European Thyroid Association

European Thyroid Journal (2024) **13** e240142
https://doi.org/10.1530/ETJ-24-0142

Receive 7 May 2024
Accepted 31 October 2024
Available online 1 November 2024
Version of Record published 25 November 2024

RESEARCH

Development of an enzyme-linked immunosorbent assay for newborns dried blood spot thyroglobulin

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Abstract

Background: Thyroglobulin (Tg) is a biomarker of iodine status. Newborn Tg is a more sensitive marker than neonatal TSH in detecting variations in iodine intake. This study aims to validate an enzyme-linked immunosorbent assay (ELISA) for Tg determination on dried blood spots (DBS) in newborns. This study also sets out to assess the stability of Tg and the influence of newborns' hematocrit on Tg determination.

Methods: A commercially available ELISA Tg assay was adapted for use on DBS. DBS-Tg in cord blood was measured in 209 newborns delivered from healthy euthyroid pregnant women. Sensitivity, linearity, repeatability, and intermediate fidelity were determined using the appropriate standards and quality control materials.

Results: The limit of detection of the DBS-Tg assay was 2.4 µg/L, and the limit of quantification was 5.8 µg/L. Repeatability and intermediate fidelity were 7.7–8.3% and 11.0–11.2%, respectively. The median cord plasma Tg and DBS-Tg values in newborns were not significantly different, 30.2 (21.3–44.4) µg/L and 31.6 (19.3–48.7) µg/L ($P = 0.48$) with the ELISA, respectively, and 76.5 (40.0–101.5) µg/L with the Elecsys assay with an $R = 0.88$. DBS-Tg concentrations decrease with increasing hematocrit values ($P < 0.05$). DBS-Tg values were stable at a concentration of 25 µg/L for 12 months at −20°C and 4°C.

Conclusion: This DBS-Tg assay demonstrated good analytical performance over a wide range of Tg concentrations, suggesting it is well suited to detecting variations in Tg concentrations. Studies comparing populations with different prevalence of anemia should consider the effect of hematocrit on DBS-Tg determination. The availability of a DBS-Tg assay for newborns makes it possible to integrate iodine status monitoring with newborn screening for inherited metabolic diseases.

Keywords: thyroglobulin; dried blood spot; iodine; ELISA; newborn

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https://doi.org/10.1530/ETJ-24-0142

Introduction

Inadequate iodine intake is one of the main determinants of thyroid diseases (1, 2). Despite progress in controlling iodine deficiency, many countries, including those in Europe, remain iodine deficient (3).

Monitoring is crucial in order to optimize iodine intake, but the process remains challenging because all biomarkers of iodine status have their limitations (4). Urinary iodine concentration (UIC), the most frequent index of iodine status, is a sensitive indicator of iodine intake during the days immediately preceding sample collection but not for assessing long-term iodine intake (5). Neonatal TSH (nTSH) has also been used to monitor iodine status (6). It is easily collected during screening programs for congenital hypothyroidism, making it an accessible biomarker. When iodine deficiency is severe or moderate, nTSH increases but is less sensitive when iodine deficiency is mild (7). This is because several pre-analytical factors influence nTSH independently of iodine status, including prematurity, the timing of the neonatal blood sample collection, seasons, maternal and newborn exposure to iodine-containing antiseptics, and the TSH assay used (7).

Thyroglobulin (Tg) is a sensitive biomarker of iodine status (8). In the absence of thyroid disease, circulating blood Tg reflects thyrocyte cell mass and stimulation by TSH, both of which increase in iodine-deficient subjects (9). Serum Tg concentrations in iodine-deficient children and pregnant women decrease rapidly after iodine supplementation (10, 11). In regions of mild iodine deficiency, serum Tg is normally the only thyroid parameter associated with UIC in adults (12, 13). In mildly iodine-deficient newborns, cord serum Tg concentrations are significantly higher than those found in iodine-sufficient newborns (14).

Interestingly, unlike nTSH, which decreases exponentially after birth, Tg concentrations remain stable for 96 h. This represent an advantage since the influence of the timing of sample collection on Tg concentrations will be far less critical (15). These considerations suggest that neonatal Tg may be a better tool for assessing iodine status. Although a validated Tg assay in dried blood spots (DBS) has already been used in iodine surveys across several populations (11, 16, 17, 18), a Tg determination of cord DBS in newborns using an ELISA has not previously been described. Consequently, this study aimed to validate a Tg ELISA assay for use in Tg determination on DBS and to evaluate the feasibility of using this assay to measure Tg in newborns. In addition, it assessed the effect of hematocrit on DBS-Tg concentrations and the DBS-Tg stability over time.

