg values less than 3 ng/ml (range 0.09–3.0 ng/ml). Results of the Passing-Bablock regression analysis and Bland Altman analysis are shown in Fig. 3 and are summarized in Table 1.

3.2.3. Samples of cured DTC patients

Furthermore, we compared Tg values of serum samples from DTC patients who were thyroidectomized with no evidence of tumor persistence or recurrence (cohort D). This cohort was further subdivided into patients WITH adjuvant radioiodine therapy (subgroup D1) in whom Tg was measured both under TSH suppressive thyroid hormone medication (subgroup D1a) and under maximum TSH stimulation (subgroup D1b), patients WITH adjuvant radioiodine therapy who were selected according to the criterion that they all had Tg values in the measurable range above the LoQ of our Medizym® Tg REM assay (subgroup D2), and patients classified as "cured" WITHOUT adjuvant radioiodine ablation (subgroup D3). The range of Tg values obtained with the three assays are depicted in Table 2. Results of the Passing-Bablock regression analysis and Bland Altman analysis are summarized in Table 3. Results showed that the comparability of the three assays in the lower measuring range of these non-tumor specific Tg values is poor. As expected, inaccuracy was worst especially in the lowest values in all comparisons.

3.2.4. Analysis of clinical differences between the cohorts

To analyze whether clinical differences between patient cohorts are correctly reflected by the different assays, we performed Mann

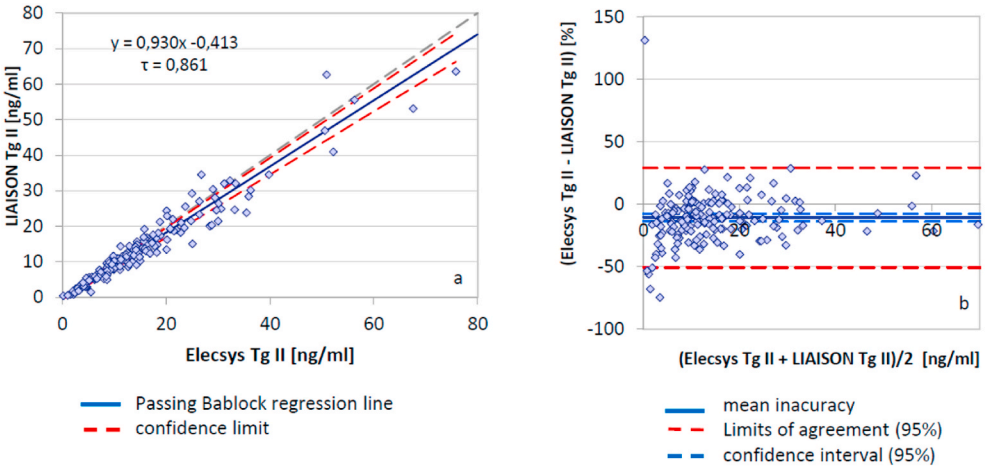

Fig. 1. Passing Bablok regression (a) and Bland Altman analysis (b) of the Elecsys® Tg II and LIAISON® Tg II Gen values in healthy persons (cohort A; n = 166).

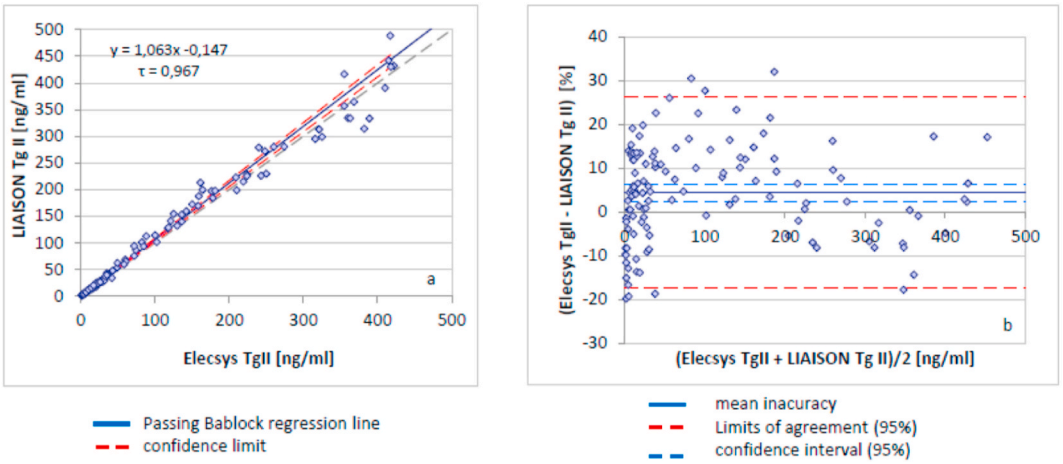

Fig. 2. Passing Bablok regression (a) and Bland Altman analysis (b) of the Elecsys® Tg II and LIAISON® Tg II Gen values in samples from patients with DTC (cohort B; n = 126 from 29 patients).

Whitney U-tests of the indicated groups within one assay. For this purpose, all values below the LoQ (0.09 ng/ml for the Medizym® Tg Rem assay and 0.1 ng/ml for the DiaSorin and the Roche assay) were set empirically to half of the value of the LoQ (0.045 ng/ml resp. 0.05 ng/ml).

In subgroup D1 (cured thyroidectomized DTC patients WITH radioiodine therapy) we analyzed if the Tg values under TSH suppressive conditions (subgroup D1a) and under maximal TSH stimulation (subgroup D1b) differ significantly when measured with the three assays. We found that for all three assays mean Tg values in subgroup D1b (under max. TSH stimulation) are significantly ($p < 0.0001$) higher than those in subgroup D1a (under TSH suppressive conditions; Table 4).

We divided subgroup D1b further into patients in whom the final radioiodine whole body scintigraphy showed residual radioiodine accumulation in the thyroid bed (n = 26) and those in whom this was not the case (n = 78) in order to analyze, whether Tg values of these subgroups differ significantly. Distribution of Tg values in the different assays are shown in Table 5. Comparison of Tg values between these two groups revealed that Tg values in patients with residual radioiodine accumulation are significantly higher with all 3 assays ($p < 0.05$; Table 4).

Additionally, we compared Tg values of subgroup D1a (thyroidectomized DTC patients WITH additional radioiodine therapy) with those Tg values of subgroup D3 (thyroidectomized DTC patients WITHOUT additional radioiodine therapy), which were measured at a comparable time interval after thyroidectomy (median 10 versus 11 months, p = 0,226). Tg values between these two groups of patients under TSH suppressive thyroid hormone medication differed highly significantly ($p < 0.001$) in each of the three assays with lower values in subgroup D1a (Table 4).

Next, we tested whether all assays correctly reflect an increase in Tg values in patients with a clinically proven tumor recurrence (cohort E). For this purpose, we selected 29 serum pairs from 13 patients with an increase of Tg Rem values above the critical difference of the Medizym® Tg Rem assay in the lower range (55% for values of 0.20 ng/ml). In the LIAISON assay, an increase of ≥55% was detected in 21 of the 29 sample pairs, while in the Elecsys assay 25 serum pairs showed this increase.

For 12 of these patients, at least 3 Tg values were available for each assay. In samples of these 12 patients, we analyzed if an increase of ≥55% was detected starting from the nadir over time. Besides in the Medizym® Tg Rem assay as a reference, this was the case for all samples in the Elecsys® Tg II assay, while in the LIAISON® Tg II Gen assay, samples from one patient did not met these criteria. Thus, in most cases, serial Tg measurements over a longer follow-up period showed, as expected, a continuous increase in all assays.

In samples of patients with measurable TgAb levels (cohort C) we compared Tg values of the different assay to test for assay-specific interferences of TgAbs. Sample characteristics are shown in Table 6.

In 5 of the 10 patients with localized structural tumorous disease all Tg values were below the LoQ in the Elecsys® Tg II assay, while in only one of these patients all Tg values were below LoQ in the LIAISON® Tg II Gen assay. In all 17 samples of patients without structural disease Tg values were below 0.10 ng/ml in the Elecsys® Tg II assay while only 3 samples from 2 patients were below 0.10 ng/ml in the LIAISON® Tg II Gen assay (range: <0.10–0.33 ng/ml).

Mann-Whitney U-tests were performed to compare Tg values of patients with structural tumorous disease with Tg values of patients without tumorous disease. For both the Elecsys® Tg II assay and the LIAISON® Tg II Gen assay, difference between these two groups were highly significant ($p < 0.01$).

Due to the low number of measurable Tg values, we further compared values qualitatively.

In 13 samples from 3 patients with structural tumorous disease, values measured with the Elecsys® Tg II assay were at least 40% higher than those measured with the LIAISON® Tg II Gen assay.