Materials and methods

Subjects

Cord blood samples from the umbilical vein were obtained from 209 newborns delivered at the H.U.B. – Hôpital Erasme by healthy euthyroid pregnant women with no history of thyroid diseases. Cord blood was collected instead of blood directly from newborns because validating the method implies repeating the Tg determination several times, requiring sufficient blood, which is easier to obtain from cord blood. The ULB Ethics Committee at the Erasme Hospital approved the study protocol (P2019/151). Informed written consent was obtained from all pregnant women.

Sample preparation and storage

Cord blood samples were collected in heparin-treated vacutainer tubes. The whole blood was centrifuged at 1260 × ***g*** (Allegra X-15R Centrifuge) for 10 min, and the plasma was removed and transferred into cryotubes of 2 mL and stored at −80°C until analysis. For the DBS samples, eight drops of cord blood were spotted onto filter paper (903 Zentech Blood Spot Diagnostic Card B), and the spots were allowed to dry at room temperature (RT) for 24 h before they were stored in sealed aluminum bags at −20°C (Foil Ziplock Biohazard Bag, GE Healthcare®).

Chemicals and materials

All buffers and reagents were prepared with ultra-pure water (18.2 MΩ; DNAase-Free; Pyrogens-Free). The coating buffer was PBS (pH 7.2 ± 0.3) – 0.15 M NaCl and 0.01 M phosphate buffer ($Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot H_2O$). The BSA (Sigma-Aldrich) solution of 1% in PBS was used for plate blocking, detection antibody dilution, plasma sample dilution, standard dilution, and streptavidin dilution. The wash buffer consisted of PBS (pH 7.2 ± 0.3) and 0.05% Tween 20 (Sigma-Aldrich). 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate supersensitive (T4444 Sigma-Aldrich) was used to produce a final blue soluble product that could be read spectrophotometrically. The TMB reaction was stopped with 1 M H_2SO_4 (95–97%; Sigma-Aldrich).

Standards and quality controls

The calibration curve was constructed with a certified reference material, thyroglobulin T-113 with a concentration of 10 µg/mL (Cerilliant®). Tg T-113 was diluted in PBS/BSA 1% to obtain concentrations ranging from 0 to 150 µg/L for plasma and DBS.

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The Liquicheck Tumor Marker (Bio-Rad, Clinical Diagnostics, LOT.94932 and LOT.94933) was used as quality control (QC) at two different levels.

ELISA kits

A commercial ELISA kit was used to determine Tg in plasma and DBS (Elisa Duoset Human Thyroglobulin Kits; R&D System). The ELISA kit uses a monoclonal mouse anti-human thyroglobulin capture antibody, a monoclonal biotinylated goat anti-human thyroglobulin detection antibody, and streptavidin-HRP (streptavidin conjugated to horseradish peroxidase). The capture antibody was diluted in PBS, and the detection antibody was diluted in PBS/BSA 1%, to a concentration of 4 µg/mL and 0.1 µg/mL per well, respectively.

ELISA procedure for plasma thyroglobulin

The determination of Tg in plasma was performed as recommended by the manufacturer.

ELISA procedure for DBS-Tg

To obtain the calibration curve, whole blood was collected from a healthy individual in heparin-treated tubes and centrifuged at 1260 × ***g*** for 10 min, and the plasma was removed. Next, the red cells pellet was washed four times by adding 10 mL of NaCl 0.9%, centrifuging the solution at 1260 × ***g*** for 10 min, and removing the supernatant. Following the last wash, standards and controls were added to the erythrocytes in a proportion of 50:50 and rotated on a blood mixer for 5–6 min, and then 55 µL were spotted onto filter paper and dried for 24 h at room temperature (RT).

A 96-well polyethylene plate (MaxiSorp-NUNC) was used to determine DBS-Tg. On the first day, the plate was coated for a minimum of 17 h at RT with 100 µL of monoclonal mouse anti-human Tg capture antibody diluted to a concentration of 4 µg/mL. At this point, filter papers corresponding to standards, controls, and samples were punched with the automatic DBS-card puncher (Analytics Sales) to produce 6 mm discs. Then, 150 µL of wash buffer (PBS/Tween 0.05%) was added to the second plate of each well and was incubated for 22 h at 4°C under constant agitation (400–500 rpm) to enable the extraction of the DBS content.

On the second day, the supernatant of each well of the first plate containing the capture antibodies was aspirated, and then 350 µL of washing buffer was added (PBS/Tween 0.05%) with a 30-s interval between each cycle, repeated four times. Then, a 300 µL blocking solution (PBS/BSA 1%) was added to each well and incubated for 90 min at RT. After the blocking time had elapsed, the washing process was repeated four times. After the last wash, a 100 µL aliquot of each well containing the DBS extract was transferred to the plate containing the capture antibodies. When all the extracts had been transferred, they were incubated for 20 h at 4°C with constant agitation (400–500 rpm).

On the third day, the temperature of the plate was first adjusted from 4°C to RT with constant agitation (400–500 rpm) for 1 h, and the washing was carried out using the same process described previously. Following the last wash, 100 µL of detection antibody was added, and incubation was initiated for 120 min at room temperature. After incubation, a diluted solution of 1% PBS/BSA streptavidin-HRP conjugate (1:40) was added and incubated at RT for 20 min, protected from light, and washed four times. Subsequently, after the last washing, 100 µL of TMB was added to each well and incubated for 90 min at RT. The reaction was stopped by adding 50 µL of 1 M H_2SO_4 per well. The optical densities (OD) were read at a wavelength of 450 nm with the reference wavelength set at 620 nm using a Biochrom Anthos MultiRead 400 Microplate Spectrophotometer.

The standards, QC, and samples were duplicated at different positions on the plate.

Validation of the DBS ELISA assay for Tg measurements

Validation of the DBS-Tg assay was performed according to the Cofrac recommendations of the technical guide for accreditation of verification/validation of medical biology methods (19).

Sensitivity

Thirty blanks were analyzed in the same series. The limit of detection (LoD) in plasma and DBS-Tg was defined as the average of these 30 values plus three S.D. ($X+3$ S.D.), and the limit of quantification (LoQ) for plasma and DBS-Tg was defined as the average of these 30 values plus 10 S.D. ($X+10$ S.D.).

Repeatability and intermediate fidelity

The repeatability of plasma and DBS-Tg was determined using two levels of control thyroglobulin concentrations with the Liquicheck Tumor Marker (Bio-Rad, Clinical Diagnostics, LOT.94932 and LOT.94933). The samples were analyzed 30 times for plasma and 15 times for DBS-Tg in the same round. The results were expressed as the mean plus standard deviation (X ± S.D.), calculating the respective coefficient of variation (%CV) and bias for each case.

In order to establish intermediate fidelity for plasma and DBS-Tg, the two Tg controls were analyzed 30 times for plasma and 15 times for DBS-Tg in 15 consecutive

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https://doi.org/10.1530/ETJ-24-0142

rounds. The results were expressed as the mean and standard deviation (X ± S.D.), calculating the respective %CV and bias for each case.

Linearity

Different Tg dilutions were prepared to assess the linearity of the results for plasma analysis and DBS-Tg. For plasma, 13 dilutions were prepared, and for DBS-Tg, seven different dilutions in series were prepared.

Effect of hematocrit on DBS-Tg

A heparinized blood sample from a healthy volunteer was transferred to a Falcon tube after homogenization. The sample was centrifuged at 1260 x ***g*** to separate plasma and red cells. The plasma was separated and spiked with commercial Tg at 75 and 112 µg/L. Red cells were washed as previously described.

Different volumes of plasma and washed red cells were mixed to obtain blood samples with hematocrit values ranging from 45% to 75% and Tg concentrations of 75 and 112 µg/L (Table 1).

For each sample, eight drops of blood of 55 µL were spotted onto filter paper, dried for 24 h, and kept at RT until analysis.

Effect of storage time on the stability of DBS-Tg

A DBS-Tg with a concentration of 25 µg/L was spotted onto filter paper (n = 4) and dried for 24 h at room temperature, protected from light and heat. The DBS were stored in airtight aluminum envelopes at −20°C and 4°C and were analyzed at 0, 3 months, 6 months, and 12 months. In addition, 27 cord blood samples were selected to assess stability at 20ºC over 20 weeks.

Statistical analysis

Statistical analyses were performed with MedCalc Statistical Software Version 22.009 (Oostende, Belgium), GraphPad Prism Version 9.1.2, and Analyse-It for Microsoft Excel (version 23). Agreement between methods was evaluated using a Bland–Altman difference plot (20) and Passing–Bablok Regression (21). Linear regression analysis was used for the analysis of linearity and stability of thyroglobulin on DBS. The Wilcoxon–Mann–Whitney tests were used to investigate the stability of DBS-Tg. Non-normal data (Tg) was expressed as the median (25–75 percentile). The coefficient of variation (%CV) of Tg for repeatability and intermediate fidelity in plasma and DBS was calculated as follows: %CV = (S.D./mean) × 100; Bias was calculated as follows: %Bias = (mean Tg-ELISA Results − true value) × 100/true value. True values were established with reference to the result obtained from the reference technique, Elecsys Tg II Roche.

Results

Plasma Tg assay performances

The LoD of the ELISA assay was 2.6 µg/L and the LoQ was 5.8 µg/L. The %CV of repeatability (n = 30) was 5% for a Tg concentration of 22.3 ± 1.1 µg/L and 3.4% for a

Table 1 Composition of samples with varying hematocrit at two plasma Tg concentrations 112 µg/L and 75 µg/L and percentage of recovery.