In 6 samples from 2 patients the LIAISON and Elecsys® Tg II assay gave comparable values.

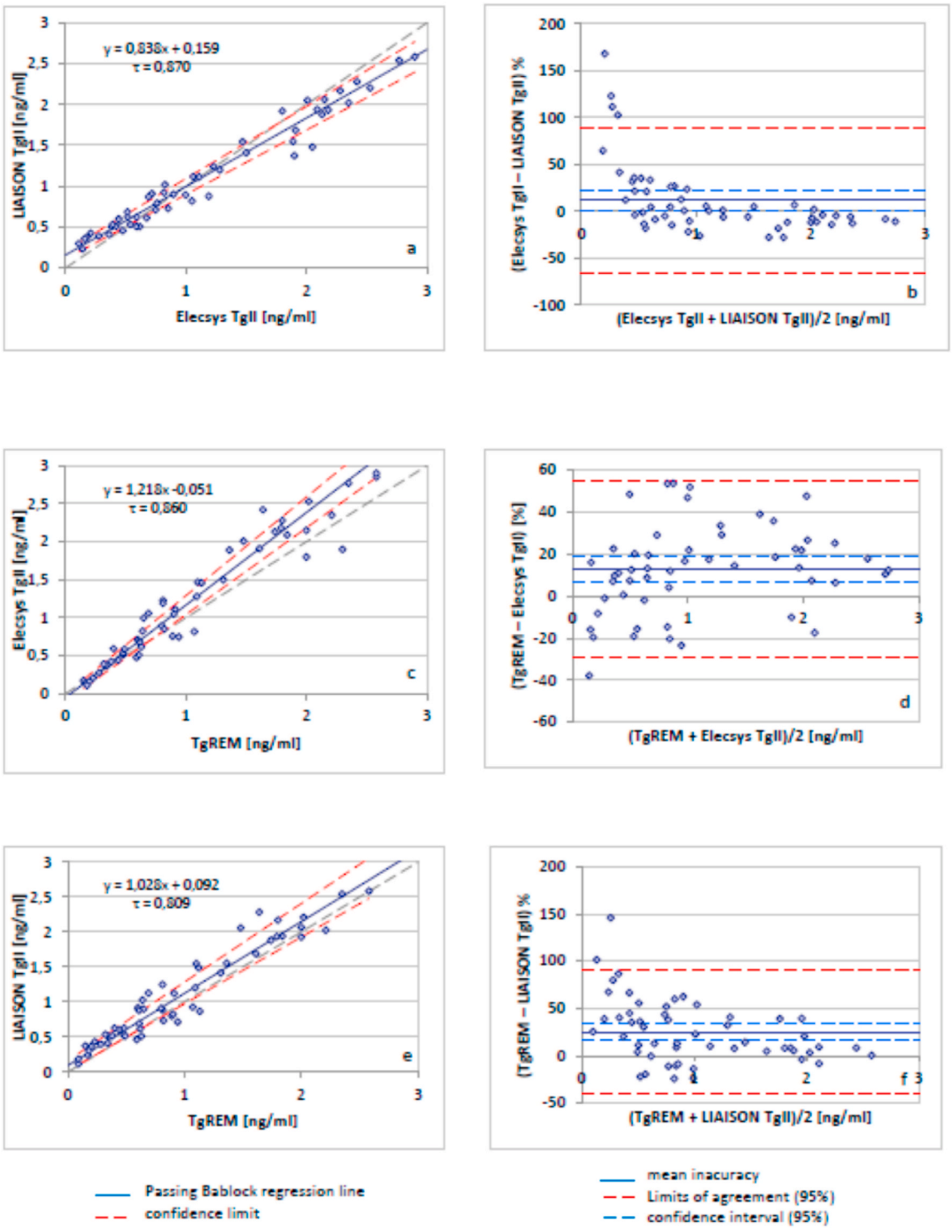

Fig. 3. Passing Bablok regression (a, c, e) and Bland Altman analysis (b, d, f) of the Elecsys® Tg II and LIAISON® Tg II Gen values (a, b), TgREM and LIAISON® Tg II Gen values (c, d) and TgREM and Elecsys® TgII values (e, f) in samples from patients with increasing Tg values and finally confirmed tumor recurrence (cohort E; n = 54 from 20 patients).