Tg conc/HT (%)	**Volume of Tg-EP** (µL)	**Volume RBC-S** (µL)	**TH-conc** (µg/L)	**EXP-conc** (µg/L)	**% Recovery**
(A): 112 µg/L					
45	550	450	61.9	55.1	89.2
50	500	400	56.2	43.7	77.7
55	450	550	50.6	39.4	77.8
60	400	600	45.0	40.1	89.0
65	350	650	39.4	38.4	97.7
70	300	700	33.7	31.5	93.5
75	250	750	28.1	23.3	82.8
(B): 75 µg/L					
45	550	450	41.2	33.4	81.0
50	500	400	37.5	28.6	76.3
55	450	550	33.7	27.6	81.6
60	400	600	30.0	23.1	77.0
65	350	650	26.3	23.0	87.6
70	300	700	22.5	19.8	88.0
75	250	750	18.7	14.9	79.7

conc, concentration; HT, hematocrit; Tg-EP, Tg-enriched plasma; RBC-S, red blood cells-suspension; TH-conc, theoretical concentration; EXP, experimental.

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Tg concentration of 50.4 ± 1.7 µg/L, corresponding to the Liquicheck Tumor Marker (Bio-Rad, Clinical Diagnostic). The bias was of −32.4% and −49.7%, respectively.

The %CV for intermediate fidelity ($n = 30$) using the same Liquicheck control was 10.2% for a Tg concentration of 23.0 ± 2.4 µg/L and 9.7% for the Tg concentration of 46.6 ± 4.5 µg/L, with a bias of −30.3% and −53.4%, respectively.

The standard curve was linear between 0 and 150.0 µg/L. The linearity for the serial dilution of the samples with different Tg T-113 concentrations was excellent, with an $R^2 \geq 0.99$ ($P < 0.0001$).

DBS-Tg assay performances

The LoD of the DBS assay was 2.4 µg/L, similar to the LoD for the plasma matrix. The LoQ of DBS was the same as the LoQ in plasma, 5.8 µg/L. The %CV of repeatability ($n = 15$) was 8.3% for the Tg concentration of 19.8 ± 1.7 µg/L and 7.7% for the Tg concentration of 43.3 ± 3.3 µg/L, with a bias of −39.9% and −56.37%, respectively. The %CV of intermediate fidelity ($n = 15$) was 11.2% for a Tg concentration of 18.7 ± 2.1 µg/L and 11.0% for a Tg concentration of 42.3 ± 4.6 µg/L, with a bias of −43.4% and −57.3%, respectively.

As in plasma, the standard curve was linear between 0 and 150.0 µg/L for DBS. The matrix plasma and DBS correlation coefficients were always ≥0.98 ($P < 0.005$).

Our method demonstrated excellent linearity for the serial dilution of DBS across different Tg T-113 concentrations, matching the performance in plasma with an $R^2 \geq 0.99$ ($P < 0.0001$). The %CV of repeatability and intermediate fidelity, as in plasma, was determined using Liquicheck Tumor Marker controls.

Comparison of cord plasma Tg ELISA with Elecsys Tg II Roche (ECLIA)

The median cord plasma Tg values in newborns were 30.2 (21.3–44.4) µg/L with the ELISA assay and 76.5 (40.0–101.5) µg/L with Elecsys Tg II Roche ($n = 92$) with an $R = 0.88$. The Bland–Altman plot (Fig. 1A) confirms higher cord Tg plasma concentrations with Elecsys Tg II Roche than with the ELISA assay, showing a mean difference ratio of +51.3 units. The limits of agreement of the two methods were +1.96 s.d. 113.6 µg/L and −1.96 s.d. −11.0 µg/L. The Passing–Bablock regression (Fig. 1B) shows a proportional and constant difference between the two methods, with 95% CI for the intercept (*A*) from 0.81 to 6.6 and slope (*B*) from 0.31 to 0.38, with a regression equation $y = 4.002 + 0.342X$. The difference between the two methods increases with Tg concentrations. Only one of the 209 newborns tested positive for Tg-Ab, so Tg-Ab interference with Tg determination is unlikely.