Table 1
Data of Passing Bablock regression, Kendall's tau and Bland Altman analyses of the Elecsys Tg II and LIAISON® Tg II Gen values, TgREM and LIAISON® Tg II Gen values and TgREM and Elecsys® TgII values in samples from patients with increasing Tg values and finally confirmed tumor recurrence (cohort E; n = 54 from 20 patients).

	Elecsys Tg II - LIAISON Tg II Gen	Tg Rem - Elecsys Tg II	Tg Rem - LIAISON Tg II Gen
Passing Bablock regression analysis:			
slope:	0.838	1.218	1.028
95% CI:	0.789 to 0.882	1.141 to 1.297	0.954 to 1.123
Intercept:	0.159	−0.051	0.092
95% CI:	0.11 to 0.21	−0.10 to −0.01	0.02 to 0.16
Kendall's tau:	0.870	0.860	0.809
Bland Altman analysis:			
med. inaccuracy:	11.8%	12.8%	25.1%
95% CI: %	0.9–22.7%	7.0–18.8%	15.9–34.2%

Table 2
Characterization of samples of cohort D.

	subgroup D1a under TSH suppressive conditions	subgroup D1 b under max.TSH stimulation	subgroup D2	subgroup D3
Serum samples (n)	104	104	46	110
TSH [mU/l]	<0.01 to 0.30	32.0 to 277.0	<0.01 to 0.46	<0.01 to 0.9
range Tg Rem [ng/ml]	<0.09 to 0.11	<0.09 to 2.50	0.09 to 1.20	<0.09 to 1.13
No. below LoQ Tg Rem (0.09 ng/ml)	102	51	0	48
range LIAISON Tg II Gen [ng/ml]	<0.10 to 0.92	<0.10 to 2.04	<0.01 to 1.06	0.10 to 1.13
No. below LoQ LIAISON Tg II Gen (0.1 ng/ml)	21	8	1	2
Range Elecsys Tg II [ng/ml]	<0.10 to 0.10	<0.10 to 2.20	<0.1 to 2.20	<0.10 to 0.95
No. below LoQ Elecsys Tg II (0.1 ng/ml)	102 (of 103; one sample missing	63	28	62

Table 3
Data of Passing Bablock regression analyses, Kendall's tau and Bland Altmann analyses of non-tumor samples (cohort D).

	Elecsys Tg II - LIAISON Tg II Gen	Tg Rem - Elecsys Tg II	Tg Rem - LIAISON Tg II Gen
Passing Bablock regression analysis:			
slope:	0.964	1.300	1.753
95% CI:	0.841 to 1.227	1.114 to 1.485	1.410 to 2.177
Intercept:	0.15	−0.05	0.03
95% CI:	0.10 to 0.18	- 0.09 to −0.01	−0.04 to −0.08
Kendall's tau:	0.591	0.705	0.451
Bland Altman analysis:			
med. inaccuracy:	75.0%	9.5%	101.6%
95% CI: %	60.5–89.6%	2.8–16.1%	88.0–115.2%
n =	107	101	162

Table 4
Comparison of Tg values in different subgroups of cohort D. P-values of the Mann-Whitney U-tests are depicted.

	Tg REM assay	LIAISON Tg II Gen assay	Elecsys Tg II assay
cohort D1a – cohort D1b	<0.0001	<0.0001	<0.0001
cohort D1b w/o radioiodine accumulation – cohort D1b residual accumulation	<0.05	<0.05	<0.05
cohort D1a – cohort D3	<0.0001	<0.0001	<0.001
cohort D1b – cohort D3	0.1683	0.1223	0.6691

Table 5
Characterization of samples of cohort D1b.

	no radioiodine accumulation in the thyroid bed cohort D1b-	residual radioiodine accumulation in the thyroid bed cohort D1b+
n =	78	26
range Tg Rem [ng/ml]	<0.09 to 2.5	<0.09 to 2.27
No. below LoQ Tg Rem (0.09 ng/ml)	43	8
range LIAISON Tg II Gen [ng/ml]	<0.1 to 1.85	<0.1 to 2.07
No. below LoQ LIAISON Tg II Gen (0.10 ng/ml)	6	2
Range Elecsys Tg II [ng/ml]	<0.1 to 1.98	<0.1 to 2.20
No. below LoQ Elecsys Tg II (0.10 ng/ml)	51	12

Table 6
Sample characteristics of cohort C.