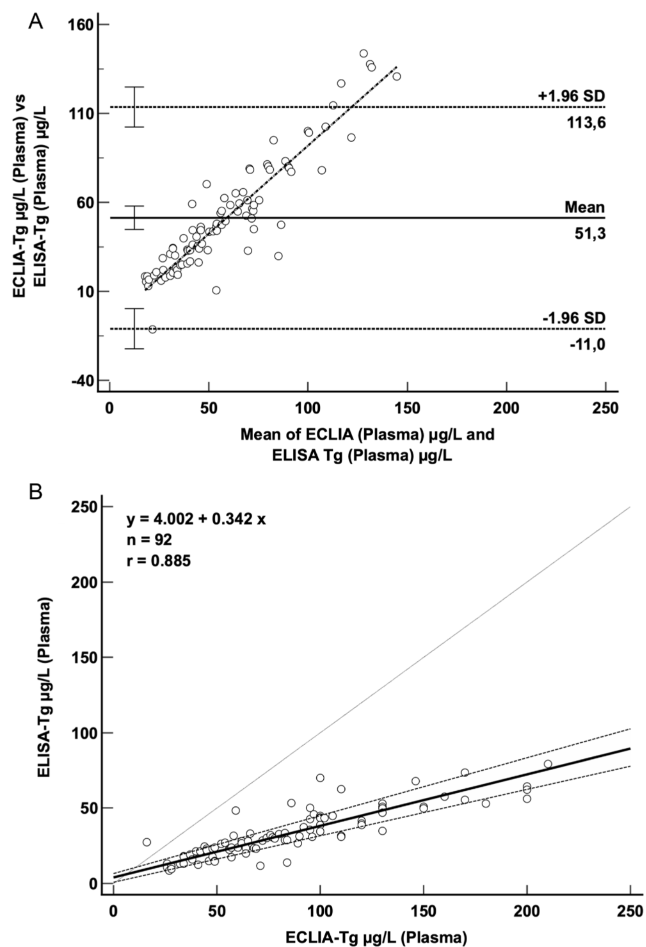

Figure 1

(A) Bland–Altman plot, comparing cord thyroglobulin plasma analysis with the reference technique Elecsys Tg II Roche (ECLIA) vs. ELISA ($n = 92$); the data points are plotted on the original scale. Solid line: mean difference ratio (ECLIA/ELISA); dashed line: 95% limit of agreement expressed as a function of the mean *X*. (B) Passing–Bablock regression, comparing cord thyroglobulin plasma ELISA assays and the Elecsys Tg II Roche (ECLIA) reference technique ($n = 92$). 95% CI of *A* (intercept) and *B* (slope) (131 × 185 mm).

Comparison of ELISA cord plasma Tg with ELISA cord DBS-Tg

The median cord DBS-Tg was 31.6 (19.3–48.7) µg/L ($n = 209$) and not significantly different from the median cord plasma Tg concentrations obtained with the ELISA assay ($P = 0.48$). The Bland–Altman plot (Fig. 2A) confirmed the mean difference of −1.4 units of cord Tg DBS compared to Tg plasma. The limits of agreement of the two methods were +1.96 s.d. 34.5 µg/L and −1.96 s.d. −37.4 µg/L. The Passing–Bablock regression (Fig. 2B) showed no constant or proportional difference between the two methods, with 95% CI for the intercept (*A*) from 7.6 to 1.8 and slope (*B*) from 1.0 to 1.2, with a regression equation $y = -4.2 + 1.13X$.

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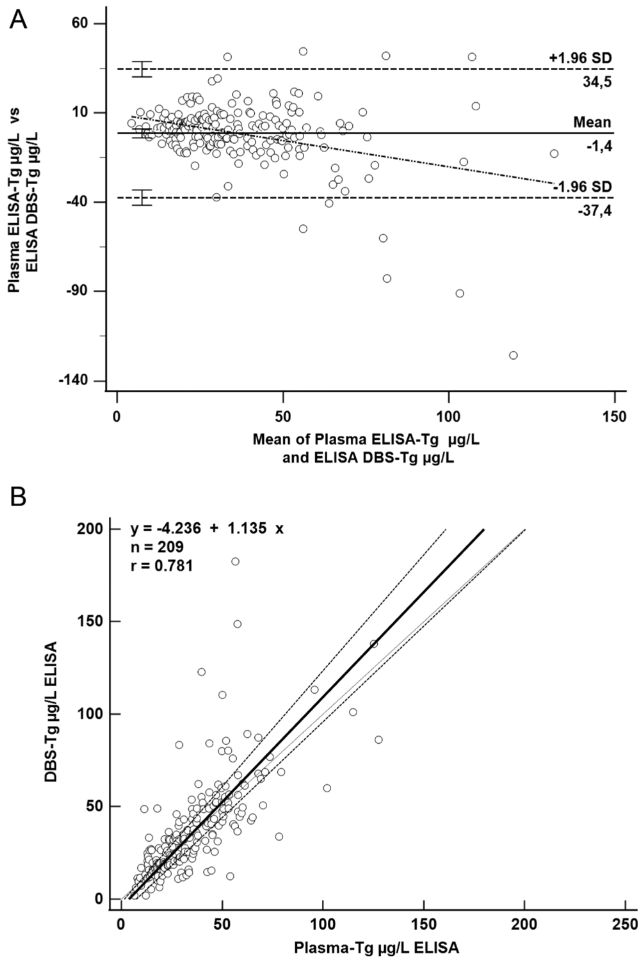

Figure 2

(A) Bland–Altman plot, comparing cord Tg-plasma ELISA and the cord Tg-DBS ELISA (n = 209). The data points are plotted on the original scale. Solid line: mean difference ratio (Plasma Tg/DBS-Tg). Dashed line: 95% limit of agreement expressed as a function of the mean *X*. (B) Passing–Bablock regression, comparing cord Tg-plasma ELISA and the cord DBS-Tg ELISA (n = 209). 95% CI of *A* (intercept) and *B* (slope) (131 × 185 mm).

Influence of hematocrit on Tg concentration measured on DBS

Hematocrit had a significant influence on DBS-Tg results, as shown in Fig. 3. DBS-Tg concentrations decreased with increasing hematocrit, independently of the Tg recovery (Table 1).

Study of Tg stability on DBS

Regression analysis for DBS-Tg with a concentration of 25 µg/L for 12 months at −20ºC and 4ºC (Fig. 4A and B) showed no significant changes (n = 4). DBS-Tg (n = 27) concentrations from cord blood samples stored for 20 weeks at 20°C showed no significant difference (P = 0.3703) (Fig. 5). The median cord DBS-Tg

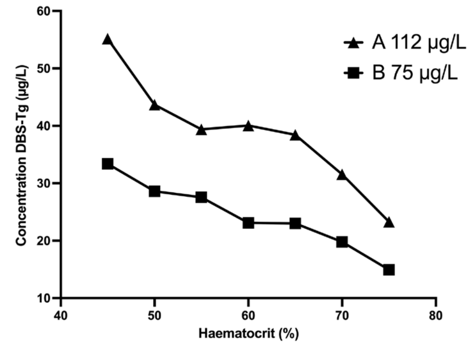

Figure 3

Effect of hematocrit plot vs. DBS-Tg, with triangles corresponding to the concentration of Tg at 112 µg/L (P < 0.001) and squares Tg concentration at 75 µg/L (P < 0.05) (143 × 108 mm).

concentration at week 0 was 32.6 (20.0–43.0) µg/L and 29.5 (21.8–38.1) µg/L at week 20.

Discussion

Our results demonstrated the excellent analytical performance of the DBS-Tg assay over a wide range of Tg concentrations, suggesting that this assay is a robust method of monitoring Tg. In addition, we have shown that hematocrit is a determinant of DBS-Tg concentrations, a finding that has implications for interpreting DBS-Tg results. Our study is the first to validate an ELISA method using cord-DBS for Tg determination in newborns.

The assay's repeatability and intermediate fidelity %CV for DBS-Tg were 11%, comparable with two other DBS-Tg methods validated for children and pregnant women (11, 16) and also with commercially available ELISA kits at <10% and <12%, respectively (22, 23).

Furthermore, the sensitivity of 2 µg/L for DBS-Tg and the limit of quantification of 6 µg/L for DBS-Tg were similar to a DBS-Tg assay developed by Stinca *et al.* (16). The Stinca assay was not validated using an existing kit but developed by selecting commercial anti-Tg antibodies and evaluated with the Immulite 2000. In this assay and also in the DBS-Tg fluoro-immunometric assay used in children (11, 17), the authors did not assess the effect of hematocrit on Tg determination.

Our study did not measure Tg concentrations in pregnant women. However, the ranges of cord Tg values that we found align with previous research. These studies consistently show significantly higher serum Tg concentrations in cord blood than in adults (15, 24, 25). The neonatal TSH surge at delivery, which stimulates the newborn thyroid, could be a critical factor in helping to explain the higher cord Tg concentrations found in neonates compared to adult blood.