n =	55
TSH [mU/l]	<0.01 to 2.7
TgAb [U/ml]	23 to 1970
range LIAISON Tg II Gen [ng/ml]	<0.10 to 11.00
No. below LoQ LIAISON Tg II Gen (0.10 ng/ml)	8
Range Elecsys Tg II [ng/ml]	<0.10 to 26.40
No. below LoQ Elecsys Tg II (0.10 ng/ml)	36

4. Discussion

4.1. Tg reference values of subjects without thyroid abnormalities

The Tg values in subjects without thyroid abnormalities (cohort A) were all within the measurable range of both tested automated hsTg assays. It is interesting to note the slight differences in the reference ranges with, on average, higher Tg values in women, as this fact is not generally established. Further studies with a greater number of samples are needed to confirm this. In the only study known to us in which similarly strict selection criteria for the normative population were applied as in our study - with additional exclusion of smokers, of patients taking any medication and with personal or familial history of thyroid disease, and of TgAb-positive sera (National Association of Clinical Biochemistry (NACB) criteria [19]) - comparable results were found when measured with the Access Tg assay (Beckmann-Coulter SA, Nyon, Switzerland): Gender-specific Tg reference ranges, calculated as mean ± 2 SD on logarithmic transformed data, were 1.40–29.2 ng/mL in males (n = 209, range 0.2–36.4 ng/mL) and 1.50–38.5 ng/mL in females (n = 229, range 0.1–48.7 ng/mL), respectively. Similar to our present study, they found no relationship between age and Tg levels in their series; all subjects were born and lived in Verona or neighboring villages.

In several studies in which the Tg values were examined as marker for the individual iodine supply of people, the Tg values were also assessed separately according to gender, but otherwise not based on the above-mentioned strict NACB selection criteria. While in a Danish [20] as well as in a Czech [21] study, median Tg values were also found to be higher in women than in men, this was not the case in a Chinese study [22]. Both ethnic and environmental (primarily iodine supply) factors are likely to play a role here.

The definition of Tg reference ranges for subjects that have been strictly preselected without any thyroid disease appears to be sensible, since benign thyroid pathologies can significantly influence the Tg levels. In studies on Tg values in various thyroid diseases [23,24], Tg was found to be significantly higher in histologically confirmed benign nodular goiter than in thyroidal healthy individuals and showed a strong overlap with thyroid nodules that were found to be DTC. Similar findings apply to other benign thyroid diseases, e.g., autoimmune thyroid diseases [23,25]. For this reason, the contribution of Tg measurement to the determination of the malignancy risk of thyroid nodules is predominantly viewed skeptically. According to the current American Thyroid Association (ATA) guideline [3] routine measurement of serum Tg for initial evaluation of thyroid nodules is not recommended.

4.2. Therapy and role of Tg measurement in confirmed DTC

The initial guideline-guided therapy of DTC consists in a total or near total thyroidectomy, if necessary combined with a stage-adapted cervical lymph node dissection (only in very-low-risk patients a partial thyroid resection may be sufficient). After surgical thyroidectomy, small thyroid remnants are usually present, which can no longer produce relevant amounts of thyroid hormone, but can still release minimal - and depending on assay sensitivity, measurable - amounts of Tg. In intermediate and higher tumor stages, thyroidectomy is followed by radioiodine therapy (possibly repeated radioiodine therapy, if persistent radioiodine accumulating tumor tissue is present). By this, not only thyroid remnants are ablated, but also possible further tumor cells contained in the thyroid remnants as well as occult and apparent DTC metastases can be eliminated. In the presence of radioiodine-accumulating distant metastases, adjuvant radioiodine therapy is the most important therapeutic tool in DTC, and significantly improve patient outcome [3, 26].

In the case of an inconspicuous course, a final radioiodine whole body scan is performed at an appropriate interval from the last

radioiodine therapy - about 1 year later in our Clinic for Nuclear Medicine - in order to visualize any remaining or recurrent iodine-accumulating tumor tissue. Subsequently, DTC patients are offered regular follow-up examinations, which include determination of Tg as well as thyroid hormone parameters and high-resolution ultrasonography of the neck. Recurrences can occur even after many years; since the chances of successful intervention increase with timely diagnosis and adequate therapy, decades of follow-up are reasonable [3].

After removal of the thyroid gland, DTC patients must take lifelong thyroid hormones. This is not only performed for hormonal replacement of the absent thyroid gland, but also for the purpose of keeping TSH levels low, as high TSH can potentially stimulate the growth of possibly persistent DTC cells. Since - on the other side – a strong TSH stimulus is required for optimal iodine uptake into the thyroid cells and the DTC cells, this must be generated briefly prior to planned radioiodine interventions. This can be done either endogenously (by thyroid hormone withdrawal for several weeks, resulting in short-term hypothyroidism) or exogenously (by repeated injection of recombinant human TSH).

Since serum Tg values are on average one order of magnitude higher under maximum TSH stimulation, Tg measurement under maximum TSH stimulation 6–18 months after completion of primary therapy is recommended for proof of curative therapy success in DTC, whereby radioiodine whole body diagnostics, which is classically performed in parallel, is partly classified as dispensable in the guidelines of recent years [3,10]. These recommendations are based on the functional sensitivity of the 1st-generation Tg assays that were exclusively available at the time (fAS approximately 0.5–1 ng/ml). The TSH stimulation factor may, however, be very variable: in less differentiated DTC <2fold, in some cases, however, >100fold of the basal Tg values [27–29].

In a meta-analysis [30] the diagnostic value of Tg was calculated on the basis of the 1st generation assays (evaluation of 1613 patients from 9 studies for the Tg under suppressive levothyroxine medication and of 1602 patients from 12 studies for the TSH-stimulated Tg). With a median cutoff of 2 ng/ml calculated by means of ROC analysis, a diagnostic sensitivity of 0.778 ± 0.023 resulted for the basal Tg (under TSH suppression); for the stimulated Tg (with a calculated median cutoff of 3 ng/ml), on the other hand, a diagnostic sensitivity of 0.961 ± 0.013 was determined. Unstimulated Tg measurements carried out with 1st generation assays accordingly have an unsatisfactory diagnostic sensitivity.

A major advantage of hsTg determination is that - at least in low- and intermediate-risk patients - it mostly eliminates the need for TSH stimulation for the exclusive purpose of increasing the diagnostic sensitivity of Tg determination [31]. In this way, impairment of the patient's quality of life (in the case of endogenous TSH stimulation) can be avoided and unnecessary costs (in the case of exogenous TSH stimulation) can be saved. Additionally, serial hsTg determinations in DTC aftercare have the potential to detect a recurrence early, at any time during long-term follow-up, based on the dynamics of the Tg development [32,33]. Since hsTg measurement is increasingly becoming the standard for the follow-up of DTC patients, it is reasonable that all widely-used laboratory automated systems offer a well-validated hsTg assay. This validation should not only include the determination of the usual assay parameters, but also the control of the plausibility of the measured Tg values in defined patient groups. In the past it has been shown that there are in some cases significant differences between the Tg values measured with different assays [34,35], even in TgAb-negative patients and when the assays are strictly calibrated to the 1st International Reference Preparation [36]. One possible reason for this are molecular variants of the individually present thyroglobulin, with variable affinity of the assay antibodies to the relevant epitopes. The interfering effects of circulating TgAb (even in lowest amounts) can also differ from assay to assay. It is therefore essential to establish assay-specific reference values and clinical cutoffs for any newly introduced Tg assay.

4.3. Comparison between the different hsTg assays

The only publication known to us that contains data on the evaluation of the new LIAISON® Tg II Gen assay comes from Cennamo et al. [37]. In their study, they compared the serum Tg values of 91 individuals with the results of the well-established Beckman Access assay. The collective comprised 21 patients with confirmed DTC, 16 patients with benign thyroid diseases and 54 apparently healthy subjects. 23 patients were positive for TgAb, 68 were TgAb-negative. In this cohort, they found also a good correlation between the two assays. However, the authors neither discriminated results from the different patient groups nor from the TgAb-positive patients so that more differentiated data are needed.

Our results, which we present here, showed a good correlation of values between different patient collectives. In the groups of healthy subjects (cohort A) and of patients with active tumorous disease (cohort B) values measured with the LIAISON® Tg II Gen assay and values measured with the Elecsys® Tg II assay showed a very good comparability. However, values compared in these cohorts covered a wide measuring range (up to 80 ng/ml resp. 500 ng/ml). In patients with low Tg values caused by tumor recurrence (cohort E; measuring range up to 3 ng/ml), we additionally compared values with the Medizym® Tg Rem assay and also found a good comparability of all three assays. This good comparability between all assays was expected since all three assays are standardized 1:1 against the first International Reference Preparation CRM-457 (now described as BCR 457).