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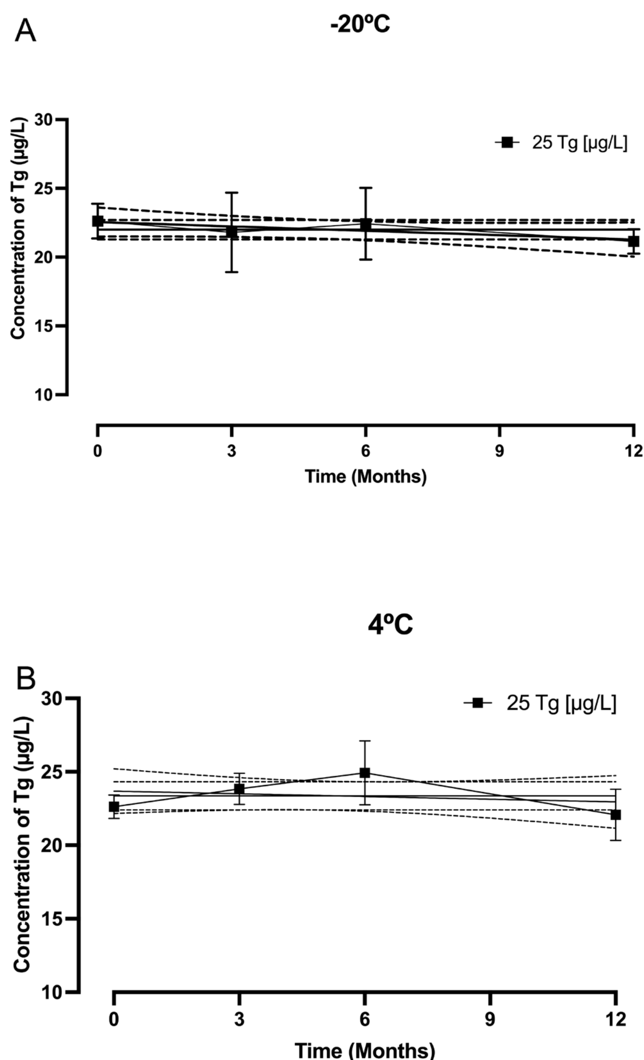

Figure 4

DBS-Tg concentrations ($n = 4$ for each point) of 25 µg/L measured over 12 months of storage at (A) −20°C ($R^2 = 0.13$; $P = 0.16$), and (B) 4°C ($R^2 = 0.02$; $P = 0.56$). Solid and dashed lines represent regression lines (147 × 243 mm).

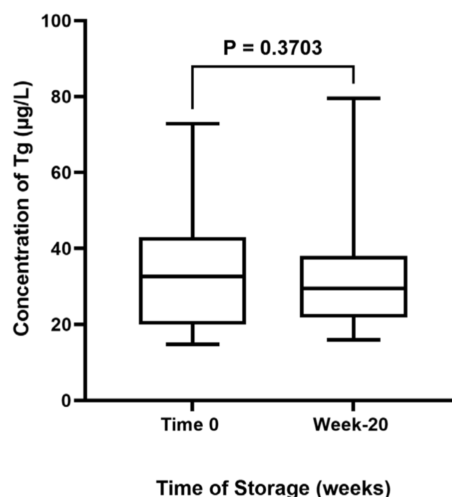

Figure 5

DBS-Tg concentrations plot of cord blood from newborns measured at week 0 and week 20 ($P = 0.3703$) of storage at 20°C (108 × 109 mm).

The limit of quantification was 5.8 µg/L for the DBS-Tg ELISA, slightly higher than the 5.0 µg/L than the one reported for RIA DBS used for the determination of Tg in 2–7-day-old infants, who also had Tg values twofold higher than cord Tg-DBS obtained with the ELISA assay (26).

The ELISA assay's limit of quantification in plasma and DBS is much higher than the 0.1 µg/L LoQ of Elecsys Tg II. However, this is not a disadvantage, since the ELISA will be used to assess high Tg concentrations, in contrast to the electrochemiluminescence immunoassay which is designed to detect low concentrations of Tg in the follow-up of thyroid cancer. Similarly to the ELISA for DBS-Tg previously described (16), we did not find significant differences between Tg in plasma and DBS-Tg. However, for plasma, we found lower Tg concentrations with the ELISA than with our reference method, Elecsys Tg II. This is distinct from the ELISA used in pregnant women, where higher Tg concentrations have been reported compared to the reference method, Immulite 2000.

Newborn Tg concentrations increase markedly 6 h after delivery and remain stable for at least 4 days (15). The newborn's thyroid gland has low iodine stores, leading to high iodine turnover and an increased thyroid weight/body weight ratio. This mimics diffuse goiter, which, probably in combination with the TSH surge, contributes to the higher Tg values in newborns than in cord Tg. However, it is important to note that not all studies have found an increase in newborn Tg compared to the level of Tg in cord blood (25).

In neonates from mildly iodine-deficient regions, cord serum Tg concentrations, but not cord TSH, were significantly higher than in iodine-sufficient newborns (14). In Denmark, TSH levels were higher, but Tg was lower, in neonates from pregnant women who took iodine supplements compared to the control group (27). These conflicting results were probably due to pre-analytical factors that predominantly may affect TSH concentrations when iodine deficiency is mild (28, 29). Included among these factors are the quality of drops collected on the filter paper, the timing of the neonatal blood sample collection, the TSH used, prematurity, mode of delivery, as well as maternal and newborn exposure to iodine-containing antiseptics (7).

Neonatal Tg concentrations, unlike nTSH, seem to vary less during the first few days following delivery. This represents an important advantage since the influence of the sample collection time on the Tg may be smaller (15).