A different picture emerged when comparing Tg values of serum samples from DTC patients who were thyroidectomized with no evidence of tumor persistence or recurrence (cohort D). As expected, Tg values of non-tumor specific Tg in these samples were in the lower measuring range of all assays (median of measurable values: 0.17 ng/ml for the Medizym® Tg Rem assay, 0.26 ng/ml for the LIAISON® Tg II Gen assay and 0.23 ng/ml for the Elecsys® Tg II assay). The comparability of this non-tumor-specific fluctuating Tg values in the very low range from the three assays is poor. The reasons for this may be the generally larger measurement deviations in the lower measuring range or physiological reasons as described in following section (4.4.).

4.4. hsTg values in thyroidectomized DTC patients with and without radioiodine therapy

In contrast to 1st generation Tg assays, hsTg assays can measure Tg values in the low range in many DTC patients, who are free from disease after successful thyroid ablation. Measured Tg values in the very low range - that are mostly only detectable by 2nd generation assays - can originate from small or smallest residual tumor tissue as well as from ortho- or ectopic residual thyroid tissue, both not necessarily localizable by medical imaging procedures. Additionally, extrathyroidal non-neoplastic sources have been discussed, e.g., Tg secretion from the thymus [38], various unspecific causes cannot be ruled out either. For instance, false positive Tg values could be caused by human anti-mouse antibodies (HAMA), other human anti-animal antibodies (HAAA), or by interference of heterophilic antibodies [39] - when these form linkages between the solid phase capture antibody and the detector antibody of the Tg assay. As different assays have varying susceptibilities against these interferences, this could explain the large discrepancies observed in some cases between the three hsTg assays examined.

In some recent guidelines, the benefit of radioiodine therapy in low-risk and in some cases even in intermediate-risk patients is questioned, as well as the requirement of a completely TSH-suppressive thyroid hormone medication [3]. Thus, in the future, the proportion of DTC patients who have only undergone surgical thyroidectomy and who receive only thyroid hormone adjustment with TSH values in the slightly subnormal to intermediate reference range will increase during follow-up [40]. These two factors have an influence on the hsTg values measured under curative conditions, which are expected to be somewhat higher on average than in patients with adjuvant radioiodine ablation or strict TSH-suppressive thyroid hormone setting. Therefore, in our study we investigated hsTg values from serum samples of both patients with and without adjuvant radioiodine therapy after thyroidectomy.

As expected, in our subgroup-analysis under TSH-suppressing conditions, it could be observed that mean Tg values in cured DTC patients after thyroidectomy but without radioiodine therapy were significantly higher than mean Tg values of patients who underwent additional radioiodine therapy, in all 2nd generation assays. Moreover, we found that for all 3 examined hsTg assays even in patients with adjuvant radioiodine therapy mean hsTg values in patients with minimal residual radioiodine accumulation in the thyroid bed were significantly higher as compared to the hsTg values in patients without radioiodine accumulation in the thyroid bed.

These findings (dependency of the measured hsTg values on the radicalness of thyroablative procedures and on the scintigraphic visualization of minimal thyroid remnant tissue) suggest that small residual thyroid tissue with preserved metabolic capability is a plausible cause for the low Tg values detectable by 2nd generation assays in cured DTC patients. This is further supported by the finding that the Tg values in the same cured patients were significantly higher under maximum TSH stimulation than under TSH suppression, as this requires differentiated thyroid tissue with TSH receptors.

In contrast to such non-tumor specific low Tg values of cured DTC patients, which can be measured more frequently with hsTg assays - and which show only moderate fluctuations without a significant increase over time or even a decreasing tendency in serial measurements [41–43] - Tg values in Patients with DTC recurrence usually describe a more or less continuous increase, which reflects the tumor progression [7,8,33]. In the latest version of the ATA Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer [3] it is postulated that in patients with Tg levels in the low measurable range the trend in serum Tg over time, recorded by serial/periodic Tg measurements under ongoing thyroid hormone medication, will typically identify patients with clinically significant residual disease (“a rising … serum Tg indicates disease that is likely to become clinically apparent”). In the ATA guidelines cited, it is also assumed that the calculation of the Tg doubling time could have a predictive value, similar to that already established for the calcitonin doubling time in medullary thyroid carcinoma.

In a previous study [33] we analyzed serial Tg measurements under ongoing thyroid hormone medication from 144 progressive DTC courses of 99 patients after radioiodine therapy. We showed that the dynamics of the Tg development in the highly sensitive measuring range ($n = 22$) was comparable with the dynamics of higher Tg values, e.g. Tg doubling time in both measuring ranges showed no significant difference in patients with progressive DTC. The data on the Tg doubling time can thus be transferred to the monitoring of relapses in the highly sensitive Tg measuring range. Our current study showed that serial Tg measurements with all 3 compared hsTg assays reflect the progress in almost all patients with confirmed relapse. There was a slightly better comparability of the Medizym® Tg Rem assay with the Elecsys® Tg II assay; the DiaSorin assay shows no increase above the suspect threshold of 55% in only one of 12 cases.

4.5. hsTg values in TgAb-positive patients

Approximately 25–30% of patients with DTC have measurable TgAbs at the time of primary diagnosis, with the TgAb level falling continuously after thyroablative treatment if the course is uncomplicated [15,44]. TgAbs can lead to a corruption of the measured Tg values. With IMAs, the measurement signal is reduced if no sandwich complexes can be formed between the capture and detector antibodies because the autoantibodies compete with the assay antibodies for the same binding sites on the Tg molecule. Various assay manufacturers have tried to develop assay antibodies against epitopes of the Tg molecule at which interference with TgAb is unlikely [45,46]; however, this problem cannot be completely eliminated [14].

Therefore, any TgAb interferences should be clarified in parallel with each Tg measurement. This can be done either by direct TgAb measurement in the patient’s serum, or by recovery tests. With the direct TgAb measurement, it is not possible in principle to differentiate whether the measured TgAbs actually interfere with the assay antibodies at the specific binding sites of the Tg molecule. However, since inadequately low measured Tg values can also be caused by in vivo effects (accelerated metabolic clearance rate of circulating Tg-TgAb complexes by the reticuloendothelial system [47], the guidelines primarily recommended direct TgAb measurement for authentication of measured Tg values. In the case of detectable TgAbs of any amount, at least doubts must be expressed that the measured Tg value actually reflects the tumor load in a representative manner.

To evaluate whether and to what extent TgAbs affect the measured Tg values of the various assays, we analyzed 55 serum samples with measurable TgAb levels (23–1970 U/ml) from 17 different DTC patients (status post thyroidectomy and radioiodine therapy; 1–7 serum samples per patient; cohort C). In the 9 patients with confirmed structural disease, the Tg values measured in both the Roche and DiaSorin assays were significantly higher than in the patients without a localizable tumor correlate, but mostly inadequately low for the existing tumor burden, in some cases even below the LoQ. Very low or - even by hsTg assays - undetectable Tg values in the presence of TgAbs despite of extensive metastases could be explained by too low Tg concentrations in the respective samples, or in vitro interferences. The significant differences in the measured Tg values between the two assays observed in some TgAb-positive samples could be due to different affinity of the TgAbs and the assay antibodies to the relevant epitopes of the Tg molecule, or a different vulnerability of the assays with regard to such in vitro interference, as described elsewhere [48].

5. Conclusion

In summary, the data from our study demonstrate that with the new LIAISON® Tg II Gen assay another assay running on an automated laboratory platform for measurement of Tg values ranging from the highly sensitive up to a pronounced increased level is available. Due to the standardizing against the 1st International Reference Preparation CRM-457 (now described as BCR® 457), we found comparable values with two established Tg assays (Medizym® Tg Rem and Elecsys® TgII) in samples from healthy subjects and in patients with DTC. In contrast, in the highly sensitive Tg measurement range for patients after thyroidectomy we found only poor comparability between the three assays tested while clinical differences between the cohorts were correctly reflected by all three assays. In TgAb sera of DTC patients depicted different results between assays indicating different interferences of TgAb's with assay antibodies.

Funding

The University Hospital Essen had a research agreement with DiaSorin (<25.000 €).

DiaSorin was neither involved in the preparation of this manuscript nor in the interpretation of the results; DiaSorin also had no influence on this.

CRediT authorship contribution statement

Martina Broecker-Preuss: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. **Irina Mehnert:** Investigation, Data curation. **Elena Gilman:** Formal analysis, Methodology. **Ken Herrmann:** Resources. **Manuel Weber:** Writing – review & editing. **Rainer Görges:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Sandra Schneider as a representative for the lab technicians for excellent technical assistance.

We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

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DOI: 10.1016/j.plabm.2021.e00250
URN: urn:nbn:de:hbz:465-20220720-133032-5