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https://doi.org/10.1530/ETJ-24-0142

Importantly, the availability of a DBS-Tg assay advances the very real possibility of integrating iodine status monitoring with the screening of newborns for inherited metabolic diseases. Determination of DBS-Tg can be done easily in healthy newborns using blood spots left over after routine screening. All these considerations suggest that newborn Tg could help to assess iodine status more frequently if implemented in maternity units and newborn screening facilities.

There are, however, some limitations to using Tg for monitoring iodine status. The interference of Tg-Ab with Tg determination can affect the accuracy of the assay. In our population, the prevalence of Tg-Ab was extremely low, as only one newborn was positive for Tg-Ab. Consequently, any interference of Tg-Ab with Tg determination is unlikely. A low prevalence of Tg-Ab in newborns from pregnant women with no history of thyroid diseases, an exclusion criterion in the iodine surveys, may be an additional advantage for using neonates' Tg. However, Tg-Ab interference may be less problematic in population studies, as previously suggested in pregnant women (16). This issue requires verification from a larger sample of newborns with positive Tg-Ab. Another area of concern is the high inter-assay variability of Tg determination, even when using the Community Bureau of Reference material (30). Consequently, the reference Tg values and the cut-off point used to define iodine deficiency are method-dependent and present a limitation when comparing the results of studies using different assays (31). The marked bias of 51% of mean plasma Tg concentration that we found between the Elecsys and ELISA assay illustrates the pronounced inter-assay variability of Tg determination between different assays. This difference arises from several factors, principally the affinity of Tg antibodies.

The influence of hematocrit on DBS immunoassays has been demonstrated before for both insulin and TSH (29, 32). Our results indicate this is also the case for DBS-Tg, where samples with low hematocrit values yielded higher Tg values. The influence of hematocrit on DBS-Tg concentrations is relevant from both methodological and physiological perspectives (33). Hematocrit has an effect on the viscosity, the homogeneity of the blood in the DBS spot, and also on the drying time, which may all affect the analytical process (33, 34). Viscosity can influence the dispersion of the blood drop in the filter paper and, thus, the spot diameter. A sample with low hematocrit will result in larger DBS per volume unit than blood with high hematocrit. This, in turn may result in an overestimation of Tg concentration (35). The effect of hematocrit on Tg concentrations should be considered when studying populations at risk for anemia. In our healthy newborns with normal hematocrit, plasma Tg concentration and DBS-Tg were not significantly different. However, DBS-Tg values will be higher than plasma Tg concentrations in newborns with a high prevalence of anemia and low hematocrit.

The impact of hematocrit is not restricted to newborns but is a matter of concern for all populations at risk from anemia. For example, women of childbearing age and pregnant women are at enhanced risk from anemia, particularly in low-income countries. The effect of hematocrit on DBS-Tg is therefore relevant when comparing the Tg values of two populations exhibiting different degrees of anemia prevalence. If this information is unavailable, determining hemoglobin and hematocrit in a subsample of DBS-collected blood using standard methods will provide this information (36). A calculated correction factor, if needed, can then be applied to the data from a sample of the population with a higher prevalence of anemia.

The stability of DBS-Tg is another critical issue in epidemiological studies. Our results indicate that DBS is stable for up to 12 months at 4°C, thus simplifying the use of this method in field studies.

In conclusion, this DBS-Tg assay shows good analytical performances across a broad range of Tg concentrations. These characteristics make it well-suited to detect variations in Tg concentrations over time. When comparing populations with different anemia prevalence, we should consider the impact of low hematocrit on DBS-Tg concentrations. We also showed that DBS-Tg is stable for 12 months at −20ºC and 20 weeks at 20°C, which has important implications for field studies. Moreover, the cost of the DBS-Tg assay, at approximately 2€, is significantly lower than that of commercial Tg assays, which are closer to 6€. Finally, the DBS-Tg assay not only expands the tools available for fine-tuning the monitoring of iodine status but also opens the possibility of integrating DBS-Tg determination and, therefore, iodine monitoring within newborn screening programs designed to detect inherited metabolic diseases.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was funded by the CMP 2019-2022 project number 2 – CMP 2022-2025 project number 14 by the Joint Cooperation Commission between Chile and Wallonia-Bruxelles International (WBI), and by an unrestricted grant from the Iodine Global Network (IGN).

Author contribution statement
CFP participated in the design, development and validation of methodology, patient recruitment, data collection, performed all data analysis, and wrote the manuscript. MCO, LM, CR, and LM participated in review, editing, and provided manuscript corrections. BH participated in the design, development, and validation of methodology, writing, editing, and review. FC participated in the development and validation of methodology and provided manuscript corrections. RMR participated in the design, development and validation of methodology, patient recruitment, supervised data analysis, and manuscript writing. All the authors read and approved the final version of the manuscript.

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Acknowledgements
We thank the Department of Gynaecology of the H.U.B. Erasme Hospital for their cooperation in this work.

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